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REPORT OF THE

WORKING GROUP ON THE APPLICATION OF GENETICS IN FISHERIES AND MARICULTURE

Bergen, Norway
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International Council for the Exploration of the Sea

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1 INTRODUCTION

As decided in C.Res. 2F03, adopted at the 2000 Annual Science Conference in Bruges, Belgium, the Working Group on the Application of Genetics in Fisheries and Mariculture (WGAGFM; Chair: M.M. Hansen, Denmark) met at the Institute of Marine Research, Bergen, Norway, 26–28 March 2001 to deal with its Terms of Reference for 2001 (Annex 1).

1.1 Attendance and Meeting Place

Fifteen persons representing eleven countries attended the 2001 WGAGFM meeting in Bergen (Annex 2). Countries represented (number of persons in parenthesis) were Belgium (1), Canada (1), Denmark (3), Finland (1), Germany (1), France (1), Iceland (1), Ireland (1), Norway (2), Spain (1) and UK (2). As for the seven previous years, the representation on the quantitative genetics was lower than on the qualitative genetics side.

The Institute of Marine Research (represented by Dr Geir Dahle) offered excellent logistics and facilities for the first part of the WGAGFM meeting. The second part of the meeting took place on a ship going from Bergen to Trondheim, sailing along the Norwegian coast; a breathtaking scenery! The logistics and facilities on board were also excellent and this rather unorthodox setting for the meeting turned out to be a tremendous success. The Working Group wants to express its gratitude to Geir Dahle and colleagues for this excellent idea and for all the work undertaken to arrange this meeting, both on land and at sea. The final part of the meeting took place at the Biological Station, Technical University of Trondheim, where the former WGAGFM Chair, Prof. Jarle Mork, was our host and had arranged an enjoyable and informative meeting; we are also very grateful to him for his efforts and kind hospitality.

1.2 Working Form

Prior to the meeting, small *ad hoc* working groups, with one main responsible person, had been established to prepare position papers related to specific issues on the Terms of Reference, and to chair the respective sessions. As in the previous year, the Chair asked the groups responsible for preparing position papers to send him electronic versions of the papers prior to the WG meeting. Most of the position papers were subsequently distributed to the meeting participants some days before the meeting. This enabled the participants to read through the papers before the actual presentation of papers at the meeting. During the meeting, the position papers were first presented and discussed in plenary. Thereafter, each topic was discussed in *ad hoc* sub-groups. Position papers were updated according to points raised in the plenary and sub-group discussions and were finally edited and included in the WG report. As a result of the new procedure, based on group work rather than having individual persons working alone on a ToR, in some cases more than one position paper was produced for each ToR, dealing with different aspects of the same issue.

M.M. Hansen chaired business and general scientific sessions (ToR point a);

E.E. Nielsen chaired ToR b: Review and report on new developments in the identification of genes of relevance to aquaculture and studies of wild populations;

E. Kenchington and M.M. Hansen chaired ToR c: Review and report on the importance of different kinds of genetic population structure in relation to human impact;

M.-L. Koljonen and M.M. Hansen chaired ToR d: Review and report on methods for estimating effective population sizes and/or changes in effective population sizes in anadromous and marine fish populations;

D.E. Ruzzante chaired ToR e: Review and report on examples where population genetics research has provided important information for the management of marine fish populations.

The session chairpersons were responsible for leading the plenary sessions and group work, and (in collaboration with their respective *ad hoc* working groups) for preparing the final report text from their sessions. All members were asked to collect national activity reports from their respective countries in advance and send them by e-mail to the Chair for inclusion in the Report. The WGAGFM decided that, as in the five previous years, the preparation of the WG Report should be done mainly by the members present at the meeting. A preliminary version of the Report was made available on the (external) WGAGFM homepage for final comments by members before submission to the ICES Secretariat.

2 TERMS OF REFERENCE FOR 2001

2.1 General Population Genetic Topics Related to Fisheries and Mariculture

This session was scattered throughout the meeting, and served mainly to identify topics for the Terms of Reference for the year 2002.

2.2 Review and Report on New Developments in the Identification of Genes of Relevance to Aquaculture and Studies of Wild Populations

Based on a position paper by Einar Eg Nielsen, Peter Bossier and Pierre Boudry, adopted by WGAGFM in Bergen 2001.

Introduction

The rapid development of molecular biological tools during the last couple of decades has opened a wealth of new opportunities for studies of genetic variation at the DNA level. For many organisms, large-scale genome projects have been initiated, which have resulted in the identification of the location and DNA sequence for many gene loci. Also, for finfish and shellfish a number of large-scale gene identification or mapping projects have been initiated (Table 2.2.1) and many more will probably follow. These projects are likely to revolutionise the aquaculture industry by providing means for faster and more effective selection programmes and increasing the potential for production of new and more efficient fish and shellfish GMOs. These methods also have many potential spin-offs for the study of genetic variation in natural populations. First of all, the new knowledge of the DNA sequence of selected loci will allow us to take population genetics "one step further" and permit us to study the mechanisms and fate of local adaptation at the gene level, not only on a spatial but also on a temporal scale, by applying analysis of historical samples. In this paper, we review methods, identified genes and current and potential use of these genes or classes of genes for aquaculture and for population genetic studies in the wild.

Table 2.2.1. Genome mapping projects for major aquacultural species (partly from Davis and Hetzel, 2000).

Species	Reference
Salmonids (salmon) SALMAP	Lie *et al.* (1997)
Rainbow trout	Young *et al.* (1998)
Tilapia	Kocher *et al.* (1998)
Channel catfish	Liu (1999)
Kurama prawns	Moore *et al.* (1999a)
Black tiger prawns	Moore *et al.* (1999b)

What is a selected gene?

Since most genes currently under study are genes under selection, it is justified to look more closely at some definitions and forms of selection before proceeding. To define what a selected gene is seems intuitively easy. However, in the scientific literature ever since the days of Darwin there has been much argument and confusion as to what "natural" (and "artificial") selection is. Following the definition by Endler (1986), natural selection (and thereby selected genes) can be defined as a process in which:

If a population has:

a) Variation among individuals in some attribute or trait: *variation*;
b) A consistent relationship between that trait and mating ability, fertility, fecundity and/or survivorship: *fitness differences*;
c) A consistent relationship, for that trait, between parents and their offspring, which is at least partially independent of common environmental effects: *inheritance.*

Then:

1) The trait frequency distribution will differ among age classes or life-history stages, beyond that expected from ontogeny;
2) If the population is not at equilibrium, then the trait distribution of all offspring in the population will be predictably different from that of all parents, beyond that expected from conditions a), and c) alone.

This definition of natural selection, which can be readily used for artificial selection as well, will be used throughout this paper.

Types of traits and modes of selection

Basically, two different classes of traits under selection are recognised. Continuously varying "quantitative traits" and discontinuously varying or "polymorphic traits". Both types of traits can be affected by different modes of selection, which needs to be considered.

a) **Directional selection**. Individuals towards one end of the distribution of the trait are favoured. In breeding practice a special case occurs which is denoted *truncation selection*, where individuals above (or below) a certain threshold level are selected/removed from the population. The trait mean will change and the variance may decrease.
b) **Stabilising selection**. Intermediate individuals do better than the extremes. The variance, but not the mean, will change.
c) **Disruptive selection**. Individuals with extreme trait values do better than intermediate. Could be caused by density-dependent or frequency-dependent selection.

Why study selected genes?

In aquaculture:

"The ultimate aim of a genetic improvement programme is to improve profit" (Davis and Hetzel, 2000). This will ultimately affect the choice of breeding objective, which means that biological traits related to income and expense will be the objectives for selective breeding. For example, income is related to growth rate and survival during the main growth phase. Likewise, expenditure is related to traits such as feed efficiency and disease resistance. This can be illustrated by looking at the breeding goals for one of the world's oldest and largest fish breeding programmes, the Norwegian breeding programme for Atlantic salmon (Table 2.2.2). This programme started in 1975 with the goal of improving growth rate (Gjøen and Bentsen, 1997). Since maturation after one year in the sea was a problem, selective breeding for late maturation was soon included in the programme. Following the large boom in salmon farming in Norway, with the resulting large number of fish and facilities, disease became a major problem for the industry, and their disease resistance was included in the breeding programme in 1993. As production capacity worldwide reached levels that fulfilled market demands, flesh quality became the major issue and has been recently incorporated (in terms of flesh colour and body composition).

Table 2.2.2. Breeding goals in the Norwegian breeding programme (from Gjøen and Bentsen 1997).

Year	Trait
1975	Growth (G)
1981	G + Age at sexual maturation (SM)
1993	G + SM + Disease resistance (DR)
1994	G + SM + DR + Flesh colour (C)
1995	G + SM + DR + C + Body composition

This programme, like many others, has been based on traditional breeding principles, i.e., estimation of breeding values of individuals from phenotypic value or by using information from all relatives of the breeding candidate. However, to be able to breed more effectively, location and characterisation of the genes responsible for the variation at selected traits will be a giant leap forward. This holds for both polymorphic and quantitative trait loci. Characterisation of molecular variation at single polymorphic gene loci or genes with major effects on the trait value will enable breeders to target the selection to allelic variants of the trait of interest. Even identification of genes that are invariable within one species can be of potential interest to aquaculture (and other industries), if the gene, when inserted into another genetic background (another species) as a transgene, leads to a change in the value of the trait of interest. For quantitative loci

(QTL) the determination of the location of a series of loci with varying effects on the selected trait can enable breeders to do marker-assisted selection (MAS), that is, the use of gene markers (microsatellites or AFLPs) linked to QTLs in genetic improvement programmes. MAS will have most application for traits that are difficult and expensive to measure and for traits that can be measured only after selection decisions are made (Meuwissen and Goddard, 1996). Several studies have indicated that integrating MAS can improve selection response (Davis and DeNise, 1998). Further, by knowing the position of the locus, the DNA sequence of the gene can eventually be determined by using standard techniques, such as "chromosome walking" (Rosenthal, 1992).

In the wild:

The increasing number of selected genes identified in finfish and shellfish have many applications in wild populations. By studying selected genes it is possible to link genetic differentiation in terms of non-coding variation with what actually "counts" in space and time, i.e., adaptive variation. For elucidating basic evolutionary processes, studies of selected genes in fishes are particularly promising. Many species show a high degree of population differentiation when studying non-coding loci, which in theory should allow local adaptations to evolve if the selection intensity for the local adaptation is stronger than migration among populations and random genetic drift (Haldane, 1930; Endler, 1986). Local adaptation has been suggested for many finfish and shellfish species. The largest number of apparent cases have been reported for salmonids (Adkison, 1995). Examples can be found also for marine fish (e.g., Nissling and Westin, 1997) and invertebrates (e.g., Johnson and Black, 2000). However, demonstrating selection in the wild is not easy (e.g., Endler, 1986). Generally, the genetic basis of the traits studied are unknown (condition *c* has not been demonstrated) and the so-called "adaptations" could be merely a reflection of phenotypic plasticity. By identifying polymorphic loci for many traits varying in natural populations, it is possible to gain much insight into the frequency of occurrence and spatial scale of these adaptations.

What do we know about selected genes in aquaculture

Fish

A number of fish enzyme loci have been cloned for use in transgenic organisms. The main reason for this is that fish enzymes generally function at much lower temperatures and at a wider temperature range than their mammalian counterparts and therefore are applicable to industrial enzyme production using microorganisms. The method of identification has in most cases been based on the known sequence of the gene of interest in other organisms, following which primers for sequencing the fish gene have been developed. Additionally, the need to improve production of GMOs in aquaculture has led to the characterisation of many loci in fishes, since fish genes work better in a fishs' genetic background. Further, sex-linked genes have been of great interest for the identification of sex in aquaculture species, which is of major interest due to the different growth characteristics of the two sexes that are often observed. Finally, with the development of linkage maps for several species, QTLs related to traits of major importance for the industry have been identified. For all classes of genes there is much research going on at the moment, so the list in Table 2.2.3 should not be viewed as exhaustive, but merely as examples of the main focus areas.

Table 2.2.3. Examples of gene loci identified for fishes and crustaceans.

Gene	Species	Function
Enzymes[1]*:*		
Transglutaminase	Sea bream	Acyl transfer catalyst
Cytochrome P450c17	Dogfish shark	Synthesis of sex steroids
Trypsin	Atlantic salmon, shrimp, (*P. vannamei*), Atlantic cod, *P. magellanica*	Protease
Antifreeze proteins[1]	Winter flounder Sea raven	Reduction of ice crystal formation within cells
Hormones[1] Growth hormones[1]	Salmon, eel, trout, tilapia, Yellow-tail flounder, Carp, sea bream, bass, yellow-fin porgy	Somatic growth, maintenance of protein, lipid, carbohydrate and mineral metabolism
MHC[2] (Class I and Class II genes)	Many species of teleostean and cartilaginous fishes. (e.g., Salmonids, Cyprenids, channel catfish, medaka, cod)	Immune response
Sex-linked genes[3]	Pacific salmon	Sex identification

Gene	Species	Function
QTLs		
Growth[4]	Kuruma prawn	
Spawning time[5]	Rainbow trout	
Upper temperature tolerance[6]	Rainbow trout	
IHN virus resistance[7]	Rainbow and cutthroat trout	

[1]Cloned for the production of GMOs (for references see Macouzet *et al.,* 1999).
[2]Selected references: McConnell *et al.,* 1998; Stet *et al.,* 1998
[3]Devlin *et al.,* 1991; Nakayama *et al.,* 1999
[4]Moore *et al.,* 1999a
[5]Sakamoto *et al.,* 1999
[6]Danzman *et al.,* 1999
[7]Palti *et al.,* 1999

Bivalves

Compared to fish, little is known about shellfish genes. This is partly due to the fact that shellfish are invertebrate species and their genome has a much lower homology to "model species" than fish have (compared to "model" vertebrate species). To date, relatively few genes have been characterised in shellfish species and, consequently, there is very little knowledge about their relevance to aquaculture and studies in wild populations.

Table 2.2.4. Non-exhaustive review of genes identified in bivalves of interest to fisheries and aquaculture.

Species	Gene	Information available	Reference
Mytilus coruscus	Mcfp1 : adhesive-plaque protein	cDNA	Inoue *et al.,* 1996a
Mytilus galloprovincialis	Mgfp2 : adhesive-plaque protein	cDNA, expression	Inoue *et al.,* 1995
Mytilus galloprovincialis	Mgpf-3 : byssal adhesive-plaque protein (= mefp3 ?)	cDNA, expression protein variants	Inoue *et al.,* 1996b; Warner & Waite, 1999
Crassostrea gigas	Gia1: Actin	cDNA , gene sequence	
Crassostrea gigas	Alpha- Amylase	cDNA	Moal *et al.,* 2000a
Crassostrea gigas	CgMT1: Metallothionein	Gene, cDNA	Tanguy *et al.,* in press
Pecten maximus	Amylase	cDNA, expression	Le Moine *et al.,* 1997
Mytilus edulis	MT-20, MT-10 IV : Metallothionein	cDNA, expression	Barsyte *et al.,* 1999; Le Moine *et al.,* 2000
Mytilus galloprovincialis	40S ribosomal (related to S27E)	cDNA	Snyder, 1999
Mya arenaria	Hsp53 and Hsp73	cDNA, expression	Kelley *et al.,* 2001
Mytilus galloprovincialis	Tropomyosin	cDNA, expression	Iwasaki *et al.,* 1997
Mytilus edulis	FMRF amide	cDNA, expression	Favrel *et al.,* 1998
Mytilus edulis	Ala-Pro-Gly-Trp amine	cDNA, expression	Favrel & Mathieu, 1996
Argopecten irradians	muscle myosin	cDNA, mutants	Goodwin, 1990; Nyitray *et al.,* 1994
Placopecten magellanicus	Tropomyosin, actin, Lim-protein	cDNA, expression	Patwary *et al.,* 1996, 1999, pers comm.
Argopecten irradians	GPI : Glucose phosphate isomerase	cDNA sequence	Krause, 1999

What do we know about selected genes in the wild?

Since the discovery of genetic markers, such as haemoglobins and allozymes for population genetic studies in the beginning of the 1960s (see Lewontin, 1991), there has been a long-standing debate concerning whether or not different alleles at these loci mainly represent neutral genetic variation or are subject to selection. The issue has not been resolved

completely (and probably never will be; see Endler, 1986), however, a series of studies have shown that in many instances different alleles at allozyme or haemoglobin loci are subject to selection. This is also the case for finfish and shellfish.

Fish

Karpov and Noikov (1981) showed that different haemoglobin alleles were associated with minimum winter temperatures for Atlantic cod (*Gadus morhua* L.). Similarly, Mork and Sundnes (1985) demonstrated significant haemoglobin allele frequency differences among year-classes of cod and assigned that to selection. For many allozyme loci it has been documented that allelic variants have different kinetic properties (Kirpichnikov, 1992). Genetic variation at these loci among wild fish populations has, therefore, been suggested to represent local adaptation (see Taylor, 1991). Temperature-dependent selection is among the causes most often suggested, i.e., as temperature varies across a species range, different alleles with different kinetic properties are favoured (directional selection). A classical example is the study by Verspoor and Jordan (1989) who demonstrated a correlation between summer temperatures and the frequency of alleles at the malic enzyme (Me-2) locus in salmon. Further, balancing selection at allozyme loci has been suggested (see e.g., Vrijenhoek, 1994; Pogson *et al.,* 1995). These types of studies have all had a major contribution to our understanding of selection in the wild. However, they all rely on the detection of selection among alleles distinguished by mobility differences on an electrophoretic gel.

The advantages of knowing the sequence of the gene coding for a specific protein are many. Primarily, it allows the detection of the mutations responsible for the apparent fitness differences among genotypes. Further, many types of population genetic studies are hampered severely by the fact that electrophoretic typing of proteins such as allozymes can only be carried out on specific tissues and therefore requires the fish to be killed and the samples to be frozen. This considerably limits sampling possibilities, especially from endangered populations. DNA-based techniques would enable routine biopsy sampling, ethanol storage of biopsies and temporal studies using archival material such as scales and otholiths. Very few DNA-based studies of selected genes have been carried out so far.

Shellfish

The impact of selective pressures on shellfish population genetics has been extensively debated. This was first generated by the frequently observed heterozygote deficiencies in marine bivalves (reviewed by Zouros and Foltz, 1984; Hare *et al.,* 1996). This has also been generated by numerous observations of positive heterozygosity-growth (or other fitness-related traits) correlation, based on allozyme markers (reviewed by David, 1998). The nature and the genetic bases of these correlations are still debated, and this topic has generated a large number of studies. The main issues in the debate have focused on whether the correlation is the result of intrinsic functional differences between enzyme variants at the electrophoretic loci scored (the "direct overdominance" hypothesis) or arises from non-random genotypic associations between these loci and others segregating for deleterious recessive genes (the associative overdominance hypothesis).

Some of these studies have focused on specific allozyme loci (i.e., the catalase locus in *Crassostrea gigas*: Fujio *et al.,* 1979; the amylase locus in *Littorina brevicula*: Park *et al.,* 1999). Pogson (1991) demonstrated overdominance activity at the Phosphoglocomutase-2 locus in *C. gigas*. Similarly, Sarver *et al.* (1992) examined a natural population sample of mussels (*Mytilus trossulus*). They revealed a significant departure from expected genotypic proportions caused by a deficiency of heterozygous genotypes for the octopine dehydrogenase (Odh). *In vitro* specific activity for this enzyme was determined and Odh heterozygotes had an average specific activity that was 19 % greater than that of apparently homozygous genotypes. They also performed the electrophoretic examination of a natural population of oysters (*Crassostrea virginica*) for the leucine aminopeptidase-2 (Lap-2) locus. *In vitro* specific activity for leucine aminopeptidase was determined. Lap-2 heterozygotes had an average specific activity that was 56 % greater than that of homozygous genotypes. In addition, the study of the effect of pollutants, such as tributyltin or herbicides, on oysters revealed genotype-dependent survival at some allozyme loci: aspartate-amino-transferase (Aat-2), adenylate kinase (Ak), phiosphogluco-isomerase (Pgi) and phosphoglucomutase (Pgm) (Tanguy *et al.,* 1999; Moraga and Tanguy, 2000).

The use of non-coding DNA markers (i.e., RFLPs or VNTRs) was proposed to determine which of these two hypotheses was correct, comparing heterozygosity-growth correlations based on allozymes with those based on non-coding DNA markers. Pogson and Zouros (1994) scored 222 individuals for their genotypes at seven allozyme loci, two non-specific protein loci of unknown function and eight nuclear RFLPs detected by anonymous cDNA probes. In contrast to the enzyme loci, no correlation was observed between growth rate and the degree of heterozygosity at the DNA markers. The authors concluded that the differences observed between the effects of allozyme and RFLP heterozygosity on growth rate provided evidence against the associative overdominance hypothesis, but this explanation must await corroboration from similar studies in different species. Besides, microsatellite-based studies (Bierne *et al.,* 1998; Launey and Hedgecock, 1999) on segregation ratios in inbred families of oysters have shown that there is

potentially a high genetic load in these species, which supports the associative overdominance hypothesis cited above. Overdominance at microsatellite loci has also been revealed in hatchery-propagated stocks of the shrimp *Penaeus stylirostris* (Bierne *et al.,* 2000).

The recent development of new molecular biology techniques and genomics should bring new insights on the genetic basis of these observations and identify selected genes in shellfish. For example, variants observed for the amylase genes have been related to differences in absorption efficiency, a trait related to growth in *Crassostrea gigas*, opening new perspectives to identify functional genetic markers that could be used in selective breeding programmes (Moal *et al.,* 2000b). In a recent paper, Floriolli *et al.* (2000) showed that individual mussels express different adhesive protein variants (Mfp-3). They conclude the following: "given that nothing is known about the genomic sequence of Mfp-3, the field of possibilities is simply too broad to entertain reasonable speculation at present".

DNA-based studies of selected genes

As can be seen from Table 2.2.5, very few population genetics studies so far have employed selected loci with known DNA sequence. Besides the non-trivial problem of demonstrating selection in the wild (Endler, 1986), this is probably because the molecular work involved in isolating and characterising gene loci is beyond the abilities (and interest) of most population geneticists. The only two good examples where population genetics interest has led to the characterisation of genes are the studies of Powell *et al.* (1992) and McMeel *et al.* (2001). Powell *et al.* (1992) developed a method for PCR screening of different *LDH-B* alleles in natural populations of *Fundulus heteroclitus.* This work has subsequently led to the identification of several genes within this species. McMeel *et al.* (2001) studied the lactate dehydrogenase (*LDH-C1*) locus in brown trout. The two most common alleles are *100 and 90*. Kinetic differences among alleles have been demonstrated (Henry and Ferguson, 1985) and their distribution in natural populations is highly informative in the study of phylogeography and population genetics of brown trout (Hamilton *et al.,* 1989). Primers for the locus were designed both by comparison to known *LDH-C* sequences from other species and from cDNA synthesised by reverse transcriptase PCR of RNA. Primers were then used to amplify and sequence the gene in homozygous individuals. A substitution leading to a shift in amino acid composition (and subsequent electrophoretic mobility was detected). The study provides an illustrative example of how polymorphic genes of interest can be identified for population genetics studies, however, it also illustrates that it is very time consuming!

Table 2.2.5. Examples of DNA-based studies of selected genes in wild fish populations.

Gene	Species	Reference
Enzymes		
Lactate dehydrogenase	*Fundulus heteroclitus*	Powell *et al.,* 1992
Lactate dehydrogenase	Brown trout	McMeel *et al.,* 2001
Growth hormones	Brown trout	Gross and Nilsson, 1995
	Atlantic salmon	Gross and Nilsson, 1999
MHC	Pacific salmon	Miller and Withler, 1996
	Chinook salmon	Miller *et al.,* 1997
	Atlantic salmon	Langefors *et al.,* 2000

Perspectives for wild populations

As mentioned previously, identifying polymorphic loci for many traits varying in natural populations will allow us to gain insight into the frequency of occurrence and spatial scale of local adaptations. For enzymes with different kinetic properties, variation in associations between environmental parameters (temperature, salinity, oxygen, etc.) and genotype can be tested. However, this requires as a prerequisite knowledge of population structure from non-coding markers (microsatellites, mtDNA) before inferences of selection can be made. Similarly, temporally collected or historical samples can be used to test if natural or man-induced changes of the environment have affected allele frequencies.

For growth hormones an interesting application would be to investigate if human disturbances in terms of fishing or hatchery operations are changing allele frequencies. For example, many populations of salmonids are subject to supportive breeding in hatcheries, which is considered a relatively harmless way to secure the populations under demographic threat. Often, such programmes are focused mainly on preserving as much neutral genetic variation as possible by securing a large effective population size in the hatchery. However, the conditions in the hatchery are much

different from the conditions in the wild. One hypothesis could be that selection for higher growth rates is altering the genetic composition of "wild" fish in the hatchery.

Some studies of MHC variability have already been undertaken in wild populations. MHC has many potential applications in wild populations. First of all for studies in differences in disease resistance (adaptations) among and within populations, but also for behavioural studies, since MHC has been associated with kin recognition and mate choice in many organisms including fish (e.g., Olsén *et al.,* 1998).

Summary

Many genes have been identified for important aquacultural species, in particular fish, due to their higher genetic homology with humans or other well-studied organisms (i.e., vertebrates). Many more will likely follow in the near future, since the general knowledge of the sequence and function of genes from many organisms is increasing dramatically in these years and many large-scale programmes for mapping and identification of genes for species in aquaculture have been (or will be) initiated. This progress will lead to more efficient selection for traits of interest to the industry, for instance by the use of marker-assisted selection (MAS). Further, it will lead to the production of new and more efficient transgenic organisms (GMOs).

Few polymorphic genes with known DNA sequence have until now been studied in wild populations, but many candidate loci have been identified from previous studies of allozyme variation. The main advantages of knowing the DNA sequence is that it allows the detection of the mutations responsible for the apparent fitness differences, allows non-lethal sampling, and the use of historical tissue collections. For studies of wild populations, the identification of genes for aquacultural (or population genetic) purposes has many interesting applications. By identifying polymorphic loci for many traits varying in natural populations, it is possible to gain insight into the frequency of occurrence and spatial scale of local adaptations in finfish and shellfish populations.

Recommendations:

- Demonstrating selection in the wild is difficult and there are many potential pitfalls. For example, it is difficult to differentiate the effects of selection from other evolutionary forces such as genetic drift and migration. We therefore recommend consulting the appropriate literature such as Endler (1986) before initiation studies of natural selection in the wild.
- Identification of selected genes in wild fish and shellfish populations is at present very time consuming. However, useful procedures and protocols have been published, e.g., by McMeel *et al.* (2001), which provides a "textbook" example on how to identify the molecular basis of a polymorphic gene with suspected selected alleles.

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Additional material on recent technical developments for screening DNA polymorphism is contained in Annex 4.

2.3 Review and Report on the Importance of Different Kinds of Genetic Population Structures in Relation to Human Impact

Based on position papers by M.M. Hansen and A. McPherson, K. Smedbol and E. Kenchington, adopted by the WGAGFM in Bergen, 2001.

General introduction

The original purpose of this ToR was to summarise current knowledge of different kinds of genetic population structures in marine fishes and to assess how human activity could have an impact on marine fishes given the different types of population structures observed. However, during the work with the ToR it became increasingly clear that only a few published studies have suggested specific models of population structure in marine fishes; most studies have not gone beyond the initial step of merely demonstrating population subdivision. This reflects the difficulties with working with genetically weakly differentiated marine fish populations. It is mostly during the past 5–10 years, primarily a result of the advent of microsatellite DNA markers, that studies have accumulated that report population subdivision. For this ToR it was therefore decided to focus on the initial question *"which models of genetic population structure are of relevance to marine fishes?"* and then to proceed more specifically with the issue of the *occurrence of metapopulation structure in marine fishes*. These two issues are represented in the following by two different position papers.

Models of genetic population structure in marine fishes

Based on a position paper by M.M. Hansen, adopted by the WGAGFM in Bergen, 2001.

Introduction

Many marine fishes, such as many commercially important species like cod (*Gadus morhua*), herring (*Clupea harengus*), etc., are characterised by huge census population sizes and presumably also huge effective population sizes. At the same time these species are often highly migratory resulting in a large potential for gene flow among populations and it is also expected that drift of eggs and larvae will result in considerable mixing (Hauser and Ward, 1998). Thus, the prediction is that there should be limited genetic differentiation among populations at neutral loci. This has been confirmed empirically by many studies showing much less differentiation in marine fishes compared to anadromous or freshwater species (Ward *et al.,* 1994). Studies based on allozyme electrophoresis have in some specific cases detected important genetic differentiation at small geographical scales (e.g., between Baltic and Atlantic cod; Mork *et al.,* 1985) and in general at very large geographical scales, such as cod through its entire range of distribution (i.e., a geographical scale of up to 10,000 km; also Mork *et al.,* 1985). However, at medium geographical scales (i.e., up to a few thousand km) allozymes have in most, but not all, cases suggested limited or no structuring (see, e.g., results for European Atlantic cod populations in Mork *et al.,* 1985). Analyses applying DNA markers have yielded highly differing results. Studies employing mitochondrial DNA have in most cases not revealed genetic differentiation at small to medium geographical scales (e.g., Arnasson and Palsson, 1996; Turan *et al.,* 1998; but see Nesbo *et al.,* 2000), perhaps due to insufficient statistical power, with only one hypervariable locus studied. However, on a global scale mitochondrial DNA analysis has proven highly informative (Grant and Bowen, 1998). Analyses of coding nuclear genes have in some specific cases revealed very high levels of differentiation even at small geographical scales (e.g., Fevolden and Pogson, 1997), which could be the result of geographically varying natural selection. Microsatellites, which are generally considered neutral (though this may not be universally true), are currently the most widely applied markers for studies of marine populations. Several independent studies of different species have detected weak (F_{ST} often < 1%) but statistically significant differentiation (e.g., Ruzzante *et al.,* 1996, 1997, 1998; Shaw *et al.,* 1999, Lundy *et al.,* 1999, 2000; Wirth and Bernatchez, 2000; Daemen *et al.,* 2001). Moreover, a recent study of cod populations based on RFLP analysis of coding nuclear genes reveals similar degrees of differentiation (Pogson *et al.,* 2001). In total, these studies suggest that marine fishes are indeed subdivided into weakly differentiated populations, but it is unclear exactly which kind of genetic population structure(s) this reflects.

Problems and pitfalls

Before discussing different types of population genetic structures in marine fish, it might be worthwhile considering if the observations of very weak but statistically significant genetic differentiation could not be explained simply by

artifacts. It may be difficult to quantify precisely the magnitude of allele frequency differences at multiallelic loci required to yield a statistically significant F_{ST} value of, say, 0.2 %. However, it is obvious that the differences must be very small. This inevitably raises the issue of whether or not simple typing error could explain the observed results. Microsatellite typing error is something of a taboo that is rarely considered in the literature (but see Marshall *et al.*, 1998; Goossens *et al.,* 1998). However, anybody who has scored microsatellite gels with, say, 20+ alleles that in many cases exhibit stutter bands knows that typing error is a fact of life.

To assess if a small percentage of typing error could result in statistically significant differentiation, a small-scale simulation experiment was carried out in which a "typical marine fish population" was simulated using the software EASYPOP (Unpublished, F. Balloux, 1999, available at http://www.unil.ch/izea/softwares/easypop.html). The effective population size was set to 10,000. Six microsatellite loci exhibiting a maximum of 50 alleles were considered which mutated according to a two-phase model with 95 stepwise mutations and 5 % non-stepwise mutations and a mutation rate of 10^{-4}. After 1000 generations, six samples each consisting of 50 individuals were taken from the population. The data sets were then manipulated by simulated typing errors, in each case amounting to 4 %. 1) For each locus in each sample, four alleles were selected at random and changed into a private allele, i.e., an allele not observed elsewhere in the data set. This sort of typing error could occur if artifact bands were present, such as PCR "ghost bands" or contamination. 2) For each locus in each sample, four alleles were selected at random and changed into an allele already present in the sample. This sort of typing error could occur if, for instance, gels are "smiling" whereby allele sizes for some alleles could be mistyped by a few basepairs. Alternatively, at heavily stuttering loci it may be difficult to separate homozygotes from heterozygotes with allele sizes separated by only 2 bp and heterozygotes may be erroneously scored as homozygotes. Both the "true" and manipulated data sets were analysed by exact tests for differences in allele frequencies (where probabilities were combined over loci using Fisher's method), both between pairs of samples and among all samples. Also, overall F_{ST} values were estimated.

Table 2.3.1. Results of analyses of genetic differentiation among simulated samples from the same population. "Type of error" refers to the sort of typing error that has been introduced, i.e., "none" denotes the unmanipulated data set, "1)" denotes manipulations where 4 % of the alleles have been changed into a private allele, and "2)" denotes manipulations where 4 % of the alleles have been changed into an allele that is already present in the data set. "F_{ST}" denotes the F_{ST} value among all samples, "Global differentiation" denotes the outcome of tests for differences of allele frequencies among all samples and "Pairwise differentiation" denotes the percentage of tests for differences of allele frequencies between pairs of samples that yielded a significant outcome. Significance levels were adjusted using the sequential Bonferroni method (initial k = 15).

Type of error	F_{ST}	Global differentiation	Pairwise differentiation
None	0	$p = 0.395$	0
1)	0.001	$p < 0.001$	27 %
2)	0.003	$p < 0.001$	20 %

The results listed in Table 2.3.1 show that a small rate of typing error (4 %) can indeed produce statistically significant differentiation of a magnitude reported in studies of marine fishes. This is, of course, not to say that all published studies merely report genetic differentiation estimates based on typing errors, but it stresses the importance of verifying the reproducibility of results, for instance by analysing two or more temporally spaced samples from each geographical locality (e.g., Ruzzante *et al.,* 1997). Also, if the results are backed up by other biological information, for instance, physiological data on the fish (Ruzzante *et al.,* 1996), or if the results otherwise make sense, for instance, by showing correlation between geographical and genetic distance between samples, this would lend further credibility to the results.

On the more statistical side, Waples (1998) pointed out the need for taking sampling error into account when estimating genetic differentiation among populations; in some cases differentiation is so small compared to sampling error that sampling error in itself may explain the observed F_{ST} value. Hedrick (1999) has pointed out that interpretation of multiallelic F_{ST} values is much more complicated than in the case of diallelic loci. Thus, in the case of diallelic loci F_{ST} may range between 0 and 1, whereas in the case of multiallelic loci the maximum value of F_{ST} can never exceed the level of homozygosity. This needs to be taken into account when analysing highly polymorphic loci in marine fish populations, as it may not be valid to compare absolute F_{ST} values across different studies and species. Finally, both authors point out the fundamental problem: When is genetic differentiation too small to be considered biologically significant, i.e., when does it reflect underlying important biological differences among populations? As described previously, the very weak genetic drift occurring in marine fishes is expected to result in very low levels of differentiation and it may be difficult to assess whether or not this reflects biologically meaningful differences among populations.

However, even with the reservations described above it must be considered a fact that statistically significant differentiation has been observed in several marine fishes, though it cannot be ruled out that typing error may have been a potent "evolutionary force" in some studies.

Different types of genetic population structures

Several types of genetic population structures have been suggested over the years. The simplest model is complete *panmixia* within a species, i.e., there is no genetic structure and each individual has an equal probability of reproducing with any other individual within the species. A variant of this would be a continuous population but with some isolation by distance, i.e., individuals from different ends of the distribution range will tend to be genetically divergent. The other extreme would be a species subdivided into several *distinct populations with complete reproductive isolation*. Such a structure is probably unlikely to occur in the marine environment except for cases of species with rare and very localised populations and limited possibility for larval dispersal.

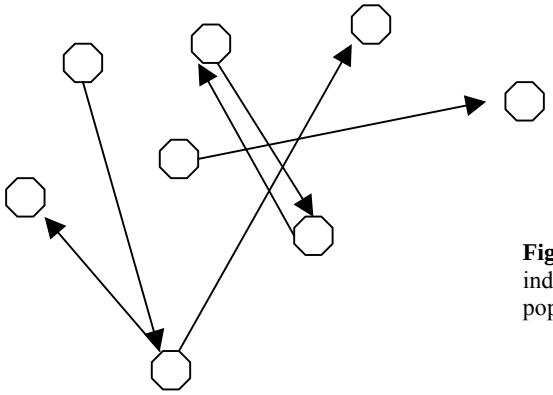

Figure 2.3.1. Island model where gene flow occurs independently of geographical distance between populations.

The most well-known model of population genetic structure is Wright's (1931) *island model*, which is a basic assumption of much population genetics theory and statistics. In this model (see Figure 2.3.1) a number of genetically distinct populations exist which are linked by gene flow. However, the probability of gene flow from one population to another is independent of the geographical distance between populations. In other words, gene flow is just as likely to occur between neighbouring populations as between geographically distant populations.

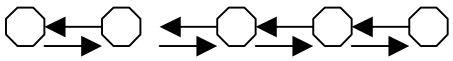

Figure 2.3.2. One-dimensional stepping-stone model, where gene flow occurs primarily or exclusively between neighbouring populations

Another important model is the so-called *stepping-stone model* (Kimura and Weiss, 1964). Again, a number of genetically distinct populations exist which are linked by gene flow. However, in this case the probability of gene flow from one population to another is dependent on the geographical distance between populations and in the most extreme case gene flow only occurs between neighbouring populations (see Figure 2.3.2). Different types of stepping-stone models have been suggested, most importantly one- and two-dimensional models. In a one-dimensional model, populations are linked in a linear fashion. This could, for instance, be salmon populations along a shoreline. In a two-dimensional model, populations are linked in a grid, which would, for instance, be cod populations spawning at different spawning grounds in the sea. In a stepping-stone model, it is expected that genetic distance between populations will increase with geographical distance, i.e., there will be isolation by distance.

In recent years the concept of *metapopulations* has become increasingly applied in evolutionary biology and ecology. In its purest form (as defined originally by Levins, 1969) a metapopulation consists of a number of populations that inhabit different patches. The populations are linked by gene flow and some turnover, i.e., extinctions and recolonisations of populations occur (see Figure 2.3.3). Since then a number of different types of metapopulations have been suggested (see Hanski and Gilpin, 1997). One of the most important of these is the *source-sink model*, where a stable population (source) contributes migrants to smaller populations (sinks) which only exist due to the recurrent contributions from the source population, i.e., the sink populations have a negative growth rate. In general, there has unfortunately been a tendency to denote any system of subdivided populations as metapopulations so that the metapopulation concept has by now become much more diffuse.

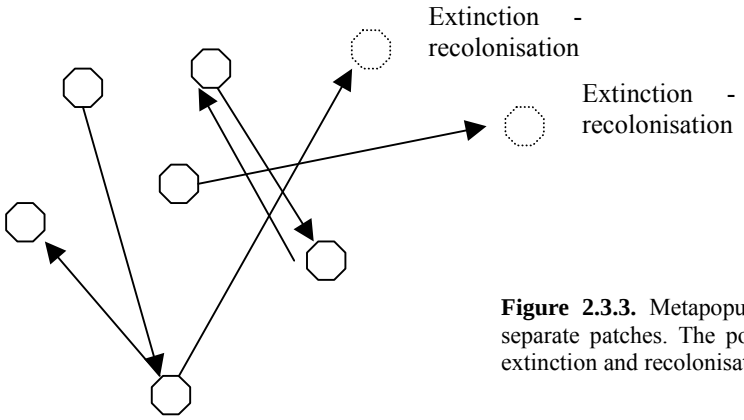

Figure 2.3.3. Metapopulation, where individual populations occupy separate patches. The populations are linked by gene flow and some extinction and recolonisation events occur.

A type of population model bearing some resemblance to the metapopulation concept is the *member-vagrant* hypothesis (Iles and Sinclair, 1982). Briefly, it is assumed that populations inhabit patches which contain suitable resources and nursery areas. The population "boundaries"are defined by these resources. Individuals that stray outside these areas and reproduce are expected to have very poor fitness and are essentially lost to the population. Consequently, there is selection in favour of being a "member" of the population which should promote homing and other efforts to remain within the population boundaries.

Types of genetic population structures observed in marine fishes

As described in the introduction, it has only recently become apparent that most marine fishes are in fact not completely panmictic but do exhibit some genetic substructuring. Even the European eel (*Anguilla anguilla*), which has often been considered an example of a completely panmictic species, appears to exhibit some kind of population subdivision (Wirth and Bernatchez, 2000; Daemen *et al.,* 2001). There are, however, many uncertainties regarding this specific case and unravelling the genetic population structure of eel would ultimately require sampling of adult eels or larvae as close as possible to the spawning places in the Sargasso Sea (Daemen *et al.,* 2001).

In most studies it has just barely been possible to detect significant genetic differentiation and only a few studies have gone one step further to suggest specific population models. Ruzzante *et al.* (1998) linked observed genetic differentiation with oceanographic data and, in some cases, spawning time differences. Herring is an extreme case in terms of spawning time differences, sometimes exhibiting sympatric spring and fall-spawning populations. Thus, a complicated spatiotemporal structure is likely to exist in this species (e.g., Shaw *et al.,* 1999).

Already the allozyme study by Mork *et al.* (1985) detected isolation by distance in cod. A recent study by Pogson *et al.* (2001) confirms this pattern (but for unknown reasons Mork *et al.,* 1985 is not cited), as they observed isolation by distance in cod populations off the North American Atlantic coast. They suggested some sort of stepping-stone model accounting for this observation and also discussed the possibility that gene flow might in fact be considerably lower than suggested by F_{ST} values, because populations are not at migration-drift equilibrium (this is in fact not what the authors say but it is the implication of what they say). This is an important point to consider in the context of many other marine fishes, where effective population sizes are very high resulting in very limited drift and only a slow rate of approach to migration-drift equilibrium.

The validity of metapopulation models to describe populations of marine fishes will be treated in more detail in another position paper. However, it is worth pointing out the paper by Grant and Bowen (1998), which reviews and synthesizes allozyme and mitochondrial DNA studies of anchovies and sardines on a global scale. Even though mitochondrial DNA

data suggest a deep phylogenetic split between the two geographically widespread genera *Sardina* and *Sardinops* (suggested to have diverged approximately 20 my ago), there is a very shallow phylogeny within *Sardinops* (all lineages are suggested to have coalesced not more than 0.5 my ago). This could be due to frequent extinction-recolonisation events, a recent rapid expansion from just one single species/population, to lineage sorting during periods of lowered population sizes or even bottlenecks, or it could be due to recurrent "sweepstakes selection" events (Hedgecock, 1994), where, despite large census population sizes, only a few individuals reproduce successfully. In anchovies (genus *Engraulis*) mitochondrial DNA data provide strong indications of extinctions and recolonisations. Thus, anchovies off the southern African coast (*E. capensis*) appear to represent a recent recolonisation event, and the founders appear to have been derived from European anchovies (*E. encrasicolus*). The authors suggest that similar shallow mitochondrial DNA phylogenies observed in many other marine fishes may indicate similar evolutionary dynamics.

Conclusions

Molecular population genetics of marine fish populations is still in its infancy. However, even though technical artifacts are a non-negligible issue, several studies demonstrate small but significant genetic differentiation. There are still relatively few studies available that attempt to describe which models of genetic population structure are of most relevance to explain observed patterns of spatial and in some cases spatiotemporal differentiation, though some studies have demonstrated isolation by distance. Classical metapopulation models involving extinction and recolonisation events may be relevant in the context of marine fishes, at least on very large spatial and temporal scales.

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Metapopulation Dynamics in Marine Species

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Metapopulation theory

Metapopulation theory addresses the long-term persistence of a species in a patchy or fragmented habitat and is maintained by a balance between local extinction and colonization (Levins, 1970). This theory is currently a major

theme in ecology and many developments have been made on the original model to incorporate a variety of constraints on immigration, emigration, and population turnover.

A metapopulation can be defined as a set of local populations or subpopulations within the range of the population, where movement from one subpopulation to another is possible (Hanski and Simberloff, 1997). Metapopulation dynamics describe the spatial dynamics of interconnected population units. The degree of segregation can range from slight to nearly total, and depends on a number of case-specific factors including the distance between areas of suitable habitat, the magnitude of variation in habitat quality, life history characteristics (e.g., discrete life stages), and the dispersive abilities of the species. This exchange of individuals prevents the development of separate, autonomous populations, and thus subpopulations may not be genetically distinct from one another, though genetic distinguishability would be indicative of very low exchange rates of effective spawners among subpopulations.

In the simplest metapopulation model first proposed by Levins (1970) there are three assumptions: 1) subpopulations are equivalent in size and degree of isolation, 2) the internal population dynamics of subpopulations are asynchronous, and 3) the rate of exchange of individuals among subpopulations is too low to affect local population dynamics. The metapopulation range is broken into areas of suitable and unsuitable habitat, wherein suitable patches may support resident subpopulations. The subpopulations within occupied patches can undergo extinction, and unoccupied patches can be colonized from extant subpopulations. The Levins model (1970) may be expressed as:

$$\frac{dP}{dt} = mP(1-P) - eP \qquad \textbf{(1)}$$

where P is the fraction of subpopulation areas occupied at time t, m is the "colonization" parameter, and e is the "extinction" parameter. The rate of colonization of empty habitats is assumed to be proportional to P $(1 - P)$, where $P < 1$. The equilibrium value of P is:

$$P_E = 1 - \frac{e}{m} \qquad \textbf{(2)}$$

and therefore the metapopulation will persist as long as $e/m < 1$ (Figure 2.3.4). For persistence to occur, recolonization must occur at a sufficiently high rate within the metapopulation to offset the rate of extinctions (Hanski, 1997). It is important to note that one of the characteristics of such a metapopulation is that even under "steady state" conditions, subpopulations are expected to undergo extinction, and unoccupied areas will be colonized. While subpopulations may go extinct, the metapopulation persists at the larger spatio-temporal scale due to the dynamic equilibrium between extinction and colonization (Figure 2.3.5).

Metapopulations that conform to the assumptions of the Levins model may be rare (Harrison and Taylor, 1997) because in most natural systems the subpopulation size and the degree of relative isolation will differ. Hanski (1991) has reported that colonization is proportional to overall metapopulation abundance, due to a "rescue effect". In the situation of high abundance, immigration levels increase, thus extending subpopulation persistence. It is more likely that existing metapopulations conform to a "mixed structure" model (Harrison and Taylor, 1997), where the metapopulation includes units with high exchange functioning as single subpopulations, together with more isolated subpopulations.

Applying the metapopulation concept to mobile marine fishes

Even with relaxing of some of the requirements of the Levins model, the life histories of most abundant, highly mobile marine fishes at first do not appear to fit under the metapopulation concept. For instance, metapopulation models usually assume that, except for dispersers, individuals remain within their home area for their entire life. However, adults of many marine fish species can traverse much of the population's range over the course of a single year, and often undergo geographically extensive spawning-feeding migrations. Yet, these populations often show some degree of spatial structure. We propose that under a strict adherence to the principles of metapopulation dynamics, most marine fishes do not constitute metapopulations. A metapopulation is not just a spatially structured population, but rather is a dynamic system of interconnected population subunits that undergo colonization and extinction. We discuss factors

limiting the determination of possible metapopulations in open marine systems, and present two example species, Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*), that may function as metapopulations.

Habitat

In metapopulation theory, subpopulations are located in areas of suitable habitat. Mobile fishes such as cod and herring are not tied to a particular geographic location within the range of the population, except during the spawning period. During this period, cod and herring show the greatest degree of spatial aggregation, and, thus, discrete spawning locations may serve as suitable substitutions within the metapopulation concept. Smedbol and Wroblewski (in press) followed this approach and proposed that within the "northern" cod (NAFO Divisions 2J3KL), the geographic location of subpopulations is associated with overwintering/spawning areas. Some of the proposed putative local populations of northern cod have distinguishing characteristics: genetic distinctiveness (microsatellite DNA allele frequency variation), phenetic distinctiveness (length-at-age, weight-at-age, fecundity-at-age), and residency in or interannual fidelity to discrete spawning areas (see Ruzzante *et al.,* 2000; Smedbol and Wroblewski, in press).

Extinction

In a metapopulation structure, subpopulations may undergo extinction, even during equilibrium conditions. A variety of mechanisms may bring about natural population extinction, but in terrestrial systems habitat loss is the most significant cause (Hanski, 1999). However, in stable habitats environmental stochasticity and anthropogenic pressure may be the most likely mechanism by which populations are driven to extinction. The marine environment is much more stable than the terrestrial environment, with relatively low temperature and salinity ranges globally, so that different extinction mechanisms are expected. However, at the boundaries of water masses, decadal-scale changes in temperature have had marked effects on the distribution of species (e.g., shrimp and snow crab show range expansion with cold water incursion events on the Scotian Shelf), and El Niño events in the Pacific regularly drive populations to extinction (directly and indirectly) over similar time scales. In coastal areas, ice scour can be a destructive force for attached plants on intertidal habitats, and disease has been known to periodically decimate populations of both plants (e.g., *Zostera marina*) and invertebrates (e.g., *Strongylocentrotus droebachiensis*). Therefore, it is possible that classic metapopulation dynamics do operate in marine species given natural mechanisms for extinction which occur periodically, albeit at different time scales from those operating on land.

Are there examples of the extinction of population subcomponents of abundant marine fish populations? It is very difficult to determine if a subpopulation of a marine fish metapopulation is truly extinct. There are many examples worldwide of exploitation reducing the abundance of local populations to very low levels, to the point where fishing is no longer economical ("commercial" extinction). There is also some limited evidence for extirpation of small, local populations (Ames, 1997). While, theoretically, extinction may have occurred during past generations, to our knowledge extinction of subpopulations in the absence of fishing has not been documented. Subpopulation extinction is likely a rare natural event given the historically large abundance of marine fish populations. However, fluctuating population size reduces genetic variance and creates a potential for inbreeding to contribute to the eventual extinction of small and isolated populations, as has been demonstrated for fritillary butterflies (Saccheri *et al.,* 1998).

The appropriate time scale for metapopulation dynamics is an important factor in the consideration of evidence for extinction of subpopulations. Cod and herring subpopulations (spawning components) are very large relative to those in most metapopulations studied. Additionally, in the Northwest Atlantic, cod and herring exhibit typical lifespans of twenty and twelve years, respectively (Scott and Scott, 1988). Given these factors, it is likely that once established, these subpopulations will persist for long periods. With low subpopulation extinction rates, metapopulation theory predicts high occupancy levels under equilibrium conditions (Figure 2.3.4). Colonization rates can thus be relatively low as well, and still result in very abundant metapopulations with high subpopulation number.

Genetic implications of metapopulation dynamics

While it has been suggested that the repeated colonization and extinction of local populations, typical of the classical metapopulation, may dramatically affect genetic structuring (Wade and McCauley, 1988), few empirical observations of metapopulation-induced effects in marine species have been documented in the literature. We suggest that this paucity of observations is due, in part, to the relatively short time scale within which many scientists work (relative to ecological time scales at which metapopulations might function) and the very few instances of veritable extinction of marine fish populations that have been demonstrated (as above).

Figure 2.3.4. Time-dependent solutions of the Levins model using several parameter values to illuminate model behaviour. Model runs begin with an occupancy of $P = 0.1$. Solid line: $m = 0.3$, $e = 0.03$; dotted line: $m = 0.3$, $e = 0.15$; dot-dash line: $m = 0.15$, $e = 0.03$; long dash line: $m = 0.03$, $e = 0.003$.

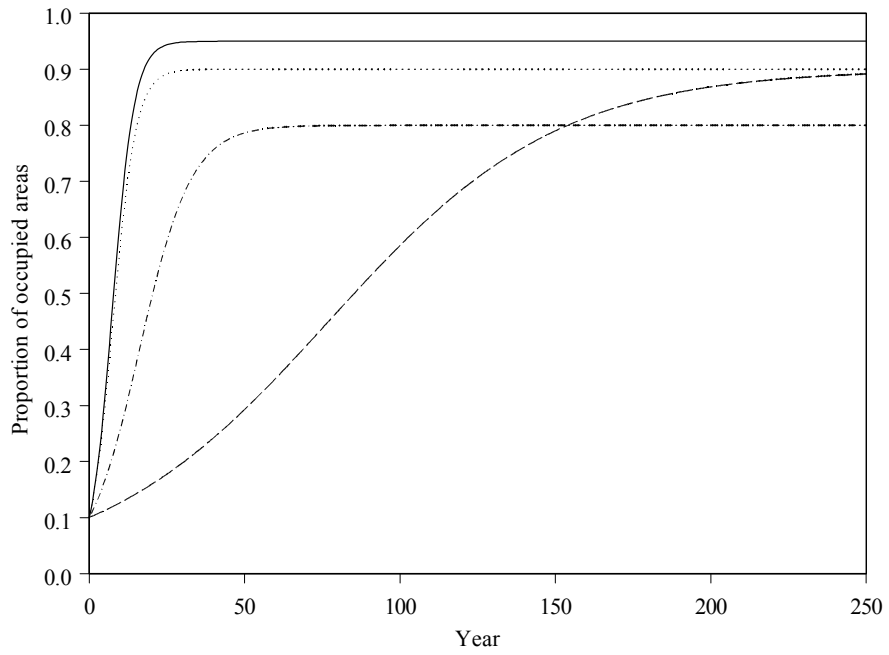
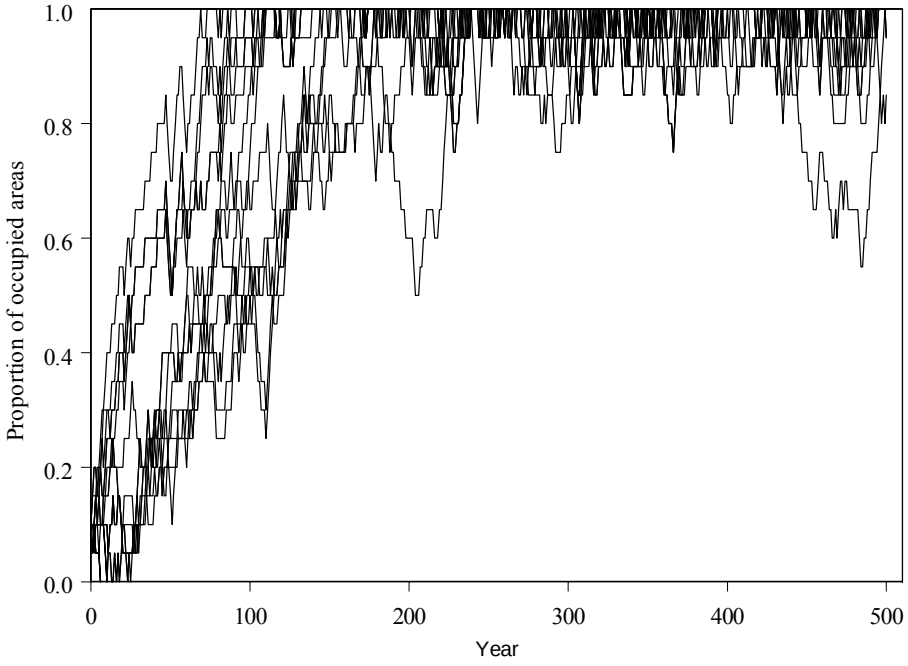


Figure 2.3.5. Multiple runs of a time-dependent, discrete approximation of the Levins model, with a term representing stochastic extinction processes added to the model. Note that several model runs result in metapopulation extinction (curves that decline to $P = 0$ occupancy). Model runs begin with an occupancy of $P = 0.1$.



Although adherence to a metapopulation model may increase or diminish levels of genetic variation (depending on the levels of gene flow and genetic drift) among local populations, models (Gilpin, 1991) have also demonstrated that metapopulation dynamics can result in reduced genetic variability within and among populations and, therefore,

evidence to suggest repeated population bottlenecks may be detected. In addition, another genetic consequence of a metapopulation may be a lack of isolation by distance as dispersal from one population is equally likely to result in colonization by any others (Levins, 1970).

Here we use genetic data presented by Pogson *et al,* (2001) for cod and unpublished analyses for herring (McPherson *et al*., unpublished) to investigate the conformance to population patterns expected in a metapopulation as metapopulation theory has been invoked in both cod (Smedbol and Wroblewski, in press) and herring (McQuinn, 1997) in an attempt to reconcile contradictory evidence suggesting both discreteness and mixing.

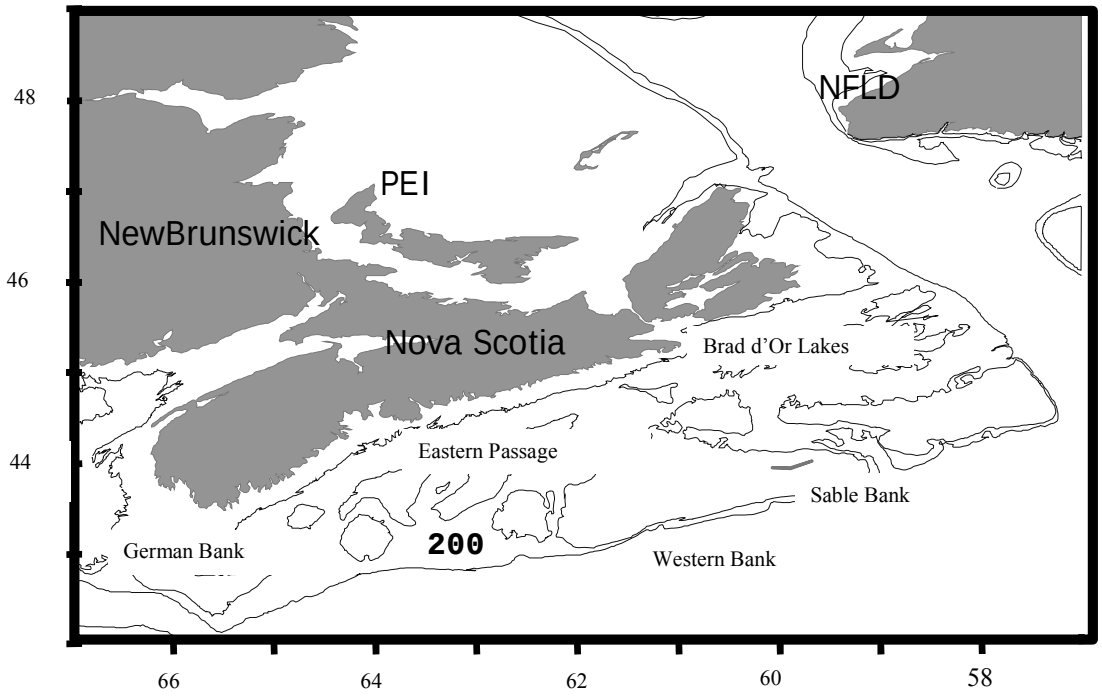
Herring and cod samples (Pogson *et al.,* 2001) were collected in approximately the same locations surrounding Nova Scotia, Canada (Figure 2.3.6). Analyses were restricted to these locations as we hypothesized that the Scotian Shelf would likely be an appropriate spatial scale for a metapopulation within the Northwest Atlantic region.

Herring data are compared directly to cod data in each of Western Bank, Bras d'Or Lakes, and Eastern Passage populations. Herring were not collected from Sable Bank; instead, German Bank was used. Cod data based on nine nuclear RFLP loci (excluding GM798) were extracted from Pogson *et al.* (2001) and are compared to data generated by nine tetranucleotide microsatellite loci (McPherson *et al.,* 2001 and unpublished). Tetranucleotide microsatellites were used to increase the distance separating alleles and eliminate PCR-induced stutter and the resulting ambiguity in allele calls often associated with dinucleotide microsatellite loci (see previous position paper "Models of genetic population structure in marine fishes"). Sample sizes ranged from 43 (Bras d'Or Lakes cod) to 138 (Western Bank cod) individuals per collection, but the average sample size was 88.

Our first test for metapopulation sub-structure was to consider characters associated with population bottlenecks and a reduced number of breeders. Low levels of heterozygosity, fewer alleles per locus, gametic disequilibrium in unlinked loci are all thought to be consistent with a population bottleneck and, therefore, may reflect metapopulation dynamics. To compare the levels of heterozygosity (averaged across loci) between cod and herring, it was necessary to standardize across species (to reflect differences inherent in the loci used) by averaging across loci and subtracting the global mean (all loci at all locations within species) from these averages. In doing so, Figure 2.3.7 reflects the heterozygosity anomaly by population and species. Although relative levels of heterozygosity are similar in both cod and herring at Western Bank and German/Sable Banks, the Bras d'Or Lakes herring and Eastern Passage cod have a lower than average heterozygosity and, conversely, the Eastern Passage herring have a higher than average heterozygosity.

Herring data are compared directly to cod data in each of Western Bank, Bras d'Or Lakes, and Eastern Passage populations. Herring were not collected from Sable Bank; instead, German Bank was used. Cod data based on nine nuclear RFLP loci (excluding GM798) were extracted from Pogson *et al*. (2001) and are compared to data generated by nine tetranucleotide microsatellite loci (McPherson *et al*., 2001 and unpublished). Again, tetranucleotide microsatellites were used to increase the distance separating alleles and eliminate PCR-induced stutter and the resulting ambiguity in allele size calls often associated with dinucleotide microsatellite loci.

Figure 2.3.6. Locations of cod and herring samples collected in the vicinity of Nova Scotia. Redrawn from Pogson *et al.* (2001).



The number of alleles per locus and population were also tabulated and again it was necessary to standardize across species (to reflect differences inherent in the loci used), by averaging across loci and subtracting the global mean (all loci at all locations within species) from these averages prior to comparisons. Both cod and herring from the Bras d'Or Lakes have fewer alleles (Figure 2.3.8) on average, when compared to the remaining populations. Given the positive anomalies at the remaining locations, it seems the Bras d'Or Lakes sample in both species has reduced the global mean allele number for each respective species.

As random genetic drift may create gametic disequilibrium between unlinked loci in populations that have become drastically reduced, evidence of gametic disequilibrium was used to investigate the likelihood of population bottlenecks. Although association of loci (as estimated by gametic disequilibrium) was not reported in Pogson *et al.* (2001) and therefore cannot be used to infer the existence of a population bottleneck in cod, three significant locus-pair associations were detected in the herring populations considered here, all of which were in the Bras d'Or Lakes population. It is thought that associations between these loci are not due to physical linkage of loci, as previous work has shown that loci were not associated in >10 herring populations previously considered. These results are consistent with a reduction in the number of breeders in the Bras d'Or Lakes herring.

Relationships between genetic differentiation and geographic distance separating populations at different spatial scales were examined in the Nova Scotia region. Mantel tests indicated a significant correlation (r^2 =0.92) between gene-flow and geographic distance between cod samples (Figure 2.3.9) based on nine loci presumed to be neutral. The isolation by distance relationship also held when Pogson *et al.* (2001) expanded the spatial scale and considered cod samples from Newfoundland.

Figure 2.3.7. Heterozygosity anomalies for cod and herring at each sample location**.**

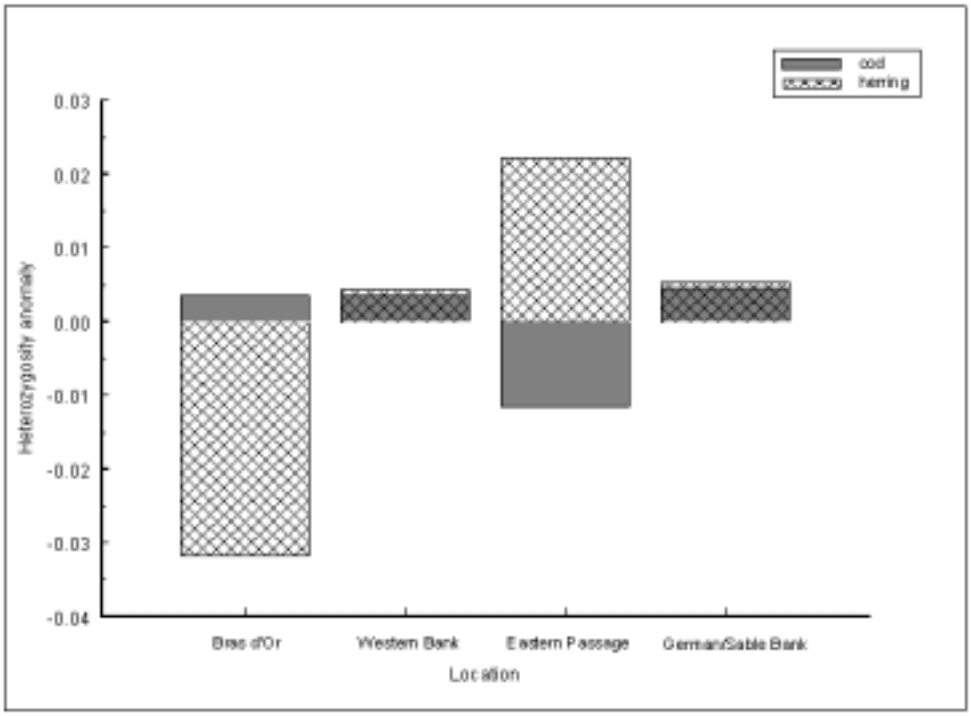


Figure 2.3.8. Allele number anomalies for cod and herring at each sample location.

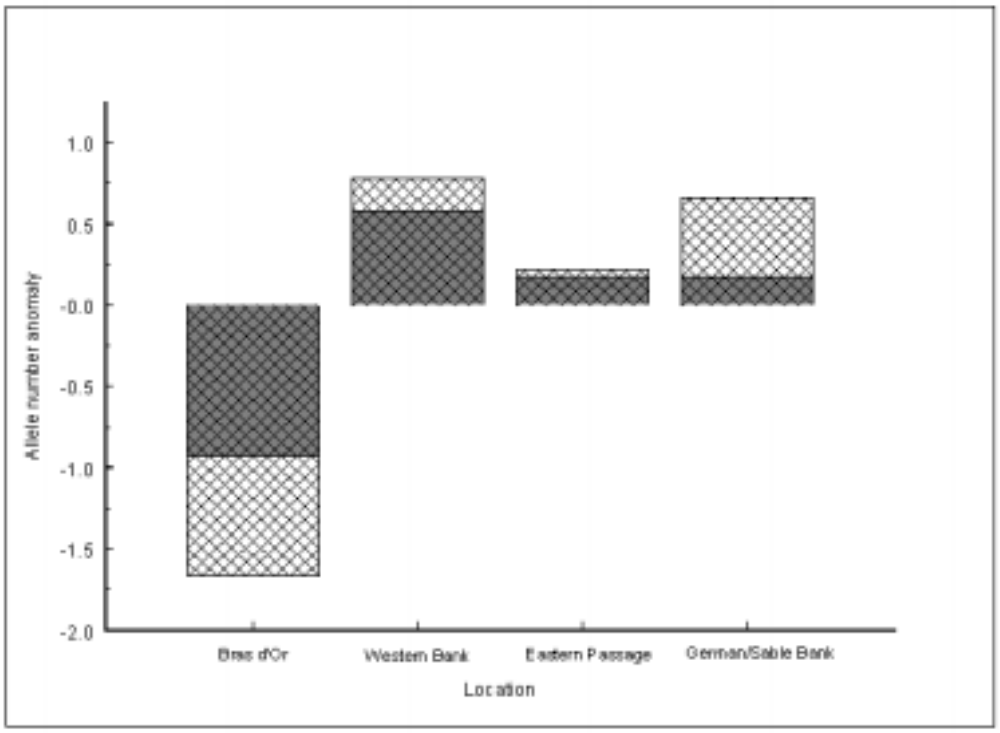


Figure 2.3.9. Relationship between log (gene-flow) and log (geographic distance) among the four NS cod populations. Open circles correspond to data generated from nine nuclear RFLP loci and the dashed line represents a regression result based on these data. Closed circles represent data based on ten loci and when data points overlap, only closed circles are visible. (Figure 2 in Pogson *et al.*, 2001).

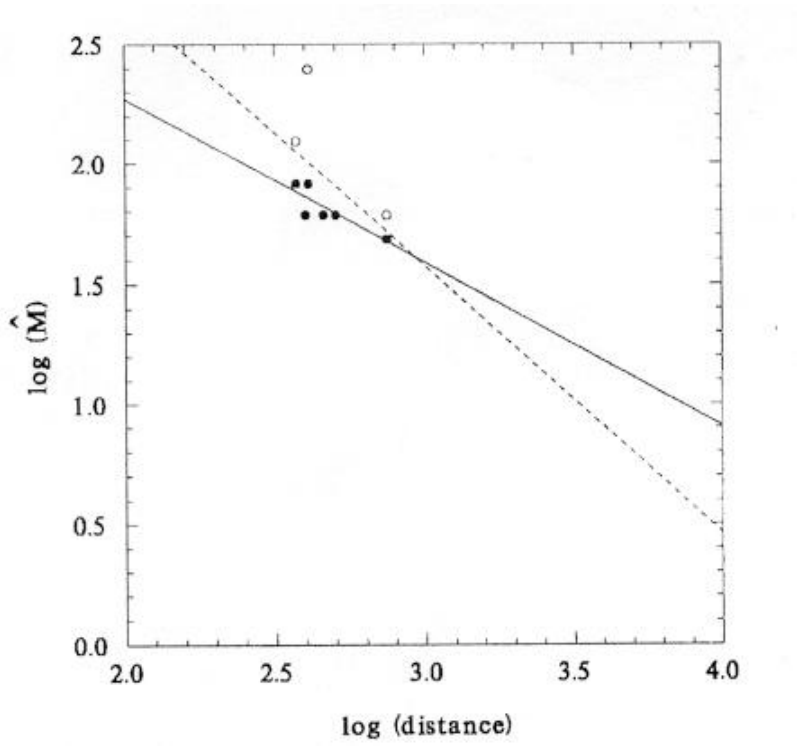
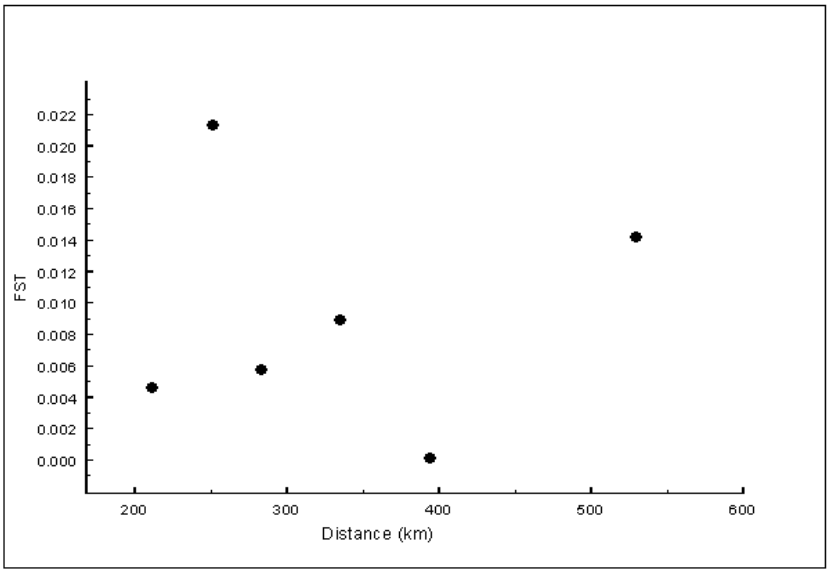


Figure 2.3.10. Relationship between F_{ST} and geographic distance among four NS herring populations.



When herring from approximately the same locations were used and an isolation by distance relationship tested among these samples (Figure 2.3.10), no significant relationship was detected (r^2 =0.01; *P*=0.531). As Pogson *et al.* (2001) log10 transformed geographic distances and expressed population differences in terms of gene-flow and not F_{ST}, Mantel tests in herring were performed with all possible combinations of transformed data with a maximum r^2 reaching 0.03.

This result is surprising as herring are thought to exhibit strong spawning-site fidelity and, therefore, we would have predicted the opposite result. While the lack of isolation by distance should not be interpreted as indicating that a metapopulation relationship does exist among samples, the significant isolation by distance observed by Pogson *et al.* (2001) at a number of spatial scales is inconsistent with a metapopulation under the strict definition of Levins (1970). However, in herring, evidence of a population bottleneck in the Bras d'Or Lakes (that was not as apparent in cod and, therefore, may not be a result of the isolated nature of the location), coupled with the lack of an isolation by distance relationship, suggests that a metapopulation population structure for this species cannot be discounted.

Conclusion

While investigating the role of metapopulation dynamics in shaping marine population structure and patterns of diversity of marine species is a useful exercise and may ultimately allow a more thorough understanding of many aspects of life history, caution must be used when applying metapopulation models to management of fishes. For example, misapplying a metapopulation model may result in decimating a local population with the assumption that replenishment from surrounding sub-populations will take place following some predictable colonization rate. In many cases, this would result in potential extirpation and the resultant erosion of diversity, which would certainly be contrary to the precautionary approach. Even if metapopulations are ultimately verified for mobile marine species, *it is almost certain that the time scale under which they operate will be much greater than the annual events documented in terrestrial systems*. We anticipate scales of decades to centuries to apply based on the limited evidence for population extirpation documented. In light of this, we recommend that scientists and managers do not use the metapopulation concept when making decisions on fishing targets. Instead, we recommend that all subpopulations identified be managed sustainably as distinct entities. Further, we advocate using genetic information to help differentiate among veritable metapopulations and others (e.g., source-sink relationships, panmixia and discrete population sub-structuring), with the hope that reconciliation of the somewhat contradictory population models developed for marine species will be achieved.

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Recommendations

1) Estimates of genetic differentiation in marine fishes are typically very low, even though the use of highly sensitive markers like microsatellites allow for detecting small, but statistically significant differentiation. However, even small percentages of typing errors may lead to erroneous conclusions regarding "small, but statistically significant differentiation". The Working Group recommends to verify the reproducibility of results, by analysing two or more temporally separated samples from each geographical locality, and by reanalysing subsets of the samples in order to provide estimates of the abundance of typing error.
2) The Working Group recommends sustainable management of all subpopulations of commercial species and that justification for non-sustainable activities (e.g., allowing the extirpation of sub-populations) based upon a hypothesized metapopulation structure be abandoned.
3) The Working Group recommends that genetic information be used to help differentiate among population models (e.g., source-sink relationships, panmixia and discrete population sub-structuring) in order to provide better advice to managers.

2.4 Review and Report on Methods for Estimating Effective Population Sizes and/or Changes in Effective Population Sizes in Anadromous and Marine Fish Populations

Based on a position paper by Marja-Liisa Koljonen, Pierre Boudry, and Michael M. Hansen, adopted by WGAGFM in Bergen, 2001.

Introduction

Effective population size, N_e, is the most important parameter of conservation genetics as the amount of maintained genetic diversity is directly related to it (Frankham, 1995a; Montgomery *et al.,* 2000). The concept of "effective population size" was first introduced by Wright (1931) in order to generalize the theoretical analysis of genetic drift and inbreeding to real biological populations. The effective population size is defined as the size of a hypothetical idealized population that has the same amount of genetic drift as the real population has. For the idealized population N is constant in time, generations are discrete, and all individuals and each sex make equal contribution to the progeny. The reproduction system of the real populations differ, thus, in several respects from the idealized populations and their effective size is also regularly markedly smaller than their census sizes (N_c). Effective population size also can be defined in relation to the rate of inbreeding, $F = 1/ N_e$, where F is the inbreeding coefficient.

The most important factors affecting N_e are sex ratio, variation in family size (i.e., reproductive success) and variation in population size over generations (Falconer and Mackay, 1996). In addition to these factors, N_e depends on some other factors of the breeding structure. Such factors are the degree of generation overlap, number of successful lifetime matings, extent of polygamy, and presence of assortative mating (Simon *et al.,* 1986). N_e can be estimated directly from the demographic data (Simon *et al.,* 1985), if the needed information is available. However, in the demographic estimation all of the components are rarely included and thus the estimates are often overestimates. Variation in family size, especially, is difficult to assess and requires tagging experiments of the wild populations. Temporal variation in population size is considered the most important factor causing deviation of N_e from N_c, and the others in the order of importance are variance in family size and unequal sex ratio (Frankham, 1995b). Genetic information provides one possibility for indirect estimation of effective population size and, in theory, genetic estimates should include all the affecting factors.

Estimation of effective population sizes of fish and shellfish populations is important as the population sizes of the wild populations are very difficult to assess and effective population sizes may be a small proportion of the actual number of fish. Even in hatchery populations N_e often deviates markedly from the actual size of the broodstocks (N_c). According to Mace and Lande (1991), the N_e/N_c ratio can usually be expected to be under 0.5 (0.2 –0.5). Nunney and Elam (1994) have concluded on the basis of theoretical studies that N_e/N_c should be between 0.25 and 0.75 for most organisms. However, it has been estimated to be as low as 0.11 in wild animal populations (Frankham, 1995b) and it can be expected to be very low for species with high fecundity and high mortality in early life stages as fishes and shellfishes have. Low N_e/N_c ratios can be expected also for species with large fluctuations in population sizes (Vucetich *et al.,* 1997).

Very high fecundity is a characteristic of many aquacultural species. It is therefore a common practice for hatcheries to produce large amounts of offspring from a limited number of parents. The genetic variability present in these offspring is of course directly related to the number of parents used, but can be also reduced by unbalanced contributions of each parent, leading to very small effective population sizes (Hedgecock and Sly, 1990). The genetic consequences of such practices are of concern, especially if some of these offspring are to be used as parents for the next generation. Inbreeding is likely to occur, leading to a decrease in performance (e.g., Kincaid, 1976; Hedgecock *et al,* 1995; Bierne *et al.,* 1998). Furthermore, a reduced genetic variability limits the possibility of future genetic improvement by selective breeding. A review of effective population size and the loss of alleles in hatchery-propagated bivalves is provided by Beaumont (2000).

In the wild, the question of whether variance in reproductive success limits effective population sizes of marine organisms was raised by Hedgecock (1994). Reproduction of marine animals is mediated by spatially and temporally varying oceanographic processes affecting most of their life cycle. Marine animals with a pelagic larval stage are likely to offer ample opportunities for differential reproductive success.

N_e estimation can be done on two time scales, either long-term operating over thousands of generations, or short-term, i.e., below approximately one hundred generations. The long-term N_e estimation requires additional assumptions to the short-term estimation. Long-term N_e can be estimated from the theory of neutral evolution (Nei and Graur, 1984), a drift-heterokaryotype disadvantage model of karyotype evolution (Lande, 1979), or DNA sequence divergence (Avise *et al.,* 1988). From the conservation genetics and population management point of view, the shorter time scale estimation methods are most relevant and thus we have concentrated on these.

Several genetic methods have been developed to estimate N_e. The estimates can be based on the rate of loss of heterozygosity or the number of alleles. The rate of loss of alleles can in principle be used to estimate N_e, as the relation between bottleneck size and the rate of loss is defined (Nei *et al.,* 1975). The formula for the relationship is

$$n = n_0 - \sum(1 - p_i)^{2Ne},$$

where n is the number of alleles after the bottleneck, n_0 after the bottleneck and p_i the frequency of the ith allele before the bottleneck (Frankel and Soulé, 1981). No practical application of this was found in literature.

From the loss of heterozygosity (H_e), N_e can be calculated from the formula $F = t/2\ln(1 - F)$, where $F = (H_o - H_t)/H_o$, in which H_o is the mean heterozygosity of the original population and H_t is the mean heterozygosity of the tth generation (Falconer and Mackay, 1996). However, these estimators are relatively uncertain as substantial changes in N_e may occur before any changes in mean heterozygosity or number of alleles could be observed and the standard errors of H_e estimates are often large, due to large variation among loci (see also later in this paper under title "Comparison of levels of genetic variation over time"). However, when the number of loci studied increases, the errors in H_e estimation will decrease. More precise estimation can be done on the basis of allele frequency changes or linkage disequilibrium.

Demographic data

The most important factors affecting N_e are variation in population size over generations, variation in family size and sex ratio (Falconer and Mackay, 1996). Their effect on N_e is assumed to follow theoretical expectations and N_e can be estimated directly if demographic data are available. Wright's (1931) formula for calculating N_e from the number of females (N_{ef}) and males (N_{em}), when sex ratio is not even is: $N_e = 4\ N_{ef}N_{em}/(N_{ef} + N_{em})$. Variance in the family size affects the N_e so as $N_e = 8N_e/(V_f + V_m+4)$, where V_f and V_m are variances of female and male offspring production (family size), respectively. Family size is often assumed to follow a Poisson distribution, when the mean and variance are the same; if so, the variance may be substituted by the mean (Tave, 1986). When the number of females and males differ and have different family sizes, the N_e for females (N_{ef}) and males (N_{em}) should be first assessed separately for each generation and then joined using an equation for uneven sex ratio: $N_{ef} = N_T/[1 - F_f + (1+F_f)V_f/u_f]$, where N_T is the number of adults of both sexes, F is Wright's inbreeding coefficient (Wright, 1921, 1922), V_f is the variance in family size of female parents and u_f size is the maternal mean family size. N_{em} for males is the same expression, with appropriate subscript changes (Simon *et al.,* 1986). When the population size also varies temporally, the N_e is the harmonic mean of the census numbers in the (t) successive generations, beginning with N_1 until N_t is $N_e = [1/t(t(1/N_1 + 1/N_{2+} ...+1/N_t)]^{-1}$ (Wright, 1939).

Effective population size of hatchery-propagated progenies can also be calculated directly using family sizes as calculated by Robertson (1961):

$N_e = (\Sigma n_{ij})^2 / \Sigma n_{ij}^2$,

where n_{ij} is the observed number of offspring of male *i* crossed by female *j* in a given progeny.

Temporal method

The amount of genetic drift in populations is directly related to their effective sizes, so the amount of drift over time can be used for estimating N_e (Nei and Tajima, 1981). Pollak (1983) was the first to develop a method to estimate N_e from allele frequency changes over time. The general prerequisites of the applications are that generations are discrete, there is no gene flow to the population, and selection and mutation do not change the allele frequencies. To observe temporal changes in allele frequencies, populations should be sampled at a minimum of two points in time (generations) and thus the method is called "the temporal method". Waples (1989, 1990, 1991) continued the work and created a more generalised version of the same method, which could also be used at least in some cases for overlapping generations. Later, Waples and Teel (1990) developed a method for semelparous fish, where year-classes were used as estimation units so that sampling intervals less than a generation could be used. Jorde and Ryman (1995) developed the method further to meet the requirements of estimation in cases when generations overlap. Differences between year classes cause additional variance, which should be corrected.

Effective population size (N_e) can be estimated on the basis of allele frequency changes using the temporal method (Pollak, 1983; Waples, 1989, 1990, 1991) with two formulas depending on the sampling scheme. In principle, if sampling takes place after reproduction or by "sampling with replacement" before reproduction occurs (i.e., non-destructive sampling), information on the actual population size is needed (plan I). Conversely, if sampling is based on young fish prior to reproduction and sampling takes place "without replacement", information on the census population size is not needed (plan II) (Waples, 1989). However, if $N_c/N_e > 2$, results from "plan I designs" converge rapidly to results obtained using the "plan II design" (Waples, 1989).

According to the simpler model (sampling plan II), $N_e = t/[2(F - (1/S))]$, where F is the standardised variance of allele frequency change, S is the harmonic mean of the sample size of the stocks, and t is the number of generations between the samples. F is estimated according to Pollak (1983), where F for one locus is $F_k = 1/(L-1) \sum \frac{(X_{0i} - X_{ti})^2}{(X_{0i} + X_{ti})/2}$ where L is the number of alleles and the X's are the frequencies of allele i (i = 1, 2, ...L) at sampling times of 0 and t.

For several loci, the estimate is the weighted mean of the single locus estimates $F = \sum(L_j - 1)F_{kj}/\sum (L_j - 1)$, where the js index the different loci (Tajima and Nei, 1984). Average sample sizes over loci can be counted as weighted averages in which the number of observations available in each locus are weighted with the number of independent alleles ($L - 1$) in the locus.

Confidence intervals for F can be calculated according to Waples (1990). The formula for the 95 % confidence interval for F is: 95 % CI for F = ($nF/\chi^2_{.025\,(n)}$, $nF/\chi^2_{.975(n)}$) for n degrees of freedom, which is the independent number of alleles over loci. The values obtained can be used in place of F to determine the confidence intervals of N_e.

For overlapping generations it has been shown that there is not necessarily a direct relationship between effective size and temporal allele frequency fluctuations (Jorde and Ryman, 1995). This is especially true when the time span studied is less than one generation. Allele frequency fluctuations depend also on the age-specific survival and birth rates of the year-classes. The difference in allele frequencies between year-classes depend on how evenly various age classes contribute to reproduction. To estimate effective sizes for overlapping generations, allele frequency changes could be considered separately for each age class and create a correction term C, which is determined by the age-specific survival and birth rates. However, since these parameters are seldom known for wild populations, it would be important to know when the temporal method can be used for overlapping generations, without that information. In practice it means either that the populations have to be sampled randomly with respect to age, or the individuals have to be aged and N_e be estimated by comparing cohorts. The bias caused by year-class changes decreases, however, markedly when time intervals more than one generation are considered and the model based on discrete generation model gives quite reliable estimates as well. Also the larger the drift, the smaller the relative error caused by the temporal shift due to year-class differences. For cases when populations cannot be aged reliably, effective population size can be estimated using allele frequencies from a single age class only for which the age is known, such as the seeds of trees (Jorde and Ryman, 1995).

Recently, a maximum likelihood estimator has been developed for diallelic markers to estimate population size from temporal allele frequency changes (Williamson and Slatkin, 1999). The maximum likelihood estimator has a lower

variance and smaller bias than the direct mean *F*-statistic estimate of N_e of Pollak (1983) and Waples (1989). This estimation cannot, however, be used for microsatellite information with several alleles per locus, without pooling of alleles. Anderson *et al.* (2000) have developed a method based on Monte Carlo estimation for multiallelic markers. In the future, Markov Chain Monte Carlo methods could also be used to increase precision and accuracy in N_e estimation. Kitada *et al.* (2000) have already developed a true Bayesian estimator for N_e.

Usefulness of the temporal method

The temporal method is very useful for estimating population size. The application area of the method is, however, limited to the population sizes in which genetic drift can be observed separately from the allele frequency changes caused by the sampling. So it is more useful for detecting small population sizes and *its usefulness for usually very large marine fish or shellfish populations is very limited*. Where the actual size limits are depends on sample sizes, number of loci studied and number of alleles per locus. The possibilities to increase the number of studied loci and number of alleles at individual loci, using microsatellites, will probably offer increased possibilities for estimating N_e. For the analysis, temporally separated samples are needed and the more generations the time span includes, the better. However, even samples of only separate year-classes can be used with certain assumptions. Numerical resampling-based estimation will in the future increase the precision and accuracy of the estimation. The expectations to be fulfilled are that the mutation and gene flow are insignificant in relation to the effect of genetic drift. For the markers and time scales usually studied, selection and mutation hardly cause a problem. Gene flow, on the contrary, may be very difficult to rule out in most marine and anadromous populations.

The temporal method has been used mainly to estimate N_e of salmonid fish populations (Jorde and Ryman, 1997; Laikre *et al.,* 1998; Hedrick *et al.,* 2000), though there is also an example of estimating N_e in Northern pike (*Esox lucius*) based on this approach (Miller and Kapuscinski, 1997). The only example available for a marine species, red drum (*Sciaenops ocellatus*), was based on mitochondrial DNA, which then results in the estimation of the female part of N_e (Turner *et al.,* 1999). In this case demographic statistics were included. The estimated effective size for the female part of the population was 14,308 (1,250 – ∞) and the N_e/N_c ratio as low as 0.004. The observed N_e is larger than could have been expected to be estimated with a method based on occurrence of genetic drift, which is indicated by the large confidence intervals as well. Jorde *et al*. (1999) have also applied the temporal method for estimating N_e from temporal shifts in dominant gene marker frequencies (RAPD). Analysis of genetic drift from dominantly expressed gene markers has, however, a number of disadvantages compared to using codominantly expressed alleles.

Linkage disequilibrium

N_e estimation by using linkage disequilibrium was first suggested by Hill (1981). It is known that if loci are unlinked the major part of gametic phase disequilibrium has been generated in the present cohort (Waples, 1991), though Pudovkin *et al.* (1996) have also pointed out that there may be reminiscences of disequilibria from previous generations. Theoretically, it is known that correlation among alleles at different loci (r) is related to effective population size (Hill, 1981). In an ideal, infinite, randomly-mating population disequilibrium (D) and correlation among alleles at different loci (r) will be 0. Linkage disequilibrium is the difference between the expected co-occurrence of two alleles at two loci and their actual co-occurrence. Estimates of D and r both measure the association of alleles at different loci. Waples (1991) has shown that although estimates from single loci are unreliable, the variance of r decreases considerably when more loci are included.

The formula for N_e(D) according to Waples (1991) is: $N_e(D) = 1/(3 \times (r^2 - 1/S))$, where r is the correlation among alleles and S is the sample size. The value for r is calculated from the relationship $r = D/(p \times (1 - p) \times q + (1 - q))^{1/2}$, where p and q are frequencies of allele A at locus 1 and allele B at locus 2, respectively, and D is Burrow's composite measure of disequilibrium (Campton, 1987).

Average correlation between alleles at a pair of loci, r, can be estimated using the program Linkdos (Garnier-Pere and Dillmann, 1992). N_e can then be estimated as: $N_e = 1 \cdot (3(R^2 - (1 \cdot S^{-1})))^{-1}$, where R^2 denotes the arithmetic mean of squared correlations between alleles, r, at all pairwise combinations of loci and S denotes the harmonic mean of sample sizes. 95 % confidence intervals can be obtained by substituting r^2 with confidence intervals for R^2 in the equation given above. For further details, see Bartley *et al.* (1992).

Waples (1991) has pointed out that this method for estimating effective numbers of breeders may be valid only for diallelic loci, which might require pooling all except the most common allele at each locus into one single composite allele. Hansen *et al.* (2000) have observed that using more than two alleles causes overestimation of N_e.

The method has found some use for estimating N_e in fish species (Bartley *et al*., 1992; Hansen *et al.,* 2000), but mainly involving captive populations.

Usefulness of linkage disequilibrium method

The clear advantage of linkage disequilibrium estimation is that it can be done from a single genotype distribution, without temporally spaced samples. The multiallelic variation at microsatellite data cannot be fully utilised. In addition, relatively large sample sizes are needed for precise estimation. Bartley *et al.* (1992) have recommended 100 or more. Even with large sample size and linked loci standards errors of the estimates are fairly large, and negative estimates are common (Beaumont, 2001). Laurie-Ahlberg and Weir (1979) have suggested that it is necessary for the sample size *n* to be clearly larger than the N_e to be able to estimate the N_e with any precision.

Oysters and other shellfish.

Most examples listed so far have concentrated on fishes. However, some efforts have also been done to estimate N_e in shellfish. In the wild, small N_e/N ratios were estimated by Hedgecock (1994) on the basis of temporal changes of allelic frequencies in the Dabob bay population of Pacific oysters (*Crassostrea gigas*). He concluded that the observed genetic drift yielded at least a 10^{-5} discrepency between estimated effective and actual population numbers. This was supported by Li and Hedgecock (1998) in a study of genetic heterogeneity among samples of larval Pacific oysters (*Crassostrea gigas*) from Dabob Bay (Washington, USA), which supported the hypothesis that larvae are produced by "relatively few parents". Besides, experimental crosses performed under controlled hatchery conditions have confirmed this supposedly high variance in reproductive success in oysters.

Hedgecock and Sly (1990), using fourteen polymorphic allozyme markers, calculated the per-generation effective sizes of two commercial stocks of Pacific oysters to be 40.6 ± 13.9 and 8.9 ± 2.2. These calculations were based on temporal variance of allelic frequencies (Pollak, 1983). The same method was used by Hedgecock *et al,* (1992), examining published data from sixteen shellfish stocks and their wild progenitors (studied species: *Penaeus japonicus*, *Mercenaria mercenaria*, *Pinctada martensii*, *Crassostrea virginica* and *Crassostrea gigas*) and by Gaffney *et al.* (1996) for wild and hatchery populations of red abalone in California. Estimates of N_e for these broodstocks were all less than 100, and thirteen were less than 50. For eight cases, estimated N_e agreed with records of census breeding numbers (in the following referred to as N_b), but in the remaining eight cases N_b lay outside of the 95 % confidence interval for estimated N_e. In four cases, N_b was smaller than the lower confidence interval of estimated N_e. This was explained by mixing of stocks propagated simultaneously in the same hatchery. For four other cases in which N_b was much larger than the estimated N_e, variance in reproductive success was proposed as the most likely explanation of loss of genetic diversity over time. This was demonstrated directly by Boudry *et al.* (1998) in a study of reproductive success in *Crassostrea gigas*. They performed a parentage analysis on two 5×5 outbred crosses of Pacific oyster, using a single microsatellite marker. This was made possible by the high polymorphism encountered at this locus and careful selection of the genitors according to their genotypes.

Results showed large variance in parental contributions at various developmental stages, leading to a strong reduction of experiment-wide population sizes. Altogether, the gametic, zygotic and genetic effects shown warn against the use of too limited a number of progenitors in breeding programmes. Segregation distortions fluctuating with time were also observed. Studying fertilisation rates in 2×2 crosses, Gaffney *et al.* (1993) demonstrated the existence of moderate gametic incompatibility and sperm-egg interactions in *C. virginica*. Additionally, non-Mendelian segregation is known to be frequent in oysters, both in inbred and random-bred families (McGoldrick and Hedgecock, 1997; McGoldrick *et al.,* 2000). This phenomenon was recently demonstrated to be the consequence of selection against deleterious mutations at early stages (Bierne *et al.,* 1998; Launey and Hedgecock, 1999). As this genetic load varies from one individual to another, it is likely that it increases the variance of reproductive success among parents.

Methods for detecting population declines and bottlenecks

Besides directly estimating effective sizes (N_e) of populations a number of statistical procedures have recently been developed for detecting *changes* of N_e without directly estimating N_e itself. Most of these procedures have been developed specifically with the use of microsatellites in mind, but in principle many of them can be used for all sorts of co-dominant nuclear genetic markers.

Comparison of levels of genetic variation over time

Perhaps the simplest way of detecting population declines would be to analyse samples from populations before and after the possible decline has taken place. It could then be assessed if there were reduced levels of variability in the most recent samples. It may be difficult to obtain data from populations prior to possible population declines, but in many cases this could be done by analysing DNA from archived samples, such as scales or otoliths (Nielsen *et al.,* 1997; Hutchinson *et al*., 1999). Alternatively, population declines could be detected through a continuous genetic monitoring programme.

The specific tests that could be applied to these kinds of sampling designs have been evaluated by Luikart *et al.* (1998a) using real and simulated microsatellite data sets. They compared Wilcoxon signed-ranks tests for reduced expected heterozyogosity, Wilcoxon signed-ranks tests for reduced number of alleles, a Monte Carlo test for reduced number of alleles, a chi-square-based numerical resampling test, a Kolmogorov-Smirnov two-sample test for change of allele frequencies and, finally, a "variance test" based on estimating N_e using the temporal method described elsewhere in this position paper. They concluded that the "variance test" was the most powerful, with a 85 % probability of detecting a bottleneck of N_e = 10, when sampling 30 individuals one generation before and after the bottleneck and analysing only five loci. Using ten or twenty loci the power was close to 100 %. The tests for detecting loss of alleles were the second most powerful, but generally >10 loci were needed in order to achieve a statistical power >50 %. Tests based on comparing expected heterozygosity had low power (<50 %) even when 20 loci were analysed. This is not a surprising outcome as it has been known for a long time that the number of alleles is a much better indicator of reduced variability compared to expected heterozygosity (Nei *et al.,* 1975). To increase the power of the tests for mean heterozygosities, McCommas and Bryant (1990) estimated that, for an 80 % probability of detecting a bottleneck at 5 % significance, fifteen loci were needed for a bottleneck size of four reproductive pairs and as many as 46 loci for sixteen pairs, when 30 individuals per population were analysed.

In principle, for testing differences in mean heterozygosities *t* test for paired observations should be used, because allele frequencies and heterozygosities of conspecific populations are historically correlated (Nei, 1987). In addition, if only differences of certain set of loci are tested, irrespective of the evolutionary forces (mutation, selection and drift) involved, only intralocus variances and normal deviate test may be used (M. Nei, personal communication). In that case, only sampling variances of the loci are included.

There are many examples of studies of fish populations comparing expected heterozygosity or number of alleles over time (e.g., Ryman and Ståhl, 1980). However, there are no examples available on the use of the "variance test" and only a few examples where reductions in numbers of alleles have been compared using numerical resampling procedures; Nielsen *et al.* (1999) compared genetic variation in a Danish Atlantic salmon population, based on samples from the 1930s and 1989, and found a limited though statistically significant loss of allelic diversity. Hansen *et al.* (2000) analysed the effect of supportive breeding on brown trout populations by testing for differences in numbers of alleles in reared offspring vs. wild spawners from the same populations and did, in some cases, observe reduced variability in reared trout.

Detection of bottlenecks by testing for deviations from mutation-drift equilibrium

Some of the most widely used methods for detecting population bottlenecks have been developed by Cornuet and Luikart (1996) and Luikart *et al.* (1998b) and can be applied using the well-known "BOTTLENECK" software (http://www.ensam.inra.fr/URLB/bottleneck/bottleneck.html) (Cornuet *et al*., 1996).

The test by Cornuet *et al.* (1996) is based on the fact that a population bottleneck reduces both the number of alleles and *expected* heterozygosity (He). However, the number of alleles is reduced at a faster rate than He (Nei *et al.,* 1975). Consequently, for some time after the bottleneck has taken place He is higher than expected given the alleles present at the loci, i.e., the population is not at mutation-drift equilibrium. Based on the alleles actually observed in the sample, the program estimates the equilibrium heterozygosity (Heq) based on simulations, assuming either an infinite allele mutation model, a stepwise mutation model, or a two-phase model of mutation. The latter model, which is presently considered the most realistic for microsatellite loci (Estoup and Cornuet, 1999), assumes that most mutations occur as stepwise mutations, but a smaller fraction (5–10 %) involves mutational changes of several repeat units. Heq for each locus is then compared to the observed He, and it is tested if a significantly high number of loci in a sample exhibits higher He than Heq.

The minimum requirements for this test are one sample of 20–30 individuals and at least five polymorphic loci. However, preferably (many) more loci should be studied to achieve high power (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998). The actual number of loci required is a complex issue, which depends on the severity of the bottleneck (i.e., the effective population size before and during the bottleneck), the time elapsed since the bottleneck and the model of mutation of the loci. As an example, Luikart and Cornuet (1998) mention that analysis of at least ten loci and a sample size of at least 30 is required to achieve statistical power > 0.80 for detecting a 100-fold decrease of effective population size.

There are still few examples of the use of this test for fish populations. However, Hansen *et al.* (2000) used the test for monitoring supportive breeding of brown trout populations and identified one case where rearing practices had clearly resulted in a bottleneck.

The test by Luikart *et al.* (1998b) is also based on the principle of a faster rate of loss of rare alleles compared to more common alleles during population bottlenecks. At mutation-drift equilibrium loci will typically exhibit a large proportion of rare alleles, whereas fewer common alleles are observed. If all alleles at all studied loci are pooled, and the number of alleles observed at different frequencies (or rather different classes of frequencies, such as >0–0.05, 0.05–0.1, 0.1–0.15, etc.) are illustrated by a histogram, this will result in a L-shaped distribution. Luikart *et al.* (1998b) showed that a bottleneck will result in a transient mode-shift and thereby a distortion of the L-shaped distribution, and this property can be used for detecting bottlenecks.

There are some recent applications of this test for studies of fish populations. For instance, Withler *et al.* (2000) used the test for assessing the genetic effects of reduced population sizes in some specific populations of sockeye salmon (*Oncorhynchus nerka*), but found no evidence of bottlenecks.

Detection of population declines and expansions by Markov Chain Monte Carlo simulations

A conceptually different method has recently been developed by Beaumont (1999). One of the advantages of the method is that it does not depend on detecting bottlenecks per se. Instead, it assesses if population declines or expansions have occurred and provides estimates of the magnitude of the decline/expansion.

The method assumes a strict stepwise mutation model and estimates the posterior probability distribution of several genealogical and demographic parameters, using Markov Chain Monte Carlo simulations, based on the observed distribution of microsatellite alleles and their repeat numbers. The most important output parameters are r, defined as No/N1, where No is the current effective population size and N1 is the effective population size at some point back in time, tf; tf is defined as ta/No, where ta denotes the number of generations which have elapsed since the decline or expansion began. Thus, the method provides information on both the magnitude of the decline/expansion and assesses the length of time the process has taken place.

As pointed out by the author, it may be a problem that the method is based on the assumption of a strict stepwise mutation model, which is not likely to be valid for microsatellite loci. It is not entirely clear how and how much deviations from this mutation model will affect the results.

Beaumont (1999) applied his method to some data sets known to represent populations that have declined or expanded. The results using his method were mainly in accordance with the results using other methods. However, there was a tendency of detecting more severe population declines taking place over longer periods than had been assumed based on available demographic information. This could perhaps be due to deviations from a strict stepwise mutation model at the microsatellite loci or, from a more positive angle, analysis using this method provided more information than methods previously applied. We are aware only of one more example of application of the method. This concerns a study by Pertoldi *et al.* (submitted), who studied the genetic effects of population declines in Danish otters (*Lutra lutra*). No loss of microsatellite diversity could be detected in samples covering the period from the 1880s to the present. However, results based on Beamont's method suggested that the present population size was only a few percent of the original population size and this decline had taken place over more than 2000 years.

Marine fish populations

It is a common feature of all the methods described that they in some way measure N_e or detect population declines by either directly measuring genetic drift or by measuring some feature related to drift, such as distortion of mutation-drift equilibrium. Genetic drift is inversely proportional to N_e.

This means that in very large populations, say, N_e = 1,000,000, there will be virtually no genetic drift, but even if N_e drops to just 10 % of that, i.e., 100,000, there will still be no detectable drift, unless extremely high sample sizes (many thousands of individuals) are analysed. In other words, it is the absolute number of N_e that is important in relation to drift, not the population decline relative to the initial population size. This makes it almost impossible to apply any of the methods described to most marine fish populations exhibiting very large population sizes, unless really extreme population declines take place, e.g., N_e declines to below 100 or less. Ironically, however, it has been shown by Ryman *et al*. (1995) that population collapses in marine fishes are likely to have a serious negative impact on the evolutionary potential of the species, as a lot of rare alleles, which may be of importance for future adaptation, will inevitably be lost. Therefore, it is important to avoid crashes in marine fish populations but, unfortunately, genetic markers cannot at present be applied to detect population declines in marine fishes.

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Recommendations

1) A number of new statistical methods are available, which are very useful for monitoring effective population sizes and for detecting population declines and bottlenecks. WGAGFM recommends a more widespread use of these methods, in particular for managing captive broodstocks.
2) The methods are also useful for monitoring wild populations, where effective population sizes are not too high (e.g., many populations of salmonid fishes).
3) In marine fish and shellfish populations exhibiting very high effective population sizes the methods are of limited or no use, because even after severe population declines effective population sizes are usually still too high to result in appreciable genetic drift. This must NOT be taken as evidence that such population declines are not detrimental to the populations and species; during such population declines many rare but potentially important alleles may be lost.

2.5 Review and Report on Examples Where Population Genetics Research has Provided Important Information for the Management of Marine Fish Populations

Position paper by Paula Alvarez, Peter Bossier, Pierre Boudry, Gary Carvalho, Geir Dahle, Anna Kristin Danielsdóttir, Michael M. Hansen, Torild Johansen, Ellen Kenchington, Marja-Liisa Koljonen, Philip McGinnity, Einar Eg Nielsen, Daniel E. Ruzzante, Jochen Trautner and Eric Verspoor.

Introduction

This ToR arose from a discussion at the 2000 WGAGFM meeting on the significance of population genetics in the context of management of marine fish populations. In the following we give some examples of the use of genetic markers for management of anadromous and marine fish and shellfish populations and point to important developments and possible future applications. This paper should not be seen as an exhaustive listing of successful applications of genetic markers in fisheries management, but rather as a statement of the present and future possibilities for improving fisheries management by increased integration and use of population genetics methods and principles.

Historically, the contribution of genetic studies to the management of marine fish species has been limited largely to issues such as species identification. This is due mainly to the limited genetic differentiation observed in many marine fish species combined with the use of genetic markers that are not fully adequate for this purpose (see ToR c: *Review and report on the importance of different kinds of genetic population structure in relation to human impact*). However, in recent years its influence has begun to expand and address other issues. In part, its increasing influence arises from the more widespread application of traditional markers such as allozymes. For the most part, it is attributable to developments in molecular biology which have allowed the rapid and more cost-effective analysis of particularly informative classes of molecular markers (e.g., microsatellites) identified by direct analysis of genetic variation at the level of the DNA. These new classes of markers are now being applied widely to the study of a range of marine species (e.g., Ruzzante *et al.,* 1998; Shaw *et al.,* 1999) and their potential for generating valuable biological insights is becoming increasingly clear.

The large number of highly variable loci provides an unprecedented sensitivity for detecting population structure in fish species. Concurrently, new statistical methods have been developed, e.g., based on Bayesian principles, Markov Chain Monte Carlo, gene genealogies, etc., that allow for addressing problems that were previously not possible using "traditional" statistics, such as determining the population of origin of single individuals, detection of population bottlenecks and assessment of rates of gene flow without the (often erroneous) assumption that populations are at equilibrium between migration and random genetic drift (see review by Luikart and England (1999) for a listing of recent statistical developments).

In this paper, we provide examples of the use of genetic markers in management of anadromous and marine fishes to address such issues as: 1) determination of genetic population structure, definition of management units and application of mixed-stock analysis, 2) forensics, 3) genetic interactions between farmed and wild salmonids and identification of indigenous populations, and 4) species and population identification in redfish.

Determination of genetic population structure, definition of management units and application of mixed-stock analysis

The population-based management of Pacific salmon supported by population genetic analysis based on allozyme markers represents a classic and perhaps the best example of the application of molecular markers (e.g., Allendorf and Waples, 1996). This application is facilitated by the high level of population differentiation displayed by this group of species. However, data from both traditional allozyme markers, but more particularly new microsatellite markers, are now providing novel insights into population structuring in a wide range of marine fish species. In many cases the genetic data have been integrated with other more traditional sources of biological insights (e.g., data on tagging, morphometrics, growth as well as other more recent innovations such as particle tracking simulations and elemental analysis of otoliths) and have had significant influence on management practise. In the following we give some examples from both salmonid fishes and a marine species, cod.

Evolutionary significant units (ESUs) in salmonid fishes

A major concern in the management of species is to make sure that as much diversity as possible within the species is conserved. In salmonid fishes, population genetics has had tremendous influence on management policies by demonstrating that the species are subdivided into many genetically distinct populations, which are likely to reflect important adaptations and biological differences among populations (see reviews by Ryman, 1983; Ferguson, 1989; Allendorf and Waples, 1996; Laikre, 1999). In other words, each single population only contains a minor part of the total genetic resources of the species. This has resulted in much more focus on the need for managing individual salmonid populations as separate units and, for instance, has led to recommendations of avoiding transfer of fish among populations and of deliberately stocking domesticated fish into wild populations (e.g., Laikre, 1999). Perhaps the most complete integration of knowledge of population subdivision into management procedures has been developed in North America through the concept of Evolutionary Significant Units (ESUs; Waples, 1995). An ESU can be defined as "an assemblage of populations that contains an important part of the evolutionary legacy of the species and which is substantially reproductively isolated from other such assemblages" (Waples, 1995). Thus, ESUs will typically consist of a group of populations sharing a long evolutionary history, for instance as a result of postglacial recolonisation from the same refuge. It should be noted that ESUs typically consist of many populations, and it is insufficient to conserve just one population in order to conserve an ESU. However, by conserving populations from all ESUs it is also ensured that as much as possible of the evolutionary legacy of the species is conserved.

The ESU concept is not only applicable to Pacific salmons. In the Atlantic region there is very detailed knowledge on both large- and small-scale differentiation in several salmonid fishes, notably Atlantic salmon and brown trout. Thus, the ESU concept easily could be applied to these species and, as an example, Laikre (1999) has suggested an ESU-based framework for focusing conservation of brown trout populations in Europe.

Application of genetic stock mixture analysis for defining coastal fishing regulation in Baltic salmon

In the Baltic Sea, Atlantic salmon from rivers draining into the Gulf of Bothnia migrate for feeding south to the Main Basin area. When they return back as spawners they follow the Finnish coast northwards. Thus, wild fish homing to those rivers have to survive a mixed fishery of wild and hatchery stocks both in offshore and coastal areas, before they reach their natal rivers. The naturally reproductive stocks compose a small minority of total catches, most fish being from sea-ranched stocks.

To ensure the maintenance of naturally reproductive stocks, since 1991 the annual salmon catch in the sea fishing has been limited by the International Baltic Sea Fishery Commission (IBSFC), which imposes a quota of total allowable catch (TAC). In 1994, the smolt production of wild stocks was down to about 17 % of the potential smolt production of present spawning habitats. Quota regulation alone was not sufficient to maintain the production of the wild stocks. Most of the salmon, 90 %, in the Baltic Sea is from sea-ranched stocks. The proportion of the wild stocks in the catches in coastal areas, however, varies as the wild and hatchery components have partly different migration behaviour. The wild fish constitute a high proportion of the catches in the springtime, when wild spawners migrate northwards.

Genetic stock composition analyses, where the contribution of individual stocks to the mixed fisheries was estimated using genetic markers, made it possible to record the timing of the untagged wild stocks' spawning migration and changes in the proportion of this group in the coastal drift-net and trap-net catches. Stock mixture analysis can be done either on the basis of genetic information alone (Koljonen and McKinnell, 1996) or by combining genetic information and smolt age distributions (Koljonen and Pella, 1997).

The wild stock group was on average slightly more abundant in trap-net catches (18 %) than in drift-net catches (12 %). The wild stocks were most abundant in spring in catches made with both gear types, indicating the time when the wild

stocks entered the coastal fishery. In drift-net catches, in 1992, the proportion of wild stocks was at its highest, 38 %, in late April (sampling 25–27 April) and early May, 33 % (sampling 5–11 May), which were the last fishing days of the drift-net season in the study area in that year. In 1993, the initial proportion of wild stocks in trap-net catches was as high as 43 % in samples taken in early May, when trap-net fishing began (Koljonen and Pella, 1997). Wild stocks, however, occurred in trap-net catches until the end of July and did not disappear from salmon catches before August. Evidence of wild stocks in autumn or winter catches was rare.

This information has been available and used together with tagging data of sea-ranched fish when planning the stepwise fishing regulation zones for coastal salmon fishery. Since 1996 a strict model of four regulation zones has been applied, in which coastal salmon fishery opens gradually from south to north, according to the expected time when the major part of the wild spawners has passed the area. The opening dates of salmon fishery for the sectors from the south to north are 16 June, 21 June, 26 June and 1 July. Since 1997 and 1998, the production of wild stocks has increased markedly, partly as a result of the TAC regulation and the exceptionally strong year-classes of spawners. However, the more strict coastal fishing regulations since 1996 has been a major factor in the recovery.

Cod in the Gulf of St. Lawrence

The following example is based mainly on the study reported by Ruzzante *et al.* (2000). It has been known for some time that cod populations spawning in the northern and southern Gulf of St Lawrence in early summer migrate from the Gulf in the autumn and overwinter in the Cabot Strait along with other putative populations. A compilation of results from tagging studies conducted in the region show migration patterns that are consistent with those outlined above. As part of an initiative to determine the relative contribution to the overwintering area in the Cabot Strait, of cod from the various management divisions, the Department of Fisheries and Oceans (DFO, Canada) funded a High Priority study to determine the composition of winter cod aggregations based on genetics, otolith microelement composition, and vertebral composition. There were some differences among the three techniques in their ability to discriminate cod aggregations from some of the regions studied, but they all agreed in that a majority of the cod caught in the Cabot Strait in the winter was migratory cod from the northern and southern Gulf of St. Lawrence rather than local, resident cod. As a direct consequence of these studies, the Department of Fisheries and Oceans ordered a shift in the timing of the survey conducted annually for stock assessment purposes. The survey is now conducted later in the year, when the migratory cod from the Gulf are presumed to have migrated back into the Gulf waters.

Forensics

Forensics is one of the areas where the application of genetic markers has received most public attention, and there are by now many examples where evidence from traces of DNA has led to conviction in criminal cases concerning, for instance, murder. In the case of food authentication and food forensics, DNA techniques are also being applied increasingly for determining the organism of origin of meat or vegetable products (e.g., Congiu *et al.,* 2000).

The first successful example of a forensics application of genetic markers in fisheries management was described by Seeb *et al.* (1990). A vessel had landed a catch of red king crabs (*Paralithodes camtschatica*) which were suspected to have been derived from an area where fishing had been closed. A sample of the catch was analysed along with samples from known geographical areas using allozyme electrophoresis. When individuals from the "unknown" sample were classified into the set of baseline samples, using discriminant analysis, it turned out that they were unlikely to have been derived from the area where they were claimed to have been caught and, on the other hand, likely to have been derived from the closed area. This evidence resulted in a penalty of 565,000 USD for the skipper and ship owner.

Assignment tests denote statistical procedures which can be used for determining the population of origin of single individuals, based on the multilocus genotype of the specific individuals and the estimated allele frequencies of the possible source populations (Waser and Strobeck, 1998). Assignment tests have huge potentials in many aspects of fisheries research and management and forensics is one of the most obvious applications (Hansen *et al.,* 2001). An example of this was recently reported by Primmer *et al.* (2000). The specific case concerned a fishing competition for landlocked salmon in a Finnish lake, where it was suspected that the winner fish had not been caught in the lake. A tissue sample was obtained from the fish and baseline samples were collected from the lake of the competition and from other populations in Finland. Assignment tests based on microsatellite DNA data showed that the fish was indeed unlikely to have been derived from the lake and the angler confessed to having bought the fish in a shop.

Microsatellite markers (Gjetvaj *et al.,* 1997), in combination with shell shape morphometrics (Kenchington and Full, 1994), have been used to identify bed of origin in cases of suspected illegal fishing of the sea scallop, *Placopecten magellanicus,* in Atlantic Canada (Kenchington, unpublished reports, pers. comm.). Sea scallop beds are managed individually with size and limit controls and fished by two separate fleets (Peacock *et al.,* 1999). During the last decade over 25 cases have pleaded guilty to illegal fishing based upon reports identifying seized scallops of unknown origin to

their location of capture. In all cases, inshore fishermen were accused of raiding offshore beds where they have no access rights. Genetic and morphometric data used in discriminant function analyses were able to classify seized stock with probabilities of greater than 95 %, proving that genetic data can be a powerful tool for enforcement of fisheries regulations on shellfish.

The sea scallop fishery is also threatened by the removal of undersized meats (adductor muscle) which are landed as *Chlamys islandica*, another commercial scallop which co-occurs with *Placopecten* but which has much smaller meats. As only the meats are landed (the scallops are processed at sea), there was no way to determine whether the catch was composed of legal *Chlamys* meats or illegal, undersized *Placopecten* meats. Kenchington *et al.* (1993) developed a genetic test based on PCR amplification and digestion of a diagnostic segment of the nuclear ribosomal RNA gene sequence, allowing clear and rapid specific identification. In both of these cases, once the fishermen were aware that these genetic techniques were available, they quickly conformed to the regulations so that the genetic technology became a deterrent to illegal fishing practices.

Genetic interactions between farmed and wild salmonids and identification of indigenous populations

Genetic interactions between farmed and wild Atlantic salmon

Atlantic salmon production from farming now exceeds natural production by two orders of magnitude. Large numbers, conservative estimates suggest around 10 % of farm salmon produced, are accidentally released into the wild. Fisheries managers have been concerned as to the possible impact of such escapes on the well being of wild populations. Until recently, it has been impossible to empirically measure, firstly, the degree of genetic interaction (evidence of introgression), and secondly, the influence of these interactions, should they occur, on important parameters such as productivity and quantitative life history traits, e.g., run timing, age of maturity, etc. As a consequence, there has been in the past a certain reticence among aquaculturalists and managers, primarily on the basis of cost, to incorporate strict regimens for controlling accidental releases.

However, a number of recent studies exploiting developments in molecular techniques, significantly their application under natural river conditions, have provided unequivocal information on farmed/wild salmon interactions. 1) It is now known that farm fish successfully spawn in the wild (Clifford *et al.,* 1998a; 1998b); 2) genetic changes occur in the recipient wild populations; 3) These genetic changes give rise to quantitative life history changes which in turn impact on survival and thus productivity (McGinnity *et al.,* 1997; Einum and Fleming, 1997; Fleming *et al.,* 2000). In light of this new information, strict regulations on aquaculture operations have been implemented; e.g., restriction of fish farming from specific sensitive areas; improved standards in salmon cage engineering for fish containment; and use of sterile fish (triploidy and all female lines).

Also on the basis of this information there has been a reassessment of deliberate inter- and intra-stock transfers for enhancement purposes and for salmon release programmes. Similar methodological approaches now are being used to determine the impact of deliberate release programmes (marine ranching) for lobster and cod, both in monitoring the success of the release programme in contributing to the relevant fisheries and determining its impact on population structure.

Identifying indigenous populations of salmon using historical samples

As a result of past escapes or deliberate stocking of farmed salmonids, it is now often not known whether extant populations are indigenous or descend from non-native farmed fish. Nielsen *et al.* (1997) used microsatellite analysis and assignment tests to assess whether or not a Danish population of Atlantic salmon was indeed indigenous. The study involved samples from the original population (DNA extracted from old scale samples from the 1930s), a sample from the contemporary population and samples from other wild populations including the geographically most proximate wild population. The results clearly demonstrated that the contemporary population was indeed original and, consequently, it was given high conservation priority. In a later study, also based on microsatellite analysis of contemporary and historical samples (from the 1910s), remnants of indigenous salmon populations were discovered in two other rivers, where it had previously been assumed that the original populations had been extirpated (Nielsen *et al.,* 2001). Unfortunately, the rivers had been stocked heavily with exogenous salmon, but assignment tests allowed for identifying individual indigenous salmon for supportive breeding. Consequently, the management of salmon in Denmark has changed from the use of exogenous fish for reintroduction to supportive breeding of indigenous individuals. Further, a national conservation plan is under construction in order to save these indigenous populations by assuring suitable habitat and limiting fishing.

Redfish — a difficult group for species and population identification

Species identification is one of the classical uses of genetic markers in fisheries management. Redfishes are a particularly problematic group, where applications of molecular markers have proven very useful.

Redfishes of the genus *Sebastes* are a group of closely related species. In the North Atlantic, four redfish species are recognised; *Sebastes viviparus*, *S. fasciatus*, *S. mentella* and *S. marinus*. *S. mentella* and *S. marinus* are distributed from Canadian waters to the Barents Sea and are the main target species in the Northeast Atlantic. *S. fasciatus* and *S. viviparus* have, respectively, westerly and easterly distribution.

Although the redfishes are known as separate species they were, for many years in the ICES fisheries statistics, referred to as redfishes only (Genus *Sebastes*), because it was difficult to differentiate the species by morphology. It was not until genetic investigations were undertaken in the 1980s that the species status was confirmed and management of the species was changed (Nedreaas and Nævdal, 1989, 1991).

Specifically in the North eastern Atlantic, the identification of the two species *S. mentella* and *S. marinus* has been difficult. It was not until the result of the genetic investigations by Nedreaas and Nævdal (1989, 1991) that the two species were treated separately in the ICES fisheries statistics. Although the genetic analyses are not used routinely, the analysis supported the morphological species identification criteria, which are now used as routine tools to separate the species in the fisheries statistics. Also, by the use of genetic markers the nursery areas of *S. mentella* were identified (Nedreaas and Nævdal, 1991).

In 1996 a new fishery along the Reykjanes Ridge below 500 metres was initiated. A *Sebastes* species similar in morphology to *S. marinus* with average length above 60 cm (called giants) was one of the main targets. In spring, summer and autumn of 1995–1997, 560 tissue and blood samples were collected as well as some whole redfish for morphometric studies. The genetic relationship of this type of redfish to related *Sebastes* species was studied by starch and polyacrylamid gel electrophoresis of haemoglobin and allozymes (Johansen *et al.,* 2000a). Although morphologically similar to *S. marinus,* these giant redfish displayed haemoglobin patterns diagnostically different from the ones usually seen in *S. marinus* and *S. mentella.* Very little *IDHP-1** variation in liver tissue was found, in contrast to the ordinary *S. marinus*. On the other hand, *MEP-2** was monomorphic as in the "ordinary" *S. marinus*. As a result of this investigation, these redfishes are managed as a separate unit in the fisheries statistics, although the younger year classes have not been identified yet. However, there has been no fisheries there since 1997.

The pelagic redfish fishery in the Irminger Sea (the deep water south of Iceland between the Reykjanes Ridge and the coast of Greenland) is another example where genetic data are available, but there is still not complete consensus on the integration of the results in the management. The fishery is believed to consist of oceanic *S. mentella* distributed mainly above 500 metres and pelagic deep-sea *S. mentella* found mainly below this depth (Magnusson, 1977, 1983; Magnusson *et al.,* 1992a, 1992b; Magnusson and Magnusson, 1995). Criteria used for separating the two types are their general morphological appearance together with their depth and geographical distributions. Also, infestation rate of parasites, length at maturity (Magnusson, 1990; Magnusson and Magnusson, 1995), and now more recently differences in their genetic composition (Johansen *et al*., 2000b) have been used. The genetic analysis of the oceanic *S. mentella* and the pelagic deep-sea *S. mentella* in the Irminger Sea showed that they differed genetically and suggested that they do not belong to the same breeding stock component. Furthermore, the oceanic *S. mentella* was found to be a more homogeneous group than the pelagic deep-sea *S. mentella*, indicating more complex stock structure of the pelagic deep-sea type (Johansen *et al.,* 2000b). Further genetic studies on the possible sub-structuring within each type are ongoing.

The advice of the ICES Advisory Committee on Fishery Management (ACFM) in the year 2000 on the management of the pelagic *S. mentella* in the Irminger Sea was to reduce catch levels in 2001. And as it says in the advice: "In addition, management measures applicable to separate fleets are required to ensure that individual stock components will not be overexploited in the pelagic fishery in the Irminger Sea" (ICES, 2001). As a result, the Icelandic Ministry of Fisheries decided to follow the advice of ICES, i.e., regulations have been made so as to manage the pelagic fishing of the Icelandic fleet from the two stock components. Other NEAFC member countries, however, have decided not to follow this recommendation for this year.

In the North west Atlantic, redfish are distributed in the south from the deep waters off New Jersey, northwards in the area off Nova Scotia and Newfoundland banks, in the Gulf of St. Lawrence along the continental slope from the southwestern Grand Banks to Hamilton Inlet Bank and in the area of Flemish Cap (Atkinson, 1986 and references therein). The main target species in fisheries in this area are the two species of beaked redfish (*S. mentella* and *S. fasciatus*). The ranges of *S. mentella* and *S. fasciatus* overlap in the waters surrounding Newfoundland, the Nova Scotian shelf, the Flemish cap and in the Gulf of St. Lawrence. Most studies of these species as well as the fisheries statistics have referred to them as redfish (Genus *Sebastes*) instead of separate species. As a result of genetic studies the

currently used methods for separating the species encompass both morphological traits and genetic markers (McGlade, 1983; Rubec *et al.,* 1991; Sevigny *et al*., 2000), however, this is not fully implemented in the management at the international level. Also, there are still some areas such as the Laurentian Channel, where the species identification is still difficult. This difficulty is due to the fact that the distribution of two or more species overlaps in these areas and that introgressive hybridisation is probably taking place (Desrosiers *et al*., 1999; Roques *et al*., 2001).

Conclusions

It is evident from the examples given in this paper that most advances in integrating population genetics in fisheries management have taken place in the case of salmonid fishes. This is no doubt a result of the pronounced genetic differentiation among populations within this group of fishes, which facilitates discrimination among populations and allows for applying statistical procedures such as mixed-stock analysis and assignment tests with high statistical confidence. Marine fish population genetics is still partly in a pioneering phase, but the ongoing development in the application of molecular markers and new statistical procedures mentioned in the introduction will undoubtedly lead to an acceleration of the use of genetic markers in the management of this group of fishes as well. As an example, the use of mixed-stock analysis based on microsatellite markers for estimating the proportion of cod from different populations in a mixed assemblage (Ruzzante *et al.,* 2000) is a development that would hardly have been considered realistic just five years ago. However, no matter which future developments will occur it should be emphasized that genetic markers are unlikely to become a tool that will completely replace other tools that are currently in use. Instead, what we will see is an increasing integration of population genetics in fisheries management along with other techniques providing complementary information, such as physical tagging and otolith microchemistry.

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3 WORKING GROUP BUSINESS

3.1 Comments on Working Group Function

Whereas the total number of participants in the WG meetings is very satisfactory, it must be considered highly problematic that there is poor and declining representation by persons with expertise in aquaculture genetics, both on the quantitative and molecular genetics side. The Chair has sent out a message to all WG members and asked for solutions to the problem, but the few responses received have not pointed in one single direction. It has been suggested that there is too much work involved in participating in the meetings (writing position papers and reports) and that a collection of abstracts and short summaries should suffice instead. Also, there are two few ToRs of direct relevance to aquaculture. This is, however, a "catch 22" situation, as the lack of ToRs directly dealing with aquaculture problems reflects the limited input and participation by aquaculture geneticists. In order to have more aquaculture ToRs for the 2002 WGAGFM meeting, all WGAGFM members have been asked for input, but so far with little success.

The new arrangement with groups rather than individual persons preparing the position papers generally worked well, and it was decided to continue with this arrangement.

3.2 Suggestions for WG ToR and Meeting Place in 2002

During discussions about the meeting place in the year 2002, WGAGFM responded positively to a generous invitation from Dr Ellen Kenchington, Dept. of Fisheries and Oceans, Bedford Inst. of Oceanography, Canada, to host the 2002 WGAGFM meeting 18–20 March 2002. Concerning Terms of Reference and meeting place for the year 2002, WGAGFM in plenary decided to recommend that:

The Working Group on the Application of Genetics in Fisheries and Mariculture (Chair: Dr Michael Møller Hansen, Denmark) will meet in Halifax, Canada, 18–20 March 2002 to:

a) assess and evaluate the utility of interspecific comparisons of population genetic parameters in understanding population structure in fish species;
b) review and report on developments in the use of DNA from archived samples (scales, otoliths, bones, etc.) for analysing fish populations;
c) review and report on the utility of molecular genetic methodologies for assessing the biological effects of contaminants on fish and shellfish;
d) review and summarize principles for minimizing diversity loss in the early generations of a captive broodstock.

Priority:	WGAGFM is of fundamental importance to the ICES advisory process.
Scientific Justification:	a) During the past few years numerous important developments have been made in the statistical analysis of genetic data, in particular microsatellite DNA, for assessing and describing the genetic structure of populations. These new statistical procedures are based on principles such as Markov-Chain Monte Carlo simulation, Bayesian statistics and coalescence theory and will undoubtedly have a profound effect on studies of the genetic structure of fish populations. However, there is so far a lack of understanding of "what to expect" from these procedures, both in comparison to "traditional" population genetics statistics and in relation to life history and other biological features of the species studied. Many marine species are particularly difficult to work with using "traditional" statistics due to weak genetic differentiation among populations, and it would be of interest to know how well the newly developed procedures perform in these cases. b) Analysis of DNA from archived samples, such as otoliths, scales and bones, is a new and very promising development in fish population genetics. This allows for studying the genetic composition of populations over much longer time spans than have previously been possible, to detect genetic changes in populations due to anthropogenic influence (e.g., loss of variability, allele frequency shifts at loci subject to selection), and to determine whether or not populations are indigenous or the result of stocked or escaped farmed fish. The WG finds that it is important to evaluate the

	utility of archived samples in studies of fish populations and to identify possible problems and pitfalls. c) This is a ToR resulting from a suggestion by the Working Group on Biological Effects of Contaminants (WGBEC). It is suggested to assess the utility of developments in molecular genetics and genomics for studying the biological effects of contaminants on fish and shellfish. The ToR builds partly on previous ToRs at the 2000 and 2001 meetings on endocrine disruptors and selected genes, respectively. d) This ToR addresses the difficulty which arises when a gene bank or supplementary broodstock is founded with a few, non-representative survivors of a dying natural population. It is often not clear what is supposed to be conserved by breeding these remnants in captivity. Standard practices for maximising effective population sizes, such as equalizing mating success and fecundities, will merely lock in the initial founder distortion. Preferentially mating some founders more than others in the early generations, e.g., to increase lineage diversity, is likely to be controversial, though it is a procedure that is used in zoos. It is important to highlight and discuss the fundamental differences, rationales and consequences of these different approaches. Also, hatchery procedures, particularly in the initial generations, should be rather different in these two cases. This is an important question in relation to management and conservation of populations and captive broodstocks. At the same time the ToR addresses an issue of importance to the maintenance of quantitative genetic variation in aquaculture stocks, particularly if aquacultural stocks are included in a comprehensive programme for genetic conservation of a species.
Relation to Strategic Plan:	Responds to Objectives 1 (d), 2 (a, d) and 4 (a).
Resource Requirements:	None required other than those provided by the host institute.
Participants:	WGAGFM members
Secretariat Facilities:	None required
Financial:	None required
Linkages to Advisory Committees:	ACME
Linkages to other Committees or Groups:	SIMWG (Delegates drew specific attention to the need to develop this link – the Chairs of these two Working Groups should correspond together to ensure that there is no unnecessary overlap in their work.)
Linkages to other Organisations:	HELCOM

ANNEX 1: TERMS OF REFERENCE FOR 2001

The **Working Group on the Application of Genetics in Fisheries and Mariculture (WGAGFM**) (Chair: Dr M. Møller Hansen) will meet in Bergen, Norway from 26–28 March 2001 to:

a) continue to review and report on the general population genetics topics in fisheries and mariculture and identify scopes for enhanced international cooperation;

b) review and report on new developments in the identification of genes of relevance to aquaculture and studies of wild populations;

c) review and report on the importance of different kinds of genetic population structure in relation to human impact;

d) review and report on methods for estimating effective population sizes and/or changes in effective population sizes in anadromous and marine fish populations;

e) review and report on examples where population genetics research has provided important information for the management of marine fish populations;

WGAGFM will report by 20 April 2001 for the attention of the Mariculture Committee, ACME and ACFM.

Priority:	WGAGFM is of fundamental importance to the ICES advisory process.
Scientific Justification:	a) WGAGFM is a relatively informal forum where members shall feel free to discuss and update each other on practical and theoretical problems related to genetics of marine species. Experience has shown that there is a need for an open scientific session on the annual meetings, where topics that are not necessarily listed in the Terms of Reference can be enlightened by the competence and experience existing in WGAGFM. Not least have those topics which need competent input from both qualitative and quantitative genetics benefited from these discussions. b) A number of new important genes have been identified and characterised in species subject to aquaculture. Further, the study of these and other coding genes in wild populations may provide important insight into the processes of selection and local adaptations. Finally, new screening techniques (so-called microarray chip techniques) are presently being developed that will allow for very fast screening of a huge number of genes, and these techniques are likely to have a profound influence on genetic research on finfish and shellfish. The WGAGFM finds it important to provide a review and update on these developments. c) Different fish species may exhibit different kinds of genetic population structure. For instance, some species are composed of distinct population units, which are subject to more or less frequent extinctions and recolonisations (so-called metapopulations), whereas other species may exhibit very weak population structure. These different kinds of population structures may lead to different kinds of effects and responses as a result of human impact and exploitation. Therefore, WGAGFM finds it important to review this topic. d) Effective population size is the key parameter determining inbreeding and loss of genetic variability. Estimation of effective population size is therefore of considerable interest both in relation to aquaculture and management of wild fish populations. New statistical developments combined with the use of genetic markers have made it possible to estimate effective population size and genetic bottlenecks. WGAGFM finds it is important to evaluate the use and relevance of these procedures for estimating effective population sizes and bottlenecks in fish populations. e) Whereas genetic research and techniques in several cases have provided important information for the management and conservation of freshwater and anadromous fish species, there are still relatively few examples of the

	successful integration of genetics in the management of marine species. WGAGFM finds it important to highlight cases where genetics has provided important results that have been used in the management of marine species in order to draw experiences for future studies.
Relation to Strategic Plan:	Responds to Objectives 1 (d), 2 (a, d) and 4 (a).
Resource Requirements:	None required other than those provided by the host institute.
Participants:	WGAGFM members
Secretariat Facilities:	None required
Financial:	None required
Linkages to Advisory Committees:	ACME
Linkages to other Committees or Groups:	SIMWG (Delegates drew specific attention to the need to develop this link – the Chairs of these two Working Groups should correspond together to ensure that there is no unnecessary overlap in their work.)
Linkages to other Organisations:	HELCOM

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ANNEX 4: ANNEX TO THE POSITION PAPER ON SELECTED GENES: RECENT TECHNICAL DEVELOPMENTS FOR SCREENING DNA POLYMORPHISM

By Peter Bossier, Adopted By WGAGFM 2002 In Bergen

The research on selected genes benefits from the prior knowledge of the gene sequence and their polymorphic sites. Once these sites are known, there is an ever-increasing amount of high-throughput techniques available to quickly screen for such sites. A number of these techniques are described briefly below. This overview does not claim to be comprehensive. Rather, it tries to illustrate the possibilities that are currently around to quickly identify and screen polymorphic sites in known genes.

Pyrosequencing

Pyrosequencing AB is a Swedish company founded in 1997 by collaborators of the Royal Institute of Technology. In late 1999, the company introduced a totally new system for DNA sequencing. The method is based on the emission of light as the consequence of the incorporation of a single base in a typical primer extension reaction.

The principles of this DNA sequencing methods are as follows:

- Formation of a typical template-primer complex in an annealing reaction
- Addition of four different enzymes
    - DNA polymerase
    - ATP sulphurylase
    - Luciferase
    - Apyrase (ATP -> ADP + Pi -> AMP + 2 Pi)
- Subsequent sequential addition of dATP, dCTP, dGTP and dTTP.

The following biochemical reactions occur and are necessary to generate the DNA sequence:

- When a particular dNTP is added and not incorporated it will be degraded by apyrase
- When a particular dNTP is added and incorporated, then the following steps take place
    - The DNA polymerase produces pyrophosphate
    - The latter is used as substrate by ATPsulphurylase making ATP
    - The enzyme luciferase uses ATP to produce light
    - Light is detected
    - Excess base is degraded by the apyrase.

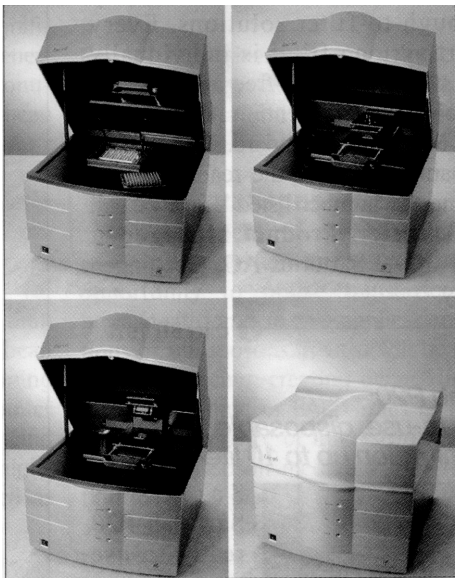
The instrumental set up is as follows:

- In liquid phase in 96-well plates, 96 reactions are executed simultaneously yielding sequence information on 96 templates without electrophoresis
- In ‘solid’ phase there is the potentiality for extention yielding an even higher sample throughput.

Table. A4.1 Potential applications of the Pyrosequencing technology.

Sequencing strategy	Application	
	Short sequence (1 base)	Medium sequence (30 bases)
Mutation analysis and polymorphism	Verification of specific alleles	Verification of specific regions
	Disease screening	Cancer susceptibility and characterisation
		Virus or bacterial typing
Screening	Virus or bacterial typing	HLA typing (transplantation antigens)
	Point mutation analysis	Drug response analysis
	Bi-allelic markers	Sequencing relevant regions to predict drug resistance
	Sequencing signature alleles and SNP's to predict drug response and resistance	Disease screening
Expression monitoring		EST analysis disease screening
High throughput		Tag sequencing to identify clones in shotgun libraries
Sequencing, genetic mapping	SNPs and bi-allelic markers for genomic scanning	Identifying genetic markers
		Mitochondrial genes

Figure A4.1. Pyrosequencing equipment.



Molecular beacons

Molecular beacons are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acids in homogenous solutions. When they bind to their target they undergo a conformational reorganisation that restores the fluorescence of an internally quenched fluorophore. They possess a stem-and-loop structure where the loop portion of the molecule is a probe sequence that is complementary to a target sequence in the nucleic acid to be detected, and the

stem is formed by annealing of complementary arm sequences that are on the ends of the probe sequence. A fluorescent moiety is covalently linked to the end of one arm and a quenching moiety is covalently linked to the end to the other arm. The stem keeps to moieties in close proximity to each other, causing the fluorescence to the fluorophore to be quenched by energy transfer. Because the quencher is a nonfluorescent chromophore that emits the energy that it receives from the chromophore as heat, fluorescence does not occur. When the probe encounters a target molecule it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid spontaneous.

The technique allows for real time detection of PCR amplicons. In the presence of the surplus of molecular beacon the fluorescent signal as measured in a spectrofluorometric temperature cycler will be correlated to the amount of PCR product available.

Also, the quencher DABCYL can be combined with several fluorophores, resulting in different couples with typical emission maxima. Hence, multiple targets can be distinguished in the same solution (multiplexing).

The power of the molecular beacon technology lies in its ability to discriminate alleles. The basis for this ability originates in a significant difference in the thermal stability of the beacon and the linear probe for a target with one mismatch. For instance, a linear and hairpin probe that forms a hybrid with their target at a T_m of 46°C will form a hybrid with a target with one mismatch at a T_m of 18°C in the case of the beacon and with a T_m of 31°C in case of the linear probe. The large difference in T_m for the beacon probe explains its enhanced specificity for target with perfect matches.

Figure A4.2. The principle of DNA detection by molecular beacons.

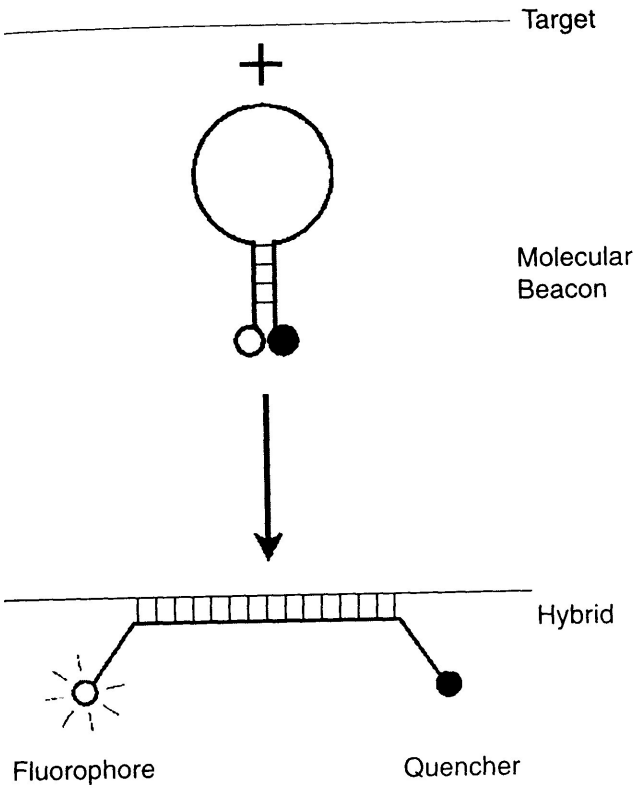
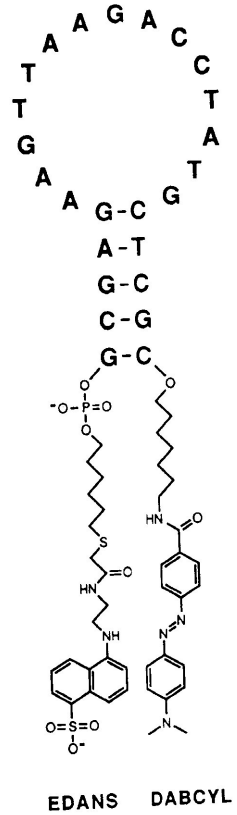


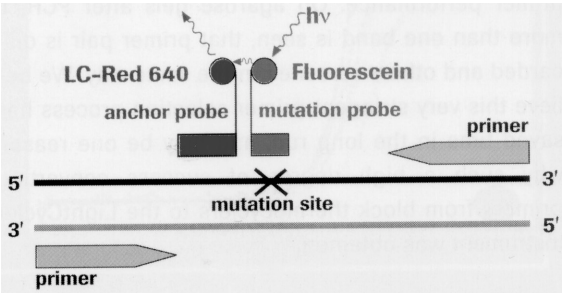
Figure A4.3. A molecular beacon showing the fluorophore EDANS and the quencher DABCYL in its hairpin-and-stem structure.



Allele discrimination by FRET

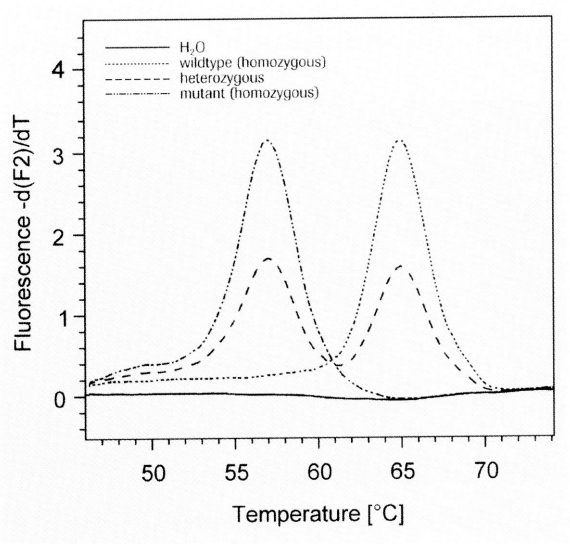
FRET stands for fluorescence resonance energy transfer. The principle is illustrated in Figure A4.4 below.

Figure A4.4. DNA and SNP detection by FRET.



To be able to perform such an analysis, a spectrofluorometric temperature cycler is necessary. The lightcyler, commercialised by Roche, is one of them. PCRs are performed in capillary tubes. This set up allows one to determine the melting curves of diagnostic primers. In a first step, a PCR fragment is amplified. Without further handling steps, the genotype is determined in the same capillary by using specific pairs of hybridisation probes. One probe, the anchor probe, is labelled with LC-Red640 and is annealing to a site where no polymorphism is expected. The other probe, labelled with fluorescein, spans the mutation site. The two probes hybridise next to each other to the target allowing for fluorescence resonance energy transfer when excited with UV, resulting in fluorescent light that is detected.

Figure A4.5. FRET melting curve on wild-type and mutant templates.

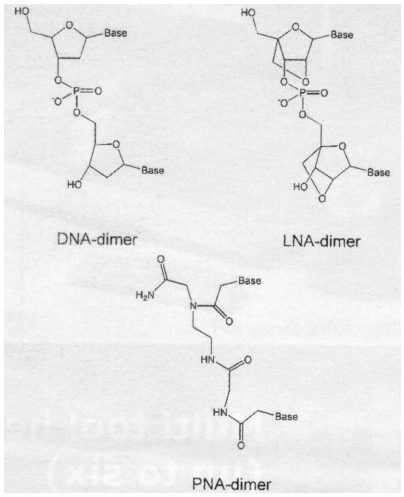


When the temperature is slowly increased during the melting curve analysis, the two fluorescent dyes melt off so that they are no longer in close proximity and the fluorescence will decrease. The melting temperature of the mutation probe is not only dependent upon length and G+C content, but also upon degree of homology between the mutation probe and the template DNA. If a mutation is present, the mismatch of the mutation probe with the target destabilises the hybrid. With a wild type genotype, mismatches do not occur, and the hybrid has a higher T_m. For mutated genotypes, this will occur at lower temperatures. The resulting melting peaks allow discrimination between homozygous (wild type or mutant) as well as the heterozygous genotype (see Figure A.4.5). The anchor probe has a higher T_m than the mutation probe, thus ensuring that the fluorescent signal generated during the melting curve analysis is determined only by the mutation probe.

Allele discrimination by LNA and ELISA

LNA consists of 2'-O,4'C methlylene bicyclonucleoside monomers (see Figure A4.6). Automated synthesis of these DNA analogues is possible, even as hybrid with DNA. LNA is soluble and resistant to 3' exonuclease activity.

Figure A4.6. The structure of LNA.



A short LNA oligonucleotide complementary to the site of the single nucleotide polymorphism (SNP) of the gene of interest is synthesised and covalently attached to the wells of a microplate by anthraquinone photocoupling method (see Figure A4.7). A short biotinylated fragment of the gene of interest generated by PCR is hybridised to the probe in the microplate. After hybridisation, the hybrid can be visualised by horseradish peroxidase streptavidin and a chromogenic

substrate. LNA obeys the Watson-Crick base pairing rules and hybridises to complementary DNA and RNA (or LNA). The LNA:DNA duplex displays a higher affinity than DNA:DNA or DNA:RNA duplexes. In the following table, melting temperatures are given for an experiment in which the temperature was increased at a rate of 1°C per min. In a typical experiment, the PCR product is hybridised to the probe in a wild type microplate and in a mutant microplate.

Figure A4.7. The principle of ELISA detection of SNP through LNA:DNA hybrid complexes.

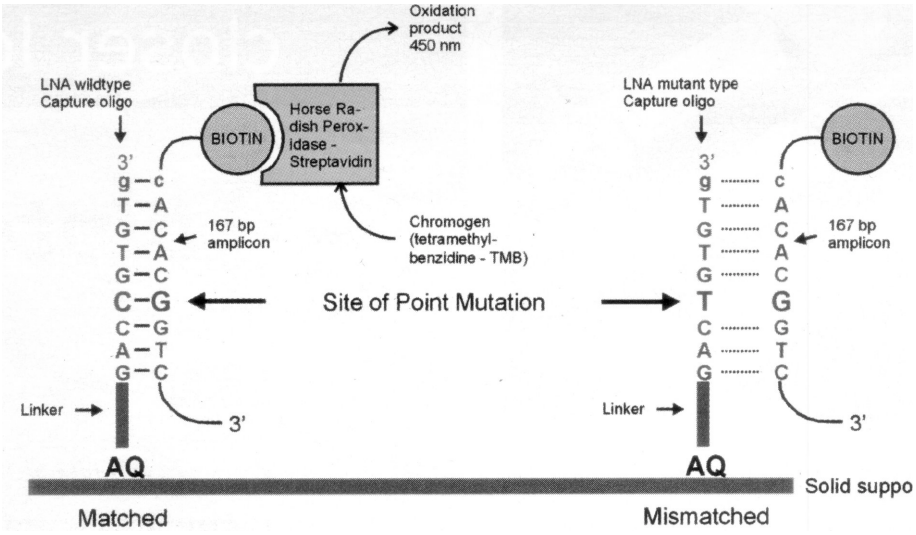


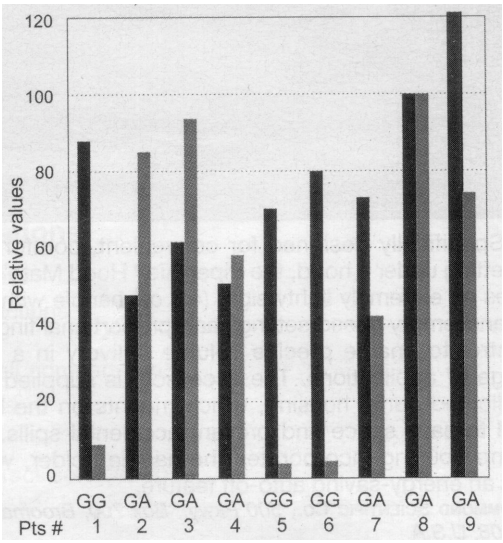
Table A4.2. Melting temperature for LNA:DNA duplexes.

Melting experiments for LNA:DNA and PNA:DNA duplexes*

	Perfect match (PM)T_m/°C	One mismatch (MM)T_m/°C	ΔT_m/°C
LNA 1 **5′-CCGCAGCC-3′**	82°C	61°C	21°C
complementary sequence	3′-ggcgtcgg-5′	3′-ggtgtcgg-5′	
LNA 2 **5′-CCACAGCC-3′**	74°C	52°C	22°C
complementary sequence	3′-ggtgtcgg-5′	3′-ggcgtcgg-5′	
PNA 1 N′-CCGCAGCC	57°C	40°C	17°C
complementary sequence	3′-ggcgtcgg-5′	3′-ggtgtcgg-5′	
PNA 2 N′-CCACAGCC	49°C	37°C	12°C
complementary sequence	3′-ggtgtcgg-5′	3′-ggcgtcgg-5′	

*LNA is in uppercase and bold, PNA is in uppercase and underlined and DNA is in lowercase. The melting temperatures (T_m values) were obtained from melting curves (A_{260} vs temperature) using 1.0 mM of each of the two complementary strands in medium salt buffer (10 mM sodium phosphate, pH 7.0, 100 mM sodium chloride, 0.1 mM EDTA) and increasing the temperature 1°C/min. All transitions were monophasic. ΔT_m values are the difference between a perfect matched duplex and a single mismatched DNA monomer within the duplex.

Figure A4.8. ELISA test on a particular human DNA fragment. Dark bar: readout from wild-type wells. Light bars readout from mutant-type wells. GG stands for homozygous wild-type persons according to DNA sequencing. GA stands for heterozygous persons.



So, the LNA comprises a novel class of nucleotide analogues that form duplexes with complementary DNA with a highly increased thermal stability and generally improved selectivity. The detection of the SNP is ELISA-based.

DNA arrays

An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. An array experiment can make use of common assay systems such as microplates or standard blotting membranes, and can be created by hand or make use of robotics to deposit the sample. In general, arrays are described as macroarrays or microarrays, the difference being the size of the sample spots. Macroarrays contain sample spot sizes of about 300 microns or larger and can be easily imaged by existing gel and blot scanners. The sample spot sizes in microarray are typically less than 200 microns in diameter and these arrays usually contain thousands of spots. Microarrays require specialized robotics and imaging equipment that generally are not commercially available as a complete system.

DNA microarray, or DNA chips, are fabricated by high-speed robotics, generally on glass but sometimes on nylon substrates, for which probes with known identity are used to determine complementary binding, thus allowing massive parallel gene expression and gene discovery studies. An experiment with a single DNA chip can provide researchers information on thousands of genes simultaneously - a dramatic increase in throughput.

There are two major application forms for the DNA microarray technology: 1) Identification of sequence (gene/gene mutation); and 2) Determination of expression level (abundance) of genes. Both types of applications can be useful for the study of selected genes since mutations in the coding part of a gene as well as mutation in promoters (influencing expression levels) can be responsible for differences in traits.

There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity. Either cDNA (500–5,000 bases long) is the probe and they are immobilized on a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method, “traditionally” called DNA microarray, is widely considered as developed at Stanford University. The second DNA array contains an array of oligonucleotide (20–80-mer oligos) probes. It is synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to be labeled sample DNA, hybridized, and the identity/abundance of complementary sequences are determined. This method, "historically" called DNA chips, was developed at Affymetrix, Inc., which sells its photolithographically-fabricated products under the *GeneChip*® trademark.

Further reading

http://www.gene-chips.com/: a website containing general info and lots of interesting links.

http://www.nature.com/cgitaf/DynaPage.taf?file=/ng/journal/v21/n1s/full/ng0199supp_42.html&filetype=pdf: a website containing a review article on the use of microarrays to detect single nucleotide polymorphisms.

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Tyagi, S., and Kramer, F.R. 1996 Molecular Beacons: Probes that fluoresce upon hybridisation. Nature Biotechnology 14: 303–308