

Fish Population Genetics and Molecular Markers: II- Molecular Markers and Their Applications in Fisheries and Aquaculture

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Abstract

Genetic diversity or variation and its measurement have vital importance in interpretation, understanding and management of populations and individuals. Development of allozyme electrophoresis and chromosomal techniques has significantly increased ability to observe the genetic variation and the former has for many years been the standard tool in genetic studies of wild and cultured fish stocks, but in recent years, it has been increasingly replaced by DNA markers. These molecular markers combined with new statistical developments enable the determination of differences and similarities between stocks and individuals, and the population of origin of single fish, resulting in numerous new research possibilities and applications in practical fisheries and aquaculture stock management. Various molecular markers, proteins or DNA (mitochondrial DNA or nuclear DNA such as minisatellites, microsatellites, transcribed sequences, anonymous cDNA or RAPDs) are now being used in fisheries and aquaculture. Unfortunately, the terminology used is sometimes confusing. The techniques are leading misunderstandings particularly between the senior scientists and, field researchers or end users of their research. More importantly the choice of these markers for particular applications is not straightforward one and is often based on the prior experience of the investigators. It has therefore become crucial that fisheries and aquaculture researchers and managers have a basic understanding of molecular tools, and their assumptions, strengths and weaknesses. In this review, we have provided basics of molecular genetics (DNA) and overview of commonly used molecular markers, and presented a critical review of the applications and limitations of major classes of these markers in fisheries and aquaculture related studies.

Key Words: Allozyme, aquaculture, fisheries, genetics, microsatellite, minisatellite, mitochondrial DNA, molecular markers, nuclear DNA

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1. Introduction

Population genetics in itself can be defined as the science of how genetic variation is distributed among species, populations and individuals, and fundamentally, it is concerned with how the evolutionary forces of mutation, selection, random genetic drift and migration affect the distribution of genetic variability (Hansen, 2003). Patterns of *genetic diversity* or *variation* among populations can provide clues to the populations' life histories and degree of evolutionary isolation. Genetic differences are expressed as differences in the quantity and quality of alleles, genes, chromosomes, and gene arrangements on the chromosomes that are present within and among constituent populations (Williamson, 2001; Çiftci and Okumuş, 2002).

Measuring genetic diversity in wild fish populations or aquaculture stocks is essential for interpretation, understanding and effective management of these populations or stocks. Genetic diversity has been measured indirectly and inferentially through controlled breeding and performances studies or by classical systematic analysis of phenotypic traits. Ecological, tagging, parasite distribution, physiological and behavioural traits, morphometrics and meristics, calcified structures, cytogenetics, immunogenetics and blood pigments are among the diverse characteristics and methods used to analyze stock structure in fish populations (Ihssen *et al.*, 1981). Unfortunately the relationship between genes and their phenotypic expression is complex and often significantly interacted by environmental variables. Thus, the population geneticists mainly focused on Mendelian traits in species widely used in laboratory studies or on available pure breeds of few species. The methods used in these studies were not suitable for wild populations and have found very limited applications in fisheries science and fisheries management (Hallerman, 2003). New methods developed in the later twentieth century to identify, characterize, measure and analyze the genes. These are resulted from the discovery and accurate description of currently accepted model of DNA structure during the early 1950s and *molecular genetics*, the study of the structure, function, and dynamics of genes at the molecular level, has recognized as a powerful branch of genetics.

Initial studies in molecular genetics in the 1960's were limited with proteins such as haemoglobin and transferrin, but attention quickly turned to enzymatic proteins, allozymes (Ferguson *et al.*, 1995), and allozymes was the dominant method employed during 1960s and beginning of 1980s (Williamson, 2001). The development of DNA amplification using the PCR (Polymerase Chain Reaction) technique has opened up the possibility of examining genetic changes in fish populations over the past 10 years (Ferguson *et al.*, 1995). Today,

many molecular methods are available for studying various aspects of wild populations, captive broodstocks and interactions between wild and cultured stocks of fish and other aquatic species. Increasing number of methods and their modifications, biological and other material requirements, widening applications areas, advantages and disadvantages of the methods arisen from their nature or in their applications in different laboratories and huge number of terminology sometimes cause confusions and even misunderstandings especially between the senior scientists and, field researchers or end users of their research. The choice of markers for particular applications is not also straightforward and mostly depends on the experience of the investigators, laboratory facilities and available fund. Thus, there is a need for occasional reviews of the developments in techniques, applications and interpretations of the data gathered. This the second part review on population genetics (see Çiftci and Okumuş, 2002) and molecular genetics, and here we have attempted to provide an overview of currently available molecular markers and the application of these molecular techniques to problems in fisheries and aquaculture. Particular aims of the current review are: i) to review basics of commonly used molecular markers; ii) evaluate the potential and limitations of molecular markers in fisheries and aquaculture science and iii) evaluate the various applications in fisheries and aquaculture and their interactions.

2. Overview of Molecular Markers Used in Fisheries and Aquaculture

The history of molecular genetics goes back to early 1950 when F. Crick, J. Watson and M. Wilkins established the currently accepted model of DNA structure (the double helix). This was a Nobel Prize winning discovery in Chemistry (Hallerman *et al.*, 2003). Since then details of structure and function of DNA and genes have been clarified and started to use in determining the genetic diversity. Methods for DNA cloning, sequencing and hybridization developed in the 1970s and DNA amplification and automated sequencing during 1980s led to the development of various classes of DNA markers. The classical molecular technique for studying genetic variation at co-dominant Mendelian inherited loci is allozyme electrophoresis. The technique was developed in the 1960s and was dominating until the early 1990s. In the early 1980s the first population genetic studies based on analysis of mitochondrial DNA emerged (Avisé *et al.*, 1979). Later, with the advent of the PCR a number of different techniques emerged, ranging from sequencing of the DNA of interest to methods analysing length polymorphisms, such as microsatellites (Hansen, 2003).

A *molecular marker* is a DNA sequence used to "mark" or track a particular location (locus) on a particular chromosome, i.e. marker gene. It is a gene

with a known location or clear phenotypic expression that is detected by analytical methods or an identifiable DNA sequence that facilitates the study of inheritance of a trait or a gene. The markers must be readily identifiable in the phenotype, for instance by controlling an easily observable feature or by being readily detectable by molecular means, e.g., microsatellite marker (Williamson, 2001; Zaid *et al.*, 1999). Today, many molecular methods are available for studying fish populations but they are basically categorized under two types of markers, protein and DNA. There are three general classes of genetic markers that are routinely used in population genetic and phylogenetic studies: (1) allozymes, (2) mitochondrial DNA, and (3) nuclear DNA. They have been subject to a number of recent reviews (e.g. Avise, 1994; O'Reilly and Wright, 1994; O'Connell and Wright, 1997; Parker *et al.* 1998; Sunnucks, 2000; Hallerman, 2003).

2.1. Allozyme

Allozyme electrophoresis denotes the technique for identifying genetic variation at the level of enzymes, which are directly encoded by DNA. The allelic variants give rise to protein variants called *allozymes* that differ slightly in electrical charge. Allozymes are co-dominant Mendelian characters (both alleles are individually expressed in a heterozygous individual) and characters passed from parent to offspring in a predictable manner (May, 2003). Allozyme variation provides data on single-locus genetic variation and these locus genetic data allow us to answer many basic questions about fish and fish populations. The term allozyme refers to only those genetically different forms of an enzyme that are produced by different alleles at the locus and often detected by protein electrophoresis. Since allelic variation reflected in an enzyme may result in different properties, it is possible to identify different alleles by electrophoresis; tissue extracts are applied to a gel and an electrical current is applied. Different allelic variants of an enzyme may then migrate through the gel at a rate determined by the net charge and conformation of the enzyme. Finally, enzyme-specific histochemical staining is used to visualise specific enzymes, and different alleles are identified from different banding patterns.

The general method for detecting allozyme variation includes extraction, electrophoresis and detection. Electrophoresis is a separation technique based on the motion of charged particles in an electric field. Tissue extracts are introduced into a solid support medium (a gel) at the Origin. As matrix (gel) for electrophoresis various media can be used such as acrylamide, cellulose acetate and hydrolyzed potato starch. Acrylamide typically has the best resolving power, however it is the most difficult to handle and also toxic (May, 2003), cellulose acetate electrophoresis is not only simpler and more rapid,

but that it is also quite sensitive and provides good resolution (Easteal and Boussy, 1987). Potato starch has been the predominant medium of choice particularly in applications requiring large amounts of data. Starch gels are easy to prepare and use can be sliced for the staining of 4-6 different enzymes, and 40-50 individuals can be analyzed per gel (May, 2003). When an electrical field is applied, most proteins have a net negative charge and will migrate from the Origin at the cathode ("*negative*") end towards the anode ("*positive*") end of the field. The positions of the protein products are detected either directly, or by coupled enzymatic reactions. For a monomeric enzyme, individuals that are homozygous each show a single band; those that are heterozygous show two bands.

The simplicity, speed, relatively low cost, little specialized equipment requirement (Park and Moran, 1995; Ward and Grewe, 1995) and general applicability of the technique have made this the most widely studied form of molecular variation. Any source of soluble proteins, from bacterial cultures to animal fluids, is in principle suitable for allozyme analysis. The protocols of electrophoretic separation and staining are easily adjustable from species to species. No marker development phase is necessary and the analysis can start for any species immediately after samples have been collected from the field. The genetic interpretation of allozyme profiles is also straightforward. It allows for screening a large number of loci, often more than 30-40. However, the technique has also some certain limitations. One major drawback has been the inability to read genotypes from small quantities of tissue, which makes allozymes inapplicable for small organisms (e.g. larvae). One disadvantage that appears to be difficult to overcome is that only a small fraction of enzyme loci appear to be allozymically polymorphic in many species. There are important demands concerning the freshness of tissue samples and many loci exhibit tissue-specific expression (e.g. some loci are only expressed in heart tissue). Thus the invasive tissue sampling method, which requires sacrificing the fish and the need for cryogenic storage to preserve enzyme activity are serious constraints. In addition a given change in nucleotide sequence may not result in a change in amino acid at all, and thus would not be detected by protein electrophoresis. Furthermore, a change in the DNA that results in a change in an amino acid may not result in a change in the overall charge of the protein and, therefore, would also not be detected (Park, 1994).

In spite of these limitations allozyme analysis has had a more profound effect on fisheries research and management than all most all other genetic tools. It has demonstrated that genetic markers can be useful in stock identification as evidenced by scores of studies that document differences in protein allele frequencies between stocks. As can be seen in the Practical Applications section below, there are wide

spread applications of allozyme analysis in fisheries (see also reviews of Shaklee and Bentzen, 1998; Grant *et al.*, 1999; May, 2003). These are; systematic, population structure, conservation genetics, mixed-stock fishery analysis (e.g. salmonids), forensic (species, population and individual – specific identification for fisheries law enforcement), hybridization and inbreeding, individual identification and inheritance and genome mapping.

2.2. Mitochondrial DNA (mtDNA)

By the early 1980's examination of the gene itself became possible by determining directly or indirectly differences in the nucleotide sequence of DNA molecule. A small portion of (<1%) of the DNA of eukaryotic cells is non-nuclear; it is located within organelles in the cytoplasm called mitochondria. The major features of mtDNA: a) in general maternally inherited a haploid single molecule; b) the entire genome is transcribed as a unit; c) not subject to recombination and provides homologous markers; d) mainly selectively neutral and occurs in multiple copies in each cell; e) replication is continuous, unidirectional and symmetrical without any apparent editing or repair mechanism; and f) optimal size, with no introns present (Billington, 2003). However, selective neutrality and strictly non-recombining nature of mtDNA has been questioned during recent years. This may lead to severe biases in phylogeny reconstruction (Hansen, 2003). mtDNA is physically separate from the rest of the cell's DNA and it is relatively easy to isolate. Any tissue or blood can be used for isolation of mtDNA. Like nuclear DNA the genome includes coding and non-coding regions and later evolves much faster than coding regions of DNA (Avice 1994). One consequence of maternal transmission is that the effective population size for mtDNA is smaller than that of nuclear DNA, so that mtDNA variation is a more sensitive indicator of population phenomena such as bottlenecks and hybridizations. Sex-specific differences in gene flow could also be revealed by contrasting nuclear with mitochondrial DNA.

The rapid rate of evolution, the maternal mode of inheritance and the relatively small size of mtDNA make the RFLP (Restriction Fragment Length Polymorphism) analysis of this molecule one of the methods of choice for many population studies (Ferguson *et al.*, 1995). RFLP is a polymorphism in an individual defined by restriction fragment sizes of distinctive lengths produced by a specific restriction endonuclease. Variation at mtDNA may be analysed mainly with two different approaches. i) RFLP analysis of whole purified mtDNA obtained from fresh tissue (usually liver or gonad) by digesting it with restriction endonucleases; ii) RFLP analysis or DNA sequencing of small segments of the mtDNA molecule obtained by means of PCR amplification (Billington, 2003). The first and earliest approach

requires some very laborious steps of purification of mtDNA, separating the mtDNA from nuclear DNA, followed by restriction analysis and subsequent electrophoresis. The technique requires large amounts of DNA material which may be invasive and lethal to small aquatic organisms. The second approach (PCR fragment analysis) use mtDNA bands resolved from total DNA digests (obtained fresh, frozen, alcohol preserved tissue or blood) by the southern blot procedure. The technique yielding maximum resolution and maximum amount of information has made examination of mtDNA variation considerably easier and faster (Ferguson *et al.*, 1995), but the major disadvantage being that this is costly both in terms of time and money invested, in particular if large sample sizes are required. This is a commonly applied procedure now, particularly using extremely small amount of tissues. RFLP analysis in mtDNA studies have several advantages, including quite high levels of detectable variation, evolving of a high mtDNA, high genetic drift and low gene flow, possibility of reconstructing the phylogenetic history of mtDNA and thus populations, analysis of very small tissues (Hansen and Mensberg, 1998; Nielsen *et al.*, 1998; Hansen *et al.*, 2000).

mtDNA is so intensively studied and sequences in some parts of the molecule are highly conserved across species. Thus several sets of "universal primers" have been developed that allow for analysing the same mtDNA segments in a variety of species. In initial studies with fish (e.g. salmonids) universal primers of Cronin *et al.* (1993) for analysing the mtDNA ND-1 and ND-5/6 segments were used. Smaller fragments of the mitochondrial genome (D-loop region) have also been targeted by probing or PCR and findings have indicated that it may be best to concentrate on the 'slow evolving' coding sequences using 6 base cutters for species comparisons, and to use the 'fast evolving' non-coding regions with 4 base cutters for population investigations. The D-loop region of the mtDNA is practically the only non-coding region in the entire mtDNA of vertebrates. Some markers have targeted the D-loop region is highly variable in mammals but for some fish species little polymorphism is shown in the D-loop. Studies with a number of fish species (*Salmo trutta*, Hall and Nawrocki, 1995; *S. salar*, McConnell *et al.*, 1995; *Anguilla anguilla*, Daemen *et al.*, 1996) have actually shown less variability in the non-coding D-loop region than elsewhere in the mitochondrial genome. Thus, the cytochrome b and dehydrogenase genes may be examined more profitably (Carr and Marshall, 1991).

Application of mtDNA in animals, including fishes has some major problems as well. The major disadvantages are the strict requirement of fresh or frozen tissue, the need for more sample than most DNA methods (Park and Moran, 1995), the necessity to sacrifice the animals, and the low level polymorphism in some species and populations

(Ferguson *et al.*, 1995). The recent demonstration of the presence of mitochondrial *pseudogenes* in the nuclear genome of a wide range of organisms is, for population studies, an unwanted reality (Zhang and Hewitt, 2003). The effectiveness of using mtDNA in population-genetic studies has been greatly weakened by this fact. In addition mtDNA data on their own have some important limitations. Firstly, mtDNA represents only a single locus. So we can look only through a single window of evolution. This window reflects at best only the maternal lineal history (Skibinski, 1994; Magoulas, 1998), which could well differ from that overall of populations or species. Therefore, the inference we make on species/population history is likely to be highly biased and the need for independent, genomic molecular markers to support mtDNA analysis is clear. Second, the effective population size of mtDNA is only a fourth of that of nuclear autosomal sequences; that means mtDNA lineages have a much faster lineage sorting rate and higher allele extinction rate (Zhang and Hewitt, 2003).

mtDNA have a number of applications in fisheries biology, management and aquaculture. In the past 15 years mtDNA has attracted a lot of attention in many species, especially for population and evolutionary studies (Avisé, 1994). It has become a very popular marker and dominated genetic studies designed to answer questions of phylogeny and population structure in fish for more than a decade. mtDNA studies particularly can contribute to identification of stocks and analysis of mixed fishery, provide information on hybridization and introgression between fishes, serve as a genetic marker in forensics analysis and provide critical information for use in the conservation and rehabilitation programmes (Billington, 2003).

2.3. Multiple Arbitrary Primer Markers

Assays that target a segment of DNA of unknown function are included under this category. A general class of PCR-based techniques used to detect such anonymous, or arbitrary, sequences is called "multiple arbitrary amplicon profiling" or "anonymous nDNA markers". The major methods are randomly amplified polymorphic DNA (RAPD, read rapid) and amplified fragment length polymorphism DNA (AFLP)

Randomly Amplified Polymorphic DNA (RAPD): RAPD is a random amplification of anonymous loci by PCR. It has several advantages and has been quite widely employed in fisheries studies. The method is simple, rapid and cheap, it has high polymorphism, only a small amount of DNA is required no need for molecular hybridization and most importantly, no prior knowledge of the genetic make-up of the organism in question is required (Hadrys *et al.*, 1992). RAPD markers allow creation of genomic markers from species of which little is

known about target sequences to be amplified. This methodology has some disadvantages which include difficulty in reproducing results, subjective determination of whether a given band is present or not, and difficulty in analysis due to the large number of products. This is because RAPDs are not sensitive to any but large-scale length mutations. Therefore, variation might be underestimated (Brown and Epifanio, 2003).

The technique is based on the PCR amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence. First of all RAPD is generated by PCR. A small amount of genomic DNA, one or more oligonucleotide primer (usually about 10 base pair in length), free nucleotides and polymerase with a suitable reaction buffer are major requirements. The main drawback with RAPDs is that the resulting pattern of bands is very sensitive to variations in reaction conditions, DNA quality, and the PCR temperature profile (Hoelzel and Green, 1998). Even if the researcher is able to control the major parameters, other drawbacks of RAPD will remain: homozygous and heterozygous states cannot be differentiated and the patterns are very sensitive to slight changes in amplification conditions, giving problems of reproducibility (Ferguson *et al.*, 1995). These problems have limited the application of RAPDs in fish studies.

RAPDs have gained considerable attention particularly in population genetics (Lu and Rank, 1996), species and subspecies identification (Bardakci and Skibinski, 1994), phylogenetics, linkage group identification, chromosome and genome mapping, analysis of interspecific gene flow and hybrid speciation, and analysis of mixed genome samples (Hadrys *et al.*, 1992), breeding analysis and as a potential source for single-locus genetic fingerprints (Brown and Epifanio, 2003). RAPD analysis has been used to evaluate genetic diversity for species, subspecies and population/stock identification in guppy (Foo *et al.*, 1995), tilapia (Bardakci and Skibinski, 1994), brown trout and Atlantic salmon (Elo *et al.*, 1997), largemouth bass (Williams *et al.*, 1998), Ictalurid catfishes (Liu and Dunham, 1998), common carp (Bártfai *et al.*, 2003) and Indian major carps (Barman *et al.*, 2003). Naish *et al.* (1995) found the technique useful in detecting diversity within and between strains of *Oreochromis niloticus*.

Amplified Fragment Length Polymorphism (AFLP): AFLPs are dominant and, several markers and alleles are confounded in the same polyacrylamide gel. It combines the strengths of RFLP and RAPD markers and overcome their problems. The approach is PCR-based and requires no probe or previous sequence information as needed by RFLP. It is reliable because of high stringent PCR in contrast to RAPD's problem of low reproducibility. The major advantage of AFLPs is that a large number of polymorphisms can be scored in a single polyacrylamide gel without the necessity for any prior

research and development. AFLP seems to be much more efficient than the microsatellite loci in discriminating the source of an individual among putative populations (Campbell *et al.*, 2003). Similar to RAPD, AFLP analysis allows the screening of many more loci within the genome in a relatively short time and in an inexpensive way. Although the dominant nature of inheritance of AFLP markers currently limits their utility for applied population genetics, the method appears to be ideal for obtaining genomic support for intraspecific mtDNA analyses. The weakness is that they are dominant markers, thus on average half of which are useful for a given backcross reference family. The methodology is also difficult to analyze due to the large number of unrelated fragments that are visible (on the gel) along with the polymorphic fragments (as with RAPD's).

2.4. Nuclear DNA Markers

The most recent approaches to gathering data relevant to fisheries and aquaculture come from direct assessments of nuclear DNA (nDNA) sequence variation (Brown and Epifanio, 2003).

2.4.1. Restriction Fragment Length Polymorphism (RFLP)

It is used in indirect assessment of nDNA sequence variation. Because of its complexity, nDNA variation cannot be detected and quantified in the same manner as described for mtDNA. RFLPs need either probing (probes) or amplification followed by restriction digests (see Section 2.2).

2.4.2. Variable number tandem repeats (VNTRs)

The nuclear genome of eukaryotes including fishes contains segments of DNA that are repeated tens or even hundreds to thousands of time (O'Reilly and Wright, 1995). These repeated sequences are the most important class of repetitive DNAs. They repeat in tandem; vary in number at different loci and different individuals dispersed throughout the genome. Based on the size of the repeat unit two main classes of this repetitive and highly polymorphic DNA have been distinguished: a) minisatellite DNA, which refers to genetic loci with repeats of smaller length (9-65 bp), and b) microsatellite DNA, in which the repeat unit is only 2 to 8 (1-6) bp long. The term VNTR is frequently used for both mini- and microsatellite DNAs (Magoulas, 1998). They differ from each other in as much as the repeat unit in minisatellites is very simple (mostly two, but also three or more nucleotides), and the total length of the "locus" is much smaller in microsatellites. Most importantly, microsatellites are much more numerous in the genome of vertebrates. Minisatellites are also classified as multilocus and single-locus. These two markers are alternatively known by a number of synonyms. Minisatellites:

DNA fingerprinting and VNTR; microsatellites: simple sequence (Tautz, 1989), short tandem repeats (STRs, Craig *et al.*, 1988); simple sequence repeats (SSRs) (Orti *et al.*, 1997). Mini- and microsatellite markers are the most important population genetic tools and increasingly applied in fisheries and aquaculture studies.

Multilocus Minisatellites: They refer to sequences which are composed of tandem repeats of 9-65 base pair and have a total length ranging from 0.1 to 7kb (Jeffreys *et al.*, 1985). Many minisatellite loci are highly variable and useful in parentage analysis or for marking individual families. In addition to high level of polymorphism, there are major advantages such as generation of many informative bands per reaction and high reproducibility. Unfortunately the highly variable loci are less useful for discriminating populations unless large sample sizes are used. Large numbers of alleles can also lead to difficulties in scoring and interpretation of data. Another limitation is the complex mutation processes (Wright, 1993; O'Reilly and Wright, 1995).

Single-locus minisatellites: In an effort to offset the difficulties in interpreting multilocus fingerprints, work concentrated on developing single-locus minisatellite probes, which were first developed for fish by Taggart and Ferguson (1990) and Bentzen *et al.* (1991) for Atlantic salmon and tilapia species. Standard single-locus minisatellite analyses by blotting require reasonable quantities of high-quality DNA. Analysis involves the PCR amplification of the minisatellite of interest and running the amplification products in an agarose gel. The products are scored after staining the gel in ethidium bromide (Galvin *et al.*, 1995). Alternatively random nDNA bands from partial restriction digests can be cloned. Then the cloned segments that hybridize strongly to Jeffrey's probes are selected and cloned inserts are used to probe complete DNA.

Despite the initial technical problems associated with using minisatellite probes, they have proved very successful in detecting genetics variations within and between fish populations (e.g. Taggart *et al.*, 1995; Ferguson *et al.*, 1995; Taylor, 1995), microgeographical (between tributaries of a river) population differences (Galvin *et al.*, 1995) and reproductive success of farm escapees (Ferguson *et al.*, 1995). Recently minisatellites have been applied in fisheries for genetic identity, parentage, forensics, identification of varieties, estimate mating success and conforming gynogenesis (Jeffreys *et al.*, 1985; Brown and Epifanio, 2003).

Microsatellites: A microsatellite is a simple DNA sequence that is repeated several times at various points in the organism's DNA. Such repeats are highly variable enabling that location (polymorphic locus or loci) to be tagged or used as a

marker. Microsatellites have much more information than allozymes and mtDNA, yet offer the same advantages of analysis. Technical expertise required for detection and scoring/analysis once the polymorphic loci identified is similar for all the methods.

Microsatellites are thought to occur approximately once every 10 kbp, while minisatellite loci occur once every 1500 kbp in fish species (Wright, 1993), which suggests that microsatellites may be more useful for genome mapping studies (O'Connell and Wright, 1997). They are one of a class of highly variable, non-coding and considered to be selectively neutral, allowing for the assumption that the estimated amount of sequence divergence between units of interest is directly proportional to the length of time since separation (Brown and Epifanio, 2003). Microsatellites are co-dominant, inherited in a Mendelian fashion and tandem arrays of very short repeating motifs of 2-8 DNA bases that can be repeated up to ~100 times at a locus. They are among the fastest evolving genetic markers, with 10^3 - 10^4 mutations/generation (Goldstein *et al.* 1995). Their high polymorphism, and PCR based analysis has made them one of the most popular genetic markers (Wright and Bentzen 1994). With current molecular methods it is feasible to score microsatellite length polymorphisms in large numbers of individuals for genetic analyses within and between populations. Some microsatellite loci have very high numbers of alleles per locus (>20), making them very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of alleles and may be more suited for population genetics and phylogeny (O'Connell and Wright, 1997; Estoup and Angers, 1998). Primers developed for one species will often cross-amplify microsatellite loci in closely related species (Estoup and Angers, 1998).

Microsatellite markers have a number of advantages over other molecular markers and have gradually replaced allozymes and mtDNA. Microsatellite loci are typically short, this makes it easy to amplify the loci using PCR, and the amplified products can subsequently be analysed on either "manual" sequencing gels or automated sequencing. Microsatellites are relatively easy to isolate compared with minisatellites, sample DNA can be isolated quickly because labour-intensive phenol-chloroform steps can generally be eliminated in favour of a simpler form of DNA extraction. The much higher variability at microsatellites results in increased power for a number of applications (Luikart and England, 1999). Only small amounts of tissue are required for typing microsatellites and these markers can be assayed using non-lethal fin clips and archived scale samples, facilitating retrospective analyses and the study of depleted populations (McConnell *et al.*, 1995). Moreover, there is potential for significant increases in the number of samples that can be

genotyped in a day using automated fluorescent sequencers. For applications where a large number of loci are required, such as genome mapping or identification of Quantitative Trait Loci (QTL), microsatellites offer a powerful alternative to other marker systems.

There are two main techniques for microsatellite analysis. The first one requires probing complete digests of nDNA with simple sequence repeats (di-, tri-, or tetra- nucleotide repeats). Alternatively they are genotyped using the PCR using primers targeted to the unique sequences flanking the microsatellite motif. PCR can easily be semi-automated. The resulting PCR products are separated according to size by gel electrophoresis using either agarose gels or more commonly (higher resolution) denaturing polyacrylamide gels. This amplification presents a significant advantage over other non-PCR based methods because it allows the use of relatively small amounts of tissue, including that from preserved otoliths, scales, larvae, and small fry.

Despite the advantages of microsatellite markers they are not without constraints. One of the main problems is the presence of so-called "null alleles" (O'Reilly and Wright, 1995; Pemberton *et al.*, 1995; Jarne and Lagoda, 1996). Null alleles occur when mutations take place in the primer binding regions of the microsatellite locus, i.e. not in the microsatellite DNA itself. The presence of null alleles at a locus causes severe problems, in particular in individual based analyses such as relatedness estimation and assignment tests, and most researchers prefer to discard loci exhibiting null alleles (Hansen, 2003). Even though microsatellites have already proven to be powerful single locus markers for a variety of genetic studies (Queller *et al.*, 1993), the need to develop species-specific primers for PCR amplification of alleles can be expensive. However, primers developed to amplify markers in one species may amplify the homologous markers in related species as well (Morris *et al.*, 1996). Another important disadvantage of microsatellite alleles is that amplification of an allele via PCR often generates a ladder of bands (1 or 2 bp apart) when resolved on the standard denaturing polyacrylamide gels. These accessory bands (also known as stutter or shadow bands) are thought to be due to slipped-strands impairing during PCR (Tautz, 1989) or incomplete denaturation of amplification products (O'Reilly and Wright, 1995). The practical outcome of PCR stutter is that it may cause problems scoring alleles. However, trinucleotide and tetranucleotide microsatellite typically exhibit little or no stuttering (O'Reilly *et al.*, 1998).

Nuclear DNA exhibits the greatest variability of all genetic markers related to fisheries science and will be highly productive avenue for research and applications in wild and aquaculture stocks. Main applications in fisheries and aquaculture are (see reviews of O'Reilly and Wright, 1995; O'Connell and

Wright, 1997; Magoulas, 1998; DeWoody and Avise, 2000; Brown and Epifanio, 2003; Hansen, 2003); phylogenetics and phylogeography (e.g. Hansen *et al.*, 1999a; Nielsen *et al.*, 1999; Hansen, 2002; Hansen *et al.*, 2002), population genetic structure (Scribner *et al.*, 1996; O'Reilly *et al.*, 1998; Nielsen *et al.*, 1997; Shaklee and Bentzen 1998; Nielsen *et al.*, 1999), conservation of biodiversity and effective population size (Reilly *et al.*, 1999), hybridization and stocking impacts (Hansen *et al.*, 2000; Hansen *et al.*, 2001a; Hansen, 2002; Ruzzante *et al.*, 2001a), inbreeding (Tessier *et al.*, 1997), domestication, quantitative traits (Jackson *et al.*, 1998), studies of kinship and behavioural patterns (Bekkevold *et al.*, 2002). Microsatellites are also becoming increasingly popular in forensic identification of individuals, and determination of parentage and relatedness, genome mapping, gene flow and effective population size analysis (Queller *et al.*, 1993; Hallerman, 2003; Withler *et al.*, 2004).

2.4.3. Single-Copy DNA Analysis

The cloned single-copy nuclear DNA (scnDNA) is non-repetitive nuclear sequences that occur with a frequency of one per haploid genome. It provides major advantages particularly over allozymes and mtDNA. Single-copy nDNA loci are especially useful because they originate multiple unlinked genes and allow the rapid survey of many individuals for genetic variation in a short period of time. It segregates as a Mendelian trait, i.e. biparentally transmitted and expressed. scnDNA are considered selectively neutral, occur throughout the nuclear genome and are very abundant. Basically three methods have been applied to observe variability of scnDNA in fisheries and aquaculture: i) single-copy nuclear RFLP (scnRFLP); ii) PCR – RFLP, and iii) exon-primed, intron-crossing (EPIC) analysis. Applications of scnDNA in fisheries have focused on population structure and the extent of gene flow.

2.4.4. Single Nucleotide Polymorphisms

The difficulty to fully automate microsatellite genotyping has revived interest in a new type of markers. Single nucleotide polymorphisms (SNPs) are polymorphisms due to single nucleotide substitutions (transitions > transversions) or single nucleotide insertions/deletions. It is a variant of scnDNA polymorphism based on detection of individual nucleotide. These variants can be detected employing PCR, microchip arrays and fluorescence technology. Major applications of SNPs in fisheries are genomic studies and diagnostic markers for diseases. Since they are main part of the many gene chips, they are considered as next generation markers in fisheries.

3. Practical Applications in Fisheries and Aquaculture

There are several recent reviews of the applications of molecular genetic techniques in fisheries and related areas (Skibinski, 1994; Ward and Grewe, 1995; Ferguson *et al.*, 1995; Ferguson and Danzmann, 1998; Magoulas, 1998; Sakamoto *et al.*, 1999; Davis and Hetzel, 2000; Peacock *et al.*, 2001; Taniguchi *et al.*, 2002; Fjalestad *et al.*, 2003; Hallerman, 2003; Taniguchi, 2003; Cross *et al.*, 2004).

3.1. Fisheries

3.1.1. Interspecific Variations

There may be an uncoupling between genetic and phenotypic diversity. For example, some Malawi cichlids exhibit remarkably little genetic differentiation, despite marked behavioural, morphological and ecological diversity (Turner, 1999). Such uncoupling not only emphasizes the highly variable rates of molecular evolution among taxa, but also the sometimes poor correlation between traditional taxonomic characters based on phenotype, and the extent of genetic divergence as revealed by molecular tools (Bernatchez, 1995). Indeed, the neutrality of most molecular markers to selective forces often limits their ability to distinguish recently diverged taxa, whereas adaptive traits, such as morphological or meristic characters used in taxonomy, are under natural selection and thus may diverge faster. It is therefore inappropriate to assume that molecular markers can provide the final answer to species identification; they are instead an additional marker system, which can be used to increase the resolution in taxonomic research. Thus molecular genetic markers have to be interpreted in relation to specific variation in evolutionary rates, both among taxa and critically among different sets of traits (Carvalho and Hauser, 1999).

It is important to identify the various species that occur together in mixed catches, so that individual species can be managed effectively. This might be difficult to identify morphologically. Where fish products have been processed, including filleting, smoking or salting, identification by external characteristics is more difficult. Similar considerations apply when attempting to identify stomach contents of fish predators in dietary studies. Another problem is the identification of early life stages of fish, as in planktonic egg surveys or in recognising the species of origin of caviar. The latter is important to prevent exploitation of endangered sturgeon species. Even closely related species differ in nucleic acid composition at a proportion of their gene loci. Genetic differences between species are much larger than between populations within a species. This means that very small sample sizes can

be used (3 to 5 individuals). Even when two species are almost indistinguishable morphologically, they are likely to be easily distinguished genetically.

Most of the molecular markers have been used in inter- and intraspecific variations. For example, they have been applied in species and subspecies identification in tilapia using RAPD (Bardakci and Skibinski, 1994) and in Sparidae species by isozyme (Alarcón and Alvarez, 1999). Ideally, two or more diagnostic markers should be sought to exponentially decrease the chance of error, due to inadvertently choosing individuals of the same species which are homozygotes for different alleles. In the past allozymes were often used in species discrimination, but these have very stringent sample requirements. An array of DNA markers are now available (e.g. mtDNA, microsatellite loci, minisatellite loci, transcribed sequences), and a series of these should be investigated seeking the most discriminatory loci, since these techniques often have much more relaxed tissue quality and storage requirements. Mitochondrial DNA because of the haploid mode of inheritance is not useful for initially identifying F1 hybrids. However, when F1 hybrids have been identified using nuclear markers, then the maternal parent can be identified by typing for a species specific feature of the mitochondrial genome.

3.1.2. Phylogeography and Phylogenetic

Primary aim of quantitative analyses for genetic stock identification is to provide objective classifications of individuals or samples into groups. One of the goals can be make inferences about historical processes affecting relationships (phylogenetics) among groups and geographical distributions (phylogeography) of groups. Studies in this field began in the mid-1970s with the introduction of mtDNA analyses to population genetics. The analysis and interpretation of lineage distributions usually requires input from molecular genetics, population genetics, phylogenetics, demography, ethology, and historical geography (Avice, 1994). Phylogenetic classification specifically attempt to show relationships based on reconstructing the evolutionary history of groups or unique genomic lineages. A critical assumption for phylogenetic analyses is that gene flow among lineages has been rare (Shaklee and Currens, 2003). Molecular genetic data have become a standard tool for understanding the evolutionary history and relationships among species (Avice, 1994). Examples of emerging applications include the definition of conservation units (Vrijenhoek, 1998), and use of genetic data to complement inferences about ecological patterns and processes (e.g., Avice 1994; Sunnock, 2000).

Mitochondrial DNA analysis has proven a powerful tool for assessing intraspecific phylogenetic patterns in many animal species (Bernatchez *et al.* 1992; Avice, 1994). In comparison to allozyme or nDNA, the higher mutation rate, smaller effective

population size, and predominantly maternal inheritance of mtDNA are expected to provide greater power to identify population structure. Furthermore due to lack of recombination and low efficiency of DNA repair mechanisms, mtDNA evolves at a rate faster than single-copy genes in nuclear DNA, which makes this molecule extremely useful for phylogenetic analyses. Taking the 'phylogeography', for example, as pointed by Avice (1998), some 70% of studies carried out thus far involved analyses of mtDNA. For instance, among the 1758 primary papers and primer notes published in the last 9 years in the journal "Molecular Ecology", 29.8% and 42.5% are indexed with mitochondrial and microsatellite DNA markers, respectively (Zhang and Hewitt 2003). mtDNA variation can resolve relationships of species that have diverged as long as 8-10 million years before present (Peacock *et al.*, 2001). After about 8-10 million years, sequence divergence is too slow to allow sufficient resolution of divergence times. Thus mtDNA is not appropriate for reconstruction of relationships among populations, subspecies and species that diverged >10 million years ago (Peacock *et al.*, 2001). In addition, this type of marker can be used to track demographic features exclusively for the female proportion of a population (Laike *et al.*, 1998).

Nuclear DNA also provides a nearly unlimited source of essentially independent loci for phylogeographic analysis (e.g. Hansen *et al.*, 1999a; Nielsen *et al.*, 1999; Hansen, 2002; Hansen *et al.*, 2002). While individual gene genealogies for single-copy nuclear DNA (scnDNA) loci may be less informative than the mtDNA gene tree, analysis of multiple scnDNA genealogies provides a framework to assess the relevance of the individual nuclear and mitochondrial genealogies to the phylogeographic structure of the organism (Bagley and Gall, 1998).

Brown trout is one of the most widely studied species regarding to phylogenetics lineages in Eurasia. The classical study by Bernatchez *et al.* (1992), based on sequencing of the mitochondrial DNA D-loop, led to the identification of five major phylogeographical lineages, with one of them, the so-called Atlantic lineage, being the only lineage present in the previously glaciated northern Europe, whereas the other lineages are distributed in Central and Southern Europe. Later studies (e.g., Weiss *et al.*, 2000; Bernatchez, 2001) have added new important pieces to the puzzle, but have not fundamentally challenged the original suggestion by Bernatchez *et al.* (1992) of five major lineages. The phylogenetic relationship between the Pacific salmon group and the Pacific trout group were also analysed using allozyme electrophoresis and mtDNA (Kitano *et al.*, 1997), and mtDNA and one nuclear growth hormone intron (Domanico *et al.*, 1997). mtDNA - RFLP analysis was used to examine the systematic and phylogenetic status of naturally occurring cutthroat trout populations in Nevada (Williams *et al.*, 1992, 1998), and phylogenetic trees were created using genetic

distance matrices.

Phylogenetic of tilapiine species have also drawn considerable attention during recent years (e.g. Feresu-Shonhiwa and Howard, 1998; B-Rao and Majumdar, 1998; Nagl *et al.*, 2001). Feresu-Shonhiwa and Howard (1998) analysed genetic variation within and among Zimbabwean tilapias 34 populations of seven species, two in the genus *Tilapia* and five in *Oreochromis*, using electrophoresis allozymes and revealed phylogenetic relationships. B-Rao and Majumdar (1998) obtained distance matrices from allozyme studies on tilapiine fish, while Nagl *et al.* (1998) revealed classification and phylogenetic relationships of African tilapiine fishes using mitochondrial DNA sequences.

3.1.3. Population Structure: Between and within population variations

Variation within and between populations and stock discrimination within exploited species are important issues not only in fisheries management but also for conservation programmes. The main aim is to recognise groups within a species which are largely reproductively isolated from each other. Scientists also need to identify non-interbreeding populations, to assess the gene flow between different genetic stocks, and to monitor temporal changes in the gene pools (Carvalho and Hauser, 1995). Many non-genetic methods of stock discrimination are available and achieve varying degrees of success in distinguishing breeding stocks. Morphological and meristic characters have both a heritable (genetic) and non-heritable (environmentally influenced) component. However, natural selection and evolutionary history can shape morphological characters, but differences (or lack thereof) among populations, subspecies or species may also be influenced or determined by the environment. With the advent of genetic methods, stock identification based solely upon morphological and meristic differences has become rare. Instead, these data are used in conjunction with genetic data. Because morphological and meristic characters can be influenced by the environment, variation in these characters may not have a genetic basis, and these characters do not necessarily provide information on genetic and evolutionary relationships (Gall and Loudenslager, 1981). Genetic methods should be most effective in this area (Cross *et al.*, 2004) and when genetic, morphological and meristic data combined can provide reliable information on actual genetic differences and important environmental effects on phenotype.

Conventionally, at least 50 individuals are sampled from each location, to allow for statistical confirmation of observed differences. It has also been recommended that at least 20 variable loci are investigated. The life stage at which fish are sampled is also vital in discriminating populations. For example, anadromous salmon populations spawn in separate rivers but often mix on oceanic feeding

grounds, so sampling should take place in the former areas. However, this may not be an appropriate strategy for marine species where separate groups spawn in the same general area, so sampling feeding aggregations would be advised instead. This implies that the reproductive biology of species of interest must be known, when sampling is being planned (Cross *et al.*, 2004). Another important issue regarding the sampling a wild population is to avoid sampling of close relatives, for example families of freshwater and anadromous species when they are occur as separate schools during early stages. Cross *et al.* (2004) advised sampling over a large section of river (1 km in length) for Atlantic salmon fry/parr. This is also valid for sea trout and other fish with similar life history.

Allozymes have been the most widely used markers to study the genetic structure of populations since the early 1970's and still continue to play an important role in the description of intraspecific genetic diversity. Utter *et al.* (1987) provide a review of the technique using examples from Pacific salmon, and the laboratory manual of and recent allozyme studies in fisheries are reviewed by Ward *et al.* (1992, 1994). May (2003) reviewed general methods used to detect allozyme variation, genetic basis of this variation, interpretation of banding patterns and applications in fisheries.

Today allozymes has to a large extent been replaced by DNA techniques, in particular microsatellite DNA analysis (Hansen, 2003). Although DNA premise has proved correct in many cases there is still little consensus as to the most appropriate DNA method. Many freshwater and anadromous fish stocks were readily identifiable by protein electrophoresis (Utter, 1991), but until the mid 1980s, little difference was detectable between adjacent marine fish groupings (Ward *et al.*, 1994). Subsequently, DNA methods (Carvalho and Pitcher, 1994) have shown inconsistencies when different types of genetic markers were used. Low levels of differentiation in marine fish species have been attributed to the large size of most marine fish populations (i.e. little genetic drift), to limited migration between marine populations, or to the recent origin of populations. For example, although allozymes and microsatellites show similar patterns of differentiation of Irish and Spanish populations of Atlantic salmon, microsatellite loci show higher levels of variation (Sanchez *et al.*, 1996). McConnell *et al.* (1995) were able to discriminate clearly between Canadian and European salmons using microsatellites. In cod, microsatellite loci have provided evidence of population structure at a finer geographical scale than that shown by other techniques (Ruzzante *et al.*, 1996). Thus, choosing highly variable systems (preferably microsatellite or minisatellite DNA), appropriate sections of the mtDNA, and transcribed nuclear sequences have been recommended.

The application of mtDNA as a genetic marker

has become widespread for population genetic studies, particularly in salmonid fishes (Bernatchez *et al.*, 1992; Hynes *et al.*, 1996; Nielsen *et al.*, 1998). In most species mitochondrial DNA (mtDNA) is highly variable and is therefore a good marker for detecting possible genetic differentiation. Additionally, mtDNA supplies information which could not have been obtained using only nuclear markers due to mtDNA being haploid and maternally inherited. With the development of PCR, RFLP analysis of PCR-amplified segments of the mtDNA has become a common method for population genetic studies (O'Connell *et al.*, 1995).

It was thought that microsatellite and minisatellite loci would be useful in many fish species, since the flanking primers are conserved sequences. Indeed, some microsatellite loci primers isolated from bony fishes produce bands in dogfish and lampreys (Rico *et al.*, 1996). Many laboratories are now turning to enrichment procedures to increase the cloning efficiency of microsatellite and minisatellite sequences from genomic DNA of the target species. Minisatellites have been developed successfully for salmonids (e.g. Prodöhl *et al.*, 1997) and have been used to study population differences (e.g. Taylor, 1994, 1995).

Due to the high level of allelic variation, microsatellite loci are excellent markers of within-species genetic heterogeneity. Microsatellites are a more powerful tool than mitochondrial DNA in defining the geographical and spatial scales for population differentiation as well as in identifying the origins of individuals in mixed stocks of migratory fish. Wright and Bentzen (1994) recommend using microsatellites for the analysis of genetic stocks of geographically proximate populations, such as stocks from a single river system. Where historical collections of scales or otoliths exist, it is possible; using PCR based techniques, to undertake a retrospective analysis over time. This can be particularly important where fisheries have collapsed, and populations may have lost genetic variation when going through a bottleneck. Microsatellite DNA loci appear to be the most suitable markers currently available for this kind of work. Ruzzante *et al.* (2001b) reported on evidence of long term stability in the geographic pattern of genetic differentiation among cod (*Gadus morhua*) collected from 5 spawning banks off Newfoundland and Labrador over a period spanning three decades (1964–1994) and 2 orders of magnitude of population size variation. Six microsatellite DNA loci amplified from archived otoliths (1964 and 1978) and contemporary (1990s) tissue samples revealed fidelity to natal spawning banks over this period.

It is also very important combining genetic with physiological, ecological, and hydrographical information when assessing the genetic structure of highly abundant, widely distributed, and high gene-flow fish species, for example marine species.

Ruzzante *et al.* (1999) highlighted the role that oceanographic features (e.g., gyre-like systems) and known spatio-temporal differences in spawning time may play as barriers to gene flow between and among neighbouring and often contiguous cod populations in the Northwest Atlantic.

3.1.4. Mixed Fishery and Genetic Stock Identification

It is widely accepted that species are typically subdivided into more-or-less discrete subpopulations or stocks, components of species that are exploited in fisheries or actively managed. In some cases these subunits occur as mixed populations. Sustainable, long-term management of mixed fisheries (multiple species, different stocks) can be major concern for fisheries managers. Different species, life stages and/or individuals from different stocks may compose these fisheries. Typical example is salmonids in feeding grounds (e.g. off West Greenland and the Faeroes Islands) or in the lower reaches of a river during migration (Cross *et al.*, 2004). The main objective in such fisheries might be identification of individual fish to population of origin. Genetic data have increasingly been used to investigate stock structure and stock contributions to mixed-stock fisheries during the last 10-15 years (Shaklee and Currens, 2003). Genetic stock identification (GSI) is a system developed for identification of species or population.

When mixed fishery composes of only two different stocks, estimation of relative contribution is simple and straightforward. This is the situation with Atlantic salmon at West Greenland where salmon from Europe and North America mix to feed. European and North American salmon constitute different races, with several nearly diagnostic genetic differences being observed. In this case, it is possible to ascribe individual fish to continent of origin with a high degree of certainty by using these markers (e.g. minisatellite: Taggart *et al.*, 1995 and microsatellite: McConnell *et al.*, 1995).

Initially, all mixed fishery analysis has utilised allozymes for a long time for identifying subpopulations or stocks of many freshwater, marine and diadromous fish and shellfish species (Shaklee and Currens, 2003). The best known example is Pacific salmon (Utter *et al.*, 1989; Seeb *et al.*, 1998). More recently, mtDNA, mini- and microsatellites are found to give much higher degrees of accuracy and precision than those available from allozymes (Avisé, 1994; Galvin *et al.* 1995; Taggart *et al.*, 1995; Seeb *et al.*, 1998; Bernatchez, 2001). For example, Atlantic salmon shows low levels of genetic differentiation using allozymes and mtDNA. However, with microsatellites, McConnell *et al.* (1995) were able to discriminate clearly between Canadian and European fish. Other examples for employment of mini- and microsatellite DNA data for stock identification are:

Galvin *et al.* (1995), McConnell *et al.* (1995 and 1997) and Taggart *et al.*, (1995) for Atlantic salmon; Ruzzante *et al.* (1996) for Atlantic cod and steelhead and rainbow trout (Taylor, 1995).

3.1.5. Genetic Marking

Fisheries scientists and managers have marked individual fish for various purposes, including tracking movement or migration, estimating population size or estimating contributions to a mixed-stock fishery. These physical marks such as fin clips, numbered plastic tags, coded wires, fluorescent elastomer tags, visible alphanumeric plastic tags or passive integrated transponders are useful for distinguishing individuals but not heritable. Thus for each generation a large number of marks and marking efforts would be required. However, there are many contexts in which we want a heritable marker. When, for example, a cultured stock is desired to mark, this could be addressed by deliberate genetic marking of the stock (Hallerman, 2003). There can be a number of contexts that fish might be marked genetically. For example, we may want to know contribution of a hatchery programme on harvest, contribution of stocked individuals on growth of targeted population and possible genetic impact of escaped or released domesticated stocks on receiving populations. In such contexts as issue of escapes, a means of identifying domesticated or farmed fish or their progeny would prove highly desirable and only heritable markers permit identification of offspring. Hindar *et al.* (1991) stress the importance of marking released fish genetically so that future consequences of releases can be monitored.

Genetic marking is executed by breeding only individuals carrying the desired marked allele or alleles. In practise, a rare allele is chosen (in most previous studies allozyme loci were used) and heterozygote crosses are made (homozygotes for the allele being extremely rare) (Cross *et al.*, 2004). There are a number of case studies particularly on Pacific salmon and some other species as well. These were summarized by Hallerman (2003). Allozymes have been used in the past, but evidence is accumulating that many of these are influenced by selection. Thus, non-coding minisatellite and microsatellite DNA loci are recommended for future trials.

3.1.6. Forensics

One of the challenges faced by fisheries biologists is the management of fish stocks under harvesting pressure from both commercial and recreational fishermen. For monitoring and surveillance officers to positively identify fish species and stocks, those morphological characters necessary for species identifications must be present. However, when different stocks of a species are considered or when the catch has been filleted, the

morphological characters may have little use. In some cases the fillets are suspected to be a regulated species, and molecular markers must be used for specific identification.

Forensics is use of scientific methods to make inferences regarding past events, especially with regard to discussion, debate, argumentative and questions relevant fisheries, wildlife and conservation law enforcement (Hallerman, 2003). Forensics in fisheries focuses on determining and proving who perpetrated criminal acts. These acts may include misuse of fisheries resources, misinterpretation of the content of fisheries products, illegal trading in fish or fisheries products and accidental or deliberate undesirable releases or introductions into natural waters. In order to prove the misuse or illegality one must be demonstrated that the evidence constitutes a taking from a banned species or population, a taking made out of season or a taking illegally introduced into marker or wild populations. The seized material or specimen is analyzed in a laboratory and posed question "what is this?" is answered. Depending on the application, the question can be considered one species, population or individual identification. Major cases or hypothetical situations forensics involves (Hallerman, 2003):

- *Species identification:* Forensics needs species-specific diagnostic genetic markers for cases of species identification. A number of molecular markers have been used, including allozymes, mtDNA and other nDNA markers. Cases on whales and sturgeons are good examples for these applications and allozymes and mtDNA are commonly used markers.

- *Population identification:* There are a number fish stocks under strict management and conservation programmes. Exploitation of these stocks is restricted or completely banned and population-specific identification approaches are needed. Here allozymes, and particularly mtDNA, microsatellites and the major histocompatibility complex (MHC) are mostly used or potential markers.

- *Individual identification:* In some cases individual – species identification might be essential. For example, product might come from an illegally harvested individual or domesticated individual. In such cases highly polymorphic individual-specific genetic markers are needed to tie the poached product directly and confidentially to the product in the suspect's possession.

DNA methodologies of animal identification are becoming commonplace and have gained acceptance in legal proceedings (Hallerman, 2003; Withler *et al.*, 2004). Methods to identify tissue samples to species based on nuclear or mitochondrial DNA sequences have been developed for a wide variety of organisms, including commercially important fish. Genetic stock identification (GSI) of coho, chinook and sockeye salmon samples for forensic purposes is conducted using large

microsatellite and major histocompatibility complex (MHC) databases accumulated in studies of population structure (Beacham *et al.*, 2001; Withler *et al.*, 2000). The ubiquitous presence and relative stability of DNA in body tissues enables non-lethal sample collection from almost any tissue, including dried, frozen and ethanol-preserved tissues and processed (canned, smoked) food products. In a recent evaluation study Withler *et al.* (2004) reported identification of salmonid tissue samples to species or population of origin for over 20 forensic cases in British Columbia. Species identification was based on published sequence variation in exon and intron regions of coding genes, while identification of source populations or regions was carried out using microsatellite and MHC allele frequency data. DNA has been obtained successfully from salmon scale samples, fresh, frozen and canned tissue samples and bloodstains in clothing. Results from DNA analyses were used in a number of convictions. The results of these studies indicate that the genetic methodologies developed for fishery management species and stock identification of Pacific salmon are sufficiently accurate and precise to use in classification of samples collected as evidence in court proceedings.

Assigning individual fish to populations:

The individual-based population assignment test aims to quantify degrees of genetic differentiation among populations and it has proved useful in a wide variety of applications in population and conservation biology (Hansen *et al.*, 2001b; Campell *et al.*, 2003). This, in turn, has enabled researchers to determine the relative contribution of each potential source population in mixed fisheries, to assign individuals during migration, to estimate sex-biased dispersal and gene flow, to identify potential admixture between populations and to estimate the long-term effects of population stocking (Hansen, 2002). Forensic sciences have also benefited from assignment tests as they can be used to discriminate if an animal originates from an illegal source (Campell *et al.*, 2003). Assignment tests have relied mainly on the use of microsatellite loci. Although the resolution obtained with these markers is often adequate, an important constraint faced by the use of microsatellites in any type of individual-based population assignment is the lack of statistical power in situations of weak population differentiation (Campell *et al.*, 2003). Recently, Campell *et al.* (2003) reported that AFLP were much more efficient than the microsatellite loci in discriminating the source of an individual among putative populations.

Developments in information technology combined with highly polymorphic microsatellite DNA markers enable the determination of the population of origin of single fish, resulting in numerous new applications in fisheries management. Assignment tests are at present most useful for studies of freshwater and anadromous fishes owing to

stronger genetic differentiation among populations than in marine fishes. However, some genetically divergent marine fish populations have been discovered (Hansen *et al.*, 2001a).

3.2. Interaction between Fisheries and Aquaculture

Aquaculture can support the wild stocks as restocking and/or sea-ranching, called "fisheries enhancement". The possibility of enhancement of wild stocks through stocking/ranching has been attracting a good deal of attention. Systematic conservation of diadromous stocks through artificial rearing and release into natural marine environments is, as we have seen, a long established practice especially in the case of the temperate salmonids and sturgeons. Theoretically, enhancement of fisheries by release and recapture has a great potential to add to fish production. However, culture and wild stocks share the same environment and interactions in various ways are almost unavoidable. Genetically important ones are the effects of introduction through enhancement and escapes from hatcheries of fish farms (Okumuş, 2000).

3.2.1. Conservation Genetics and Hatchery Supplementation

Stocking is the release of reared fish into the wild. It is also known as enhancement when the wild fish are still present in targeted location, restoration/reintroduction if the wild population has become extinct, ranching in case of anadromous and introduction when exotic species used. Stocking of hatchery reared fishes and intentional introductions are important components of fisheries management programmes. However, concerns have been raised about the possible effects of such introductions on native fish populations. That is because in captive stocks genetic changes can occur during rearing period in a number of ways. Extinction, loss of within population genetic variation, loss of between population variations - population identity and domestication selection are the main types of genetic concerns have been raised in relation to stocking activities (Busack and Currens, 1995 cited in Miller and Kapuscinski, 2003). The well known basic principles for appropriately dealing with these concerns relate to identifying and conserving genetic resources (Hindar *et al.*, 1991; Grant *et al.*, 1999; Taniguchi, 2003). In this context it is important to supplement wild fish with fish derived from native stock. After choice of donor population an appropriate number of founder fish are sampled. Miller and Kapuscinski (2003) recommend a minimum of 50 spawners, preferably with equal numbers females and males.

Many reviews, particularly with salmonids, emphasising genetic effects have appeared in the last

decade (e.g. Miller *et al.*, 1990; Allendorf, 1991; Hindar *et al.*, 1991). Unfortunately the available evidence suggests that the impact of releases on indigenous fish populations tends to be some negative effects. Molecular techniques allow the monitoring of genetic variation between and within wild and hatchery stocks, and thus help in the detection of the negative effects on receiving wild stocks (Skibinski, 1998). Ideally genetics studies can make following contributions (Vrijenhoek, 1998):

- resolving problems with taxonomically difficult groups,
- the design of captive breeding programmes;
- understanding natural breeding systems;
- detecting diversity within and among geographical populations;
- managing gene flow; and
- understanding factors contributing to fitness.

The first valuable information on the dynamics of gene flow from domesticated fish into wild fish populations has been gained using allozyme markers. Bartley and Kent (1990, cited in Skibinski, 1998) assayed variation at 19 polymorphic allozyme loci in 13 wild and in six hatchery samples of white seabass (*Atractoscion nobilis*) derived from 20 broodstock fish maintained for several years. The hatchery samples lacked some rare alleles present in the wild samples, but at least in the short term genetic diversity was maintained quite well. Ferguson *et al.* (1991) compared levels of allozyme variation in cultured stocks of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) with the wild populations from which they were derived. Again, some rare alleles had been lost in the hatchery fish. In most cases the resolution power of allozymes has only allowed the genetic discrimination of domesticated and wild populations that are genetically strongly divergent (Largiadèr and Scholl, 1996). DNA markers are a good monitoring tool for such broodstock management. mtDNA can be particularly useful in this context (Hansen *et al.* 1995). However, mtDNA only traces female gene flow and inferences are drawn on the basis of just one haploid marker locus. Better markers appear to be highly variable nuclear DNA loci, namely microsatellite markers. In fact these two markers have been used together in most cases (e.g. Brunner *et al.*, 1998; Hansen *et al.*, 2000; Engbrecht *et al.*, 2002). Brunner *et al.* (1998) used sharing of alleles between stocked and nonstocked Arctic charr (*Salvelinus alpinus* L.) populations as indicators of introgression. Hansen *et al.* (2000) assessed the utility of polymorphism at seven microsatellite loci, combined with variation in mtDNA, to detect interbreeding between wild and stocked domesticated brown trout (*Salmo trutta* L.) in the Karup River, Denmark. In a wider genetic content Hansen *et al.*, (2001a) demonstrated the applicability of microsatellite analysis and assignment tests for monitoring gene flow from captive or translocated populations to wild indigenous populations. They

have demonstrated that microsatellite analysis provides a useful tool for distinguishing heavily introgressed populations from those unaffected by stocking. In brief, the best approach for above mentioned concerns in hatchery supplementation programmes seems to be using microsatellite and mtDNA markers together.

3.2.2. Genetic Interactions between Wild and Farmed Fish

The genetic risks facing wild populations when they are exposed to escapees from fish farms is a serious concern. The main question is “what happens when escaped fish have the opportunity to enter wild stocks?” Perhaps the most commonly-cited threat is genetic contamination and loss of natural stock identity (Okumuş, 2000). The interactions between farmed and wild fish can be problematic for many reasons:

- *Genetics*: Selectively bred, farm-raised fish that escape from aquaculture facilities and reproduce with wild fish can cause a decrease in the genetic diversity.
- *Competition*: Farm-raised fish that escape into the wild can negatively affect wild populations through competition for food, habitat, and mates.
- *Disease*: Farm environment can lead to increased levels of disease and parasites and these can be transferred to wild fish by escapees.

Genetic markers can be suitable for assessing the differences between culture stocks and wild populations and addressing concerns about escapes or releases from aquaculture farms into natural populations. So far studies of interactions have been largely confined to salmonids, because of their importance for aquaculture in Europe and North America. In some rivers, escaped farm-raised Atlantic salmon make up a majority of the salmon found. Each year several million farmed fish are believed to enter the wild through accidental escapes from fish farms. For example, in Norway, escapes of as many as 700,000 Atlantic salmon have occurred and in Washington State mishaps resulted in the release of 360,000 Atlantic salmon in 1997 and 115,000 in 1999 (Seaweb, 2004). Thus, the issue of escapes has been quite well studied in Atlantic salmon. Clifford *et al.* (1998) reported that some of the escapees may manage to reach spawning areas and inbred with wild salmon. They used two markers (mtDNA and minisatellite) that showed substantial frequency differences between these farm and wild populations. Farmed populations also showed a significant reduction in mean heterozygosity over the three minisatellite loci examined. Independent occurrence of mtDNA and minisatellite DNA markers in several juvenile samples indicated interbreeding of escaped farm salmon with wild salmon. However, according to Skaala (1994) in spite of escapees numbers (approaching 2 million) in Norwegian rivers in excess of the native (wild)

broodstock (roughly 100,000) swamping did not occur on a genetic basis.

Beside the intraspecific hybridization, interspecific gene exchanges have also been observed. For example, hybridization between rainbow trout and cutthroat trout has been found in British Columbia (Rutridge *et al.*, 2001). Allozyme and mtDNA markers have been useful markers in hybridization studies (Gall and Loudenslager, 1981; Williams *et al.*, 1992, 1998). Maternally inherited markers (mtDNA) are not useful in identifying extent of hybridization if matings are predominantly between non-native males and native females (Peacock *et al.*, 2001). Newly developed markers systems such as simple sequence repeats (SSRs) have been shown to be particularly useful for hybridization studies in salmonids (Ostberg and Rodriguez, 2002). SSRs have been developed specifically for use in rainbow-cutthroat trout hybridization studies (Ostberg and Rodriguez, 2002). Ideally a number of markers should be used to test for and monitor the extent of hybridization in critically important populations.

3.3. Aquaculture

Molecular markers also show significant promise for aquaculture applications. They can provide valuable information in aquaculture, such as: (i) comparison of hatchery and wild stocks; (ii) genetic identification and discrimination of hatchery stocks; (iii) monitoring inbreeding or other changes in the genetic variation; (iv) assignment of progeny to parents through genetic tags; (v) identification of quantitative trait loci (QTL) and use of these markers in selection programmes (marker assisted selection); and (vi) assessment of successful implementation of genetic manipulations such as polyploidy and gynogenesis (Magoulas, 1998; Davis and Hetzel, 2000; Fjalestad *et al.*, 2003; Subasinghe *et al.*, 2003). Several recent papers have reviewed the use of molecular markers in aquaculture, in particular their integration into breeding programmes (e.g. Ferguson and Danzmann, 1998; Magoulas, 1998; Sakamoto *et al.*, 1999; Davis and Hetzel, 2000; Taniguchi *et al.*, 2002; Fjalestad *et al.*, 2003; Taniguchi, 2003; Cross *et al.*, 2004).

3.3.1. Identification of Genetic Variability between and within Stocks

It is important to compare the genetic composition of hatchery strains with their wild donor populations and also within and between genetic variability in hatchery strains. Studies demonstrated considerable loss genetic variation in hatchery stocks (e.g. in salmonids) as a result of different factors including a low effective number of parents, domestication selection or the mating design (Allendorf and Ryman, 1987; Ferguson *et al.*, 1991; Pérez *et al.*, 2001). Thus hatchery stocks or strains should be monitored to detect genetic changes from

the wild donors and seek to minimise any further changes.

Many aquaculture species (e.g. salmon, rainbow trout, common carp, channel catfish, and tilapia) already have published heritability and genetic correlations parameters for major traits such as growth and maturity. However, for many aquaculture species and traits (e.g. food conversion efficiency, disease resistance, flesh quality etc) there have been no parameters published due to various difficulties. Molecular markers can be useful tools in stock identification and monitoring potential changes in broodstock, called DNA fingerprinting (Ferguson *et al.*, 1995; Reilly *et al.*, 1999; Fjalestad *et al.*, 2003).

Almost all major molecular markers from allozymes to microsatellite have been used in determination of between and within genetic variations in hatchery stocks (Ferguson *et al.*, 1991; Pérez *et al.*, 2001; Sekino *et al.*, 2002; Ramos-Paredes and Grijalva-Chon, 2003). Monitoring genetic changes in hatchery populations through variation in allozyme loci has been used (Pérez *et al.*, 2001), but recent studies mostly employed DNA markers. Most of the work using mtDNA for cultured species has been conducted on salmonids (Ferguson, 1994). Concerning marine species, Funkenstein *et al.* (1990) have reported the first mtDNA polymorphism in an Israeli broodstock of *Sparus aurata*, by using RFLP analysis of the whole mtDNA molecule. Later, Magoulas *et al.* (1995) confirmed this polymorphism in a Greek broodstock. The application of mini- and microsatellite markers to problems in aquaculture has been introduced recently, and again salmonid fish were the first to be studied (e.g., Estoup *et al.*, 1993). Sekino *et al.* (2002) assessed genetic divergence within and between hatchery, and wild populations of Japanese flounder (*Paralichthys olivaceus*) by means of microsatellite and mtDNA sequencing analysis. Desvignes *et al.* (2001) studied the genetic variability of French and Czech strains of hatchery stocks of common carp (*Cyprinus carpio*) using allozymes and microsatellites. They detected a more pronounced discrimination between the strains of the two countries by the microsatellite markers. More recently Bárfai *et al.* (2003) analyzed the whole broodstock of two Hungarian common carp farms (80 and 196 individuals) by using RAPD assay and microsatellite analysis. Microsatellite analysis revealed more detailed information on genetic diversities than RAPD assay. They also compared genotypes from the two stocks to those from a limited number of samples collected from other hatcheries and two rivers. Because allozymes cannot be assumed to be selectively neutral and the amount of their polymorphism is limited, they are not the assay of choice for the study or the discrimination of culture stocks (Ferguson, 1994; Magoulas, 1998). DNA-based techniques mainly microsatellite DNA loci are likely to be most efficient markers for above mentioned purposes.

3.3.2. Monitoring Genetic Changes in stocks

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

Allozyme assays have been very successful in detecting the genetic impact of culture. Studies have revealed retention of high enzyme heterozygosity levels in cultured rainbow trout, but there have been also cases of significant losses of allozyme variation (Ferguson, 1994). Variation in mtDNA can be a more sensitive indicator of maternal genetic history than allozymes (Ferguson *et al.*, 1993). The higher sensitivity of mtDNA to phenomena such as genetic drift and founder effect make this marker ideal for monitoring the consequences of founding and propagation in aquaculture. Microsatellite DNA analysis has been recently used for such studies. Microsatellite markers have been used for minimizing inbreeding in rainbow trout (Fishback *et al.*, 1999). Analysis of the F₁ generation of a Greek gilthead sea bream broodstock revealed a 15% reduction in the number of alleles and a homozygosity increase of 1.5% (Magoulas, 1998). Thus, hypervariable genetic markers may have important applications in monitoring inbreeding depression. For example, genetic markers can be used to locate the specific chromosomal regions responsible for inbreeding depression. This would be most feasible with cultured species where parents and their progeny can be managed and traced within a closed system. It will be possible to use mapped genetic markers to trace the inheritance of specific chromosomal arms in progeny (Ferguson and Danzmann, 1998).

3.3.3. Parentage and Pedigree Analysis in Selective Breeding

Selective breeding has been very successful in increasing production in a number of aquaculture species. Selection programmes make use of information not only on the candidates for selection, but also on their relatives in order to increase the accuracy of selection and therefore selection responses. Thus the optimal utilization of farmed stocks often requires knowledge of family relationships (parents, sibships). Ideally, individuals are identified uniquely when they are born and then the pedigrees of individual animals can be tracked

across generations (Villanueva *et al.*, 2002). However, in breeding programs, the progeny of many males and females are often reared together to minimize environmental variation. Such conditions are often imposed because of limited rearing space in small commercial facilities. Here, knowledge of family structure, i.e. pedigree system, can lead to a higher rate of genetic improvement because it becomes possible to identify the progeny of parents with desirable or undesirable characteristics (Doyle and Herbinger, 1994). Furthermore, matings between closely related individuals can be avoided. Genetic identification can also increase selection intensity for sex-limited traits (spawning date of females) (Ferguson and Danzmann, 1998). In practice, a molecular pedigree system should be able to discriminate a minimum of several hundred families and ideally it would also allow discrimination of individuals within families. The latter is important if both among- and within-family selection to be carried out, as it allows tracking individual performance and its evaluation over time. Some examples of the practical applications of molecular markers in selection programmes summarized here.

Mass spawning: One of the applications of molecular markers in broodstock management is in the identification of the contribution of possible parents in a mass spawning. Typically limited numbers of broods are used in spawning and some putative parents apparently fail to spawn. In these situations without the use of genetic markers, it is not possible to quantify the relative success of the potential parents. Parentage can be assigned using variable genetic markers, such as minisatellite or microsatellite markers after the spawning (Morán *et al.*, 1996; Thomaz *et al.*, 1997; Thompson *et al.*, 1998). The first attempt to apply this approach to a marine species was undertaken successfully for gilthead sea bream (Batargias *et al.*, 1997, cited in Magoulas, 1998). 32 breeders were put together for mass spawning, the eggs of a single-day spawning were collected, and the offsprings were reared in a common tank until the age of 6 months. At this point 150 individuals were removed at random, weighed and frozen. Another random sample of 150 offsprings was removed and treated the same way at the age of 10 months. Both samples of offsprings were genotyped for the same 4 microsatellite loci, for which the parents were scored. Both parents of the offspring were unambiguously identified in the vast majority of individuals.

Communal rearing: As mentioned earlier, one of the most important impediments to applying effective selective breeding programmes for fish is that newborn individuals are too small to be tagged physically. Thus, selective programmes making use of family information have needed to keep families separated until the fish are large enough to be

individually tagged. This is costly, limits the number of families available for selection and can induce environmental effects common to the members of the same family (Doyle and Herbinger, 1994). This problem can be resolved by applying DNA-based genetic markers. Consequently, more families can be kept in the breeding stock without the need for using separate tanks at early ages. These markers have been used to assess family/parentage identification in many species and can be used to discriminate fish in mixed family groups (e.g. Avise, 1994; Doyle and Herbinger 1994; Herbinger *et al.*, 1995; Thomaz *et al.*, 1997; Thompson *et al.*, 1998). Herbinger *et al.* (1995) were the first to demonstrate the feasibility of determining pedigrees in a mixed family rainbow trout population, using a small number of microsatellite markers.

Walk-back selection: A breeding programme can be initiated with a previously unselected farm raised strain by using a method, termed walk-back selection (Doyle and Herbinger, 1994). In general, this will involve the physical tagging and biopsy of individuals when they are large enough to be marked, with microsatellite analysis based on the biopsy used to assign individuals to family. Assuming that several hundred potential broodstock are available, fish of the same age just prior to sexual maturity is chosen. The biggest fish is typed, using non-destructive sampling, for several microsatellite DNA loci. The next largest fish is then sampled and only included in the broodstock if more distantly related than half sib to the first. This process is continued, with full or half sibs being excluded, until an adequate number of potential broodstock have been identified so as to minimise inbreeding. Thus, modified mass selection can be utilised without any need for prior pedigree information, or for the use of multiple tanks.

Development of molecular pedigreeing methods in most species has over the last decade focused on mtDNA, minisatellites and microsatellites. Ferguson *et al.* (1995) have used minisatellite-based pedigree analysis to evaluate variation in growth and seaward migration of different families of wild, farm and farm-wild hybrid Atlantic salmon families. However, use mtDNA and minisatellites has now been largely eclipsed by microsatellites. Microsatellite has been described as the most useful type of markers to assess genetic parentage (e.g. O'Connell and Wright, 1997), which have already been isolated and characterized in several fish species including salmon (McConnell *et al.*, 1997; Nielsen *et al.*, 1997; O'Reilly *et al.* 1998; Gilbey *et al.*, 2004), trout (Estoup *et al.*, 1993; Herbinger *et al.* 1995; Morris *et al.*, 1996; Estoup *et al.* 1998; Hansen *et al.*, 2000; Sakamoto *et al.*, 2000), carp (Bártfai *et al.*, 2003), turbot (Estoup *et al.* 1998; Bouza *et al.*, 2002), sea bass (Castilho, 1998; Ciftci *et al.*, 2002), sea bream (Perez-Enriquez *et al.* 1999), Japanese flounder (Coimbra *et al.*, 2001; Sekino *et al.*, 2002), cod (Ruzzante *et al.*, 1996) and tilapia (Bentzen *et al.*, 1991; Kocher *et al.*, 1998). Several studies have

empirically used microsatellite loci to successfully reconstruct pedigrees in fish populations with families mixed from hatching (Herbinger *et al.* 1995; Estoup *et al.* 1998; O'Reilly *et al.* 1998; Herbinger *et al.* 1999; Norris *et al.* 2000). Villanueva *et al.* (2002) developed deterministic predictions for the power of microsatellites for parental assignment and compared with stochastic simulation results. Their results showed that the four most informative loci are sufficient to assign at least 99% of the offspring to the correct pair with 100 crosses involving 100 males and 100 females. Doyle *et al.* (1994) used them to discriminate family groups of cod (*Gadus morhua*). In these cases, offspring assignment was to known parental types. However, with sufficient levels of variability, family or parental discrimination may also be achievable in the absence of parental information (Norris *et al.*, 2000). In this case all potential parents are characterised using a number of hypervariable loci and all resulting progeny are similarly screened. Progeny can then be assigned to particular mating, and the relative contribution of each family assessed (Norris *et al.*, 2000; Smith *et al.*, 2001).

3.3.4. Genome mapping and Detection of Quantitative Trait Loci (QTLs)

Genome or linkage mapping documents the synteny, order and spacing of genes or genetic markers on chromosomes. The genome maps facilitate the mapping of genes and markers of unknown location and also enable mapped genes in one species to be identified with homologous regions in another. They provide concrete evidence of the location of genes and markers. Thus genetic markers can be and have been used to search for the location of commercially important quantitative genes. These genes may be identified as single genes inherited in a Mendelian fashion. Alternatively they may be regions of the genome identified as accounting for a significant proportion of the variation in a trait is quantitative in nature. A single gene may control some disease resistance and morphological traits, while many traits of economic interest are controlled by many genes of small additive effects. They are considered particularly useful in breeding programmes and known as Quantitative Trait Loci (QTL). QTLs are detected by analysing phenotypes with linked marker maps and identification of markers linked to QTLs can provide significant gains for traits that are difficult or expensive to measure (feed conversion) and, can only be measured; only one sex (e.g. fecundity), after date of selection (reproductive traits), after killing the fish (flesh quality) or on fish environmentally separate from main breeding stock (disease resistance) (Davis and Hetzel, 2000). The purposes of QTL mapping are to measure genetic variability and relatedness between strains, families and individuals, and the use of that information in marker-assisted programmes to improve production related traits (Fjalestad *et al.*, 2003).

The detection of markers for QTLs, and the identification of QTLs themselves, is facilitated by the development of a genetic map. A number of different technologies are available (Park and Moran, 1995) and could be applied (Poompuang and Hallerman, 1997). The method involves isolating a large number (usually in excess of 100 loci) of highly variable markers (usually microsatellite loci, RAPDs or AFLPs) and then searching for correlation between each allele in turn and in combination, and various production traits. The most promising source of molecular markers is likely to be hypervariable microsatellite loci. These appear to be numerous in most fish species and to be widely dispersed in fish genomes based on available mapping studies. Microsatellite loci, though more time consuming and technically difficult to develop, are felt to be superior to RAPDs and AFLPs for genome mapping and the search for QTL (Sakamoto *et al.*, 1999), because the former are co-dominantly inherited, whereas the latter are inherited as dominants and recessives (Cross *et al.*, 2004).

A considerable effort is now being devoted to develop markers and to construct genetic maps for the major farmed species. Unfortunately genetic maps for important aquaculture species are partially developed. Actually in this context the zebra fish (*Danio rerio*) is only exception among fish species (Woods *et al.*, 2000). A collaborative EU FAIR project titled "Generation of highly informative DNA markers and genetic marker maps of salmonid fishes – SALMAP" ran from 1997 to 1999 and was aimed at constructing a map of major salmonid species, namely Atlantic salmon, rainbow trout and brown trout. A genetic map of rainbow trout comprising approximately 200 microsatellites was developed and published (Sakamoto *et al.*, 2000; Fjalestad *et al.*, 2003). So far for Atlantic salmon around 300 and for brown trout 232 markers, mainly microsatellites have been developed (Fjalestad *et al.*, 2003). Other genome mapping efforts are including tilapia (Kocher *et al.*, 1998), rainbow trout (William *et al.*, 1998; Sakamoto *et al.*, 2000; Nichols *et al.*, 2003), Atlantic salmon (Gilbey *et al.*, 2004), channel catfish (Liu and Dunham, 1998; Liu, 2003) and kuruma shrimp (Moore *et al.*, 1999). Most of the mapping effort has focused on microsatellites because of their hypervariability, relatively uniform distribution throughout the genome and the ability to use primer sets developed for one species on other closely related species (Ferguson and Danzmann, 1998; Jackson *et al.*, 1998; Sakamoto *et al.*, 1999; Davis and Hetzel, 2000; Perry *et al.*, 2001). Kocher *et al.* (1998) constructed a genetic map for a tilapia (*Oreochromis niloticus*) using DNA markers, microsatellite and anonymous AFLPs. They have identified linkages among 162 (93.1%) of these markers. 95% of the microsatellites and 92% of the AFLPs were linked in the final map. Sakamoto *et al.* (2000) reported a genetic linkage map for rainbow trout, using 191 microsatellite, 3 RAPD, 7 ESMP, and 7 allozyme

markers. Coimbra *et al.* (2001) identified twenty microsatellite markers in Japanese flounder. More recently Nichols *et al.* (2003) updated previously published rainbow trout map adding more AFLP markers, microsatellites, type I and allozyme markers, and Gilbey *et al.* (2004) described a linkage map of the Atlantic salmon consisting of 15 linkage groups containing 50 microsatellite loci with a 14 additional unlinked markers (including three allozymes).

AFLPs are another type of genetic marker in vogue for gene mapping. Actually in some crustaceans AFLP has been preferred since the development of microsatellite markers is difficult (Moore *et al.*, 1999; Davis and Hetzel, 2000). The appeal of AFLPs is that a genetic map can be constructed within a relatively short period of time (months). Indeed, a rainbow trout linkage map is now available that is composed of approximately 500 markers, with the majority of markers being of the AFLP type (Young *et al.*, 1998). One potential drawback to AFLP markers for QTL mapping and pedigree analysis is that many appear to be dominantly expressed. The lack of co-dominant allelic expression for these regions will necessitate densitometric PCR quantitation methods to discriminate between homozygous and heterozygous genotypes (Ferguson and Danzmann, 1998).

The detection of QTL markers in fish and their use in selective breeding programmes have recently been reviewed by Poompuang and Hallerman (1997). Increasing number of molecular markers for QTLs in fish has been reported in the literature. Jackson *et al.* (1998), Sakamoto *et al.* (1999) and Perry *et al.* (2001) published the first result of a genetic map in rainbow trout to locate QTL for growth, spawning date, and upper temperature tolerance, while Ozaki *et al.* (2001) described QTLs associated with resistance/susceptibility to infectious pancreatic necrosis virus (IPNV) in same species. Genomic research team in Auburn University have identified one marker linked to growth rate, three markers that are linked to feed conversion efficiency, and one putative marker that is linked to disease resistance to enteric septicemia of catfish. Genome-wide QTL scan using AFLP markers has been conducted for this species (Liu and Dunham, 1998; Liu, 2003).

In view of the limited success of traditional breeding methods, knowledge of linkage associations between marker loci and QTL can be integrated into selective breeding programs. Such an approach, called marker-assisted selection (MAS), is expected to increase genetic response by affecting intensity and accuracy of selection (Falconer and Mackay, 1996; Ferguson and Danzmann, 1998).

Marker-assisted selection (MAS) is the use of gene markers linked to QTLs in genetic breeding programmes. Although the term QTL strictly applies to genes of any effect, in practice it refers only to major genes, as only these are of a large enough size to be detected in mapping (Fjalestad *et al.*, 2003). MAS will have most application for traits that are

difficult and expensive to measure. For example, in fish resistance to diseases is measured in selected candidates but in their progeny. Similarly, flesh quality traits can only be measured after scarifying the fish. It has been reported that MAS can increase selection response, in particular for traits that can only be measured after selection decisions are made (Davis and Hetzel, 2000). A variety of techniques exist for incorporating marker information into genetic evaluation systems. However, the majority of work has focused on the marker as fixed effects in a normal genetic evaluation analysis that produces BLUP (Best Linear Unbiased Prediction) EBVs (Estimated Breeding Values) for the residual breeding value and an EBV for the QTL effect (Davis and Hetzel, 2000).

3.3.5. Assessment of Genetic Manipulations

A variety of methods exist for genetic manipulation of fish, such as polyploidy and gynogenesis. Genetic markers have been successful in conforming desired manipulations. For example, microsatellite loci are ideal for confirming the triploidy, because their high polymorphism makes it possible to detect triploid animals, by the observation of three-allele genotypes at certain microsatellite loci. The detection of paternal inheritance in gynogenesis is of critical importance and can most effectively be accomplished by using genetic markers. This has been done by allozyme markers in several cases, but VNTR loci, especially the single-locus ones, are very suitable for such assays (Ferguson, 1994). Highly polymorphic genetic markers can also be used for the discrimination between meio- and mitogynes, by assessing the level of homozygosity, which is expected to be complete in the case of mitogynes.

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

3.3.6. Disease and Parasite Diagnose

In recent years, molecular techniques have been increasingly developed for disease diagnosis purposes in aquatic animals. PCR assays have now become relatively inexpensive, safe and user-friendly tools in many diagnostic laboratories (Belak and Thoren, 2001; Louie *et al.*, 2000). However, with one or two exceptions, molecular techniques are currently not acceptable as screening methods to demonstrate the absence of a specific disease agent in a fish population for the purpose of health certification in connection with international trade of live fish and/or their products (OIE, 2003).

DNA-based methods have been used in

diagnosis and for detection of many economically important viral pathogens of cultured finfish and shrimp. For finfish, tests have been developed for pathogens such as channel catfish virus (CCV), infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), viral hemorrhagic septicemia virus (VHSV), viral nervous necrosis virus (VNNV) and *Renibacterium salmoninarum*. PCR has been used in Japan to screen striped jack (*Pseudocaranx dentex*) broodstock for VNNV, permitting selection of PCR-negative spawners as an effective means of preventing vertical transmission of this pathogenic virus to the larval offspring (Muroga, 1997). DNA-based detection methods for detection of penaeid shrimp viruses are now used routinely in a number of laboratories around the world. These include probes for such diseases as white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hematopoietic and infectious hypodermal and haematopoeitic necrosis virus (IHHNV) and Taura syndrome virus (TSV) which pose the greatest threat to world shrimp culture production (Lotz, 1997).

By identifying molecular markers for disease resistance in fish, the efficacy of selective breeding programmes in aquaculture will be increased by allowing the maintenance of fewer fish, reducing time and cost of producing resistant brood stock, and minimizing the effort of monitoring stock resistance. The major histocompatibility complex (MHC) genes can also be targeted for selection. Functionality of MHC molecules is dependent on their structural polymorphism which, in turn, depends upon variability in the DNA which codes for MHC molecules. In a population of fish, thanks to a particular MHC molecule, some fish may be naturally resistant to a particular pathogen. By comparing the MHC genes of fish that are resistant/susceptible to the pathogen, using a process called oligo-screening, broodstock that possess only the resistant gene can be selected. Thus MHC genes are likely candidates for identifying markers associated with disease resistance.

Few studies have yet addressed the functional aspects of MHC molecules in fish. In rainbow trout, class I genes are expressed in all tissues, and class II are mainly expressed in lymphoid tissue (Hansen *et al.*, 1999b). MHC class II beta-chain haplotypes were found to be associated with differences in magnitude of immune response in fish (Wiegertjes *et al.*, 1996). Grimholt *et al.* (2003) evaluated the association between disease resistance and MHC class I and class II polymorphism in Atlantic salmon. Palti *et al.* (2001) claimed that MHC DNA polymorphism can be used in linkage and association studies of disease resistance in rainbow trout. Identification of MHC haplotypes composed of such polymorphism may provide a more powerful tool for analyzing associations between MHC and immunity to infectious diseases.

4. Choice of Markers

The increasing availability molecular markers provide a universally applicable and objective approach for the comparative analysis of species, population/stock and individual identity. However, there is a significant subjective element in the choice of markers. Thus the choice of marker will have a significant effect on divergence estimates obtained (Park and Moran, 1995; Carvalho and Hauser, 1999).

The genetic marker and method of analysis proposed for a study must be appropriately matched (Parker *et al.*, 1998; Sunnucks, 2000). Thus when choosing a genetic marker it is critical to consider: (i) the evolutionary time frame of the question being asked, (ii) the rate and mode of evolution of the genetic marker, and (iii) mode of inheritance (e.g., maternal, biparental) and expression (dominant, co-dominant) (Table 1). The fast rate of evolution will erase the phylogenetic history; in other words, the genetic divergence among populations results in virtually no shared alleles. Conversely, genetic markers with slow rates of evolution are inappropriate markers to resolve relationships among more recently isolated populations or recently diverged subspecies or species (e.g., 10,000-250,000 years). When dealing with questions of contemporary gene flow, population isolation, and recent speciation events, a highly variable marker with a fast rate of evolution can increase resolution significantly (Peacock *et al.*, 2001).

To date no single molecular technique has proven to be optimal for resolving major genetic concerns in fisheries and aquaculture stock management. Each technique has strengths and weaknesses generally based upon the equilibrium between repeatability, cost and development time and the detection of genetic polymorphism (Table 1 and 2). Main considerations in the choice of a marker can be summarized as (Sunnucks, 2000; Cross *et al.*, 2004):

- *Availability of samples*: Fresh or -40°C stored tissue samples are required for protein analysis. DNA can be extracted from most tissues. Particularly with PCR, there are much less stringent requirements in terms of sample quality. PCR can be performed on alcohol preserved samples or even, but with more difficulty, on dried samples like scales or otoliths.

- *Sensitivity*: A marker must have the correct sensitivity for the question. Among markers with suitable resolution, choices can be made on more pragmatic bases.

- *Availability of markers*: The markers of choice currently should be used being in the local laboratory or available from other laboratories.

- *Rapid development and screening*: Genetic markers have already been developed or can be transferred from earlier work, and the possibility of rapid screening can yield important savings in resources.

- *Multilocus or single-locus?* Usually, there is a trade-off between practicality and accuracy of genetic markers. One manifestation of this is the dichotomy between multilocus DNA techniques (usually RAPDs and AFLPs) and single locus techniques (microsatellites and scnDNA). Multilocus approaches are technically convenient, but have some marked weaknesses and limitations, e.g., most of the variation detected non-heritable. A fundamental limitation is dominant inheritance. Thus single-locus markers are far more flexible, informative and connectible, because they can be analysed as genotypic arrays, as alleles with frequencies and as gene genealogies. Since fisheries scientists often need high-resolution genetic markers, the most useful techniques are those that produce a large number of alleles at a single locus and/or many loci with two or more common alleles (Sunnucks, 2000).

- *Relative costs*: The relative cost of development and use of different markers should be considered. It is sometimes asserted that multilocus techniques are more economical, but this is doubtful, especially per unit information. However, single-locus markers are even more economical when the value of comparing data sets is considered.

- *Organelle and nuclear DNA*: As it has been mentioned previously mtDNA has a lower population size than nuclear markers, and consequently mtDNA variants become diagnostic of taxa more rapidly. Comparison of nuclear and mitochondrial genotypes can help recognize hybrid individuals, asymmetrical mating preferences and stochastic effects on variants for which ancestral taxa were polymorphic.

The choice of a particular DNA technique usually involves a series of trade-offs. Some techniques are easily implemented with little or no prior information, but are more difficult to interpret than other methods. Methods that target mtDNA are relatively easy to develop and interpret, yet the information derived represents only a single locus. Other classes of markers provide more information from more loci, but involve extensive cloning and sequencing, and therefore substantially more lead time. Another trade-off is in the development of non-destructive markers. Unfortunately in most cases fish have to be sacrificed, at least in early stage (larva and juvenile). This might be very critical for species of special concern where lethal sampling may not be possible. Research on these species requires some non-destructive PCR-based method that allows sampling of a few scales or minute fin clips, but again, this requires more sequence information and more development time. In case of limited funding, careful choices must be made, and the value of a particular technique should not be considered independent of its application (Moran, 1994).

Allozymes are the most widely used method in fish (Laike, 1999). In spite of new molecular genetic techniques protein electrophoresis must still be considered a very valuable tool. Extensive reference

Table 1. Major attributes of molecular markers commonly used in fisheries and aquaculture genetics (Park and Moran, 1995; O'Connell and Wright, 1997; Parker *et al.*, 1998; Sunnucks, 2000).

Marker	General ease	PCR	Expression	Loci	Genome	Overall Variation	No. loci readily available	Comparison of data among studies
Allozymes	electrophoresis; analysis straightforward	No, protein	Co-dom	Single	~Nuclear	Low	Moderate	Direct
mtDNA RFLPs	extraction, digestion, electrophoresis, gel blot hybridization; analysis straightforward	No	Varies /Co-dom	Single	Organellar	Variable (Low-moderate)	Single	Direct
mtDNA sequence	extraction, PCR, prepare DNA template, electrophoresis; analysis straightforward	Yes	Varies /Co-dom	Single	Organellar	Variable (Low-high)	Single	Direct
RAPDs/AFLPs	extraction, digestion, electrophoresis, gel blot hybridization; analysis problematic but developing	Yes	Dom	Multilocus	~Nuclear	High	Many	Limited
Multilocus mini-and/or microsatellite 'fingerprints'	extraction, digestion, electrophoresis, gel blot hybridization; analysis problematic	No	Dom	Multilocus	Nuclear	High	Many	Limited
Nuclear RFLPs	extraction, digestion, electrophoresis, library screening, gel blot hybridization; analysis straightforward	No	Co-dom	Single	Nuclear	Variable	Many	Direct?
VNTRs: Minisatellites	Mostly similar to microsatellites	Few	Co-dom	Single	Nuclear	High	Moderate	Indirect
VNTRs: Microsatellites	Primer development may be long (library screen, sequencing); extraction, PCR, electrophoresis; analysis straightforward	Yes	Co-dom	Single	Nuclear	High	Many	Indirect
Anonymous scn	Similar to microsatellites	Yes	Co-dom	Single	Nuclear	Moderate?	Many	Indirect

Table 2. Evaluation of molecular markers with regard to practical applications in fisheries and aquaculture (Park and Moran, 1995; O'Connell and Wright, 1997; Parker *et al.*, 1998; Sunnucks, 2000).

Marker	Tissue requirements	Sacrifice of specimens	Major applications				
			Population structure	Stock/strain identification	Individual Identification	Parentage and pedigree analysis	Gene mapping
Allozymes	Stringent	Often	Moderate/High	Moderate	Low	Low	Low
mtDNA RFLPs	Moderate	Often	Moderate/High	Moderate/high	Inappropriate	Low/Moderate	Low
mtDNA sequence	Relaxed	No	Moderate/High	Moderate/high	Cumbersome	Moderate	Low
RAPDs/AFLPs	Relaxed	No	Low	Low	Moderate	Moderate	Moderate-RAPD High - AFLP
VNTRs: Multilocus minisatellite 'fingerprints'	Stringent	Yes/No	Low	Moderate	Moderate	High	-
VNTRs: Minisatellites	Stringent	No	High	Low	Moderate/high	High	High
VNTRs: Microsatellites	Relaxed	No	High	High	High	High	High
Anonymous scn	Relaxed	No	High	High?		High	-

data sets are available for allozymes, allowing comparisons between samples collected from populations separated in space and/or time.

mtDNA has proven useful for identifying major evolutionary lineages (Bernatchez *et al.*, 1992). In addition it can be used to track demographic features exclusively for the female proportion of a population (Laikre *et al.*, 1998). Taking the 'phylogeography', for example, some 70% of studies carried out have used mtDNA (Avisé, 1998).

Microsatellites have enabled the assessment of genetic variations at much smaller scales than has been possible with other markers (Parker *et al.*, 1998; Sunnucks, 2000). The amount of genetic variation found at these loci has increased the power to resolve relationships between individuals, as well as between populations and closely related species. They are optimal for mapping "causal" genes, whether these are responsible for single or multifactorial traits (QTLs). They are also the best markers for determining parenthood in mass spawning and/or rearing (Colbourne *et al.*, 1996; Morán *et al.*, 1996; Thomaz *et al.*, 1997; Thompson *et al.*, 1998), tracing escapes from contained to wild populations and estimating coefficients of kinship among individuals drawn from a population (Brunner *et al.*, 1998; Hansen *et al.*, 2001a). Statistical tests that are particularly suitable for mini- and microsatellites have been developed for detecting recent population bottlenecks (Goldstein *et al.*, 1995; Slatkin, 1995; Raymond and Rousset, 1995; Goudet, 1995). Their basic drawback remains the high cost and labour-intensity during the first phase of the technique, i.e. the development of primers. Another disadvantage is the existence of null alleles that are alleles that do not amplify in PCR reactions (O'Reilly and Wright, 1995).

In fish population genetics there is a large potential in establishing coordinated databases containing DNA sequence data and, in particular, allelic frequency data. Unfortunately when genetic databases are considered some genetic markers may not be suitable for such purpose. In general, the banding patterns obtained by RAPDs, AFLPs and multilocus fingerprinting are probably too complex to allow for comparisons of results among laboratories. The most suitable and relevant genetic markers for genetic databases are allozymes (currently a large amount of allozyme data for many species exist), microsatellites, DNA sequences (both nuclear and mtDNA), RFLP (PCR - amplified segments) and SNPs.

5. Concluding Remarks

The main purpose of this review was to provide an overview of molecular markers widely used in fisheries and aquaculture. The questions being tried to answer were: what are they? What are main advantages and disadvantages? Where can they be used? These rapidly developing markers can identify closely related species, populations/stocks, genetic

strains, families and individuals. Thus, it is becoming increasingly important for fisheries and aquaculture stock managers to understand and evaluate genetic data.

We have described the types of molecular markers that seem to be suited for different levels of applications. Unfortunately the information one needs to begin using a new technique is not fully reported in journal articles, but rather exists in the written or oral traditions of different laboratories. However, it is not possible to describe the details of these techniques in such review. Thus extensive recent references have been provided. It is apparent that no one molecular marker is superior for all common applications and it is not possible to say that no variation exists, on the basis of evidence from one or more markers. Unless the entire nuclear and mitochondrial genome is considered, this conclusion cannot be reached. Unfortunately increasing the number of alleles per locus does not always increase the probability of detecting significant differences. Thus it is recommended that at least two markers, mitochondrial and nuclear, should be utilised for each case. Once again which technique is most appropriate for a particular situation depends upon the extent of genetic polymorphism required the analytical or statistical approaches available for the potential techniques and the pragmatics of time and costs of materials. Finally, for routine applications in fisheries and aquaculture it may be better to focus on markers that are in widespread use by the scientific community rather than trying to develop novel markers. This is because the methodology for markers in widespread use will already have been optimised.

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