The Open Access Israeli Journal of Aquaculture – Bamidgeh

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Use of Molecular Tools for Research and Improvement of Aquaculture Stocks

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Key words: Genetic markers, parentage assignment, QTL detection, marker-assisted selection, gene expression profiling, genetic marking

Abstract

Development of molecular genetic markers provides aquaculture with tools for a number of research and practical applications. Genetic marking of experimental groups allows their evaluation in the same rearing units, increasing statistical power within limited research infrastructure. Parentage can be inferred for individuals in mixed-progeny groups, quantifying the contributions of individual parents and supporting the estimation of sire and dam effects. Building upon parentage assignment, walk-back selection entails retention of the best members of each family as broodstock for the next generation. Molecular markers can be used to detect the segregation of quantitative trait loci (QTL) and knowledge of such linkages can be used for marker-assisted selection. Gene expression profiling can identify genes affecting traits of interest, providing candidates for QTL or functional analysis. Purposeful genetic marking can be used to identify proprietary stocks, marketed products, and fish out-planted or escaping into natural ecosystems. Although each application has been demonstrated, genetic markers are not routinely used in commercial aquaculture. The limited practical application can be explained by the limited development of broodstocks for most aquaculture species, the small size and limited scope of most aquaculture operations, and the costs of genetic screening.

Introduction

Although quantitative and molecular genetics developed independently, over the past 20 years their interface has become an area of rapid advancement. Molecular genetic markers have a wide range of applications in aquaculture research and for improvement of aquaculture stocks (Hallerman and Beckmann, 1988; Poompuang and Hallerman, 1997; Liu and Cordes, 2004; Chistiakov et al., 2006).

Here, I discuss key applications, including parentage assignment, walk-back selection, quantitative trait loci (QTL) detection and marker-assisted selection, gene expression profiling, and genetic marking. I illustrate the potential use of these applications by means of case studies.

Use of genetic markers in aquaculture research has a longer history than many read-

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ers may realize, a history that started in Israel in the heyday of isozyme research. Moav et al. (1976) and Brody et al. (1976, 1980, 1981) used allozyme markers to mark genetic lines and assess outcomes of a classic series of common carp breeding experiments. Among their applications, they identified full-sib families nested within half-sib families, identified families within 2 x 2 diallel crosses, and marked full-sib families to assess the breeding value of hybrid crosses. Using markers to identify membership of individual fish within a particular group allowed the authors to use powerful "common garden" experiments that both decreased the number of experimental units needed and increased the statistical power of their experiments. This path-breaking work showed the utility of molecular markers as an aid to breeding.

Looking from the context of our own times, however, it is clear that this classical marker-assisted breeding work was limited by the properties of the protein-level markers then available. There were not many markers, and they tended to be diallelic. Subsequent advances in molecular genetics brought powerful new methods for observing genetic variation. Among the many sorts of molecular tools, three seem particularly useful for aquaculture genetics.

The first is microsatellite DNA markers. Scattered about the genome are huge numbers of "microsatellite" loci bearing motifs of simple short sequences, e.g., $(AC)_n$. Using primers annealing to sequences flanking microsatellite tracts, we can use the polymerase chain reaction (PCR) to amplify particular microsatellite loci and characterize the number of such repeats at a locus. Microsatellite loci often exhibit high levels of variation within and between populations. Variation across many microsatellite loci can be observed cost-effectively in a multiplex, a mix of amplification products for several loci. Microsatellite markers have been shown useful for a range of applications including population genetics, parentage assessment, family identification, and QTL detection (Wright and Bentzen, 1994; O'Connell and Wright, 1997; Chistiakov et al., 2006).

A second type of marker is newer and highly promising. Throughout the genome are innumerable instances where DNA sequences vary by but one nucleotide among sister chromosomes or among individuals. These are termed single nucleotide polymorphisms, or SNPs. SNPs are common, perhaps one per kilobase of genomic sequence, but must be discovered. Once known, they can be screened using a range of methods, including PCR- and DNA chip-based approaches (Ohnishi et al., 2001). Use of SNP screening methods opens the entire genome to screening. However, the potential of SNPs for use in aquaculture applications is largely undemonstrated (for an exception, see Glenn et al., 2005).

A new third approach for detecting expression of genes that affect a trait of interest arises following development of a breakthrough technology. A "gene chip" or microarray is comprised of thousands of gene transcripts immobilized on a solid surface (Southern, 1996; Lockhart et al., 1996). The microarray can be screened to determine the level of expression of particular genes in an organism of interest. For example, after exposing an organism to an experimental treatment, tissues of interest would be collected and mRNA would be isolated and used to screen a microarray for the species. The intensity of expression for a gene will be revealed by the intensity of fluorescence for its corresponding transcript on the microarray. A broad range of questions can be answered using this approach.

With this brief technical background, I consider the applications of DNA markers for research and improvement of aquaculture stocks.

Parentage Assignment

There are several reasons why parentage assignment is of practical interest to aquaculturists. Fish breeders may want to determine parentage relationships to know whether all broodstock indeed contributed to the progeny generation and whether their contributions were reasonably equal. Breeders will want to design future matings so as to avoid inbreed-

ing. Culturists may want to know whether the offspring maintain the genetic character of the wild or founder population. In particular, they may want to know whether genetic variability was maintained in terms of number of polymorphic loci, number of alleles per locus, and heterozygosity. Culturists should be aware of the effective population number, N_e, for their production stock. N_e is a function of the sex ratio among breeders, family size, and the number of spawners each generation. Breeders will want to know whether N_e meets some propagation target. If it does, then propagation and rearing practices are appropriate and may be maintained. If not, there are several options. The culturist may (a) equalize family size in the current generation, (b) propagate non-contributors or new broodstock in the next breeding cycle, or (c) rotate broodstock in and out of the breeding program to maximize the genetic variability in the propagated stock.

Parentage assignment has been applied for a number of aquaculture species including rainbow trout (Herbinger et al., 1995), European sea bass (Garcia de Leon et al., 1998), Atlantic salmon (Norris et al., 2000), and Japanese flounder (Hara and Sekino, 2003; Sekino et al., 2003). In a case study involving Atlantic halibut (Jackson et al., 2003), the broodstock traced back to a 1996 collection of wild fish from the Bay of Fundy in eastern Canada. The F_1 stock was the product of 80 potential crosses among 13 females and 14 males using pooled milt. 145 individuals were retained as future broodstock in three facilities. The authors sought to determine the pedigrees of these fish, in particular to determine how evenly the genetic variation in the founders was represented in the broodstock candidates. This knowledge could be used to plan future breeding, especially to avoid inbreeding. Jackson et al. (2003) screened variation at five microsatellite loci to identify full- and half-sibs and to determine whether genetic variation had been lost. The PROBMAX software (Danzmann, 1997), one of several available for the purpose, was used to assign parentage to the screened individuals. Among the results, single parental pairs

were assigned to 98%, 96%, and 100% of the progeny individuals at the respective facilities. Only 36% of the attempted crosses were represented among the broodstock candidates. N_e declined from 27 to 13 because of variation in family size among broodstock. The F_1 population showed at 26% reduction in the number of alleles. Among the practical implications of the findings, halibut producers need to better plan and monitor future matings and to introduce more genetic variation into their broodstocks.

This case study shows the utility of genetic markers for evaluating parentage in production stocks. The approach is becoming widely applied. If microsatellites are available for a given species, the cost is not prohibitive and a wide variety of producers might benefit from evaluating their broodstock. For species where markers are not available, the investment to develop and screen useful genetic markers will be in the order of US$25,000, which may affect the decision of whether to go forward.

Walk-Back Selection

Walk-back selection builds on parentage assessment to support selection of broodstock from within families (Doyle and Herbinger, 1994). Walk-back selection is best understood within the context of the definitive case study on rainbow trout (Herbinger et al., 1995). Salmonid Production Associates is a small farm in Nova Scotia, Canada, where a complete factorial cross was made between 10 sires and 10 dams. All progeny were communally reared for one year. The largest and smallest size-classes of progeny were sampled. Parentage was assessed using four or five microsatellite markers. Among the results, 91% of the fish were assigned to 1 or 2 parental pairs. There were statistically significant effects among sires and dams for growth and survival of their progeny. The progeny of inbred crosses showed depressed survival and growth. Following parentage assignment, the parental fish that produced superior offspring were re-spawned in subsequent years. At the end of the generation, the best fish in the mixed-progeny group were genotyped

one-by-one, and the best male and female from each family were retained as broodstock.

In a figurative sense, the Doyle group "walked back" sequentially from the best individual through the progeny group to identify the best broodstock candidates from each family; hence, the term "walk-back" selection. The best members from each family were crossed to minimize inbreeding. Walk-back selection supported high selection intensity with minimum inbreeding increase.

In walk-back selection, the Doyle research group demonstrated a new marker-assisted breeding concept. By using genetic markers, it was possible to practice genetic improvement within the context of commercial aquaculture production. There was no need for specialized infrastructure (i.e., replicated rearing units) or interference in commercial farm operations. Still, the walk-back selection approach has not been widely applied, suggesting possibilities for further application and evaluation.

QTL Detection and Marker-Assisted Selection

A very different approach to marker-assisted breeding uses molecular markers to reach inferences on the basis for expression of quantitative traits, and then uses that knowledge for breeding purposes. Quantitative trait loci (QTL) are genes whose expression interacts with environmental factors to determine phenotypes for measured traits such as yield. QTL are generally unknown. Detection of QTL would help us understand the genetic architecture of the trait, i.e., the numbers and relative effects of genes that determine a trait. As discussed below, such knowledge can be applied for marker-assisted selection, or MAS. There is a rich body of theory for QTL detection in plant (Paran and Zamir, 2003) and animal (Kashi et al., 1990; Weller, 1997) breeding that we can draw upon to design powerful experiments appropriate for fish (Poompuang and Hallerman, 1997).

In the classic experimental design, segregation of QTL is demonstrated by showing linkage to genetic markers. Such detection is possible wherever a marker locus (with alleles *M* and *m*) is located near a quantitative trait locus (with alleles *Q* and *q*). *Both* marker and QTL loci must be heterozygous in an individual to detect linkage in its progeny. Screening of markers and evaluation of quantitative phenotypes are carried out within families. Statistical testing assesses whether phenotypes of carriers of *M* differ from those of carriers of *m.* Significant differences imply linkage of marker and QTL alleles. When genome scans for QTL are conducted with knowledge of the genetic map for the species, interval mapping and maximum likelihood techniques (Lander and Botstein, 1990; Ott, 1991) can be applied. Detailed discussion of QTL detection is presented by Thorgaard (2006).

A number of QTL have been detected in aquacultured organisms. For example, QTL for upper thermal tolerance (Jackson et al., 1998; Perry et al., 2001), spawning time and body weight (O'Malley et al., 2003), and IPNV resistance (Ozaki et al., 2001) have been detected in rainbow trout. QTL for cold tolerance and body weight (Cnaani et al., 2003; Moen et al., 2004), sex ratio distortion (Shirak et al., 2002), sex determination (Lee et al., 2003, 2004), stress response, body weight, and sex determination (Cnaani et al., 2004), and susceptibility to inbreeding (Palti et al., 2002) have been detected in tilapia. Focusing on a case study, Cnaani et al. (2003) evaluated an F_2 hybrid (*Oreochromis mossambicus* x *O. aureus*) tilapia family for cold tolerance and growth rate, and then screened it with the UNH collection of microsatellite markers. A first scan was based on a family of 60 fish screened with 20 microsatellites, and a second with 114 fish screened for six microsatellites in one key linkage group. Within linkage group 23 (Fig. 1), a QTL for cold tolerance was detected near the *UNH879* locus and one for body size near *UNH130*. Sex determination was associated with marker *UNH879*, and survival with markers *UNH130*, *UNH180*, and *UNH907*. Genome scans for QTL have been carried out for relatively few traits and species, and there is much useful work yet to be done.

Knowledge of marker-QTL linkages and the strengths of QTL can be applied through
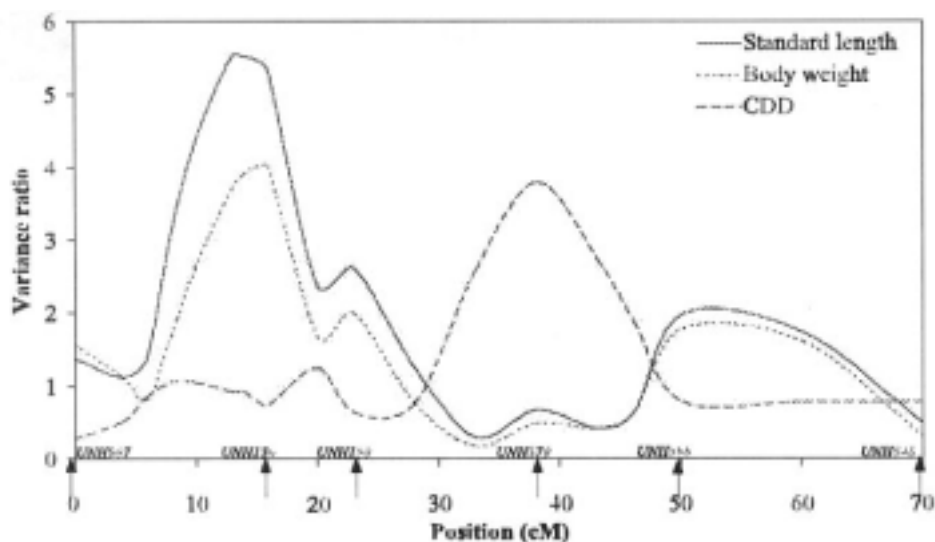
Fig. 1. Interval mapping of tilapia linkage group 23, showing approximate locations of quantitative trait loci (QTL) for standard length, body weight, and cold tolerance (CDD; Cnaani et al., 2003).

marker-assisted selection. Two modes of application may be anticipated. First, we can select directly upon the family material on which QTL mapping was done. I anticipate that this mode will have limited applicability, mostly for basic research. Second, we can select upon existing commercial broodstocks – I anticipate that this mode will have more general applicability. Commercial broodstock will be screened for segregation of QTL of interest and to determine the coupling of marker and QTL alleles specific to families within that broodstock. MAS has been well demonstrated in plant crop systems (Collard et al., 2005) such as corn (Yousef and Juvik, 2002) and millet (Serraj et al., 2005). If the gene directly affecting a trait is known (as opposed to a genetic marker linked to that gene on the chromosome), then gene-assisted selection (GAS) can be applied. In agricultural animal systems, MAS is being applied to increase litter size in pigs (Rothschild et al., 1996; Visscher and Haley, 1998). GAS is being applied to increase scrapie resistance

(DEFRA 2006) and to decrease incidence of spider syndrome in sheep (R. Lewis, Virginia Polytechnic Institute and State University, pers. comm.). To my knowledge, neither GAS nor MAS have yet been applied to fish.

A key practical question is whether MAS can accelerate genetic progress to the degree that it is cost-effective. The power of selective breeding plus MAS will have to be demonstrated relative to conventional, phenotype-based breeding alone. The efficacy of MAS depends on three factors, the heritability of the trait, the proportion of genetic variance associated with marker(s), and the selection scheme at issue (Fig. 2; Lande and Thompson, 1990). The relative efficiency of MAS relative to conventional selective breeding is highest for low heritability traits when selecting on the basis of an individual-based index combining both genetic marker and phenotypic information. Development of selection indices combining phenotypic and marker information depends upon the relationships among individuals, their breeding
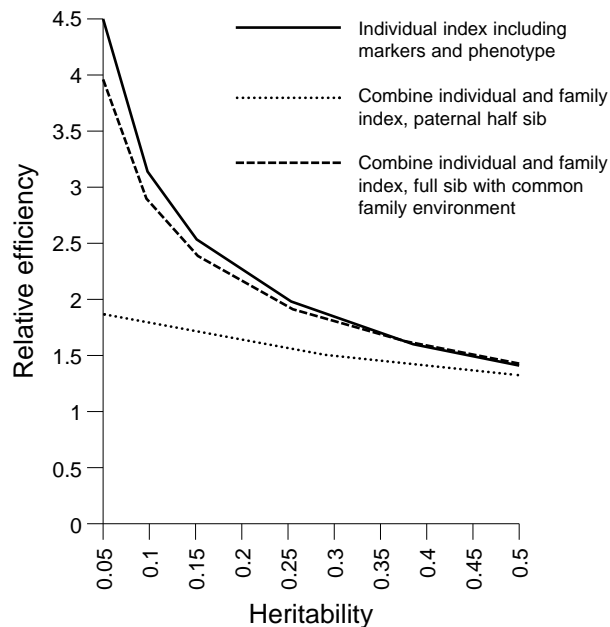
Fig. 2. Relative efficiencies of marker-assisted selection as a function of the heritability of a trait of interest and experimental design (Lande and Thompson, 1990; Poompuang and Hallerman, 1997).

values as estimated using classical animal models, and the phenotypic effects of marked QTL (Spelman and Garrick, 1998). The theory for estimating selection indices has been particularly well established for dairy cattle (Hoeschele, 1993; Weller, 1997). Since fish families can be large and reared in single units, the parameters entering the analysis presumably can be estimated with considerable precision. Still, the theoretical basis for development of selection indices for fishes needs more theoretical work.

MAS cannot be cost-effectively applied for every trait. High heritability traits might best be improved by classical, phenotype-based selection. Traits for which MAS would be most appropriate include sex-limited traits, traits expressed late in life, carcass traits, and low heritability traits (Poompuang and Hallerman, 1997). Given that many key traits, such as growth rate, often have high enough heritability to be improved using classical selective

breeding, cost considerations might dictate that MAS will be used to develop resource lines (e.g., disease-resistant lines) as opposed to general production lines. These resource lines might be crossed into production stocks as needed to improve targeted traits for which QTL detection and MAS are cost-effective.

Gene Expression Profiling

Application of microarray techniques is an area of great potential for understanding gene expression. For example, aquaculture scientists may want to identify the molecular genetic pathways through which animals respond to environmental stressors such as temperature, salinity, nutrient limitation, or disease. Some individuals might be exposed to a stressor of interest, while other, control individuals would not. By comparing the profile of gene expression among these individuals, genes that are differentially expressed in the two groups

could be identified. These genes could be assessed for known function or, if their function is unknown, these results might suggest their function.

As a case study, I consider the issue of how a cultured fish responds physiologically to its diet (S.R. Craig, unpubl. data). Groups of tilapia were fed one of four diets that were isoenergetic but varied in protein or lipid contents for six weeks. After the feeding trial, the fish were sacrificed and an anterior segment of the intestine was dissected out. mRNA was isolated and used to screen a microarray for zebrafish. Expression of particular genes was compared among treatments, that is, among control, low protein, high lipid, high protein, and low lipid diets. Profiles of gene expression differed dramatically among diets. Focusing on the effects of the high lipid diet on gene expression, five genes exhibiting at least 1.5-fold increases in expression for the high lipid diet (*arf1*, *aporE*, *PI3KC*, *RFP2*, and *LRP1*) seemed clearly related to the dietary treatment. Others did not seem to be directly related, but may have had subtle functional connections to the diet. Several functional categories of genes (e.g., receptors, energy-related, signal transduction, transcription factors) were impacted by the high lipid diet.

Microarrays provide cutting-edge technology for research on gene expression. A range of applications in aquaculture can be identified, including development of advanced diets based on eliciting the function of key metabolic pathways, and identification of candidate genes for QTL or functional analysis.

Genetic Marking

Aquaculturists may want to mark cultured fish to show persistence of stocked fish in a targeted ecosystem, assess the recruitment of escapees from aquaculture systems into wild populations, or establish a proprietary mark for a cultured stock. Culturists might apply physical marks such as fin clips, tags, brands, or dye to the fish, but such practices mark just the one generation at issue. Better, culturists might apply genetic marks by selecting to dramatically increase the frequency of rare allele(s) in the population at issue. The advan-

tage is that this selection yields a heritable mark that will be expressed by the descendents of the selected stock. Hatchery stocks of pink salmon, chum salmon, red drum, lobster, and other species have been genetically marked in this way (Hallerman, 2003). The most efficient marking strategy depends on genetic variation within and among populations, the effort devoted to marking the population, and the effort devoted to detecting the mark in the mixed population (Gharrett and Seeb, 1990).

A case study involves red abalone in California for which numerous outplantings of small hatchery-derived red abalone seed exhibited poor survival. In 1979, the California Department of Fish and Game outplanted 42,000 3-cm abalone at San Miguel Island, California. Allozyme frequencies (Gaffney et al., 1996) showed that outplanted abalone dominated the catch for years afterwards. However, because the offspring of relatively few individuals had been stocked, the N_e for the population (<10) actually was *reduced* by outplanting. This finding raised the question of whether stock enhancement had really been achieved. The key point in the context of this review is that the effects of stocking were inferred by use of genetic markers.

A variation of genetic marking termed "family-printing" (Letcher and King, 1999) uses multilocus microsatellite genotypes to identify offspring from a specific targeted stock of randomly-mated parents. In the context of restoring a lost run of Atlantic salmon, the authors suggested that whole families could be stocked in given tributaries and family-printing used to see which tributaries supported high recruitment, thereby indicating which ecosystems should be targeted for further restoration. Genetic markers also could be used to determine whether the restoration of the population met programmatic goals. A case study involved Connecticut River Atlantic salmon. Spidel et al. (2004) screened the 1996-1999 runs of restored salmon populations at nine microsatellite loci. They found that heterozygosities were similar, although allele frequencies differed among source and reestablished populations. A healthy level of

genetic variation was observed in the restored Connecticut River population of Atlantic salmon.

Discussion

This review has emphasized potential applications of genetic markers and presentation of case studies from research rather than general aquaculture practice. At this time, genetic markers are not routinely utilized in commercial aquaculture. Why are genetic markers not more widely utilized in commercial aquaculture? Beyond the simple explanation that there is a time lag for adoption of any new technology, the structure of key sectors of the aquaculture industry may also prove important. (1) There is but limited development of broodstocks for most aquaculture species. For most species, we are in the stages of domestication or characterization of the inheritance of key quantitative traits. The most notable exception to this generality is salmonids. (2) The small size and limited scope of most aquaculture operations constrains their capacity for long-term planning and investment in selective breeding, including development and use of genetic markers. Again, the notable exception to this is salmonids and, more recently, penaeid shrimps. (3) The costs of developing and screening genetic markers are considerable relative to the budgets of most aquaculture operations. Again, the notable exception is salmonids and, possibly, penaeid shrimps.

Given this background, it is not surprising that the greatest progress in development of genetic markers and screening for purposes of genetic improvement has been achieved for salmonids. It seems to me that a key challenge facing aquaculture genetics is to overcome these challenges for more aquaculture sectors. Strategic planning and collaborative research among the research and commercial sectors will be needed to fully realize the benefits posed by use of molecular markers.

Acknowledgements

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