

Application of DNA-Based Markers in Stock Enhancement Programs

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Introduction

Aquaculture and fisheries management require tools for identifying individuals or groups of aquatic organisms for the purpose of monitoring performance (growth, survival and behavior) and stock structure. In aquaculture research, commercially important traits of tagged individuals are assessed to generate supportive data for selective breeding, genetic improvement and commercial-scale fish-farming. Fisheries management employs identification systems for the evaluation of stock abundance, population dynamics and documentation of wild and hatchery-bred stocks. Stock structure analysis is useful in the planning and implementation of sound stock management and more importantly, in stock enhancement programs. Blankenship and Leber (1995) underscored the inclusion of tagging/marking strategies for released hatchery stocks in the guidelines for responsible marine stock enhancement. Identifying and keeping track of introduced stocks in release habitats allows an assessment of their adaptability in the wild (Allendorf et al 1988) and the success of the reseeded and/or restocking effort.

Although often used interchangeably, the terms “tags” and “markers” differ by definition. Tags are artificial or synthetic materials that are attached to the aquatic organism to allow individual or group identification while markers are traits or characters either applied or inherent to the organism (Thorsteinsson 2002). Tags/markers are essential in evalua-

ting resource distribution patterns, behavior, migration and movement of stocks, dynamics of exploited aquatic populations and evolutionary processes, all of which comprise baseline information for any stock management, enhancement and conservation program in aquaculture and fisheries (Allendorf et al 1988, Mulvey et al 1998).

Tags and Markers

Before choosing an ideal marker or tagging method, the program or research objectives should be clearly defined. The type of identification system should suit the purpose for which the tags are used. The species, size and number of organisms that will be tagged or marked, should also be known as some tags are size- and/or species-specific. Preliminary studies on the different types of identification techniques should be conducted to assess their performance and reliability. Through these preliminary trials, the preferred mode of attachment, duration of handling and effect of tagging on the identified organism can be determined. Tagging/marking should be done within a short period to avoid adverse effects on the tagged organism. Fouled tags and even the presence of some tagging/marking materials have been found to affect the health, behavior and growth of the identified animal (Thorsteinsson 2002). Finally, one should also take into account the cost of tagging/marking. A cost-benefit analysis should be conducted before finally deciding on a tagging/marking method that is cost-efficient and reliable.

Tag/marker types, advantages and limitations

There are four types of tags/markers used in fisheries research and these are – external tags, external markers, internal tags and internal markers (Thorsteinsson 2002, AFS et al 2004).

External tags

These are externally applied, visible tags which carry an individual and/or batch code (AFS et al 2004). Sometimes external tags also carry instructions on how recaptured organisms should be reported. Many of the conventional tags are classified as external tags. Examples are the ribbon, wire or spaghetti tags, Petersen disk and plates. Dangling tags (flag tags or Carlin tags), hydrostatic tags (anchor tags, floy T-bar tags and arrow tags), internal anchor tags, strap tags and jaw tags are also some examples of external tags. The internal anchor tag is a tag that is implanted in the body cavity of the animal but part of the tag hangs like a floy tag that is visible from the outside as well.

External tags are inexpensive and are therefore cost-effective. They are very detectable and can be applied to a large number, size and range of species. Apart from the number code, external tags also carry relevant reporting instructions. Many of these tags remain attached to the animal for a long period of time, hence the possibility of providing a large number of returns is high. External tags are ideal for providing information on broad geographical and seasonal return distribution.

Although the tags carry batch or individual code and reporting instructions, no data between release and recapture of the restocked individual can be obtained from the tags. Another disadvantage of the external tags is that their mere presence affects

the animal, as reviewed by Thorsteinsson (2002). Long-term use can likewise expose the tag to fouling by macroalgae and attachment of other debris. Fouling makes the tag heavier, slowing down the movement of the animal. High tag loss or shedding is also possible due to fouling thus making this tagging method unreliable. Another limitation of external tags is that some may not be applicable to small animals.

External marks

External markers are external characters or modifications that are found on or applied to the organisms for identification purposes (AFS et al 2004). Some types of external marks have been used in studies related to taxonomy and systematics. Examples of these are the meristic (e.g., number of fin rays, spines, teeth) and morphometric characters (truss patterns, length measurements, scale shape, etc.). Some physical characters are modified and used as external marks. Fin-clipping, pinching and operculum punching are typical examples of these external marks. Pigments, dyes, stains and brands (hot and/or cold branding) are also used as external marks.

Like external tags, external marks are inexpensive, easy to apply and useful in identifying separate populations or groups. They can be applied to any species, size and number of organisms. Unlike external tags, external marks have little, if any, effect on the tagged organism. External marks can also have a long retention time depending on the type of marker used.

Although external marks allow batch identification, the number of marking combinations and codes may be limited. For marks like fin clips, the clipped fins regenerate later and could cause confusion in identifying or discriminating marked organisms. Some external markers, especially paints or dyes, may also deteriorate with time. Given the problems

associated with external marks, recapture/return data from a broad geographical range may prove to be difficult.

Internal tags

These tags are injected or inserted into the body cavity, muscle or cartilage of aquatic organisms and are carried internally for individual or group identification (AFS et al 2004). Most of these tags require reading devices that will enable the detection of the implanted or inserted tags. Examples of these are coded wire tags (CWT), coded radio tags (CRT), coded acoustic tags (CAT), magnetic cavity tags, passive integrated transponder (PIT) tags, sonar transponding tags, transmitter tags, data storage tags (DST), and visible implant tags (VIT).

Internal tags allow individual identification and may be applied to any size and species of aquatic organism. They have a long retention time and have little effect on the growth, survival and behavior of tagged organisms. Likewise, repeated and non-destructive recoveries are possible. Using special fully automated equipment, a large number of individuals may be tagged at a single time.

Compared to external tags and marks, internal tags are costly. They require the use of detecting devices apart from trained people who apply the tags. Recovery of tagged specimens, retrieval and identification of the tags are labor intensive. Since the tags are kept inside the organism for a long time, tag migration within the organism is possible. As for some internal tags like VIT, the transparency of the tag may deteriorate through time. Moreover, internal tags do not carry sufficient information about the tagged organism.

Internal marks

Internal marks are marks that are either intrinsically found in the organism or are artificially produced (AFS et al 2004). These

marks characterize the organisms individually or collectively. Chemical/thermal marks on otoliths and other bony structures as well as biological markers (e.g., type of parasites found on specific groups or individual aquatic organisms) are considered as internal markers. Other examples of internal marks are externally detectable elastomer marks that are injected into the organism. With the recent introduction of the polymerase chain reaction (PCR) technology and advances in molecular genetics, DNA-based genetic markers have been developed and used as internal markers.

Except for genetic markers, most of the internal marks are inexpensive, simple and readily applicable to a wide range of animal sizes. Some of the marks are visible for a long period and are repeatedly recognizable without causing any damage to the organism. Individual and group identification is possible with internal markers since one can use of a wide array of colors (especially for chemical marks) and marking positions.

Although internal markers keep for a longer period in the organism, the transparency of the marks may change through time, become less visible, and thus can be easily overlooked. In some cases, marker injection tools are required and special devices are necessary for marker detection.

Conventional markers like physical tags are commonly applicable in a) estimating growth in farmed and natural populations of aquatic organisms, b) evaluating survival, migration and behavior of restocked organisms, c) calculating recapture rates, d) comparing tag reliability and performance, and d) monitoring introductions, stock transfers and species conservation. These tagging/markings methods are efficient only when tagging losses and recovery errors are minimized. If tagging problems are not addressed, these methods might find limited use in population or stock structure analysis.

Genetic Markers

Traditional tags such as spaghetti and floy tags have been used for decades in both fish culture and stock management. Several types have evolved since then – from the very crude simple ribbon or wire tags to recent applications such as biochemical or genetic markers. Physical tags carry very limited information. More advanced markers such as genetic markers not only provide individual or batch identification, but also describe the genetic information of each marked individual and in general, the genetic structure and integrity of the stocks being monitored (Allendorf et al 1988, Ward and Grewe 1994, Mulvey et al 1998, Sweijd et al 2000).

Genetic markers are selectively neutral markers. They are biochemical attributes that remain as discrete character units or combinations of such units that are detectable as protein or deoxyribonucleic acid (DNA) variants (Benzie 1994). Genetic markers are indelible, present in all members of a population at all ages, and can be used to determine the source and relationships (parentage and kinship) among aquatic organisms (Pella and Milner 1987, O'Connell and Wright 1997). In this sense, genetic markers are unique and are able to discriminate between individuals and groups when traditional morphological differences are unclear (Sweijd et al 2000).

To fully understand what genetic markers are, we recall some basic concepts in genetics. Each cell in any organism contains chromosomes. Chromosomes are made up of long DNA molecules that are complexed with protein. Each DNA molecule has many genes that are the basic physical and functional units of heredity. A gene is a specific sequence of nucleotide bases (adenine, guanine, cytosine and thymine). Alleles are biochemically different forms of the gene while a locus is the specific location of a gene on the chromosome. The order of nucleotide

bases along the sugar-phosphate backbone of DNA is referred to as the DNA sequence. Sequences carry information for constructing proteins that provide the structural components of cells, tissues and enzymes for essential biochemical reactions. Hence by definition, the DNA sequence specifies the exact genetic instructions for creating a particular organism each with its own unique set of traits. In developing DNA markers, DNA sequence information is a prerequisite. With the use of modern molecular genetics equipment like the PCR or thermal cycler, electrophoretic apparatus and DNA sequencer, DNA profiling and genetic marker development have been made possible.

Genetic markers can be classified into: a) maternally inherited mitochondrial DNA (mtDNA) markers, and b) biparentally inherited nuclear DNA markers. MtDNA sequence data and mtDNA-restriction fragment length polymorphism (mtDNA-RFLP) are classified as mtDNA markers while allozymes, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellite DNA (msDNA) markers are nuclear DNA markers.

Two of the commonly used markers are mtDNA-RFLP and msDNA. MtDNA analysis is particularly useful for genetic marking as applied in gene conservation or the use of genetic information to measure and manage genetic diversity, and for molecular ecology (Moritz 1994). MsDNA markers are markers that follow a Mendelian codominant inheritance pattern (e.g., the two alleles in a gene pair, each associated with a different phenotypic substance or expression, appear together in a heterozygote). MsDNA markers are effective tools for the assessment of genetic divergence and pedigree analysis in the broodstock management of aquatic species (Taniguchi 2003). These two methods essentially follow similar protocols.

Genetic marker methods are non-invasive unlike physical tagging/markings. Molecular marker analysis can be carried out on fresh, frozen or alcohol-preserved tissue (e.g., scales, fin clips, pleopods) from the aquatic organism. Small tissue samples (from at least 30 individuals per stock) are collected and preserved. DNA extracted from each sample are PCR amplified using a thermal cycler and later processed for either mtDNA or msDNA analysis. MtDNA-RFLP marker analysis is a method where genomic DNA is cut with restriction enzymes and the products are separated by size through agarose gel electrophoresis. The presence or absence of multiple restriction sequences around a given DNA region represent haplotypes (Silva and Russo 2000). These haplotypes are scored from fragments that are visualized as bands on stained agarose gels. Genetic marker variability data are based on the scored haplotypes.

Meanwhile, microsatellite DNA are short stretches of DNA composed of tandemly repeated arrays of di-, tri- or tetranucleotides (Wright and Bentzen 1994). Changes in each microsatellite locus are usually noted as insertions or deletions during DNA replication or by recombination between DNA molecules (Goldstein and Scholterer 1999). These allele length polymorphisms that result from changes in the number of repeats are quantified by sizing PCR-amplified copies of the DNA on a polyacrylamide gel (Stepien and Kocher 1997). MsDNA on the gel are blotted and hybridized onto nylon membranes. Genetic marker data based on msDNA variation is obtained from scoring banding patterns visible on nylon membranes that have been earlier stained through a chemoluminescent method. MsDNA markers exhibit high levels of genetic variability. These markers have the potential to isolate large numbers of gene loci and therefore provide a marker system that can detect differences even among closely related populations (O'Connell and Wright 1997).

Genetic markers – uses, advantages and limitations

In stock enhancement programs, genetic markers are extremely useful in marking stocks, studying the population structure of threatened or endangered species (Ward and Grewe 1994), measuring genetic differences and changes within and between released hatchery-bred stocks and wild populations, determining the fate of reared animals after deliberate or inadvertent release in the wild (Cross 2000), and identifying the presence of intraspecific hybrids (between wild and hatchery stocks) in the release habitat. They are also generally useful in studies on fish populations especially in delineating the relative roles of microevolutionary forces that shape population structures (Sweijid et al 2000).

Genetic markers are heritable, stable and are non-invasive hence they do not affect the growth, health and behavior of the organism. Unlike physical tags or markers that should be applied prior to the release of tagged individuals in the wild, genetic marker information can be obtained from the individual organism even after they have been released and recaptured. Genetic markers therefore provide a means of tracking stocked animals without necessarily handling them individually before release. How is this done? In stock enhancement, the parental stocks that are used to breed the released hatchery stocks are genetically documented using molecular markers. The hatchery-bred individuals are then released and allowed to grow in the natural habitat. After some time, samples from stocks in the natural habitat are collected and the individuals are genetically marked and their genotypes compared with the parental stocks to trace the pedigree of both the wild and released hatchery stocks. This method is referred to as parentage analysis or pedigree tracing.

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Although use of genetic markers present several advantages, there are also limitations to the application of genetic markers. When the level of detectable genetic variation within and between stocks is small and negligible, genetic marker methods may not be suitable. Furthermore, genetic marker analysis is quite costly and requires technical expertise.

DNA marker analysis data

DNA sequence information can be determined from individual organisms using modern molecular genetic methods and equipment. Several parameters (e.g., allele frequency and levels of heterozygosity) that measure genetic variation can be inferred from DNA marker analysis. These indices of genetic variability can be measured at the individual, stock and species levels. The inbreeding coefficient within a particular stock or population and through subsequent generations can be inferred from DNA markers, particularly through microsatellite marker analysis. Based on data generated from genetic or DNA marker analysis, stock structure analysis is possible, genetic diversity can be conserved and other genetic concerns can be addressed if all these genetic variability indices are known.

Genetic Markers in Stock Enhancement

The success of any stock enhancement program lies heavily on the assumption that released hatchery-bred stocks can adapt well and survive in the natural environment. Genetic diversity is an important component of adaptation and evolutionary success. A change in the genetic structure of a particular organism or group of organisms is equivalent to a change in the fitness or the ability of the population to thrive in the release habitat. Loss of genetic diversity often occurs in small populations with few founder stocks (such as hatchery stocks). It

is likely that the individuals in these populations are closely related to each other and when mated through generations, the probability of inbreeding becomes higher. Inbreeding causes inbreeding depression that may be expressed as poor fitness, apart from abnormalities and slow growth. Low genetic variability may thus result in poor adaptability or reduced fitness (Moritz 1994). Hence hatchery-bred stocks with low genetic diversity may not survive well in wild or natural habitats (Allendorf et al 1988). It is in this regard that genetic diversity should be conserved if only because genetic variability contributes directly to fitness-related traits. Genetic diversity or variability can be evaluated and monitored through genetic marker analysis. Hence this emphasizes the significant role of genetic markers in conservation and stock enhancement programs.

Genetic Concerns in Stock Enhancement

There are several genetics issues which must be fully understood and considered in any stock enhancement program. The evolution of genetic differences between wild and hatchery stocks is a concern. Hatchery-bred stocks genetically differ and sometimes fare poorly compared to wild stocks. Several studies have shown that some hatchery stocks suffer loss of genetic variation due to random genetic drift, domestication and inbreeding (Allendorf and Phelps 1980, Cross and King 1983, Doyle 1983, Ståhl 1983, Taniguchi et al 1983, Ferguson et al 1991).

Both domestication and inbreeding contribute to the continuous erosion of genetic variation within the hatchery stocks. How does this occur? The inbreeding coefficient is inversely proportional to effective population size or $F = 1/(2N_e)$ (Falconer 1981). The higher the effective population size, the possibility of inbreeding would be lower. In contrast to the actual population size (N)

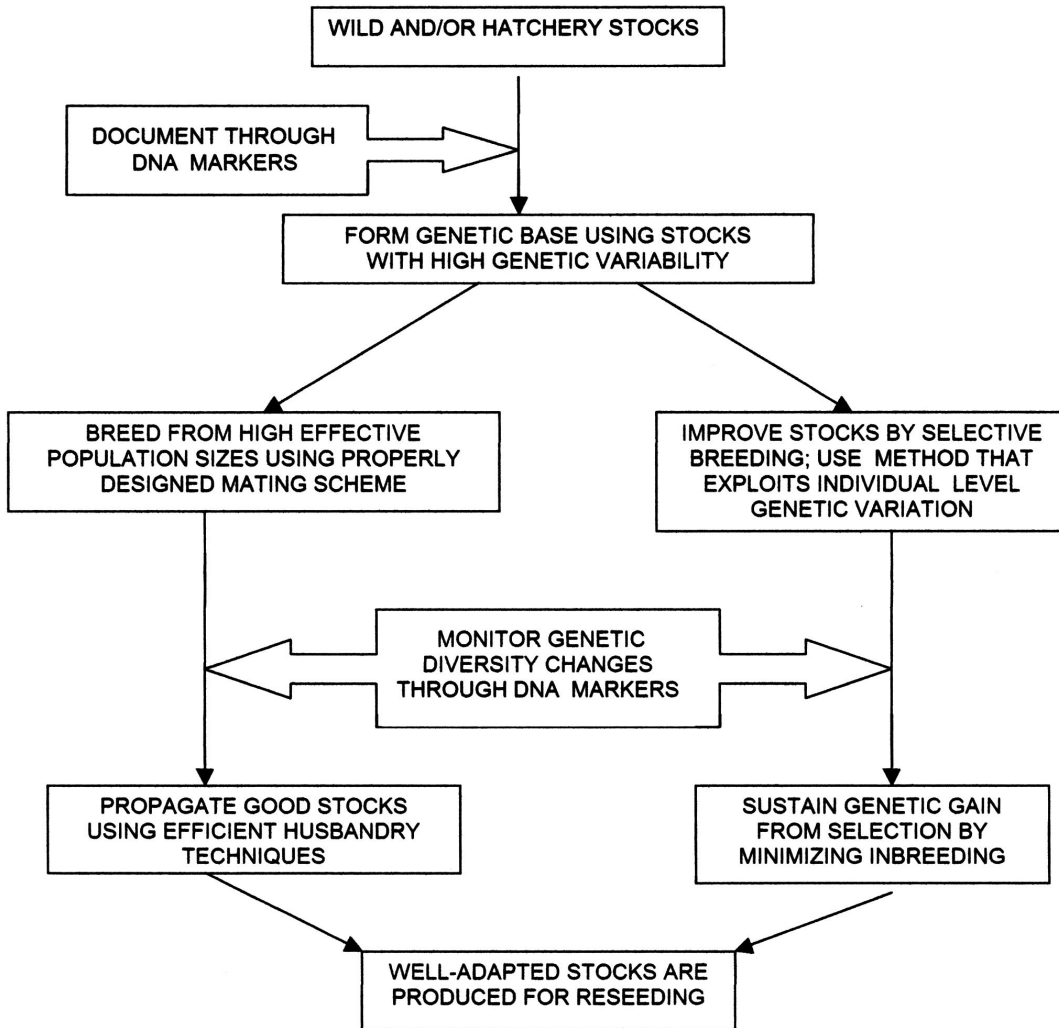


Fig. 1. Recommended scheme for the sustainable management of wild/hatchery stocks used for reseedling.

which is the number of male and female breeding individuals that are set up as broodstock to produce the next generation, effective population (N_e) is the total number of males and females that actually mate and contribute to the gene pool of subsequent generations. Fish breeding in the hatchery is normally based on few founder stocks. This alone already limits the number of effective females and males that mate and contribute to the next generation so the situation hastens the loss of genetic diversity in hatchery-bred stocks. The genes (and therefore the traits

that are expressed) present in the hatchery stock that are most suitable to hatchery conditions will increase in frequency (Allendorf et al 1988). The individuals possessing the so-called “hatchery-adapted” traits will fail to survive or grow well in the natural habitat.

This issue can be addressed by either a) increasing the effective population size in the hatchery that produces the seedstock for release in the wild (Taniguchi 2003) or by b) collecting wild broodstock for breeding in the hatchery and releasing their young in

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the natural habitat. The second method is known as supportive breeding.

A third main concern is the genetic “contamination” of the wild stocks in the area where stock enhancement efforts are being implemented. It is believed that if hatchery-bred aquatic organisms have low genetic variability, when these stocks are released in the wild, introgression between the highly variable wild stock and the genetically depauperate hatchery stocks would inevitably occur because of interbreeding. When gene introgression happens, there is concern that the frequency of nondaptive “hatchery” genes might increase (Allendorf et al 1987). The occurrence of interbreeding can be checked by examining the genetic diversity and structure of stocks found in the release habitat through genetic markers.

Recommendations

Figure 1 shows the recommended scheme for the sustainable management of wild/hatchery stocks used for re-seeding. In a stock enhancement program, both wild and hatchery stocks can be documented genetically through DNA markers. From this genetic database, stocks that have high genetic variability can be chosen and utilized as the founder stocks. If enough individuals can be used as founder stocks, a high effective population size would be desirable to use for breeding in the hatchery. Genetic changes are again monitored in the hatchery-bred offspring. The progeny are then reared onwards using efficient husbandry techniques. These purportedly well-adapted stocks can be used for reseeded purposes. On the other hand, if the size of the population is insufficient to provide a high N_e , the existing hatchery stocks can be further improved through selective breeding. These stocks are continuously monitored for genetic changes using DNA marker analysis. Monitoring genetic changes in stocks can help determine whether the aquaculture stocks can still be used in propagating seedstock for stock enhance-

ment. If the aquaculture stocks can still be used, selective breeding can be done while simultaneously adopting mating schemes that can minimize inbreeding. Well-adapted stocks may be chosen from the progeny and reared onwards for use as seedstock in stock enhancement activities.

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