Review

DNA marker technologies and their applications in aquaculture genetics

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Abstract

The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. With DNA markers, it is theoretically possible to observe and exploit genetic variation in the entire genome. Popular genetic markers in the aquaculture community include allozymes, mitochondrial DNA, RFLP, RAPD, AFLP, microsatellite, SNP, and EST markers. The application of DNA markers has allowed rapid progress in aquaculture investigations of genetic variability and inbreeding, parentage assignments, species and strain identification, and the construction of high-resolution genetic linkage maps for aquaculture species. Well-designed studies using these genetic markers will undoubtedly accelerate identification of genes involved in quantitative trait loci (QTL) for marker-assisted selection. In this review, the principles, potential power, requirements, advantages, and disadvantages of the various marker types are discussed, along with their applications in a variety of aquaculture studies. Included are discussions on how to efficiently exploit research progress made from the Human Genome Project and from other model species such as zebrafish for the benefit of aquaculture genomics and aquaculture genetics research.
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1. Introduction

DNA marker technologies have revolutionized the way aquaculture genetics research is conducted. The dramatic development of molecular genetics since the first widespread use of allozymes in the 1970s, and currently exemplified by the Human Genome Project and other equally ambitious undertakings, has laid the groundwork for genomics. Broadly defined as the study of genes and their functions, genomics is rapidly impacting many facets of life, from health care and food safety to reproduction and law enforcement.

Keys to the emergence of genomics were advances in DNA marker technology. These advances have resulted in a wealth of genetic markers (defined and discussed below) including allozymes, mtDNA, RFLPs, RAPDs, AFLPs, microsatellites SNPs, and ESTs with potentially widespread utility in a variety of aquaculture endeavors. In this review, the principles, potential power, advantages and disadvantages, and requirements for use are discussed for the various marker types, along with their application in assessments of genetic variability and inbreeding, parentage assignment, species and strain identification, hybridization, and marker-assisted identification of quantitative trait loci (QTL) through the construction of genetic linkage maps. This review article is intended to provide a systematic introduction of DNA marker technologies and their

relevant applications to the general audience working in aquaculture; therefore, specific details are provided concerning the genetic principles and inheritance involved in different DNA marker systems. Also discussed are future trends in DNA marker technology and means of exploiting research progress from the Human Genome Project and from model species such as zebrafish for the benefit of aquaculture genomics and aquaculture genetics.

As this review focuses on DNA markers and their applications in aquaculture, and our point of view could be biased by our research experience, readers are referred to other reviews concerning application of DNA marker technologies (Rafalski and Tingey, 1993; Buitkamp and Epplen, 1996; Dodgson et al., 1997; Beuzen et al., 2000; Rafalski, 2002; Vignal et al., 2002).

2. Types of molecular markers and their principles

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species, and higher order taxonomic groups. For this variation to be useful to geneticists, it must be (1) heritable and (2) discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable through molecular techniques. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. Through long evolutionary accumulation, many different instances of each type of mutation should exist in any given species, and the number and degree of the various types of mutations define the genetic variation within a species. DNA marker technology can be applied to reveal these mutations. Large deletions and insertions (indels) cause shifts in the sizes of DNA fragments produced upon digestion by restriction enzymes, and are among the easiest type of mutations to detect, mainly by electrophoresis of the fragments on an agarose gel; smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Inversions and rearrangements that involve restriction sites can be easy to detect because they disrupt the ability of a restriction enzyme to cut DNA at a given site and thus can produce relatively large changes in DNA fragment sizes. Point mutations are more difficult to detect because they do not cause changes in fragment sizes.

Several marker types are highly popular in aquaculture genetics. In the past, allozyme and mtDNA markers have been popular in aquaculture genetics research. More recent marker types that are finding service in this field include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers. Table 1 summarizes the basic properties of these marker types, and each is discussed in detail below.

Table 1
Types of DNA markers, their characteristics, and potential applications

Marker type	Acronym or alias	Requires prior molecular information?	Mode of inheritance	Type	Locus under investigation	Likely allele numbers	Polymorphism or power	Major applications
Allozyme		Yes	Mendelian, codominant	Type I	Single	2–6	Low	Linkage mapping, population studies
Mitochondrial DNA	mtDNA	No[a]	Maternal inheritance	—		Multiple haplotypes		Maternal lineage
Restriction fragment length polymorphism	RFLP	Yes	Mendelian, codominant	Type I or type II	Single	2	Low	Linkage mapping
Random amplified polymorphic DNA	RAPD, AP-PCR	No	Mendelian, dominant	Type II	Multiple	2	Intermediate	Fingerprinting for population studies, hybrid identification
Amplified fragment length polymorphism	AFLP	No	Mendelian, dominant	Type II	Multiple	2	High	Linkage mapping, population studies
Microsatellites	SSR	Yes	Mendelian, codominant	Mostly Type II	Single	Multiple	High	Linkage mapping, population studies, paternity analysis
Expressed sequence tags	EST	Yes	Mendelian, codominant	Type I	Single	2	Low	Linkage mapping, physical mapping, comparative mapping
Single nucleotide polymorphism	SNP	Yes	Mendelian, codominant	Type I or type II	Single	2, but up to 4	High	Linkage mapping, population studies?
Insertions/deletions	Indels	Yes	Mendelian, codominant	Type I or type II	Single	2	Low	Linkage mapping

[a] Conserved PCR primers can be adopted from sequence information from a related species.

2.1. Type I versus type II markers and polymorphic information content (PIC)

Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments (O'Brien, 1991; Table 1). Under this classification, most RFLP markers are type I markers because they were identified during analysis of known genes. Likewise, allozyme markers are type I markers because the protein they encode has known function. RAPD markers are type II markers because RAPD bands are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). AFLP markers are type II because they are also amplified from anonymous genomic regions. Microsatellite markers are type II markers unless they are associated with genes of known function. EST markers are type I markers because they represent transcripts of genes. SNP markers are mostly type II markers unless they are developed from expressed sequences (eSNP or cSNP). Indels are becoming more widely used as markers since they often are discovered during genomic or transcriptomic sequencing projects; they can be either type I or type II markers depending on whether they are located in genes.

The significance of type I markers was not fully appreciated in the early stages of aquaculture genetics, though it is becoming clear that these markers are extremely important. In addition to their functions as markers in population studies, type I markers are becoming very important in studies of genetic linkage and QTL mapping (discussed in Section 4.5). Type I markers have utility in studies of comparative genomics, genome evolution, candidate gene identification, and enhanced communication among laboratories. Due to evolutionary constraints on the genome, many genes and their organization are conserved among species. Comparative genomics deals with the similarity and differences found among genomes. Much time, money, and effort can be saved in developing markers for use in aquaculture genetic studies if genetic information is already available for closely related species. To date, full understanding of aquaculture genomics depends heavily on information from well-studied species such as human, mouse, and zebrafish. Type I markers serve as a bridge for comparison and transfer of genomic information from a map-rich species into a relatively map-poor species. Such interspecific comparisons can also be made based on type II markers, but the extent to which the comparison can be made is limited to closely related taxa. The requirement for such comparisons lies in sequence conservations. For the most frequently used microsatellite markers, such comparative studies depend on conservation of the flanking sequences used for the design of PCR primers. In contrast, sequence conservation within genes are high, allowing type I markers to serve as anchor points for genomic segments to be compared among species. For instance, if 15 genes are located between type I markers A and B in zebrafish, it is likely that the majority of the 15 genes also reside between markers A and B in catfish, even though the exact number of genes, gene order, and orientation are not necessarily identical. Currently, large insert bacterial artificial chromosome (BAC) libraries are already available for several leading fish species in aquaculture genomics including channel catfish (http://bacpac.chori.org/catfish212.htm; Quiniou et al., 2003), tilapia (Katagiri et al., 2001), Atlantic salmon (http://bacpac.chori.org/salmon214.htm), and rainbow trout (Thorgaard et al., 2002). In the near future, the linear arrays of the large insert clones will be ordered into clusters of overlapping clones known as BAC contigs. BAC contigs will allow develop-

ment of large numbers of molecular markers within a region of interest (presumably containing important QTL). Further analysis of the DNA sequences surrounding the trait-linked markers will allow identification of genes representing QTL.

In general, type II markers such as RAPDs, microsatellites, and AFLPs are considered to be non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies whose characterizations of genetic diversity and divergence within and among populations are based on assumptions of Hardy–Weinberg equilibrium and selective neutrality of the markers employed (Brown and Epifanio, 2003). Type II markers also have proven useful in aquaculture genetics for species, strain and hybrid identification, in breeding studies, and more recently as markers linked to QTL.

The usefulness of molecular markers can be measured based on their polymorphic information content (PIC, Botstein et al., 1980). PIC refers to the value of a marker for detecting polymorphism in a population. PIC depends on the number of detectable alleles and the distribution of their frequencies, and equals 1 minus the sum of the square of all allele frequencies. For instance, the PIC of a microsatellite marker with two alleles of frequency 0.5 each should be $1 - [(0.5)^2+(0.5)^2] = 0.5$, while PIC for a microsatellite marker of two alleles with allele frequencies of 0.9 and 0.1 is 0.18. Thus, the greater the number of alleles, the greater the PIC; and for a given number of alleles, the more equal the allele frequencies, the greater the PIC. Comparison of PIC values can give researchers a rough idea of the power of the various marker types discussed below to address specific questions in aquaculture genetics.

2.2. Allozyme markers

Allozymes are allelic variants of proteins produced by a single gene locus, and are of interest as markers because polymorphism exists and because they represent protein products of genes and are thus type I markers. Since the 1960s, starch gel electrophoresis of allozymes has been the most commonly employed molecular method in fishery genetics (Ryman and Utter, 1987; Hillis et al., 1996). Still in widespread use, allozymes were among the earliest markers used in aquaculture genetics (May et al., 1980; Seeb and Seeb, 1986; Johnson et al., 1987; Liu et al., 1992, 1996; Morizot et al., 1994).

Amino acid differences in the polypeptide chains of the different allelic forms of an enzyme reflect changes in the underlying DNA sequence. Depending on the nature of the amino acid changes, the resulting protein products may migrate at different rates (due to charge and size differences) when run through a starch gel subjected to an electrical field. Differences in the presence/absence and relative frequencies of alleles are used to quantify genetic variation and distinguish among genetic units at the levels of populations, species, and higher taxonomic designations. Allozymes found use in aquaculture for tracking inbreeding, stock identification, and parentage analysis. In a few cases, correlations existed between certain allozyme markers and performance traits (Hallerman et al., 1986; McGoldrick and Hedgecock, 1997). Their use in linkage mapping has been demonstrated in studies of salmonids (Pasdar et al., 1984; May and Johnson, 1993) and poeciliids (Morizot et al., 1991), but the limited number of available allozyme loci precludes their use in large-scale genome mapping. Disadvantages associated with allozymes include heterozygote deficiencies due to null (enzymatically

inactive) alleles and the amount and quality of tissue samples required. In addition, some changes in DNA sequence are masked at the protein level, reducing the level of detectable variation. Some changes in nucleotide sequence do not change the encoded polypeptide (silent substitutions), and some polypeptide changes do not alter the mobility of the protein in an electrophoretic gel (synonymous substitutions). Although 75 isozyme systems representing several hundred genetic loci are currently available (Murphy et al., 1996), the relatively modest number of loci usually employed and the low number of alleles (usually two or three) exhibited by most loci tend to keep the PIC of these markers fairly modest. Low levels of genetic variation revealed in many allozyme studies of marine fish populations (e.g. Siddell et al., 1980; Mork et al., 1985; Crawford et al., 1989) prompted a continued search for markers with greater genetic resolution. In spite of their strength as codominant type I markers, ease of use, and low cost, their use in aquaculture genetics has become limited.

2.3. Mitochondrial DNA markers

Studies of vertebrate species generally have shown that sequence divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Brown, 1985). This has been attributed to a faster mutation rate in mtDNA that may result from a lack of repair mechanisms during replication (Wilson et al., 1985) and smaller effective population size due to the strictly maternal inheritance of the haploid mitochondrial genome (Birky et al., 1989). Almost the entire mtDNA molecule is transcribed except for the approximately 1-kb control region (D-loop), where replication and transcription of the molecule is initiated. In general, non-coding segments like the D-loop exhibit elevated levels of variation relative to coding sequences such as the cytochrome *b* gene (Brown et al., 1993), presumably due to reduced functional constraints and relaxed selection pressure.

Analyses of mtDNA markers have been used extensively to investigate stock structure in a variety of fishes including eels (Avise et al., 1986), bluefish (Graves et al., 1992), red drum (Gold et al., 1993), snappers (Chow et al., 1993), and sharks (Heist and Gold, 1999). Mitochondrial markers are quite popular among aquaculture geneticists, in part due to their use in identification of broodstocks (e.g. Benzie et al., 2002). In the early days of molecular analysis, the high levels of mtDNA polymorphism relative to allozymes were exploited in aquaculture genetics for population differentiation. Technically, mtDNA markers are RFLP markers (see below) except that the target molecule is mtDNA rather than nuclear genomic DNA (Cronin et al., 1993). Although mtDNA loci can exhibit large numbers of alleles per loci, the limited number of markers available on the mtDNA molecule positions its PIC values higher than those for allozymes but lower than highly variable nuclear markers such as RAPDs, microsatellites, AFLPs, and SNPs.

Due to its non-Mendelian mode of inheritance, the mtDNA molecule must be considered a single locus in genetic investigations (Avise, 1994). In addition, because mtDNA is maternally inherited, the phylogenies and population structures derived from mtDNA data may not reflect those of the nuclear genome due to gender-biased migration (Birky et al., 1989) or introgression (Chow and Kishino, 1995). In addition, mtDNA markers are subject to the same problems that exist for other DNA-based markers, such as back mutation (sites that have already undergone substitution are returned to their original

state), parallel substitution (mutations occur at the same site in independent lineages), and rate heterogeneity or mutational hot spots (large differences in the rate at which some sites undergo mutation when compared to other sites in the same region).

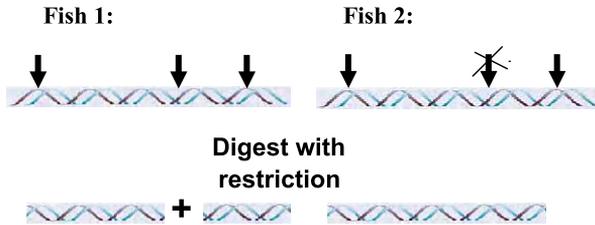## 2.4. Restriction fragment length polymorphism (RFLP)

RFLP markers (Botstein et al., 1980) were regarded as the first shot in the genome revolution (Dodgson et al., 1997), marking the start of an entirely different era in the biological sciences. The procedures and principles of RFLP markers are summarized in Fig. 1. Restriction endonucleases are bacterial enzymes that recognize specific 4, 5, 6, or 8 base pair (bp) nucleotide sequences and cut DNA wherever these sequences are encountered, so that changes in the DNA sequence due to indels, base substitutions, or rearrangements involving the restriction sites can result in the gain, loss, or relocation of a restriction site (Fig. 1). Digestion of DNA with restriction enzymes results in fragments whose number and size can vary among individuals, populations, and species. Traditionally, fragments were separated using Southern blot analysis (Southern, 1976), in which genomic DNA is digested, subjected to electrophoresis through an agarose gel, transferred to a membrane, and visualized by hybridization to specific probes.

Most recent analyses replace the tedious Southern blot method with techniques based on the polymerase chain reaction (PCR). If flanking sequences are known for a locus, the segment containing the RFLP region is amplified via PCR. If the length polymorphism is caused by a relatively large (> approx. 100 bp depending on the size of the undigested PCR product) deletion or insertion, gel electrophoresis of the PCR products should reveal the size difference. However, if the length polymorphism is caused by base substitution at a restriction site, PCR products must be digested with a restriction enzyme to reveal the RFLP. With the increasing number of 'universal' primers available in the literature, a researcher can target DNA regions that are either relatively conserved or rapidly evolving, depending on the amount of variation observed and the taxonomic level under examination. In addition, PCR products can be digested with restriction enzymes and visualized by simple staining with ethidium bromide due to the increased amount of DNA produced by the PCR method.

The potential power of RFLP markers in revealing genetic variation is relatively low compared to more recently developed markers and techniques discussed below. Indels and rearrangements of regions containing restriction sites are perhaps widespread in the genomes of most species, but the chances of such an event happening within any given locus under study should be rare. Similarly, in a given genome of 10^9 base pairs, approximately 250,000 restriction sites should exist for any restriction enzyme with a 6-bp recognition sequence (that accounts for 1.5×10^6 bp or 0.15% of the entire genome). Base substitutions within these restriction sites must be widespread as well, but again, the chances that such base substitutions would occur within the locus under study would be relatively small.

The major strength of RFLP markers is that they are codominant markers, i.e., both alleles in an individual are observed in the analysis. Because the size difference is often large, scoring is relatively easy. The major disadvantage of RFLP is the relatively low level of polymorphism. In addition, either sequence information (for PCR analysis) or probes
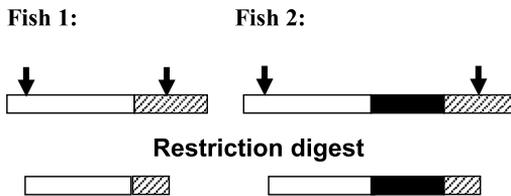
A. Base substitutions at the restriction sites

Fish 1: **Fish 2:**

Digest with restriction

Gel electrophoresis and Southern blot

B. Insertions or deletions

Fish 1: **Fish 2:**
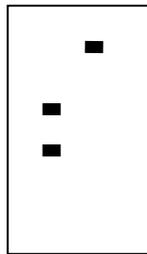
Restriction digest

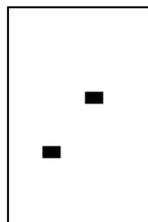**Gel electrophoresis and Southern blot**

Fig. 1. Molecular basis of restriction fragment length polymorphism (RFLP). Genomic DNA is digested with a particular restriction enzyme (arrows). (A) Base substitution within the restriction site can knock out the site (conversely, new sites can arise by base substitutions as well). Loss of a restriction within the locus of interest leads to loss of one fragment and increase of fragment size that can be resolved by gel electrophoresis. (B) Insertion of a piece (black bar) between two restriction sites within the locus leads to an increase in the fragment size that can be resolved by gel electrophoresis (reversely a deletion should reduce the fragment size). In both cases, classical means of detection relies on Southern blot using specific probes.

(for Southern blot analysis) are required, making it difficult and time-consuming to develop markers in species lacking known molecular information.

2.5. Random amplified polymorphic DNA (RAPD)

RAPD procedures were first developed in 1990 (Welsh and McClelland, 1990; Williams et al., 1990) using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 8–10 bp in length (Fig. 2). Because the primers are short and relatively low annealing temperatures (often 36–40 °C) are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus. Because most of the nuclear genome in vertebrates is non-coding, it is presumed that most of the amplified loci will be selectively neutral. Genetic variation and divergence within and between the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the DNA sequence at each locus. RAPD polymorphisms can occur due to base substitutions at the primer binding sites or to indels in the regions between the sites (Fig. 3). The potential power is relatively high for detection of polymorphism; typically, 5–20 bands can be produced using a given primer pair, and multiple sets of random primers can be used to scan the entire genome for differential RAPD bands. Because each band is considered a bi-allelic locus (presence or absence of an amplified product), PIC values for RAPDs fall below those for microsatellites and SNPs, and RAPDs may not be as informative as AFLPs because fewer loci are generated simultaneously.


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acacgacatg-3′              catgtcctgt
| | | | | | | | | |        | | | | | |   | | |
tgtgctgtac                 3′-gtacagcaca
```
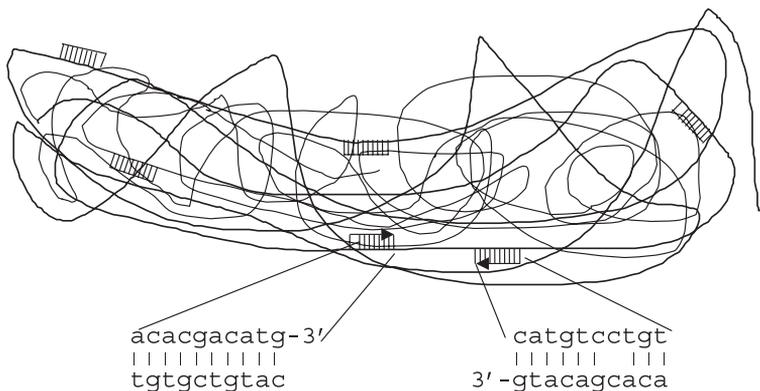
Fig. 2. Schematic presentation of the RAPD procedure. Genomic DNA (indicated by long strings of lines) is used for PCR using two arbitrary short primers of identical sequences (indicated by short segments annealing to their complementary sites in the genome either perfectly or non-perfectly) under low annealing temperatures. When the two primers bind to sites close enough (often less than 2000 base pairs) on opposite strands of DNA (indicated by arrowed segments with base pairing), a PCR product results. Random primers of 10 bases by chance have about 1000 perfect binding sites on each strand of genomic DNA for a genome size of 1×10^9 base pairs. It should be noted that non-perfect binding of primers to genomic DNA templates (e.g., 9 out of 10 bases pair with genomic DNA, as shown with the primer on the right) may also lead to production of PCR products if the 3′ end of the primer has strong base-pairing.

A. Base substitutions at the primer binding sites
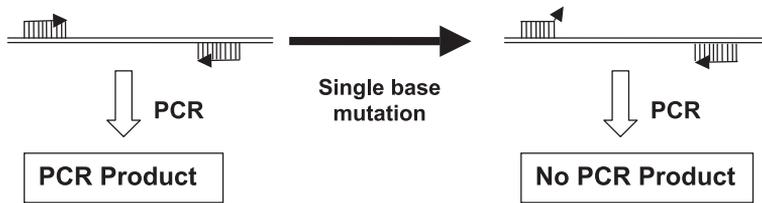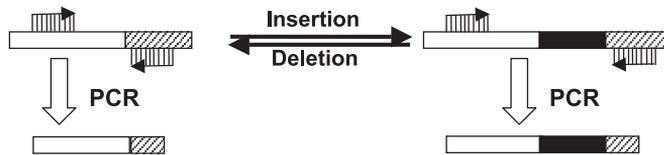

B. Insertion/deletion between two RAPD primers

Fig. 3. Molecular basis of RAPD polymorphism. (A) Base substitutions in the primer binding sites, especially at the 3′ end of the primer binding sites may lead to decrease (as shown) or increase of the number of RAPD bands. (B) Insertion or deletion between two primers may lead to increase or decrease of fragment sizes.

RAPD markers are inherited as Mendelian markers in a dominant fashion (Fig. 4) and scored as present/absent. A RAPD band is produced by homozygotes as well as heterozygotes, and though band intensity may differ, variations in PCR efficiency makes scoring of band intensities difficult. As a result, distinguishing homozygous dominant from heterozygous individuals is not generally possible. In addition, it is difficult to determine whether bands represent different loci or alternative alleles of a single locus, so that the number of loci under study can be erroneously assessed. This is especially true if the RAPD is caused by deletion or insertion within the locus rather than at the primer binding sites. In breeding studies, the number of RAPD bands seen in the F1 generation should equal the sum of the bands seen in the parents, assuming parental homozygosity at each locus; polymorphic RAPD then segregates in a 3:1 ratio in F2 populations (Fig. 4, Liu et al., 1998a, 1999b).

RAPDs have all the advantages of a PCR-based marker, with the added benefit that primers are commercially available and do not require prior knowledge of the target DNA sequence or gene organization. Multilocus amplifications can be separated electrophoretically on agarose gels and stained with ethidium bromide, although higher resolution of bands has been achieved with discontinuous polyacrylamide gel electrophoresis (dPAGE) and silver staining (Dinesh et al., 1995), a somewhat costlier and more labor-intensive method. Other advantages of RAPDs are the ease with which a large number of loci and individuals can be screened. RAPD makers have been used for species identification in fishes (Partis and Wells, 1996) and mollusks (Klinbunga et al., 2000; Crossland et al., 1993), analysis of population structure in black tiger shrimp (Tassanakajon et al., 1998) and marine algae (Van Oppen et al., 1996), analysis of genetic impact of environmental stressors (Bagley et al., 2001), and analysis of genetic diversity (Wolfus et al., 1997; Hirschfeld et al., 1999; Yue et al., 2002).

A. Identification of alleles of dominant markers
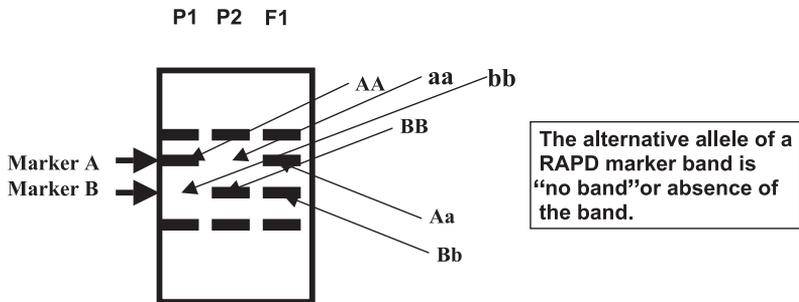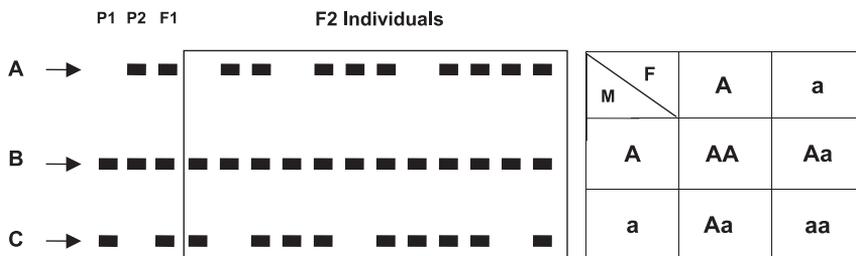

B. Dominant marker inheritance

Fig. 4. Allele identification and inheritance of dominant markers. (A) Identification of alleles of dominant markers. For a mating between parent 1 (P1) and parent 2 (P2) to produce F1, two bands are different between the two parents and therefore can be followed as markers. For dominant markers, the alternative allele of a band is the absence of the band. For instance, for marker A, parent 1 is homozygous *AA*; the genotype of P2 at the locus is *aa* for its absence of the band. In spite of production of bands by P1, P2, and F1 at both locus *A* and locus *B*, their genotypes are different (*AA* vs. *Aa*, respectively, in P1 and F1 at locus *A*; and *BB* vs. *Bb*, respectively in P2 and F1 at locus *B*). Heterozygotes cannot be distinguished from dominant homozygotes with dominant markers although band intensities may differ (see text). (B) Inheritance of dominant markers. Three loci (bands) A, B, and C are under consideration (arrows on the left margin). If bands are homozygous in both parents P1 and P2, a sum of all bands show up in F1 individuals (heterozygous bands should segregate among F1 individuals). In F2 individuals, locus B does not segregate because it is homozygous in both parents. Different bands A and C between the two parents segregate in a 3:1 ratio. A Punnett square is shown on the right for illustration with marker A. In the square, F stands for female and M stands for male. Two types of gametes are predicted from the heterozygous F1 individual, A and a. Three genotypes should result in F2 population: 1 *AA*, 2 *Aa*, and 1 *aa*. *AA* and *Aa* genotypes produce bands while *aa* genotype does not, in dominant marker systems such as RAPD and AFLP. Similarly, a 3:1 segregation is expected for marker C.

Shortcomings of this type of marker include the difficulty of demonstrating Mendelian inheritance of the loci and the inability to distinguish between homozygotes and heterozygotes. In addition, the presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus) limits the use of this marker. Finally, RAPD markers are subject to low reproducibility due to the low annealing temperature used in the PCR amplification. These difficulties have limited the application of this marker in fisheries science (Wirgin and Waldman, 1994).

2.6. Amplified fragment length polymorphism (AFLP)

AFLP is a PCR-based, multi-locus fingerprinting technique that combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods discussed previously (Fig. 5). Like RFLPs, the molecular basis of AFLP polymorphisms includes indels between restriction sites and base substitutions at restriction sites; like RAPDs, it also includes base substitutions at PCR primer binding sites. The unique feature of the technique is the addition of adaptors of known sequence to DNA fragments generated by digestion of whole genomic DNA. This allows for the subsequent PCR amplification of a subset of the total fragments for ease of separation by gel electrophoresis. Its primary target of genetic variation is the same as RFLP, but instead of analyzing one locus at a time, it allows for the analysis of many loci simultaneously.

First employed by Vos et al. (1995), AFLP generation begins with the digestion of whole genomic DNA with two enzymes (most often *Eco*RI and *Mse*I). Since sequences for the resulting DNA fragments are unknown, adaptors of known sequence are ligated to the ends of the fragments and used as primer sites for PCR amplification. Since this would result in the production of millions of PCR fragments, the number of amplified fragments is reduced by adding known bases to the 3′ of the PCR primers. Since these bases extend past the ligated site and into the DNA fragment, the primer will only anneal if the fragment has the correct sequence. Since a given DNA site can contain one of four bases (A, T, G, or C), adding one known base to one of the primers will reduce the number of amplified fragments 4-fold. Adding one base to both primers should reduce the fragment population 16-fold; adding three bases to each PCR primer should result in a 4096-fold reduction, and so on. In an AFLP analysis, only the *Eco*RI primer is labeled. Digestion with this enzyme should result in approximately 250,000 fragments from a genome of 10^9 bp, or 500,000 *Eco*RI–*Mse*I fragments total since most, if not all, *Eco*RI fragments will be further digested by *Mse*I (*Mse*I is a 4-bp cutter while *Eco*RI is a 6-bp cutter). Thus, the three-base addition to the PCR primers should reduce the *Eco*RI–*Mse*I fragments to about 122 bands on average (500,000/4096), a number amenable to resolution through gel electrophoresis.

The power of AFLP analysis is tremendously high for revealing genomic polymorphisms. All of the approximately 500,000 fragments generated by *Eco*RI–*Mse*I digestion of a 10^9-bp genome caused by deletions, insertions, and primer site base substitutions can be revealed by a full AFLP scan of the 4096 possible primer combinations. Of course, the potential power is endless since different enzymes can be used to scan the genomes. Though possible, a full scan using all 4096 primer combinations would be prohibitively time-consuming and expensive, and the 100 or so polymorphisms generated in the procedure outlined above would normally be sufficient for most applications. For instance, Young et al. (2001) used the AFLP technique to generate 133 polymorphic markers, 23 of which were diagnostic in distinguishing rainbow trout, coastal cutthroat trout, and their hybrids. AFLP markers also have been used for analysis of meiogynogens and androgens (Young et al., 1996; Felip et al., 2000). Other applications (including generation of high-resolution linkage maps) are discussed in a review by Blears et al. (1998).
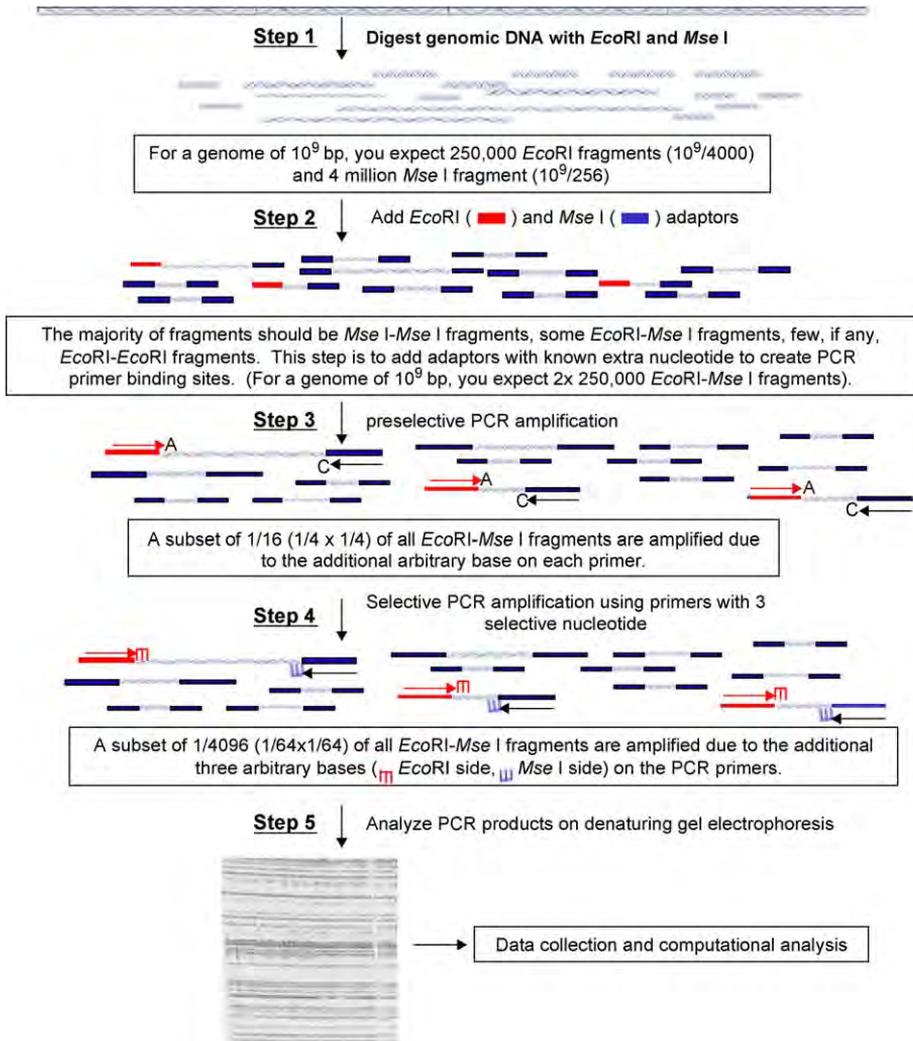
Fig. 5. Schematic representation of amplified fragment length polymorphism (AFLP) analysis. Step 1, genomic DNA is digested by *Eco*RI and *Mse*I into many fragments of various sizes; step 2, adaptors are ligated to the ends of the DNA fragments; step 3, selective amplification of a subset of the restriction fragments by adding an extra arbitrary base at the 3′ end of the PCR primers, which leads to 1/16 of the fragments being amplified; step 4, selective amplification of a subset of the restriction fragments by adding two additional arbitrary base at the 3′ end of the PCR primers, which leads to 1/4096 of the fragments to be amplified; step 5, PCR products are resolved on a sequencing gel.

Like RAPDs, AFLP markers are inherited as dominant markers, although software packages are now available (AFLP QuantaPro, Key Gene) for co-dominant scoring of AFLP bands. Co-dominant scoring is possible when using well-characterized families, but is difficult for population studies. This can be overcome by cutting out individual

bands of interest from an AFLP gel, sequencing them, and designing locus-specific primers for each band, in effect generating anonymous, single-copy nuclear DNA markers. Although time-consuming, this results in one or more co-dominant markers that can be scored using traditional analyses. Such markers have been used to investigate hybridization between native and introduced trout in California waters (B. May, Department of Animal Science, University of California, Davis, unpublished data). As a conservative approach, AFLPs should be treated as dominant markers. If parents are homozygous, all the bands summed from the maternal and paternal parent will show up in an F1 generation, while heterozygous bands will segregate (Liu et al., 1998b, 1999c).

The major strengths of the AFLP method include large (over 100) numbers of revealed polymorphisms, high reproducibility due to high PCR annealing temperatures, and relative economy on a per marker basis. It is more expensive than RAPDs, but because large numbers of loci can be analyzed from a single run, the cost per marker is reduced significantly. Like RAPDs, it does not require any prior molecular information and thus is applicable to any species, including less well-studied fish species. Also like RAPDs, AFLP bands are considered to be bi-allelic and therefore have relatively low PIC scores, but the larger number of loci that can be simultaneously scored greatly increases their utility. Its major weakness includes the need for special equipment such as automated gene sequencers for electrophoretic analysis of fluorescent labels; traditional electrophoretic methods can also be employed but they require the use of radioactive labels or special staining techniques such as silver staining.

2.7. Microsatellites

Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (e.g., ACA or GATA; Tautz, 1989; Litt and Luty, 1989). Abundant in all species studied to date, microsatellites have been estimated to occur as often as once every 10 kb in fishes (Wright, 1993). Microsatellites tend to be evenly distributed in the genome on all chromosomes and all regions of the chromosome. They have been found inside gene coding regions (e.g. Liu et al., 2001c), introns, and in the non-gene sequences. The best known examples of microsatellites within coding regions are those causing genetic diseases in humans, such as the CAG repeats that encode polyglutamine tract, resulting in mental retardation. Most microsatellite loci are relatively small, ranging from a few to a few hundred repeats. The relatively small size of microsatellite loci is important for PCR-facilitated genotyping. Generally speaking, microsatellites containing a larger number of repeats are more polymorphic, though polymorphism has been observed in microsatellites with as few as five repeats (Karsi et al., 2002b).

Microsatellite polymorphism is based on size differences due to varying numbers of repeat units contained by alleles at a given locus (Fig. 6). Microsatellite mutation rates have been reported as high as 10^{-2} per generation (Weber and Wong, 1993; Crawford and Cuthbertson, 1996), and are believed to be caused by polymerase slippage during DNA replication, resulting in differences in the number of repeat units ((Levinson and
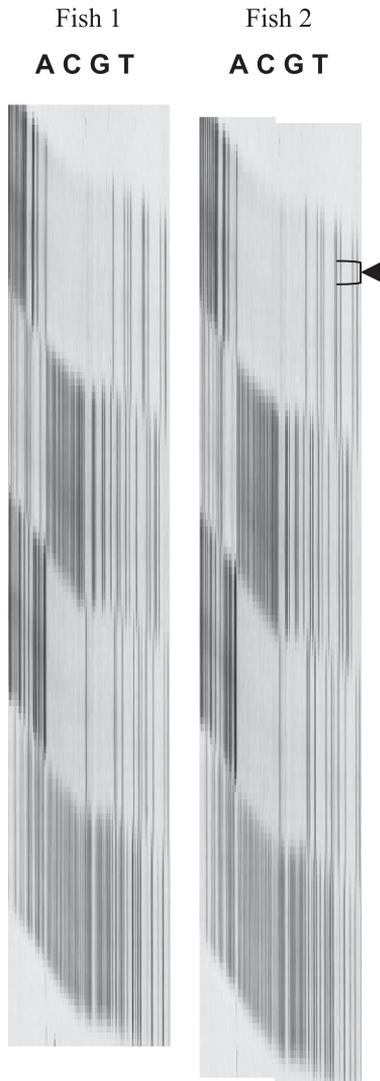
Fig. 6. The molecular basis of microsatellite polymorphism is the difference in repeat number among alleles numbers. Shown are sequence gels of two individual catfish that have identical flanking sequences, but differ by three AG repeats (indicated by the bracket and the arrow).

Gutman, 1987; Tautz, 1989). Direct studies of human families have shown that new microsatellite mutations usually differed from the parental allele by only one or two repeats (Weber and Wong, 1993), favoring a stepwise mutation model (see review by Estoup and Cornuet, 1999). However, in a few fish species, we have observed alleles with very large differences in repeat numbers, predictive of an infinite allele model (Balloux and Lugon-Moulin, 2002). Regardless of specific mechanisms, changes in

numbers of repeat units can result in a large number of alleles at each microsatellite locus in a population.

Microsatellites are inherited in a Mendelian fashion as codominant markers (Fig. 7). This is another strength of microsatellite markers in addition to their abundance, even genomic distribution, small locus size, and high polymorphism. However, use of microsatellite markers involves a large amount of up-front investment and effort. Each microsatellite locus has to be identified and its flanking region sequenced for the design of PCR primers. For most efficient marker development, microsatellite-enriched genomic DNA libraries are made (Ostrander et al., 1992; Kijas et al., 1994). Due to polymerase slippage during replication, small size differences between alleles of a given micro-satellite locus (as little as 2 bp in a locus comprised of dinucleotide repeats) are possible. Because of this, PCR-amplified microsatellite DNA was traditionally labeled radioac-tively, separated on a sequencing gel, and then exposed on X-ray film overnight (Sambrook et al., 1989). Significant increases in the number of samples that can be typed in a day have been achieved by using automated fluorescent sequencers coupled with computer imaging systems (O'Reilly and Wright, 1995).

The large number of alleles per locus results in the highest PIC values of any DNA markers. Microsatellites recently have become an extremely popular marker type in a wide variety of genetic investigations, as evidenced by the recent debut of the journal *Molecular Ecology Notes*, dedicated almost entirely to publishing primer and allele frequency data for newly characterized microsatellite loci in a wide range of species. Over the past decade, microsatellite markers have been used extensively in fisheries
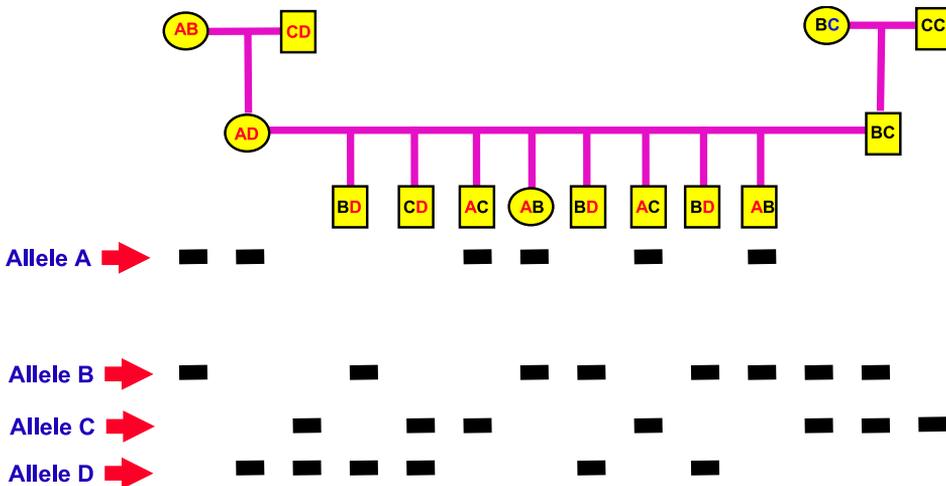
Fig. 7. Schematic presentation of co-dominant marker inheritance. Two pairs of matings were used to produce the F1. In the first pair, the female has genotype AB at the locus; the male has genotype CD; and their F1 (one female individual) has genotype of AD. In the second pair, the female has genotype BC at the locus; the male has genotype CC; and their F1 (one male individual) has genotype BC. The F2 individuals produced from the two F1 individuals (AD and BC) had all four possibilities of genotypes under independent segregation: AB, AC, BD, and CD. Note that both alleles are present for co-dominant markers. If only one band is observed, it is homozygous at the locus.

research including studies of genome mapping, parentage, kinships, and stock structure [see O'Connell and Wright (1997) for review]; a cursory online literature search produced over 500 entries since 1998 involving the utilization of microsatellites in such studies.

2.8. Single nucleotide polymorphism (SNP)

Single nucleotide polymorphism (SNP) describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. Such sequence differences due to base substitutions have been well characterized since the beginning of DNA sequencing in 1977, but the ability to genotype SNPs rapidly in large numbers of samples was not possible until the application of gene chip technology in the late 1990s. SNPs are again becoming a focal point in molecular marker development since they are the most abundant polymorphism in any organism, adaptable to automation, and reveal hidden polymorphism not detected with other markers and methods.

Theoretically, a SNP within a locus can produce as many as four alleles, each containing one of four bases at the SNP site: A, T, C, and G. Practically, however, most SNPs are usually restricted to one of two alleles (most often either the two pyrimidines C/T or the two purines A/G) and have been regarded as bi-allelic. Obviously, their PIC is not as high as multi-allele microsatellites, but this shortcoming is balanced by their great abundance. SNP markers are inherited as co-dominant markers.

Several approaches have been used for SNP discovery including SSCP analysis (Hecker et al., 1999), heteroduplex analysis (Sorrentino et al., 1992), and direct DNA sequencing. DNA sequencing has been the most accurate and most-used approach for SNP discovery. Random shotgun sequencing, amplicon sequencing using PCR, and comparative EST analysis (see below) are among the most popular sequencing methods for SNP discovery.

Despite technological advances, SNP genotyping is still a challenging endeavor and requires specialized equipment. Traditional methods available for SNP genotyping include: direct sequencing, single base sequencing (reviewed by Cotton, 1993), allele-specific oligonucleotide (ASO, Malmgren et al., 1996), denaturing gradient gel electrophoresis (DGGE, Cariello et al., 1988), single strand conformational polymorphism assays (SSCP, Suzuki et al., 1990), and ligation chain reaction (LCR, Kalin et al., 1992). Each approach has its advantages and limitations, but all are still useful for SNP genotyping, especially in small laboratories limited by budget and labor constraints. Large-scale analysis of SNP markers, however, depends on the availability of expensive, cutting-edge equipment.

Several options are available for efficient genotyping using state of the art equipment. Particularly popular are methods involving Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Ross et al., 1998; Storm et al., 2003), pyrosequencing (Ahmadian et al., 2000; Alderborn et al., 2000; He et al., 2003a,b), Taqman allelic discrimination (Li et al., 2004), real-time (quantitative) PCR (Nurmi et al., 2001), and the use of microarray or gene chips (Hacia et al., 1999). Mass spectrometry and microarray technologies require a large investment in equipment.

The equipment for pyrosequencing and quantitative PCR is generally under US$100,000, and should be more affordable to many laboratories working in the area of aquaculture genetics. Another consideration is the expense of genotyping in relation to sample sizes. Microarray (gene chip) technology and quantitative PCR are particularly useful in medical and clinical settings where large numbers of samples (thousands of individuals per locus) are involved and that can justify the cost involved in the development of the gene chips and hybridization probes. Mass spectroscopy and pyrosequencing are relatively cost-effective (after acquisition of the equipment) when working with relatively small sample sizes (e.g., hundreds of individuals per locus), as is most likely the case in aquaculture research. For more detailed information concerning SNP genotyping, readers are referred to a recent review by Vignal et al. (2002).

2.9. Expressed sequence tags (ESTs)

Expressed sequence tags (ESTs) are single-pass sequences generated from random sequencing of cDNA clones (Adams et al., 1991). The EST approach is an efficient way to identify genes and analyze their expression by means of expression profiling (Franco et al., 1995; Azam et al., 1996; Lee et al., 2000). It offers a rapid and valuable first look at genes expressed in specific tissue types, under specific physiological conditions, or during specific developmental stages. ESTs are useful for the development of cDNA microarrays that allow analysis of differentially expressed genes to be determined in a systematic way (Schena et al., 1996; Wang et al., 1999), in addition to their great value in genome mapping (Boguski and Schuler, 1995; Hudson et al., 1995; Schuler et al., 1996).

For genome mapping, ESTs are most useful for linkage mapping and physical mapping in animal genomics such as those of cattle and swine, where radiation hybrid panels are available for mapping non-polymorphic DNA markers (Cox et al., 1990). A radiation panel is composed of lines of hybrid cells, with each hybrid cell containing small fragments of irradiated chromosomes of the species of interest. Typically, the cells from species of interest are radiated to break chromosomes into small fragments. The radiated cells are unable to survive by themselves. However, the radiated cells can be fused with recipient cells to form hybrid cells retaining a short segment of the radiated chromosome. Characterization of the chromosomal break points within many hybrid cell lines would allow linkage and physical mapping of markers and genes. In spite of its popularity in mammalian genome mapping (Yang and Womack, 1998; Amaral et al., 2002; Korwin-Kossakowska et al., 2002; McCoard et al., 2002), radiation hybrid panels are not yet available for any aquaculture species. Development of radiation hybrid panels from aquaculture species is not expected in the near future, given the fact that physical mapping using BAC libraries can provide even higher resolution and the fact that BAC libraries are already available from several aquaculture species. Therefore, ESTs are useful for mapping in aquaculture species only if polymorphic ESTs are identified (Liu et al., 1999a). Additionally, ESTs can be mapped to physical maps by hybridization, and integration of physical and genetic linkage maps would in turn anchor the ESTs to the linkage maps (see below). Likewise, ESTs can be mapped to genetic linkage maps if they are found to be associated with microsatellites. In this context, microsatellite-containing ESTs are rich resources of type I markers (Serapion et al., in press).

3. DNA marker development in aquaculture

3.1. Recently developed markers in aquaculture species

Much progress has been made in the development of DNA markers in aquaculture species including catfishes, tilapias, salmonids, oysters, and shrimps, whose genome research has been included in a regional project (NE-186) in the United States and are now a part of the national project NRSP-8. Large efforts are also under way for striped bass. A large number of markers representing various marker types have been developed, including microsatellites in Atlantic salmon (Slettan et al., 1997), catfish (for review, see Liu, 2003; Waldbieser and Bosworth, 1997; Liu et al., 1999d,e, 2001c; Tan et al., 1999; Serapion et al., in press), tilapias (Lee and Kocher, 1998; Carleton et al., 2002; Palti et al., 2001; Streelman and Kocher, 2002; Cnaani et al., 2002), penaeid shrimps (Xu et al., 1999), common carp (Tanck et al., 2001), chinook salmon (Williamson et al., 2001; Naish and Park, 2002), rainbow trout (Rexroad et al., 2001, 2002a,b), and oysters (Reece et al., 2002; Hubert and Hedgecock, in press; Peatman et al., in press). AFLPs have been developed for channel catfish and blue catfish (Liu et al., 1998b, 1999c), rainbow trout (Young et al., 1998), and oysters (Li and Guo, 2004; Yu and Guo, 2003). RAPDs have been developed for catfish (Liu et al., 1998a, 1999b) and Asian arowana (Yue et al., 2002). SNPs have been developed for catfish (He et al., 2003b). At least several hundred molecular markers are now available for the above-mentioned aquaculture species. A recent Canadian government genome initiative called the Genome Research on Atlantic Salmon Project (GRASP) accelerated efforts of DNA marker development and genome mapping. Much progress has been made in the analysis of ESTs. Tissue analysis of ESTs and expression profiling has been conducted in channel catfish (Karsi et al., 1998, 2002a; Ju et al., 2000; Cao et al., 2001; Kocabas et al., 2002b), and has been one of the most efficient means of systematic gene cloning and gene expression analysis (Liu et al., 1997, 2001a,b; Karsi et al., 2002b; Kocabas et al., 2002a; Patterson et al., 2003), for development of polymorphic markers (Liu et al., 1999a), and for the development of cDNA microarrays (Ju et al., 2002; Kocabas et al., in press; Rise et al., 2004). Recently, major progress has been made toward EST development in several aquaculture species, especially in Atlantic salmon and rainbow trout, where over 100,000 ESTs have been sequenced (Davey et al., 2001; Martin et al., 2002; Davidson, 2003; Rexroad, 2003; Rexroad et al., 2003; Rise et al., 2004). A French group has also deposited a significant number of rainbow trout ESTs to GenBank (Y Guiguen, INRA, France, unpublished).

The value of EST resources is perhaps underestimated currently in the aquaculture genetics community, primarily because of the lack of bioinformatics capabilities. A greater level of applications of bioinformatics in aquaculture genetics/genomics is inevitable, and it is expected that various EST databases will serve as rich sources of genomic information not only for aquaculture geneticists, but also for aquaculture physiologists, immunologists, biotechnologists, and the like.

3.2. Trends in marker usage

The trend of marker use in research is reflected in the number of publications. Results of a simple literature search using the CAB Abstracts Database are shown in Fig. 8.
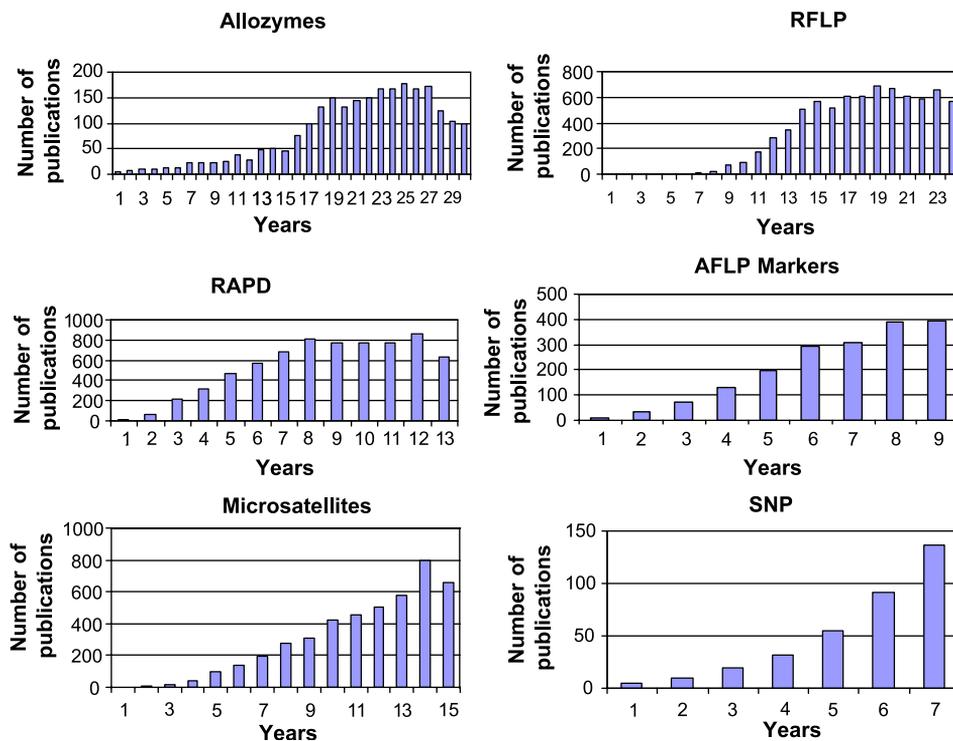
Fig. 8. Number of marker type publications detected in CAB Abstracts, a bibliographic database which covers research literature in the fields of agriculture, forestry, human health, human nutrition, animal health, and management and conservation of natural resources. All searches were conducted using the key words of the marker included in abstract, title, original title, or heading words. Literature searches had start dates of: Allozyme, 1973; RFLP, 1980; RAPD, 1990; AFLP, 1995; microsatellites, 1989; SNP, 1996. Numbers under the X-axis are years after the start; numbers on the Y-axis are numbers of publications. Note the difference in scales for each marker type.

Several points can be made from the results of this search. First, literature on allozymes and RFLPs is on the decline (as judged by at least 3 years of decrease in the number of studies using these markers), and the use of RAPD markers has probably reached its peak. AFLP, microsatellites, and SNP markers are still in their log phase of growth. These markers are also likely to be the major driving forces of the genomics revolution. Second, the speed of marker application varied greatly: it took over 13 years for RFLPs to reach 300 publications per year; 9 years for microsatellites, and an as yet unknown number of years for SNPs; but it only took 4 years to reach the same level with RAPDs, and 7 years for AFLPs (Fig. 8). Clearly, this is a reflection of the technical simplicity and applicability of the respective marker systems. Large amounts of time and effort are involved in the development of RFLP, microsatellites, and SNP markers, while little or no upfront work is required for RAPDs and AFLPs. RAPDs in particular were quickly applied because they do not require much in up-front resources, equipment, or technical expertise. Although the number of laboratories using AFLPs, microsatellites, and SNPs

may be small because they require much investment in equipment or technical expertise. These markers will probably have the largest impact on genome mapping even if only a few laboratories can develop a large number of these markers. As a consequence, these markers may have a large impact on aquaculture genetics, since aquaculture genome projects requiring linkage maps are the driving force behind marker development in this field.

Although applications of genomics in aquaculture such as parentage assignment and variability assessment have found great utility to date, the key component of aquaculture genomics in the near future may be QTL mapping. Aquaculture genomics is especially interested in performance and production traits that are unique to individual species, and the mapping of specific QTL related to such traits in targeted species can be greatly accelerated when coupled through comparative genomics to established genomic information from model species. With the availability of the entire genome sequences from both zebrafish and pufferfish, much can be learned from well-conserved parts of the genome, at least within these taxonomic orders. Whole-genome sequencing of Atlantic salmon and rainbow trout, among others, is already underway; see the Genomes Online Database at http://igweb.integratedgenomics.com/GOLD for updated lists of ongoing projects. White papers advocating sequencing whole genomes of tilapia, catfish, shrimps, and oysters have been filed with various funding agencies. As more genome sequences are completed, the advantages of comparative genomics will see greater application in a wider variety of aquaculture species. Based on the emerging importance of QTL mapping and marker-assisted selection (MAS), it is likely that the demand for type I genetic markers will rapidly increase.

4. Applications of DNA markers in aquaculture genetics

4.1. Choice of marker systems

One of the questions at the beginning of any genome research is what type of marker is most suitable given the project at hand and the species of interest. There is no simple answer to this question, and much depends on the specific objectives of the study (Table 2). However, with a good understanding of the DNA marker technol-

Table 2
Applications of DNA markers in aquaculture genetics

Tasks	Recommended marker system	Other useful marker types
Species identification	RAPD	AFLP, microsatellites, isozymes
Strain identification	AFLP, microsatellites	RAPD
Hybrid identification	RAPD	AFLP, microsatellites, mitochondria[a]
Paternity determination	Microsatellites	
Genetic resource/diversity analysis	AFLP, microsatellites	RAPD
Genetic mapping	Type I markers, Microsatellites SNP	AFLP, RFLP
Comparative mapping	Type I markers	ESTs, conserved microsatellites

[a] Use of mitochondrial markers should also allow determination of maternity.

ogies, appropriate decisions can be reached. In this regard, one has to know if there is already molecular information available when choosing a marker system. RAPD and AFLP markers do not require prior molecular information for the target species. Comparing between RAPDs and AFLPs, levels of polymorphism and power of differentiation are much greater for AFLPs than for RAPDs. Choosing between these two marker systems depends on the tasks involved and the equipment available. If the objective is to reveal whether fish are pure species or F1 hybrids, RAPDs are probably sufficient, since the simplest method with the minimum requirement for resources is the best method if the same objective can be met. If the analysis requires a more powerful approach to reveal higher levels of polymorphism, AFLPs would provide a greater level of differentiation than RAPDs. For genome mapping, AFLPs are highly superior to RAPDs because of their greater reliability (i.e., better reproducibility).

Use of microsatellites requires upfront development of the microsatellite markers. If there is time and resources to develop such markers, it is worthwhile in the long run, especially for genome mapping. However, if genome mapping is the primary goal, consideration should be given from the start to the development of type I microsatellites, i.e., microsatellites associated with genes of known functions. Another technical shortcoming of microsatellites is the difficulty involved in nonspecific amplification (amplification of secondary DNA products other than the targeted microsatellite locus due to nonspecific binding of the PCR primers), although most researchers overcome this problem by optimizing PCR conditions. In addition, genotyping with microsatellites (especially those with dinucleotide repeats) is often complicated by the presence of so-called stutter bands. Stutter bands are caused by polymerase slippage during PCR amplification, which results in secondary products containing one or more repeat units less than the primary allelic band. Stutter bands can sometimes equal the intensity of the primary band, making it difficult to accurately characterize genotypes, particularly in population studies. In gene mapping, the genotypes of parents are already known, so segregation of alleles in the progenies is relatively straightforward. In population studies where relatedness is not known, interpretation can prove problematic. For population studies, one other consideration is the high level of microsatellite polymorphism. Sample sizes must be large enough to adequately characterize the genetic variation both within and among populations, thereby ensuring that apparent differences among populations are not due to sampling error.

SNP markers are perhaps most powerful for genome mapping and identification of candidate genes for QTL, but their discovery requires great economic investment. Efficient genotyping also requires expensive equipment. Mass spectrometers cost over US$300,000, pyrosequencers cost about US$100,000, and quantitative PCR equipment costs about US$50,000. Additionally, processing currently costs about US$1 per genotype for SNPs. In spite of the cost, it is predicted that SNPs will be the future marker of choice in biotechnology-related industries due to their nearly unlimited power and adaptability to automation. While it is not so certain that SNPs will become popular in aquaculture genetics due to the financial limitations listed above, it is almost certain that RFLPs and allozymes represent markers of the past.

4.2. Species, strain, and hybrid identification

Genetic identification of species or strains is sometimes required in an aquaculture setting. Because of the major genetic differences among most species, their identification using DNA markers is relatively straightforward. RFLP, RAPD, AFLP, and microsatellite markers are all applicable, but RAPD analysis probably provides the least expensive, yet reliable identification of species if no prior molecular information is available. Each species will generally exhibit a RAPD profile with unique binding patterns, and a simple comparison of profiles generated using one or two primers should be sufficient for species identification.

Species identification is often required for determining whether fish stocks are pure species or hybrids, a problem often seen in tilapia (Bardakci and Skibinski, 1994). Both RAPD (Partis and Wells, 1996) and AFLP (Congiu et al., 2001; Young et al., 2001) analyses can provide rapid solutions; the dominant nature of these markers means that hybrid fish should have a gel profile that combines the unique dominant bands from each parent (Fig. 4). Use of both RAPDs and AFLPs for the analysis of situations involving hybridization and introgression beyond the F1 generation is more complicated, however, due to the unresolved nature of the dominant bands (some bands may be different alleles of the same locus, while most bands are considered to be products of unique loci), so that breeding studies to determine the Mendelian inheritance of species-specific bands becomes mandatory.

Strain identification is more complicated, since fixed, strain-specific markers are not usually available for strains within a species. The amount of genetic variation among strains may be limited, and may require DNA markers and techniques with higher resolution than traditional markers such as allozymes, RFLPs, or RAPDs. Both micro-satellites and AFLPs have been shown to provide sufficient power for the determination of strains in aquaculture fish species. The use of allele frequency analysis across multiple microsatellite loci is a powerful approach for delineation of individual strains. Allele frequencies for each microsatellite locus are estimated for each strain involved and those microsatellites that have highly differential allele frequencies among strains are used for strain identification. This approach has been used in strain identification of catfish (Waldbieser and Wolters, 1999). For more technical details, readers are also referred to similar applications in microorganisms (Hennequin et al., 2001; Sampaio et al., 2003). In contrast to microsatellites, AFLP markers typically have only two alleles per locus, and are treated as dominant markers with either the presence or absence of the band. The large number of loci typically generated simultaneously in an AFLP analysis makes up for the lack of large numbers of alleles per locus. As in the case of microsatellites, allele frequencies of multiple loci are combined to delineate strains. The AFLP approach has been used to identify strains of common carp (David et al., 2001) and channel catfish (Mickett et al., 2003).

4.3. Genetic diversity and resource analysis of aquaculture stocks

In spite of a long aquaculture history, aquaculture broodstocks are not well character-ized genetically. Many strains/lines of a specific aquaculture species may be used, and the

precise genetic relationship among strains is most often unknown. Questions arise as to how diverse is the genetic background of aquaculture broodstocks and how the domestic stocks differ from their wild counterparts. As with strain identification, analyses of genetic diversity and resources require methodologies that exhibit high powers of resolution to reveal genetic variations among the stocks. Traditionally, allozymes and mtDNA have been most frequently used in fishes, but their differentiating power is limited compared to more recently developed markers such as RAPDs, AFLPs, and microsatellites (as discussed earlier under the sections dealing with marker types). Among these newer marker systems, RAPDs have the least differentiation power. AFLPs and microsatellites should be highly powerful in revealing genetic diversities. In the author's (Z.J.L.) experience, AFLP markers have proven very useful for genetic diversity analyses because of the large number of loci that can be screened simultaneously. Bagley et al. (2001) compared the use of RAPDs and AFLPs in rainbow trout and concluded that AFLP is the method of choice for the analysis of genetic diversity. Bagley et al. (2001) also increased reproducibility of the AFLP fingerprints to nearly 100% by excluding 10% of the largest and smallest bands, as well as bands that comprised less than 1% of total intensity in the AFLP analysis.

4.4. Parental assignments and reproductive contribution

Fishes have some of the most complex mating systems known in the animal kingdom. Effective methods of traceability are required for basic research, different types of aquaculture operations, and to control the trade in aquatic animals and products. With the advent of powerful genetic markers and an emerging mathematical framework to calculate parentage (e. g., Queller and Goodnight, 1989; Danzmann, 1997), it is now possible to analyze genetic relatedness and inheritance in these systems (for review, see Hastein et al., 2001). For parental assignments, microsatellites provide the best results, since genetic variation among individuals is extremely high with microsatellites, while polymorphism in other types of markers is generally low among individuals of the same strain. The large numbers of alleles and high levels of detectable polymorphism exhibited by microsatellites make obtaining unique genotypes for every individual in a study feasible. Of course, the larger the population, the greater the number of required microsatellite loci. For instance, Neff (2001) successfully determined paternity and reproductive contribution in natural populations of bluegill sunfish using 11 microsatellite loci. In contrast, Herbinger et al. (1995) used just four microsatellites to identify both parents and their reproductive contribution in a breeding study of hatchery rainbow trout, while Norris et al. (2000) was able to correctly identify the parental pair for offspring of farmed Atlantic salmon with 95% accuracy even in the absence of pedigree data.

4.5. DNA markers, quantitative trait loci (QTL), and marker-assisted selection (MAS)

Benefits of the expanded molecular genetic technologies currently available include their potential for greatly enhancing traditional breeding techniques. Since most, if not all, performance and production traits are controlled by multiple genes and therefore inherited as quantitative traits, analysis of their associated quantitative trait loci (QTL) is emerging as

a very important part of aquaculture genetics/genomics. QTL are largely unidentified genes that affect performance traits (such as growth rate and disease resistance) that are important to breeders. Relative chromosomal positions of QTL in a species genome can be identified in a two-step process that begins by constructing a genetic linkage map. Genetic linkage maps are constructed by assigning (mapping out) polymorphic DNA markers (such as microsatellites, SNP, or AFLPs) to chromosome configurations based on their segregation relationships. This requires two elements: polymorphic DNA markers, and families in which these markers segregate. Once a linkage map has been constructed for a given species, it can be used in combination with studies of breeding and assessment of quantitative traits to identify markers that are closely associated (linked) to QTL of interest, thus allowing the QTL to be positioned on the linkage map. This information can then be used to aid aquaculture personnel in efficiently crossing different strains of cultured species to maximize growth, disease resistance, or some other desirable trait through marker-assisted selection (MAS). Typically, evenly spaced markers covering the entire genome are selected for screening of trait-linked markers, and this process is known as a genome scan of QTL. Once the QTL are mapped to a chromosomal region, fine mapping can be conducted using polymorphic markers near the chromosomal regions containing the QTL.

Genetic linkage and QTL mapping in aquaculture species are not as advanced as they are in other production species such as tomato (Weller, 1987), soybean (Caetano-Anolles et al., 1993) cattle (Bishop et al., 1994) and pig (Rohrer et al., 1994). Currently, medium-density framework linkage maps are available for salmon (Stein et al., 2001), rainbow trout (Young et al., 1998; Sakamoto et al., 2000), catfish (Waldbieser et al., 2001; Liu et al., 2003), tilapia (Kocher et al., 1998; Agresti et al., 2000; McConnell et al., 2000), oysters (Yu and Guo, 2003; Li and Guo, 2004; Hubert and Hedgecock, in press), and shrimps (Wilson et al., 2002), and fine mapping of selected genome regions is under way.

A number of QTL have been mapped and characterized in aquaculture species. In rainbow trout, QTL for upper thermal tolerance, spawning time, and embryonic development rate have been mapped (Jackson et al., 1998; Danzmann et al., 1999; Sakamoto et al., 1999; Robison et al., 2001; Perry et al., 2001). In tilapia, a collaborative group involving Israeli and US scientists has developed a hybrid tilapia stock for the analysis of linkage mapping and QTL analysis (Agresti et al., 2000; Cnaani et al., 2003). In addition, loci associated with deleterious alleles and distorted sex ratios (Shirak et al., 2002), QTL controlling body color and sex determination (Howe and Kocher, 2003; Lee and Kocher, 2003) and QTL controlling a number of biochemical parameters related to innate immunity response to stress (Cnaani et al., in press) have been recently identified in tilapia. Several markers have been identified in catfish that are linked to feed conversion efficiency (Karsi et al., 2000). Several groups are now working on QTL for disease resistance (Ozaki et al., 2001; also reviewed by Gibson, 2002), and two putative QTL have been identified to be associated with resistance/susceptibility to infectious pancreatic necrosis virus (IPNV) in rainbow trout (Ozaki et al., 2001). With the availability of resource families and DNA markers, it is expected that greater successes will be achieved in the near future in QTL mapping in aquaculture species, which will eventually lead to marker-assisted selection (MAS).

MAS refers to a selection process in which future breeders are chosen based on genotypes using molecular markers. To implement MAS (reviewed by Poompuang and

Hallerman, 1997), researchers need to produce high-resolution linkage maps, understand the number of QTL affecting a given performance or production trait and their mode of inheritance and relative contribution, determine the linkage and potential interactions of different QTL for the trait and for other traits, and estimate the economic importance of each trait. Selection of one trait may be made at the expense of another, and a well-planned MAS program should take all economically important traits into consideration. A selection index may be useful in achieving a balanced approach in cases where contradictory decisions are called for regarding different traits.

As reviewed by Hulata (2001), most of the genetic improvements of aquaculture broodstocks to date have been through the use of traditional selective breeding techniques such as selection, crossbreeding, and hybridization. DNA marker technology and gene manipulations have yet to significantly affect the aquaculture industry. However, genomic research and especially QTL mapping will eventually lead to MAS for efficient and precise selection. Therefore, the construction of high-density genetic linkage maps is a long-term objective. In addition to adding more markers to the linkage maps, several other approaches should be taken to significantly enhance the resolution of linkage maps. The first is to develop physical maps and integrate them with linkage maps using common sets of markers. The second is to conduct comparative mapping by mapping sets of type I markers to linkage maps of aquaculture species and then making comparative maps with map-rich species such as zebrafish and pufferfish. Since the major focus of aquaculture genomics in the near future probably will be QTL analysis, comparative mapping and physical mapping when integrated with linkage mapping should increase our abilities to identify and eventually clone the candidate genes controlling performance and production traits. This would lead to even more precise selection by gene-assisted selection (GAS), in which future breeders could be chosen according to favorable genotypes based on genes directly controlling performance traits, rather than on neutral markers associated with those traits via linkage. To accelerate the widespread development and use of MAS and GAS programs in aquaculture, imperative objectives for each species of interest include developing type I markers, establishing bacterial artificial chromosome (BAC) libraries, constructing BAC contigs (physical maps), and the integration of physical and genetic linkage maps.

4.6. EST markers and their uses in BAC contig mapping and integration of maps

BAC contigs are constructed by analysis of overlapping genomic segments cloned into BAC vectors. Each array of linearly arranged BAC genomic segments is defined as a BAC contig. Many contigs collectively cover the entire genome. BAC contig construction is a part of physical mapping. BAC contig construction is important for several reasons. Upon initial identification of QTL from QTL mapping by genetic linkage, QTL should be further mapped to delineate the exact genomic location. Genomic regional markers are needed to map the QTL with high resolution. Such regional markers can be developed from the nearby BAC clones. BAC contigs also serve as a physical hub connecting genetic linkage mapping, QTL mapping, and comparative mapping. To integrate these different maps, a common set of type I markers (type II markers are fine for integration, but are not suitable for comparative mapping among distantly related species) should be mapped on both

linkage maps and BAC contigs. This can be accomplished by mapping polymorphic ESTs to genetic linkage maps and hybridization of the same set of ESTs to BAC clones. When the same set of ESTs is mapped to genetic linkage maps by polymorphism (e.g., SNP polymorphism), the BAC contigs can be anchored onto the linkage maps. Mapping of the same sets of genes in aquaculture species would allow comparative mapping among aquaculture species and also between aquaculture species and other systems such as human and zebrafish.

4.7. Future applications of DNA markers in aquaculture genetics

In addition to genome mapping and the other applications discussed in this review, DNA markers are likely to prove useful in many other aspects of aquaculture. The development and application of DNA marker technologies already underway in other areas such as molecular systematics, population genetics, evolutionary biology, molecular ecology, conservation genetics, and seafood safety monitoring will undoubtedly impact the aquaculture industry in unforeseen ways. Already, lessons learned from studies in population and conservation genetics are changing the very role that hatcheries and aquaculture play for augmentation and restoration of wild fish stocks such as salmon and trout. Advances in aquaculture genomics are also likely to affect other areas utilizing molecular markers as well. Although it may take some time to implement marker-assisted selection in aquaculture, the techniques of genome mapping and QTL analysis used to support MAS will eventually also be used to identify and clone genes that could prove to be economically important outside of the aquaculture arena, and find applications in medicine and other bio-related industries.

Zhang; and to our highly dedicated station workers such as Randell Goodman and Renee Beam.

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