Population Structure of Atlantic herring, *Clupea harengus* L., in the Northeast Atlantic using Phenotypic and Molecular Approaches

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by

Cemal Turan (BSc)

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Supervisor: Professor Gary R. Carvalho

University of Hull

Department of Biological Sciences

To Mum and Dad
also
To all members of my family

SUMMARY

Most genetic attempts to study the population structure of herring (*Clupea harengus* L.) have been limited by the low levels of genetic differentiation observed among discrete spawning aggregations over large geographic scales. Thus, the population genetic structure of Atlantic herring remains undefined. Three sets of phenotypic (meristics, morphometrics, otolith shape) and molecular (allozymes, mitochondrial DNA RFLPs, microsatellites) markers were simultaneously used to investigate the morphological and genetic structure of herring populations in the Northeast Atlantic and also to assess the relative usefulness of phenotypic and genetic markers in population identification. Samples were collected from the Celtic Sea, North Sea and fjords, Baltic Sea, Norwegian Sea, Barents Sea, and Pacific Ocean (Pacific herring, *Clupea pallasi* L.).

The Truss morphometric method was very sensitive in detecting morphological differences, revealing significant differences among all discrete spawning aggregations. Otolith analysis showed a lower discriminatory ability than the morphometrics, differentiation of more widely separated populations, revealing a clear discreteness in the Icelandic, Baltic and Trondheimsfjord herring populations. Meristic analysis was more effective for the identification of different species rather than conspecific populations, exhibiting a clear divergence of the Trondheimsfjord and Icelandic herring samples.

With allozyme electrophoresis, twenty-eight putative enzyme-coding loci were examined, and the result was in accordance with previous allozyme studies, showing genetic homogeneity among widely separated populations and localised

heterogeneity in the Norwegian fjords (Trondheimsfjord). Also, an allozymically unique Norwegian spring-spawning (NW1) population was detected off the northern Norwegian coast. The number of low-frequency alleles apparently was a major problem with allozymes, limiting the overall ability to detect weak differences in allele frequencies between populations.

PCR-based RFLP analysis of ND 3/4 and ND 5/6 regions of mtDNA with six restriction enzymes revealed significant genetic discreteness of the Baltic, Icelandic and Norwegian spring-spawner (NW1) herring. The results also showed a high level of haplotype diversity at the ND genes which contrasts with low levels of genetic divergence. This is apparently due mainly to the high number of unique haplotypes, and low number of common haplotypes detected, which reduced the power of the statistical test.

Microsatellites were the most effective molecular marker, revealing genetically distinct Icelandic, Trondheimsfjord, Balsfjord and Norwegian spring-spawner (NW1) herring populations. The detected a high number of alleles and heterozygosity at microsatellite loci provide a new perspective on past estimates of detectable low levels of genetic differentiation among Atlantic herring populations.

In identification of Atlantic and Pacific herring, meristic characters is most effective among phenotypic markers, allozymes and mtDNA are good choices among molecular markers.

The significance of these findings in terms of stock separation and management are discussed.

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CONTENTS

CHAPTER-1

GENERAL INTRODUCTION

1.1 The role of genetics in fishery management

The attainment of a maximum sustainable yield, the level of catch taken from a stock without adversely affecting future reproduction and recruitment, has for a long time been a common and traditional objective of fisheries management. It was embedded in the minds of both biologists and politicians as the appropriate basis for the scientific management of fisheries through ensuring the perpetuation of fish resources. However, the only reliable way to identify the sustainable catch level is by exceeding it, and many fisheries have collapsed in the face of sustainable yield management (Glantz, 1983; Anonymous, 1988; Beverton, 1990). In this perspective, fisheries managers often have a very short-term view, concerned essentially with short-term demographic changes in terms of the harvest determining the subsequent years yield, and are thus mainly interested in abundance, recruitment, growth and mortality. Fisheries managers usually deal with the amount of fish caught, and the consequent economic benefits of that catch. Fishery biologists are thus interested mainly in quantitative changes in exploited populations, that is, the pattern of changes of fish abundance in order to predict future availability under various fishing regimes.

Traditionally, therefore there has been little direct considerations of changes in the qualitative aspect of populations, that is, changes in the

phenotypic and genetic characters of populations. Phenotype is the product of the environment, the genotype, and the interaction between the two. Hence the marked phenotypic variation observed in populations is not necessarily associated with genetic variability. Fish generally reveal more phenotypic variability than other vertebrates (Allendorf *et al.*, 1987) with especially large differences in growth rate and body size between as well as within populations. For example, the size range between populations of Arctic char is over 4000%, which compares with 250 % between species of Darwin's ground finches (Allendorf *et al.*, 1987). On the other hand, heritability for similar traits such as body length and weight are generally much lower within fish populations than within populations of other vertebrates (Purdom, 1979; Kirpichnikov, 1981). The strong influence of environmental factors on phenotypic variation may result in an underestimation of the extent and significance of genetic variation. Non-genetic changes in phenotypic or life history characters such as time of spawning, fecundity, growth rate, onset of sexual maturity of fish populations in response to environmental changes (Anokhina, 1971; Ricker, 1981; Stearns, 1983; Aneer, 1985; Kapuscinski & Lannan, 1986; Allendorf *et al.*, 1987; Robinson &. Wilson, 1995) reveal the fact that there may be considerable variation of life history characters even in the absence of genetic variation. Nonetheless, there have been numerous reports of genetic changes in life history characters associated with environmental alterations (Schaffer & Elson, 1975; Beacham, 1983a, 1983b; Templeman, 1987; Reznick *et al.*, 1990; Gharret & Smoker, 1993; Smith, 1994).

Management of populations based only on phenotypic structure or life history characters would be ineffective to ensure perpetuation of stocks, since some changes in genetic structure such as the loss of allelic diversity may be irreversible, and so affect future viability and persistence. In this respect, fisheries managers should deal also with both quantitative and qualitative changes (Carvalho & Nigmatullin, 1997), and how changes in the former influence abundance, and vice versa.

In the literature, the role of microevolutionary forces (selection, genetic drift, migration) in affecting intraspecific variability and eventually the performance of fish populations has been more strongly recognised for fish than for other vertebrates (Allendorf *et al.*, 1987). An example is the fish 'stock' concept demonstrating the general recognition of genetic differentiation among conspecific populations. In order to ensure perpetuation of fish populations, it is important in terms of productivity to minimise genetically-based changes in life history characters such as fish size, fecundity, and age at sexual maturity caused by human activities. In this regard, fishing or harvesting can be a strong selective force which causes non-random survival of genotypes or individuals in populations. Thus fishing can change genetic composition or gene frequencies of populations over time because of catching particular sub-groups of fish (Ricker, 1982; Beacham, 1983a, 1983b, Mathesion, 1989; Policansky, 1993). For example, by increasing fishing efforts, desirable older and larger fish are usually removed, and a higher proportion of faster growing young fish are left (Borisov, 1979; Rowell *et al.*, 1989; Policansky, 1993) which bring about associated effects such as a decrease in average size and age at sexual maturity (Ricker,

1982; Beachham, 1983a, 1983b, Kirpichnikov *et al.*, 1990). This non-random change in the composition of genotypes and any associated changes in fitness may result in a loss of genetic diversity, with a reduction in the ability of populations to survive or adapt and evolve in response to short and long-term environmental change. In addition to ensuring appropriate recruitment, an often understated objective of capture fisheries management is to minimise or prevent non-desirable genetic changes and to conserve genetic diversity (Ryman, 1991; Carvalho, 1993). There is no benefit in ending up with populations exhibiting smaller size or lower fecundity, since many such changes are irreversible.

The relevance of genetic management to capture fisheries can be understood by considering genetic variation, which is an important feature of populations both for the short-term fitness of individuals and long-term survival of populations, through allowing adaptation to changing environmental conditions.

1.1.1 Intra-population genetic diversity

Genetic diversity within populations is important for the evolutionary success of a species (Fisher, 1930). Allelic diversity and heterozygosity are useful universal indicators of genetic diversity in natural populations, though genes occurring at a low frequency contribute very little to heterozygosity. Maintaining allelic variants in populations leads to greater genetic diversity and, thus in a sense, preadapting the population to future evolutionary change, if different alleles are favoured in different environments (Allendorf & Leary,

1986; Lannan *et al.*, 1989; Ryman, 1991; Carvalho, 1993; Ryman *et al.*, 1995). Although rare alleles may not contribute much to the value of heterozygosity, they may play a vital role in adaptation to diseases or parasites under changing environmental conditions (Frankel & Soulé, 1981; Ryman *et al.*, 1995). Therefore, the reservoir of genetic variation represented by such low-frequency alleles is also important. Genetically very homogenous populations may have reduced performance in the long run (Soulé, 1986), whilst heterozygous populations perform better in fitness parameters such as growth (Mitton & Grant, 1984; Kirpichnikov *et al.*, 1990) disease resistance (Ferguson & Drahushchak, 1990), body size (Danzmann *et al.*, 1989; Danzmann & Ferguson, 1988), and survival (Allendorf & Leary, 1986) revealing a positive relationship between heterozygosity and fitness.

However, genotypes that have an advantage in some aspect of fitness may have a disadvantage in other fitness components (Allendorf & Leary, 1986). There are many well documented examples of heterozygote superiority, but there are fewer examples of disadvantages of heterozygosity in fitness traits (Allendorf & Leary, 1986).

1.1.2 Inter-population genetic diversity

Fish species, in common with most animals, are often divided into more or less reproductively isolated subpopulations or local populations. Subdivision of a species has several benefits through the occupation of different environments which may lead to local adaptation by favouring new combinations

of genes through processes such as epistasis and genetic drift (Wright, 1969; Cavalli-Sforza & Bodmer, 1971). Response to local selection pressures are assumed to enhance epistatic interactions among genes leading to increased fitness (Hindar *et al.*, 1991; Taylor, 1991; Carvalho, 1993). Thus coadapted gene complexes (genes between which there is specific interaction leading to high fitness; Wallace, 1968) may be specific to a local population due to local environmental pressures, and over generations may represent a concerted population response to specific environmental conditions, leading to inter-population differentiation.

Due to the extensive phenotypic plasticity usually detected among fish populations, inter population differentiation has mostly been attributed to environmental rather than genetic influences. Nevertheless in recent years, evidence for local genetic adaptation is increasing (Ricker, *et al.*, 1981; Hindar *et al.*, 1991; Taylor, 1991; Thompson, 1991; Carvalho, 1993). The existence of a large number of genetically distinct populations (Smith & Chesser, 1981; Hindar *et al.*, 1991) indicates the ecological importance of genetic variation and the presence of local adaptation. Since survival of fish incorporates a whole range of performance traits, it can thereby be a indicator of local adaptation. Better survival may be related to a better performance of local fish in aspects like enzyme physiology (Powers *et al.*, 1991), swimming stamina (Green, 1964) and aggressive behaviour (Rosenau & McPhail, 1987). Saunders (1981) reported evidence that genetic differences between local populations of Atlantic salmon (*Salmo salar*) were adaptively related to environmental differences, and also that stocks differed in their resistance to vibrio, a bacterial disease.

A contemporary fishery management viewpoint is that stocks are genetically distinct and optimally adapted for survival and reproduction in their environments (FAO/UNEP, 1981; Helle, 1981; Ryman, 1991). As a result, individual stocks often differ considerably in their biological characteristics including recruitment and mortality, and therefore may respond independently to exploitation thereby requiring independent management.

A sound genetic approach to management is thereby dependent on a knowledge of the extent of genetic diversity distributed within and between populations.

1.1.3 Importance of genetic conservation

Genetic conservation is concerned with the maintenance of genetic diversity between populations and between individuals within populations, as well as with species richness. By appreciating the importance of genetic variability both within and between populations, it becomes apparent that fish represent a resource that is only partly renewable: losses in numbers can be replaced by natural recruitment, however losses in genetic diversity may be irreversible, especially as they relate to genotypic variance in fitness. A reduction in genetic variability in a population can, in the absence of migration, only be compensated through mutations on evolutionary time scale. Such reductions in genetic diversity may render a population less able to adapt to man-made or natural changes in the environment and thus more likely to undergo severe population fluctuations or eventual extinction (Nelson & Soule, 1987; Witte *et*

al., 1992). Therefore existing genetic variability can play a vital role in adaptive evolutionary change.

Thus genetic conservation is an important goal in long-term fisheries management. Indeed, such genetic conservation must be non-specific through maintaining as much genetic variation within and between populations as possible, because very little is known about the economic, ecological or evolutionary value of individual genes or populations (Ryman, 1991).

From a genetic perspective, genetic management can be defined as the incorporation of information on the levels and distribution of genetic variability into a management programme, with the overall aim of conserving genetic resources (levels of allelic diversity and the associated genotype variance in ecologically significant traits; Carvalho, 1993). On the basis of this perspective, management authorities should devise appropriate and effective management strategies on the basis of existing genetic diversity in order to conserve pronounced genetic variability within and between populations. Such strategies are the central notion of the stock concept.

1.2 The stock concept

As previously explained, fish species are often composed of discrete stocks with a definable pattern of recruitment and mortality. Thus the stocks may react to harvesting more or less independently, therefore, requiring management below the species level (Altukhov, 1981; MacLean & Evans, 1981; Sinclair, 1988; Gauldie, 1991; Carvalho & Hauser, 1994). A fundamental

problem for fisheries managers is the definition of stocks for management, especially since it is difficult to get a consensus on what constitutes a stock (Gauldie, 1991; Carvalho & Hauser, 1994). A range of stock definitions exist in the literature (Gulland, 1969; Larkin, 1972; Jamieson; 1973; Booke, 1981, Ihssen *et al.*, 1981b; Smith *et al.*, 1990), and they differ in their emphasis on the degree of homogeneity within stocks, the extent of reproductive isolation, and their relevance to exploitation. The application of the stock concept in fisheries management becomes more complicated by interactions with political, social and economic factors (Fig. 1.1). Although there are many definitions and uses of the term "stock" in the literature there is no universally excepted definition. This terminology is, in fact, confused due to taking different perspectives for practical purposes, because it is dependent on who is defining it and why. The definitions and uses can be reduced to three categories. The first could be termed the "pragmatic" use where a group of fish is exploited in a particular geographic area or by a particular fishing method ("fishery stock"; Smith *et al.*, 1990). For example, if the maximum range of fishing boats from a certain port is 30 miles, then all of the fish within a radius of 30 miles represents the "stock" to which the ships of that port and its fisheries managers have access. Changes in the size of such a "fishery stock" are determined largely by only economic and political interests, with little or no consideration of biological or genetic heterogeneity among areas; the biological or genetic substructuring of fish species is not taken into account.

Gauldie (1988) proposed another stock definition, "harvest stock", largely based on sustainable yield as "locally accessible fish resources in which

fishing pressure on one resource has no effect on the abundance of fish in another contiguous resource". This definition which is concerned especially with demographic changes in stocks, implies a group of individuals with different recruitment and mortality rates caused by differential fishing, and thus shows how much effect of fishing on one stock has on surrounding stocks. By this definition, stocks are isolated in a fishery management sense if the proportion of fish which, on average migrate from one stock to another is less than the proportion lost due to fishing pressure (Gauldie, 1988). Nevertheless with this definition, biological or genetic differentiation of putative stocks is neglected.

Genetic discreteness of local stocks implies a restriction of gene flow by a variety of geographical (spatial) and biological (temporal) isolation mechanisms. Therefore various biological stock definitions have been proposed to establish this perspective. A fruitful definition has been put forward by Ihssen *et al.* (1981b): "a stock is an intraspecific group of randomly mating individuals with temporal and spatial integrity". This definition takes many others into account; the aspect which varies is the degree of spatial and temporal integrity of stocks. Accordingly, the level of integrity is low in the harvest stock and absent in the fishery stock. The problem with the above definitions is that they permit considerable gene flow among stocks without considering its genetic consequences; whereas a low level of gene flow (few migrants) among stocks, even if they differ in biological characters such as spawning time and locations, may serve to prevent the development of genetic differentiation (Gyllensten, 1985; Wables, 1987; Ward *et al.*, 1994b). For example, anadromous salmonid

populations appear to exchange genes (migrants) at a frequency of greater than zero which is sufficient to prevent local differentiation (Hindar *et al.*, 1991).

The third category of the terms could be termed the "genetic stock" with a high degree of integrity defined as "a reproductively isolated unit, which is genetically different from other stocks" (Jamieson, 1973; Ovenden, 1990). Here, the degree of reproductive isolation is typically partial, unless of course where populations are entirely allopatric, where distinct intraspecific stocks exhibit markedly less genetic differentiation than among separate species (Thorpe, 1983).

There are many further stock definitions given by fishery biologists with differing stock integrity from the harvest stock to the genetic stock. The incorporation of these two definitions, the harvest and the genetic stock, into management decisions contribute to the two extremes of time scale of fisheries management (see section 1.1). In the short term, the goal of fishery management is the perpetuation of benefits from a local fish stock by preventing overexploitation and maintaining a sustainable yield. Thus, the harvest stock that has differential harvesting rates in different localities are the units of management. This definition can be used as a practical approach to identify a particular fishery. In the long term, fisheries management should maintain stock integrity and the existing level of genetic diversity between and within populations, and here the genetic stock concept becomes an applicable definition. Thus, they are equally applicable to managing the harvest of surplus production on the one hand, and to genetic conservation on the other.

Effective management does, however, require sufficient information about the extent of biological differentiation among discrete stocks. Therefore effective management depends on the amount of population data available for each stock separately. Often different stocks are treated as one stock due to an absence of information such as catch and effort data required to assess each stock separately. For example, the Western North Atlantic population of blue fish (*Pomatomus saltatrix*) is probably divided into several stocks, but is considered as one stock owing to the absence of data (Brown *et al.*, 1987). In some cases, although available evidence indicates stock separation for a species, fishery managers are often not able to elucidate complex problems posed by such a fishery due to insufficient information or being practically uneconomic. For example, plaice (*Pleuronectes platessa*) in the English Channel possibly has three harvest stocks. Owing to their overlapping distribution, they are treated as one management unit though independent management of the three harvest stock is considered impossible with the information available (IFREMER/MAFF, 1993), or would be too expensive to implement practically.

Incorrect methods of data collection may not elucidate stock structure due to inadequate material available. For example, the British sea bass (*Dicentrarchus labrax*) fishery, which has been divided into five main geographic regions, each of which defined by its fishing pattern rather than the integrity of its bass population (Pawson & Pickett, 1987). Either obtaining more fine-scaled information on population parameters or reorganising the previously collected data to current applications or techniques would overcome the

complex problems in the application of stock structure data (Carvalho & Hauser, 1994).

Socio-economic factors are also very important in the application of research results, and are often detrimental to the exploited stock, even where the stock structure of a species is well described and all the necessary data are available (Pitcher & Hart, 1982). It is difficult to impose catch quotas or restrictions if there is a benefit to other groups exploiting common resources. This situation occurs particularly in vagile pelagic fish like billfish, swordfish and tuna. Although each species has to be managed throughout their distributional range by large multinational bodies, it is very difficult due to different management strategies of corresponding countries (Gulland, 1983). However, if the distribution of stocks overlap with the fishing grounds, the management of exploited species would be greatly simplified. The king mackerel (*Scomberomorus cavalla*) fishery in the West Atlantic and Gulf of Mexico is a good example of the effects of political and socio-economic considerations on management practices (Brown *et al.*, 1987). Two king mackerel groups characterised with seasonal migration between the regions are differentially harvested in the two different regions. There are much more restrictive quotations on catches from the Gulf stock due to recent overexploitation. However, due to socio-economic conditions of the Louisiana fishermen, an artificial separation line between the two stocks was drawn at the Alabama-Florida border to give them more access to the fishery. In this case, the management decisions were based on socio-economic and accessibility factors,

and any phenotypic or genetic structure of stocks were neglected. Thus, there are complexities in both the definition and implementation of the stock concept.

Despite this, there is an even increasing array of phenotypic and molecular tools being used to elucidate stock structure. Such information can provide indications of migration patterns, population identity and the extent of population and genetic differentiation.

1.2.1 Stock structure analysis and stock identification techniques

The genetic structure of populations and the extent of stock separation can change in a matter of decades (Gharret & Thomason, 1987; Smith *et al.*, 1990; Vuorinen *et al.*, 1991) which can be associated with changes in population fitness (Hindar *et al.*, 1991; Taylor, 1991; Carvalho, 1993), and are therefore relevant to fishery management even in the short-term. Stock structure analysis is thereby an important task for fisheries management to detect putative stocks and thus to conserve existing levels of genetic diversity and divergence. Stock structure analysis can play a role in monitoring both phenotypic and genetic changes in populations. Various characteristics and methods have been applied in stock identification and stock structure analysis including the use of ecological studies, tagging, distribution of parasites, physiological and behavioural aspects, morphometrics and meristics, calcified structure, cytogenetics, immunogenetics, blood pigments, allozyme electrophoresis and nucleic acid analysis (Ihssen *et al.*, 1981b; Kumpf *et al.*, 1987; Ryman & Utter, 1987; Ovenden, 1990; Ward & Grewe, 1994; Carvalho & Pitcher, 1994).

The applicability of phenotypic markers, particularly at the intraspecific level, is complicated by the fact that the phenotypic variation is not directly under genetic control but subjected to environmental modification (Allendorf *et al.*, 1987), so is not necessarily heritable. They are usually uninformative and non-discrete with respect to genetic characteristics of a stock, that is, the alleles, their combinations, and their frequencies (Ryman, 1991). Phenotypic markers, do however, continue to play a central role in stock structure analysis, especially when used in conjunction with genetic methods.

Mixed-stock fisheries have generated particular interest in the application of genetic markers. When two or more biologically distinct local stocks are simultaneously harvested and where the origin of individual fish cannot be readily identified on the bases of their morphology (Levery & Shaklee, 1991; Campton *et al*, 1992), fishery managers often need to estimate the relative contribution of the various local stocks to the catch (Utter, 1991; Utter & Ryman, 1993). In the management of Chinook salmon fisheries, for example, genetic markers permitted precise and timely estimates of the stock composition of specific fisheries that were previously impossible to obtain from traditional markers (Miller *et al.*, 1983; Lincoln, 1987). The several advantages of molecular genetic approaches such as being discrete, heritable, universal (same genetic material, DNA, in all animals), and increasingly being non-destructive (especially important for endangered and expensive fish) provide important insights into population studies.

The molecular genetic approach was initiated in fisheries in the 1950s. The primary studies were of blood group variants, which demonstrated the

utility of genetically controlled variation in the analysis of population structure (Ligny, 1969). However, these serological procedures were not widely adopted by fisheries biologists and most were progressively abandoned in favour of electrophoretic procedures providing genetically determined protein polymorphism. Allozyme electrophoresis has found its widest application in the management of mixed-stock fisheries and played a central role in the development and continuous application of genetic markers (Utter & Ryman, 1993). Nevertheless, protein or allozyme electrophoresis provides an indirect assessment of nuclear DNA (nDNA) variability. Therefore, many recent studies have concentrated on the direct examination of DNA, and several techniques have been developed to exploit the nucleotide sequence variation of nDNA and mitochondrial DNA (mtDNA) among populations (Grewe *et al.*, 1990; Taggart & Ferguson, 1990a & 1990b; Hansen & Mensberg, 1996; Hansen & Loeschcke, 1996; Bentzen *et al.*, 1996; García et al., 1997).

1.3 Brief review of molecular genetic techniques

In the early 1930's, red blood cell antigen frequencies were studied as possible markers for identifying genetically isolated races (Ligny, 1969). Red blood cells have surface antigens and the blood plasma is comprised of the complementary immune antibodies in solution. Different red blood cell antigens are regarded as allelic variants of one or more loci; consequently, red blood cell antigen frequencies can provide the basis to analyse genetic structure of populations. This initial genetic approach was introduced into fisheries biology

in the 1950s, primarily in tunas (Cushing, 1956), catfish (Cushing & Durall, 1957), herring (Ridgway, 1958), and salmonids (Ridgway & Klontz, 1960), and successfully revealed the existence of genetically controlled variation which could be used in the identification of different populations. However, many geneticists reported (Robson & Richards, 1936; Stormont, 1961) that blood group genetic variation had no adaptive significance. Additionally it was shown (Hougie, 1972) that the proteins were systematically altered by various posttranslational modifications, therefore detected red blood cell surface antigen frequencies would very often be dependent on the physical state and disease history of the fish.

Consequently these serological procedures were not widely adopted by fisheries biologists and fell into disuse especially with the development of starch gel electrophoresis (Smithies & Walker, 1955) coupled with histochemical staining (Hunter & Markert, 1957). Allozyme electrophoresis provided the detection of enzyme and other protein polymorphisms and supplied the first wide scale and readily applicable abundant genetic markers on natural populations.

Molecular genetic markers will be divided into two categories, protein and DNA-based.

1.3.1 Protein variants: Allozyme electrophoresis

Allozyme electrophoresis uses polymorphic proteins as genetic markers for stock identification. The proteins used most often in electrophoretic studies are enzymes. When enzymes differ in electrophoretic mobility, as a result of

allelic differences at a single gene, they are called allozymes (Richardson *et al.*, 1986). Isozymes are enzymes that promote the same chemical reaction but are the products of alleles at different loci, and may have non-genetic origins. Such non-genetic or epigenetic variability must be excluded when isozymes are applied as genetic markers.

Five of the 20 common amino acids, which make up all proteins, have different net electrical charges and move at different rates through a gel (or cellulose acetate strips) when exposed to an electric current (Utter *et al.*, 1987; Avise, 1994). Three of the five amino acids (lysine, arginine, and histidine) have a positive net charge, and the remaining two (aspartic acid and glutamic acid) a negative net charge. Enzymes are usually extracted from tissues such as muscle, heart, liver, eye, or brain, and centrifuged to remove tissue debris, and the samples are then inserted into small slots in a gel, across which a direct electric current is applied. The net charge of the protein, which varies with the pH of the running conditions, determines the protein's movement toward the anode or cathode in the gel. Protein size and shape also influences the distance of protein in the gel, that is its "electrophoretic mobility". After electrophoresis, the enzymes are visualised using histochemical staining methods. The resultant banding patterns, or "zymograms" which identify the locations of various forms of a single type of protein of an individual, are scored on the gel so providing information on individual genotypes for that specific locus.

Due to the DNA control of protein synthesis, mutational changes at the nucleotide level may bring about corresponding changes in net charges, size or shape of the protein, producing enzymes with different electrophoretic mobilites.

Such genetically controlled variants (Markert & Moller, 1959), provide estimators of genetic diversity and differentiation, and can also provide data on mating patterns in relation to the Hardy-Weinberg paradigm (Richardson *et al.*, 1986). It is thereby possible to determine whether individuals in a sample are drawn from a large, randomly mating population with equilibrium genotype frequencies, or whether samples contain an assemblage of genetically distinct units. Such information is valuable for both stock structure analysis and the conservation of genetic resources, where the extent of fisheries activities (e.g. harvesting, size-selective mortality; Hilborn & Walters, 1992) and genetic component of population differentiation (Taylor, 1991; Carvalho, 1993) are significant management considerations.

The utility of the above applications, speed of processing large samples, and relatively low cost still renders allozyme electrophoresis the first choice for many fishery biologists (Ryman & Utter, 1987). There are, however, several limitations and drawbacks (Richardson *et al.*, 1986; Hillis & Moritz, 1990; Smith *et al.*, 1990). Firstly, studies on population structure require a sufficient level of intraspecific variation. The percentage of the genes that are transcribed and translated into proteins is very small, hence the variation detected at protein gene loci may not be representative of the genome as a whole. Furthermore, only a certain number of products from such loci can be resolved on electrophoretic gels. Secondly, allozymes are phenotypic expressions of genotypes and do not necessarily equate to allelic variation (Lewontin, 1974). 15 of the 20 common amino acids are electrostatically neutral, so some base substitution that results in different amino acids being attached to the

polypeptide chain will not be detected on the gel. Further, seventy percent of third codon base changes causes no modification to the amino acid sequences of proteins due to the redundancy of a large amount of genetic code, and only 32% of amino acid substitution alters the electrostatic charge and are detectable by electrophoretic techniques (Moritz *et al.*, 1987; Meyer, 1993). Thirdly, selective neutrality at allozyme loci is a basic assumption when using allele frequency data in the analysis of genetic population structure and in the estimation of migration between populations. However, allele frequency differences between populations may arise from differential selection pressures rather than the cumulative effects of mutation, gene flow and random genetic drift, though some alleles and genotypes may have selective advantages under specific environmental conditions (e.g. Koehn *et al.*, 1980; Ward, 1989). Fourthly, difficulties in the interpretation of gels can occur if alleles are present that have no expression (null alleles), or two loci produce allozymes of the same electrophoretic mobility (Murphy *et al.*, 1990).

Despite these limitations, allozyme electrophoresis, coupled with histochemical staining of specific enzymes, still provides a simple but powerful tool for estimating genetic variation and population divergence (Smith, 1990; Jorstard *et al.*, 1994; Bembo *et al.*, 1996a, 1996b; Edmands *et al.*, 1996).

1.3.2 Direct DNA approaches

Over the past 17 years, increasing emphasis has been given to direct DNA examination, initially mtDNA (Avise *et al.*, 1979; Brown *et al.*, 1979) and then, as molecular techniques developed, nuclear DNA. The potential amount of genetic variation detectable by DNA methods largely exceeds the amount detectable by protein methods, because DNA sequences are assayed directly. Thus, since fishery geneticists have posed an increasing number of questions that cannot be resolved by allozymes (Ward & Grewe, 1994), DNA methods have received increasingly more attention.

1.3.2.1 Mt DNA

One of the most studied portions of the genome in animals for population or evolutionary studies is the mtDNA genome (Wilson *et al.*, 1985). Mitochondria are the cytoplasmic organelles in eukaryotic cells where respiration takes places. Mitochondria have their own DNA, which contains 38 genes, 13 genes coding for proteins, two genes coding for ribosomal RNAs, 22 genes coding for transfer RNA, and one major non-coding region, vital for cell respiration and other functions. It is physically isolated from the rest of the cell's DNA, which resides within the nucleus, and this physical separation makes it relatively easy to isolate the 1600 to 2000 base pair closed circular mt DNA molecule from the billions of other nucleotides in the genome. The neutrality of

coding and even non-coding mtDNA regions is debatable (Avise *et al.*, 1987; MacRae & Anderson, 1988, Nigro & Prout, 1990) though they may be under selective constraints relating to function in gene expression and or DNA replication (Park & Moran, 1994). There is evidence for selection acting on some regions of the mtDNA in some species, indicating that haplotype frequencies may therefore not be at the stable neutral distribution (Ballard & Kreitman, 1995)

As well as being compact in size, mtDNA is haploid; that is, each mitochondrion contains only one type of mtDNA. However, a few studies (Moritz *et al.*, 1987; Bentzen *et al.*, 1988; Moritz, 1991) have reported heteroplasmy, the presence of more than one type of mtDNA in an individual. Heteroplasmy has occasionally been found in several fish species including bowfins (Bermingham *et al.*, 1986), shad (Bentzen *et al.*, 1988), Atlantic cod (Arnason & Rand, 1992), sturgeon (Buroker *et al.*, 1990) and anchovy (Magoulas & Zouros, 1993). Mitochondria are cytoplasmically inherited, and as the cytoplasm of an ovum is derived from the female, thus mtDNA is predominantly inherited maternally. There is little or no paternal contribution of mtDNA in most organisms, and no known recombination between mitochondrial genomes (Avise, 1994). The maternal inheritance dictates that the mixture of mitochondrial lineages passed to the next generation depends on the relative reproductive success of each female (Ovenden, 1990).

Maternal inheritance and haploidy combine to reduce the effective population size for mtDNA to one quarter of that for the nuclear genes of the same organism (Nei & Tajima, 1981; Ward & Grewe, 1994). A smaller effective

population size means that genetic drift can cause frequency differences between isolated gene pools more readily in mtDNA than in nDNA. In many organisms, mtDNA also appears to accumulate mutations 5-10 times more rapidly than single copy nuclear genes (Brown *et al.*, 1979; Ferris & Berg, 1987; Moritz et al., 1987). In other words, it provides markers with greater variability and sensitivity to drift, and it is therefore more likely to show differences between closely related populations and species; this makes the mitochondrial genome attractive for both systematic (Shedlock *et al.*, 1992; Lockwood *et al.*, 1993; Moran *et al.*, 1994) and population genetic studies (Avise *et al.*, 1986; Heist *et al.*, 1995; McVeigh *et al.*, 1995; Hansen & Loeschcke, 1996). Indeed, in many fish species, mtDNA differentiation was found where there was no allozyme differentiation (Bentzen et al., 1989; Ward et al., 1989; Gonzalez-Villasenor & Powers, 1990, Mulligan et al., 1992; Smolenski et al., 1993), though the converse has also been found (Ferguson et al., 1991; Ward et al., 1994a; Turan *et al.*, 1997). This may be partly due to the fact that mtDNA is inherited as a single unit and is therefore treated as one locus in analyses, which is a distinct disadvantage when compared to multilocus allozyme or nuclear DNA assays.

Mt DNA variability can be assayed by restriction fragment length polymorphisms (RFLPs) and direct sequencing. Early studies of mtDNA variation required large tissue samples and time-consuming protocols and many such studies were inadequate with respect to sample size examined. Polymerase chain reaction (PCR) amplification of selected regions has made examination of the mtDNA variation considerably easier and faster. Universal vertebrate primers (GenBank/EMBL) can be used successfully to amplify various mtDNA regions,

or more specific fish primers can be designed, and also complete mtDNA sequences for several fish species are available in public-access databases. 1-3 KB fragments of the mtDNA genome can be PCR-amplified readily which involves three steps; (i) denaturation of double-stranded DNA by heating; (ii) annealing of primers to the sites flanking the region to be amplified; and (iii) primer extension, in which strands complementary to the region of interest between flanking primers are synthesised under the influence of a DNA polymerase (*Taq*) which is thermostable. This entire process is repeated around 30 cycles and the resulting PCR product is either sequenced or examined for RFLPs. In mtDNA analysis, the most widely used technique has been the digestion of the total genome with restriction enzymes, with application of PCR at one or a few regions of the mtDNA genome. These enzymes (restriction endonucleases) recognise a specific sequence of bases (usually 4-6) called a recognition site, and cut DNA wherever the recognition sites occurs. The resulting restriction fragments can be separated by gel electrophoresis and differences between the fragment patterns occur due to the gain or loses of restriction sites by base substitution or length mutation (Upholt, 1977). If a base substitution occurs in the recognition sequence of a particular restriction site, the enzyme will no longer cleave the DNA at that position, producing a single large fragment rather than two smaller ones. Conversely, a base substitution might result in the creation of a recognition site, and a large fragment will be digested into two smaller fragments. Six base cutting restriction enzymes typically produce 1-10 fragments, whereas four base cutting restriction enzymes yield 10-50 fragments (Gyllensten *et al.*, 1991). The increased data from four base

restriction enzymes may be offset by difficulties in scoring the gels and determining fragment homology (Avise & Lansman, 1983).

Sequencing is the most sensitive method for detecting variation at the DNA level. However it requires much more laborious techniques and also is restricted to short parts of the DNA and relatively small sample sizes. In contrast, RFLP analyses is relatively fast and cheap, allowing examination of large numbers of fish.

Since different regions of the mtDNA evolve at different rates (Meyer, 1993), certain regions of the mtDNA have been targeted for certain types of studies. The cytochrome *b* and ND genes have been examined in a number of species (Carr & Marshall, 1991; Brown *et al.*, 1993; Cronin *et al.*, 1993; Bembo *et al.*, 1995; Hauser, 1996) as they are reported to exhibit variability at the population level. The D-loop (or control region) has also been targeted for population studies because it is highly variable in mammals, though this is not necessarily the case in fish (Nielsen *et al.*, 1994; Park *et al.*, 1993). For example, no variation was found in brown trout from the Atlantic basin by sequencing of the D-loop (Bernatchez *et al.*, 1992), but extensive variation among Atlantic brown trout has been found in the ND1, 5 and 6 and 16sRNA genes (Hall & Nawrocki, 1995; Hynes *et al.*, 1996).

1.3.2.2 Nuclear DNA

The nuclear genome in bony fishes is about 0.3-4 billion base pairs in size (Ohno, 1974) which comprises repetitive and non-repetitive DNA. Non-repetitive DNA (or single copy nDNA) comprises 70% of the total mammalian genome (Alberts *et al.*, 1983), the other part of the genome comprise repetitive DNA that has from a few, to thousands of copies of various sequences, which usually occur in non-coding regions of the genome (Park & Moran, 1994). The existence of repetitive DNA sequences in eukaryotic genomes was documented during the 1960s (Britten & Kohne, 1968). The advent of DNA fingerprinting (Jeffreys *et al.*, 1985) has revealed the existence of an extensive class of genetic loci that are sufficiently polymorphic to serve as markers. DNA fingerprints (Jeffreys *et al.*, 1985) highlight loci containing arrays of tandemly repeated short DNA sequences in which differences between alleles are generated by variation in the number of repeating units. Such loci are known as variable number of tandem repeats (VNTRs).

Several particular features of VNTRs render them valuable for examining fish population structure. Firstly, they are usually non-coding, and therefore the variation should be largely independent of natural selection, except where they are closely linked to adaptively significant coding sequences. Secondly, allozyme studies require fresh and frozen tissue, often causing problems of sampling logistics, whereas small amounts of blood or other tissues preserved in alcohol are adequate for analysing repetitive DNA. Furthermore, such tissues can be obtained without killing the fish. Thirdly, the high level of heterozygosity,

ranging from 59 to 90 % (Taggart & Ferguson, 1990a; Wright, 1993; Brooker *et al.*, 1994; Bentzen *et al.*, 1996), usually ensures the provision of abundant variants to characterise populations (Carvalho & Hauser, 1994).

Multilocus fingerprinting reveals many VNTR loci simultaneously and provides multi-banded DNA profiles (Jeffreys *et al.*, 1985). Restriction enzymes chosen to cut the genomic DNA usually do not have a recognition sequence within the tandem array of VNTRs. Total genomic DNA is fractionated by agarose gel-electrophoresis, Southern blotted, and hybridised under conditions of low stringency to either a cloned minisatellite VNTR, or a core sequence present only in this class of minisatellites. Discrete bands detected by autoradiography identify the allelic variants at the minisatellite VNTR loci. In most cases the complexity of allelic pairs of bands specific to individual loci can not be identified (Bentzen *et al.*, 1991; Wright, 1993). However, the inherent difficulties in the interpretation of multi locus fingerprints can be avoided by examining VNTR loci individually (Bentzen *et al.*, 1991).

A major class of VNTR loci comprises the minisatellites which consist of DNA sequences of typically 9-65 bp in length which are tandemly repeated from two to several hundred times in a locus (Jeffreys *et al.*, 1985; Jarman & Wells, 1989). Microsatellites are also known as simple sequence units of 1 to 6 bp in length such as (GT)n or (AT)n and are tandemly repeated up to 100 times at a locus. Microsatellites are thought to occur approximately once every 10 bp while minisatellite loci occur every 1500 kbp in fish species (Wright, 1993). Microsatellite loci are highly abundant and dispersed throughout the genome (Royle *et al.*, 1988; Jeffreys *et al.*, 1991). Individual alleles at a locus differ in

the number of tandem repeats of the unit sequence and as such can be differentiated by electrophoresis according to their size.

Mutation rates are high in microsatellite loci, estimated around 0.05 to 0.2% (Huang *et al.*, 1992). High levels of length mutation were reported for microsatellite arrays which lead to extensive allelic variation and a high level of heterozygosity (Wright, 1993; Wright & Bentzen, 1994). In the Atlantic cod (*Gadus morhua*), average number of alleles was 41 at per microsatellites locus, and heterozygosity exceeded 89% (Bentzen *et al.*, 1996). Mutations occur in microsatellite arrays due to length changes arising from slipped-strand misspairing or slippage during DNA replication, while in minisatellite arrays, mutations are thought to occur mainly due to DNA recombination (Wright, 1993).

Since many different alleles are possible, and mutation rates are often high, these highly variable regions potentially overcome the problem of low number of alleles which may limit allozyme and other protein studies.

Attention has now turned to the development and application of single locus minisatellite probes and to development of PCR primers for individual minisatellites and microsatellites (Bentzen *et al.*, 1991; Ward & Grewe, 1994; Wright & Bentzen, 1994). Individual microsatellite loci can be studied either by developing primers specific to unique flanking domains of individual microsatellite loci, allowing amplification and description of individual alleles, or by cloning the entire VNTR or one or both domains of unique flanking nDNA and using this to probe Southern blots of the total genomic DNA. However, such single-locus probes or primers currently have to be developed anew for

each species, or group of closely related species, and the development phase can take several months of skilled and expensive labour.

The study of VNTRs has generated a great deal of interest in fisheries research and aquaculture (Wright, 1990; Franck *et al.*, 1991; Galvin *et al.*, 1995; Angers *et al.*, 1995; Tessier *et al.*, 1995). McConnell *et al.* (1995) found significant allele frequency heterogeneity between European and North American Atlantic salmon populations. In contrast to surveys of mtDNA (Smith *et al.*, 1989; Carr & Marshall, 1991) and allozymes (Pogson *et al.*, 1995) significant microsatellite heterogeneity was detected among Atlantic cod populations (Bentzen *et al.*, 1996). Also, single locus minisatellite analysis has demonstrated its potential by detecting high levels of genetic variability, which has not been detected by other markers, among Atlantic cod populations (Galvin *et al.*, 1995).

In summary, it is important to choose the simplest, most informative tools initially, and then proceed to more complex approaches if required (Carvalho & Hauser, 1994), because there is no advantage to looking directly at DNA variation if the genetic diversity can be screened sufficiently by protein electrophoresis. Phenotypic and genetic markers are often applied independently at different times for the same populations of a species. Hence, when significant phenotypic heterogeneity is detected between populations it is difficult to determine whether it has a genetic basis unless genetic markers are applied to the same sample set. Also, there are an increasing number of cases where a lack of concordance between molecular markers has been observed (Ferguson *et al.*, 1991; Ward & Grewe, 1994; Ward *et al.*, 1994a; Turan *et al.*, 1997). Therefore,

the simultaneous application of molecular markers on the same sample set would provide a valuable approach to determining their utility in describing population structure.

1.4 Relevant features of the biology of Northeast Atlantic herring

Atlantic herring, *Clupea harengus,* has been for many years commercially one of the most important commertial fish species in the North-east Atlantic. It is not surprising, therefore, that it has been the subject of intensive research by fishery scientists of many European countries during this century (Svetovidov, 1963; Parrish & Saville, 1965 & 1967; Jakobsson, 1985; Blaxter, 1985; Haegele & Schweigert, 1985). Although studies have focused on the main features of the biology and ecology of exploited populations, and the factors governing long-and short-term variations in the productivity, the population structure of Atlantic herring is still far from resolved.

1.4.1 Reproductive biology and spawning behaviour

The name 'herring' refers to a group of closely related species of marine fish in the family Clupeidae. Herring are pelagic, free swimming and schooling, and live at depths to 200m. In herring, there is sexual differences in spawning behaviour, and towards the end of spawning, males dominant the spawning grounds (Ewart, 1884). Spawning grounds are located in high-energy

environments (intensive plankton), favouring larvae for feeding and growth, either nearshore for spring spawners or hydrophysically active areas for autumn spawners. Spawn is deposited on marine vegetation or on bottom substrate, such as gravel, which is free from silting (Haegele & Schweigert, 1985).

Temperature and salinity are not documented as critical to the successful spawning (fecundity) of herring; though both factors may influence spawning in time and space (Blaxter, 1985). Eggs are tolerant to temperatures in the range of 5-14 °C and salinities in the range of 3-33 (Haegele & Schweigert, 1985). However, there is an inverse relationship between egg size and fecundity, e.g.; winter-spring spawners have large eggs and low fecundity, and summer-autumn spawners have small eggs and high fecundity. It is thought that large eggs and low fecundity are an advantage where food is in short supply and at low predator density conditions, and in contrast, small eggs and high fecundity have greater adaptive value in conditions of good larval food supply and high predator density (Hempel & Blaxter, 1967). Egg mortality results mostly from suffocation due to high egg densities, silting and predation, and in the intertidal spawn, from stresses imposed by exposure to air and from egg loss by wave action (Haegele & Schweigert, 1985).

Spawning grounds are widely distributed ranging from about 50° to 80° N. The timing of spawning depends on temperature conditions ranging from 0°C in spring spawning and to 20 °C in some autumn spawning, e.g. spawning is early in high temperatures and late in low temperatures. It is common in herring that spawning can occur over a period of a few weeks on any given ground. This flexible annual spawning pattern is coupled with greater flexibility in terms of

resistance to environmental conditions and in terms of adaptation to optimum egg size-fecundity (Lambert, 1984). Herring larvae become widely distributed as they drift away from the spawning grounds, and some migrate into estuaries at the end of spring and summer and remain in estuaries for feeding. They may remain in estuaries until early autumn, depending on favourable temperatures, before emigrating back towards the sea (Blaxter, 1985; Haegele & Schweigert, 1985).

1.4.2 Distribution of spawning grounds and stock structure

The first world-wide record of herring was proposed by Svetovidov (1963) who classified herring into two subspecies which, in turn, were divided into a number of forms: the first subspecies is the Atlantic herring, *Clupea harengus harengus* (North Atlantic and Barents Sea), and its form is Baltic herring, *Clupea harengus harengus n. membras* (Baltic Sea). The second subspecies is Pacific herring, *Clupea harengus pallasi* (Pacific, Arctic and adjoining seas), and its forms are *Clupea harengus pallasi n. maris-albi* (White Sea), *Clupea harengus pallasi n. suworowi* (Chesha Bay area). These subspecies are divided mainly geographically, however they have also differences in meristic characters, body dimensions, rate of growth, and size at sexual maturity (Svetovidov, 1963).

In the North-east Atlantic, herring (*Clupea harengus* L.) consist of both winter-spring (spawning between January and May) and summer-autumn (spawning between August and December) spawning groups, each characterised

by oceanic and shelf populations respectively (Parrish & Saville, 1965). The subdivision of north-east Atlantic herring populations into these major groups has been made from observations on the morphological (the number of vertebrate, keeled scales, fin rays and gill rakers), physiological (spawning time, maturity cycle, pattern and rate of growth etc.) and ecological (distribution, population structure, environmental relationships) characters of herring spawning at different times and localities (Parrish & Saville, 1965).

Oceanic populations are divided into five major stocks (Fig. 1.2): (i) the Norwegian winter-spring spawning stock; spawning grounds encompass the west coast of Norway from the Lofoten Islands to the entrance of the Skagerrak; adult feeding grounds in the Norwegian Sea to the north and east of Iceland. (ii) Icelandic winter-spring spawning stock; spawning ground off the south and west coast of Iceland. These spawners differ in the meristic characters, growth pattern and scale structure from Norwegian winter-spring spawners, and are suggested to separate into distinct stocks (Johansen, 1926; Runnström , 1936; Fridriksson, 1944, 1958). However, the Icelandic winter-spring-spawners collapsed completely in the late 1960s and have not recovered (Iceland Marine Research Institute, *Personal Communication*). (iii) Icelandic summer-spawning stock; spawning grounds in the same localities as the winter spring spawners; adult feeding grounds in the Norwegian Sea to the north and east of Iceland. This group differs consistently from the Icelandic and Norwegian winter-spring spawners in several morphological and physiological characters (Johansen, 1926; Fridriksson, 1944, 1958; Liamin, 1959). These three groups are collectively, called "Atlanto-Scandian herring" (Fridriksson, 1944,

1958; Parrish and Saville, 1965, 1967; Haegele & Schweigert, 1985). (iv) Scottish west coast winter-spring spawning stock; spawning grounds in the outer reaches of the Clyde, off the north west coast of Ireland, off the Scottish mainland in the North Minch and to the north of the Hebrides; adult feeding grounds on or close to continental shelf to the west coast of Scotland. (v) Southern Irish (Dunmore) winter-spring spawning stock; spawning grounds off the Irish south-east and south coast; adult feeding grounds in the southern Irish and Celtic Seas. These spawners are considered distinct in several biological characters such as fecundity, vertebral number, egg size distinct from the northern Irish Sea (Isle of Man) summer autumn spawners (Parrish & Saville, 1965).

Subdivision of shelf populations is unclear, though it is suggested according to available evidence that the shelf populations can be subdivided into six major groups (Fig. 1.3): (i) Central and northern North Sea (Bank), summer-autumn spawning stock; spawning grounds from the Shetlands in the north to Dogger Bank in the south probably extending to North Minch (Buchan) and eastern North Sea; adult, non-spawning distribution in the northern and central North Sea, to the north of Scotland and extending into Skagerrak and Minch. There may be partial segregation of the groups spawning in the northern (Buchan), central (Dogger) and eastern North Sea. (ii) Southern North Sea and eastern Channel (Downs) winter-autumn spawning stock; spawning ground in the southern North Sea (Sandettie) and eastern English Channel; adult non-spawning distribution in the central and northern North Sea. In the North Sea, commonly three spawning groups in terms of their spawning time are identified:

Buchan (from August to September), Dogger or Bank (from September to October), Downs (from November to January) (Cushing, 1968). Studies of morphological characters and population parameters (age composition, growth, occurrence of year classes, maturity stage) revealed a distinction between the Bank and Downs spawners, and it has been suggested that they be treated as a distinct units for fisheries assessment and management (Cushing, 1955; Cushing & Burd, 1957; Ziijlstra, 1958). (iii) North-eastern Kattegat, summer autumn spawning stock; spawning grounds along Swedish Kattegat coast; adult non-spawning distribution in Kattegat, Skagerak and eastern North Sea. Also it is proposed that they constitute a separate stock, distinct from the North Sea and Sound, Belt Sea, and Baltic Stock (Parrish & Saville, 1965). This group also exhibited clear distinction in meristic characters from the Baltic spawners (Parrish & Saville, 1965) (iv) Sound, Belt, and southern Baltic summer-autumn spawning stock; spawning grounds in the Belt Sea, Sound, south western and southern Baltic; adult non spawning distribution mostly confined to these localities to the central Kattegat and Baltic. (v) Scottish west coast (Minch), summer-autumn spawning stock; spawning ground in the North and South Minch; adult non spawning distribution in Minch and on continental shelf to west of Scotland. Also it is reported that some mixing of spawners with the North Sea Bank herring may occur. This group differs morphologically and in population structure (fecundity, egg size) from others (Parrish & Saville, 1965). (vi) Northern Irish Sea (Isle of Man), summer autumn spawning stock; spawning grounds off the south east coast of the Isle of Man; adult non spawning distribution occurs in the Irish Sea, and may extend through to North Channel to

northern Irish coast and South Minch. This group differs from the southern Irish Sea (Dunmore) and Clyde, winter spring spawners in morphological (vertebral number, otolith structure) and population parameters (egg size, fecundity) was reported (Parrish & Saville, 1965).

In the White Sea, spawning occurs in the Gulfs of Dvina, Onega, and Kandalaksha both during the spring and summer. These spawners are morphologically similar to Pacific herring (Svetovidov, 1963; Soin, 1971), and also are referred as a subform of Pacific herring (*Clupea harengus pallasi n. maris-albi*) (Svetovidov, 1963).

Surprisingly, despite all these different characters among herring populations in the Northeast Atlantic, most genetic attempts have revealed genetic homogeneity among these vastly separated aggregations including North West Atlantic populations (Anderson *et al.*, 1981; Jørstad & Nævdal., 1981; Kornfield *et al.*, 1982; Ryman *et al.*, 1984; Grant, 1984; Jørstad & Pederson., 1986; King *et al.*, 1987; Kornfield & Bagdanowicz, 1987; Dahle & Erikson, 1990; Jørstad *et al.*, 1991) revealing non genetic based differentiation.

Genetic attempts to define stock structure in herring are largely based on allozyme studies (Anderson *et al.*, 1981; Jørstad & Nævdal., 1981; Kornfield *et al.*, 1982; Ryman *et al.*, 1984; Grant, 1984; Jørstad & Pederson., 1986; King *et al.*, 1987; Jørstad *et al.*, 1991). There are limited data using DNA (mtDNA) analysis (Kornfield & Bagdanowicz, 1987; Dahle & Eriksen, 1990) confirming a similar lack of genetic differentiation.

This general lack of genetic differentiation in herring on a geographic scale contrasts with localised genetic heterogeneity in Norwegian fjords (Jørstad

& Nævdal., 1981, 1983; Jørstad *et al.*, 1994). Balsfjord herring are particularly genetically distinct from Atlantic herring, and show an apparently higher genetic similarity to Pacific herring (Jørstad *et al.*, 1994).

1.5 Aims

While many studies, employing a variety of stock identification techniques across different sample sets have been undertaken in the Northeast Atlantic throughout the population range, the stock structure of Atlantic herring is still far from resolved. Moreover, such independent phenotypic and genetic studies make it very difficult to infer the level of migration between putative stocks. The present study aims to elucidate the population structure of North East Atlantic herring using three different phenotypic markers (morphometrics, meristics, and otoliths) and three different molecular markers (allozymes, mtDNA RFLPs, microsatellites) on a set of samples collected from throughout the Northeast Atlantic, including Icelandic waters and the Norwegian fjords. Such studies using more than one data set to describe population structure have the potential to describe population interactions more fully than would be possible using a single marker set.

Further, the literature indicate that the relative usefulness of different molecular methods depends largely on the species and geographic scale to which they are applied, as well as on the question asked. Therefore, it is valuable to compare the information generated by three different molecular markers simultaneously on the same set of samples.

Consequently, the objectives of this study is as follows:

1) to analyse the morphological and genetic structure of Northeast Atlantic herring populations;

2) to compare the data collected with existing stock structure data described elsewhere;

3) to compare phenotypic and genetic data provided by nuclear and mitochondrial genes;

4) to assess the relative usefulness of the phenotypic and the genetic markers in population identification;

5) to investigate the utility of PCR-based microsatellite loci analysis and RFLP analysis of mtDNA ND3/4 and 5/6 genes for the analysis of herring population structure.

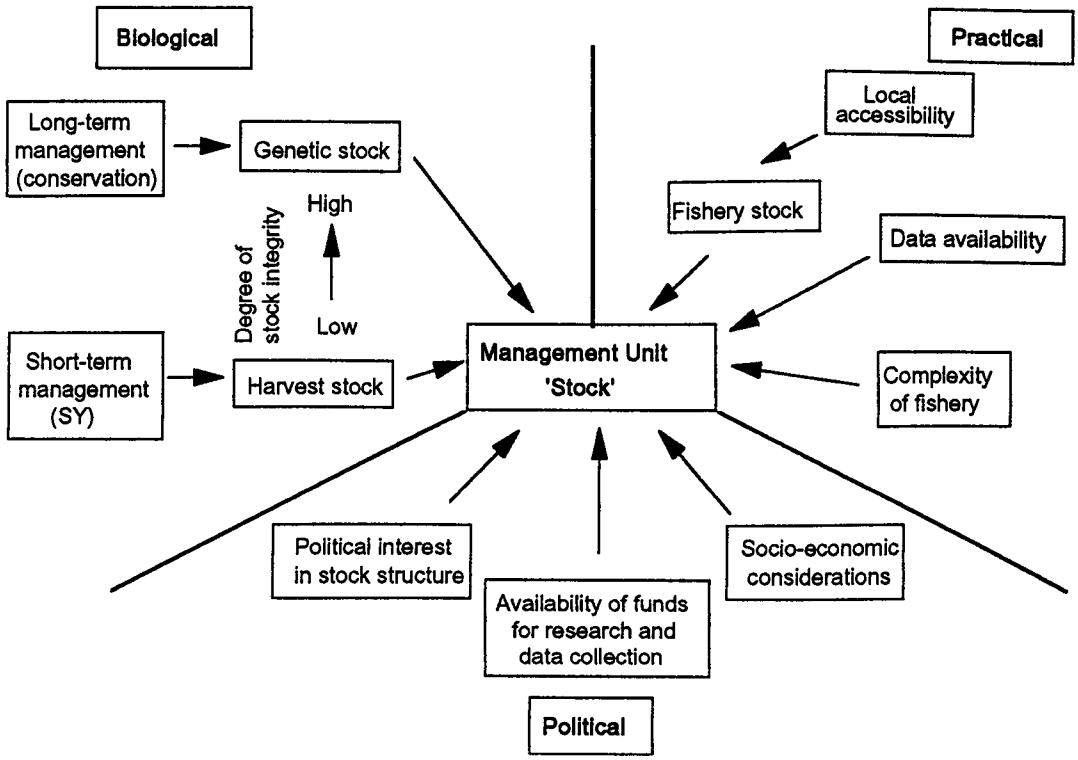

Figure 1.1. Schematic illustration of factors affecting the application of the stock concept. Three types of stocks are variously described (fishery, harvest and genetic stock). From Carvalho & Hauser (1994).

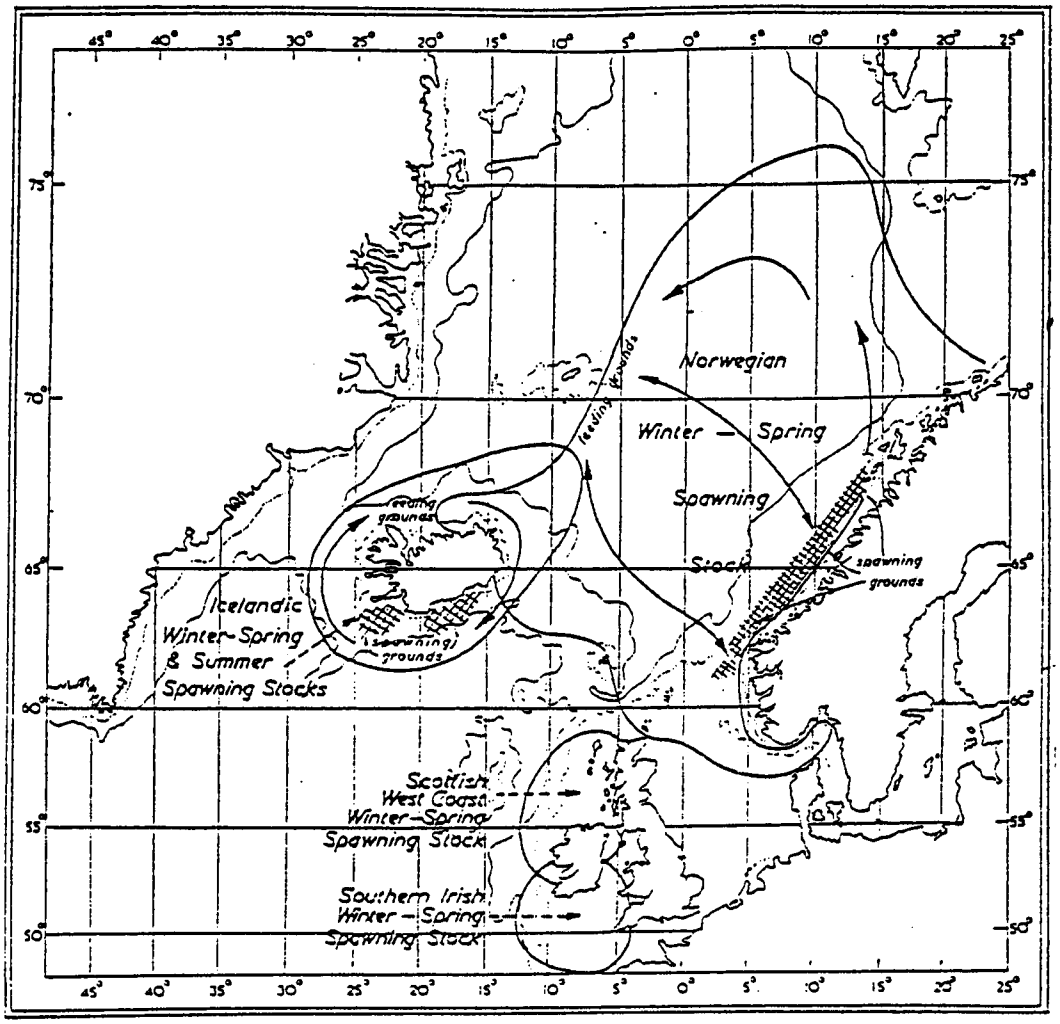

Figure 1.2. Geographic distribution of 'Oceanic' populations of herring in Northeast Atlantic. From (Parrish & Saville, 1965).

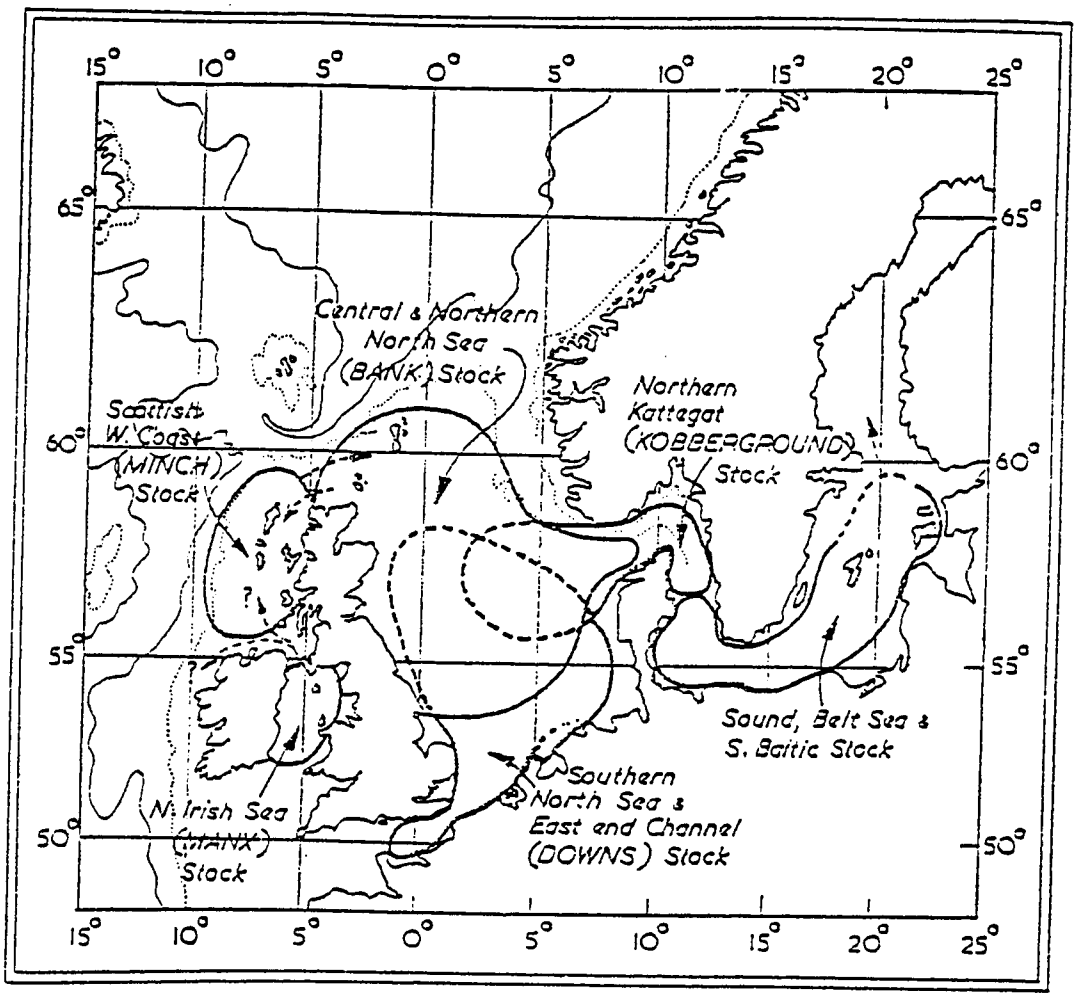

Figure 1.3. Geographic distribution of 'Shelf ' populations of herring in the Northeast Atlantic. From (Parrish & Saville, 1965).

CHAPTER 2

SAMPLING OF NATURAL POPULATIONS

2.1 Geographic location of samples

A prerequisite for effective stock structure analysis is to obtain a representative sample of putative stocks of a species. It is important to undertake complementary spatial and temporal analysis to provide a measure of integrity, especially when analysing the stock structure of commercially important and highly mobile pelagic fishes. In the present study, samples were collected from spawning aggregations throughout the Northeast Atlantic Ocean covering the Celtic Sea, North Sea, Baltic Sea, Norwegian Sea and Barents Sea. However, it was not easy to collect samples at regular intervals and from exact/precise locations throughout the range, due to the limited availability of sampling vessels and presource of fish. In the present study, sampling at a similar location in space and time was achieved just with the Icelandic samples. In order to obtain samples from multinational waters correspondence was with national fisheries institutes, universities and MAFF laboratories. Sample sizes, gear, depth and source of samples are given in Table 2.1.

Statistical considerations suggest that a sample size of at least 50 individuals per sample is generally necessary to provide a representative level of

genetic variation of a specific population, and the magnitude of allele frequency differences expected between samples (Shaklee, 1983).

Spawning areas of Celtic (CS), Downs (NSD), Dogger (NSC), Buchan (NSN1 & NSN2), Baltic (BA), Icelandic (IC1 & IC2), Norwegian (NW1, NW2, NW3, NW4) samples are different from each other (Haegele & Schweigert, 1985).

Icelandic samples (IC1 & IC2) composed of summer-spawners were sampled from the same location (two times, February 1994 and November 1995) (Fig. 2.1 & Table 2.1).

Norwegian spring-spawning samples were collected from oceanic (NW1-Barents Sea, NW2) and shelf or fjord (NW3, NW4) locations (Fig 2.1 & Table 2.1).

The Baltic Sea sample (BA), probably representing spring-spawners of the Baltic herring, was collected in the vicinity of Muskö island (Fig. 2.1 & Table 2.1).

From the North Sea, one sample represented the Bank group herring (NSN2) collected from the Northern North sea, and another collected from the Southern North Sea presumably represented the Downs group herring (NSD). Also, samples were collected from the east coast of Shetland (Northeast North Sea) (NSN1) and off the coast of Durham (Dogger Bank) (NSC) (Fig. 2.1 & Table 2.1), though these samples (NSN1 & NSC) were not used for genetic analysis due to poor storage conditions.

From the Celtic Sea, the sample (CS) was caught off the south coast of Ireland (Dunmore) and represented the southern Irish winter-spring spawning stock (Fig. 2.1 & Table 2.1).

Pacific herring samples (PC) were collected in the northern part of the Strait of Georgia and represented spring-spawning herring (Fig 2.1 & Table 2.1).

2.2 Biological data of samples

Collection data for all samples is reported in Table 2.1 and location of samples is given Fig. 2.1. Age, size, spawning condition and sex of samples (Table 2.2) were recorded as biological background for interpreting the spatial distribution of populations. In the Norwegian samples (NW1, NW2, NW3), with the exception of Trondheimsfjord (NW4) fish, the sex was recorded at Trondheim Biological Station. No age data were available for these samples. Age was determined directly from otoliths using a binocular microscope after removing blood and debris from otoliths with glycerol and 0.5 % thymol. For example each annual ring on the otolith was counted as one age. The age varied between samples, the oldest fish were recorded in Icelandic and Pacific samples, while the youngest fish were recorded in Baltic samples (Table 2.2).

Standard length (mm) was taken from the truss network measurements (landmark distance between a and f, see chapter 3). The mean standard length of three Norwegian samples (NW1, NW2, NW3) was recorded at Trondheim Biological station. The largest standard length was recorded in the Norwegian

spring-spawner (NW2) and Icelandic (IC1 & IC2) samples. Higher body depth, especially the post-orbital side of the head, was also observed in Icelandic samples. In contrast, a clear shallow body form was observed in Baltic herring.

Sex could be determined only from mature individuals from the condition of the gonads (Table 2.2). Spawning conditions was determined as either ripe-running or non-spawning. Sex ratios varied among samples, with the Norwegian spring-spawning sample (NW2) highly dominated by females (Table 2.2).

The mean standard length of fish demonstrated significant differences for the same or similar age classes between populations sampled (Table 2.2 & Fig. 2.2). For example, Trondheimsfjord fish comprised smaller standard length than other samples except Baltic sample, with most frequently 2 years old fish. However, Buchan herring samples (NSN2) consisted of larger fish (standard length), with most frequently 1 year old fish. The differences in length characteristics of the samples may be stock-specific, which may indicate a differential response to environmental effects on the growth and body shape of herring. A possible environmental factor which may cause the differentiation in length characteristics of populations may be differences in the availability of food or water temperature between habitats. For example, the small size of Trondheimsfjord herring, despite its higher age, may be due to its cold habitat reducing the basal metabolic rate, thus causing a reduced feeding rate (Bone *et al.*, 1995). On the other hand, the observed differences between populations may have a genetic basis which may be revealed by the application of molecular markers.

2.3 Sample transportation and storage

After sample collection, herring were placed individually into plastic bags, and kept frozen at -20°C until transportation. For transportation the samples were put either into a polystyrene box or tissue samples were placed into eppandorfs. Frozen samples were stored at -80°C until dissection.

For the three Norwegian samples (NW1, NW2, NW3), dissection of liver and muscle tissues for allozyme analysis were carried out at the Trondheim Biological station in Norway. The dissected samples were stored at -20°C until transportation. Samples of muscle tissues for DNA analysis were also dissected from the same fish, and put in eppendorf tubes filled with 90 % ethanol.

On arrival in the laboratory, fish were defrosted and morphometric (Truss) measurements were made, including eye diameter and head width. Thereafter, samples of liver, eye were taken and muscle tissues were dissected quickly along the lateral line of the fish, and then all samples were snap-frozen in liquid nitrogen. All samples were stored at - 80°C until examined for allozyme analysis. For DNA analysis, white muscle samples were removed along the lateral line of the same fish and stored in eppendorf tubes filled with 90 % ethanol. Meristic counts (dorsal, pectoral, anal, pelvic fin rays and gill rakers) were taken from the dissected fish using a binocular microscope for meristic analysis. Finally, sagittal otoliths were removed from the cranium of the fish and stored in envelopes for subsequent otolith analysis.

Table 2.1. Collection date, location, size, gear and depth of herring samples used in this study.

Name of sample	Abbreviation	Collection date	Latitude and longitude	Sample size	Gear	Depth (m)	Source
Icelandic summer-spawners (first year)	IC1	02.02.1994	64° 33' N 12° 19' W	50	Pelagic trawl	15-20	Jakop Jacopsson[1]
Icelandic summer-spawners (second year)	IC2	20.11.1995	64° 33' N 12° 19' W	48	Pelagic trawl	15-20	Jakop Jacopsson
Norwegian spring-spawners (northeast Norwegian Sea)	NW1	28.08.1996	71° 05' N 29°10' E	50	Pelagic trawl	15-20	Jarle Mork [2]
Norwegian spring-spawners (central Norwegian Sea)	NW2	15.08.1993	68° 45' N 9° 50' W	46	Pelagic trawl	15-20	Jarle Mork
Norwegian spring-spawners (Balsfjord)	NW3	26.09.1992	69° 30' N 19°40' W	50	Pelagic trawl	15-20	Jarle Mork
Norwegian spring-spawners (Trondheimsfjord)	NW4	17.10.1994	63° 50' N 10° 40' W	50	Bottom trawl	30-35	Jarle Mork
Baltic herring	BA	22.10.1995	58° 95' N 18° 50' E	50	Gillnets		Nils Ryman[3]
Buchan herring (northmost North Sea)	NSN1	27.10.1991	60° 20'N 0° 40'E	28	Bottom trawl	30-35	David Thomson[4]
Buchan herring (northern North Sea)	NSN2	19.11.1994	57° 85'N 1° 50'E	25	Bottom trawl	30-35	David Thomson
Dogger herring (central North Sea)	NSC	15.08.1994	54° 80'N 1° 50'W	27	Bottom trawl	30-35	David Thomson
Downs herring (southern North Sea)	NSD	23.10.1994	52° 80'N 3° 50'E	28	Bottom trawl	30-35	David Thomson
Dunmore (Celtic Sea)	CS	20.03.1994	51° 80'N 8°10'W	50	Bottom trawl	30-35	Tony Child[5]
Pacific herring	PC	8.03.1996	49° 35' N 124° 40' W	30	purse seine		Dog Hay[6]

[1] Hafrannsoknastofnunin, PO Box 1390, Stulagata, 121 Reykjavik, Iceland
[2] Trondhjem Biological Station, Bynesveien 46, N-7018 Trondheim Norway
[3] Division of Population Genetics, Stockholm University, S-106 96, Stockholm Sweden
[4] Fisheries Directorate, MAFF Labarotory, Pakefield Road,Lowestoft, Suffolk, NR3 OHT U.K.
[5] Fisheries Directorate, MAFF Labarotory, Benarth Road, Conwy, LL32 8UB U.K.
[6] Departmant of Fisheries and Oceans, Pacific Biological Station, 3190 Nanaimo, Canada

Table 2.2. Length, age, spawning condition and sex of *Clupea harengus* samples used in this study. Standard deviations of means are given in brackets. Samples referred to in the text were Icelandic summer-spawners (first year, 1994) (IC1),Icelandic summer spawners (second year, 1995) (IC2), Norwegian spring-spawners (Northeast Norwegian Sea) (NW1),(NW3), Norwegian spring-spawners (central Norwegian Sea) (NW2), Balsfjord (NW3), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (northmost North Sea) (NSN1), Buchan herring (northern North Sea) (NSN2), Dogger herring (NSC) Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC). The abbreviation of techniques are :MO, morphometrics; ME, meristics; OT, otoliths; ALO, allozymes; RFLP, restriction fragment length polymorphism, MC, microsatellites. * denotes employment of ND 5/6 region and @ denotes employment of ND 3/4 region of mtDNA for corresponding sample.

Sample	Mean standard length (mm)	Age (between years) / mean age	Spawning condition	Sex (male:female)	Techniques employed
IC1	254 (+/- 15)	2-4 / 2.77 (+/- 0.69)	non-spawning	--	MO, ME, OT, ALO, RFLP*,@, MC
IC2	259 (+/- 24)	2-4 / 3.02 (+/-0.54)	non-spawning	--	MO, ME, OT, ALO, RFLP*
NW1	151 (+/-6)	--	ripe-running	28: 22	ALO, RFLP*, @, MC
NW2	347 (+/- 29)	--	ripe-running	7: 39	ALO, RFLP*, MC
NW3	151 (+/- 26)	--	ripe-running	25: 25	ALO, RFLP*, MC
NW4	167 (+/- 8)	2-3 / 2.10 (+/- 0.30)	ripe-running	16: 25	MO, ME, OT, ALO, RFLP*, @, MC
BA	129 (+/- 8)	1 / 1	non-spawning	--	MO, ME, OT, ALO, RFLP*, @
NSN1	227 (+/- 8)	2 / 2	non-spawning	--	MO, ME, OT
NSN2	198 (+/- 12)	1-2 / 1.08 (+/-0.28)	non-spawning	--	MO, ME, OT, ALO, RFLP*
NSC	223 (+/- 13)	1-3 / 1.32 (+/-0.58)	ripe-running	16: 29	MO, ME, OT
NSD	201 (+/- 15)	1-2 / 1.38 (+/-0.50)	non-spawning	--	MO, ME, OT, ALO, RFLP*
CS	191 (+/- 14)	1-2 / 1.25 (+/-0.44)	non-spawning	--	MO, ME, OT, ALO, RFLP*, @
PC	195 (+/- 20)	2-4 / 2.87 (+/-0.63)	non-spawning	--	MO, ME, OT, ALO, RFLP*, @, MC

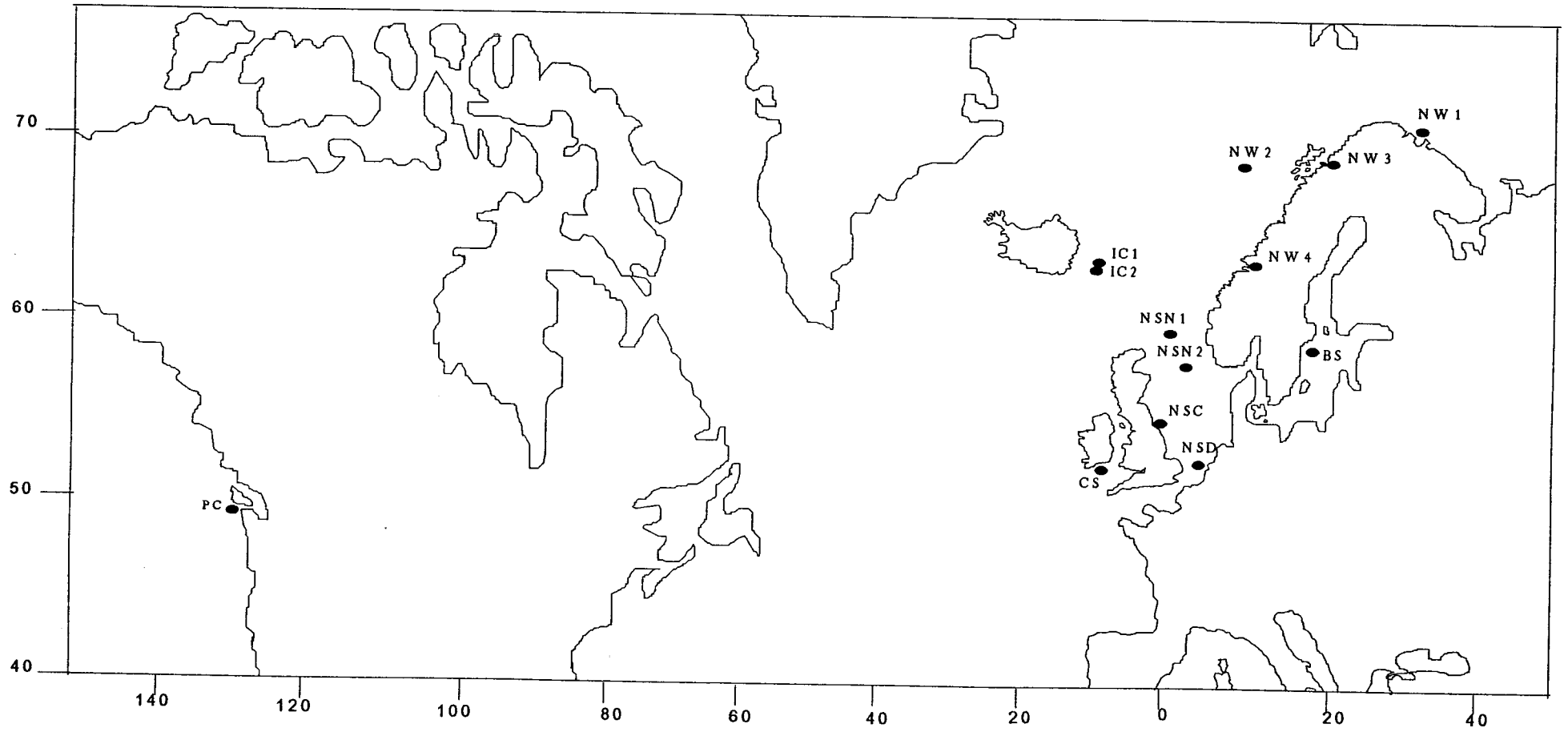

Figure 2.1. Map of the sampling locations of herring. Samples referred to in the text are Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring -spawners (NW1), Norwegian spring-spawners (NW2), Balsfjord (NW3), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (NSN1), Buchan herring (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC).

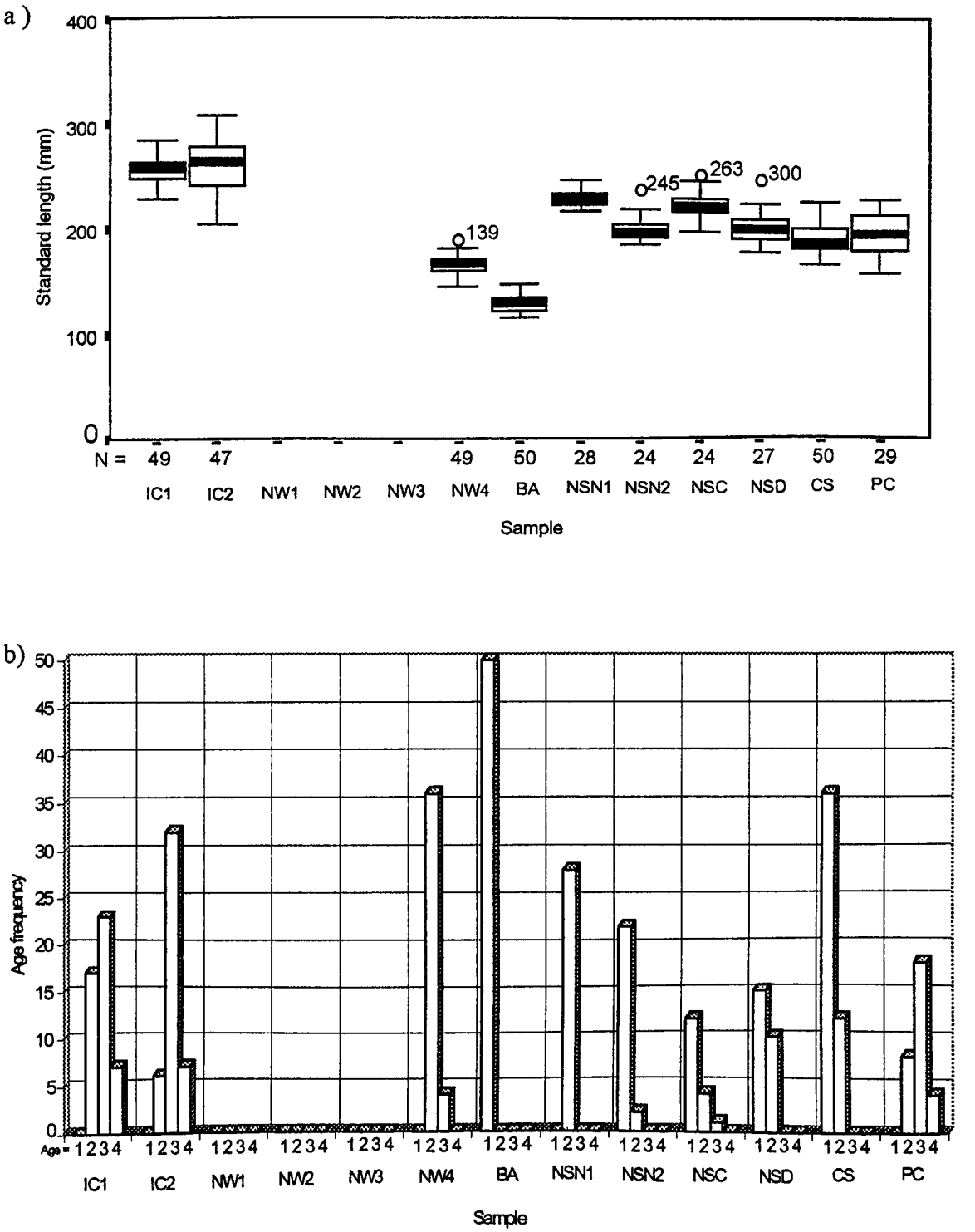

Figure 2.2. Standard length (a) and age (year) (b) distribution of the samples are shown in the box-and-whiskers plot and bar chart respectively. Samples are identical defined in Figure 1 and Table 1. Age are not available for NW1, NW2, NW3 samples (see Chapter 2.2.). N; sample size.

CHAPTER 3

PHENOTYPIC VARIATION: MORPHOMETRIC ANALYSES

3.1 Introduction

Morphological characters have been traditionally used in fisheries biology to measure discreteness and relationships among various taxonomic categories. There are many well documented morphometric studies which provide evidence for stock discreteness (Cataudella *et al.*, 1987; Corti *et al.*, 1988; Villaluz & Crimmon, 1988; Shepherd, 1991; Haddon & Willis, 1995; Bembo *et al.*, 1996a). However, the major limitation of morphological characters at the intra-specific level is that phenotypic variation is not directly under genetic control but subjected to environmental modification (Clayton, 1981). Phenotypic plasticity of fish allows them to respond adaptively to environmental change by modification in their physiology and behaviour which leads to changes in their morphology, reproduction or survival that mitigates the effects of environmental variation (Stearns, 1983; Meyer, 1987). Such phenotypic adaptations do not necessarily result in genetic changes in the population (Ihssen *et al.*, 1981b; Allendorf, 1988), and thus the detection of such phenotypic differences among populations cannot usually be taken as evidence of genetic differentiation. For example, Swain *et al.* (1991) used the truss system in identification of hatchery and wild populations of Coho salmon

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1996a). The truss network system covers the entire fish in a uniform network, and theoretically should increase the likelihood of extracting morphometric differences within and between species. A regionally unbiased network of morphometric measurements over the two-dimensional outline of a fish should give more information about local body differences than a conventional set of measurements (Strauss & Bookstein, 1982; Winans, 1984). There is evidence that the truss method is much more powerful in describing morphological variation between closely related fish taxa compared to traditional measurements (Strauss & Bookstein, 1982; Winans, 1984; Cataudella *et al.*, 1987; Corti *et al.*, 1988).

Morphometric characters have been successfully used for stock separation in many fish species, including Pacific salmon (*Oncorhynchus tshawytscha;* Winans, 1984), Atlantic cod (*Gadus morhua;* Pepin & Carr, 1993), White fish (*Coregonus huntsmani*; Edge *et al.*, 1991), Pacific herring (*Clupea pallasi*; Meng & Stocker, 1984), and anchovy (*Engraulis encrasicolus;* Bembo *et al.*, 1996a). Winans (1984) investigated three coastal stocks of Chinook salmon, *Oncorhynchus tshawytscha,* in Oregon using the truss network system. Discriminant function (DFA) and principal component analysis (PCA) were performed to summarise variability and covariability of morphological variables. Truss data revealed more specific information concerning shape changes than previous studies and produced significant between-group differences. Roby *et al.* (1991) used conventional and truss morphometric analyses together with allozyme analyses to show the degree of differentiation between capelin, *Mallotus villosus,* populations in the Estuary and Gulf of St.

Lawrence. It was concluded that the results of both morphometric and genetic analyses were congruent, and a better discrimination between samples was obtained with the truss approach than with the conventional approach. Bembo *et al.* (1996a) investigated two stocks of the European anchovy (*Engraulis encrasicolus)* in Adriatic waters using the truss network system. Significant differences were detected between two stocks; 98.6 and 88.3 % of north-western and central-southern anchovies, respectively, were correctly assigned by canonical analysis (CA). Importantly their morphometric data was in accordance with significant allozymic differentiation of the two Adriatic stocks.

Studies describing phenotypic structure of herring usually consider morphometric, meristic and also otolith characters together in multivariate analyses (Parrish & Saville, 1965; Rosenberg & Palmén, 1982; Ryman et al., 1984; King, 1985). Nevertheless there is very limited published information on morphometric variation in herring populations in the Northeast Atlantic. Oyaveyer (1980) investigated morphometric differentiation between 1970 year-classes of the spring and autumn Baltic herring. Substantial differences were found between the spawning aggregations, which was related to differences in the larval development conditions and the adaptation of these aggregations to different ecological niches. On the other hand, there are several morphometric studies on pacific herring (*Clupea pallasi*), revealing significant inter-population differentiation among regions (Meng & Stocker, 1984; Kanno, 1989a, 1989b). Therefore this study constitutes a first detailed attempt in analysing morphometric variation in Northeast Atlantic herring.

The purpose of this study is to:

1) to examine stock structure using morphometric data for Northeast Atlantic herring populations, and

2) to test the utility of the truss network system for stock identification.

3.2 Materials and methods.

3.2.1 Laboratory procedure

Herring samples were collected from the nine local Atlantic and one Pacific herring populations reported in section 2.2.

Truss network measurements are a series of measurements calculated between landmarks that form a regular pattern of contiguous quadrilaterals or cells across the body form. Cells and truss characters are referenced according to the scheme of Strauss and Bookstein (1982), for example, the distance between landmark a and b is a truss character in first quadrilateral or cell (landmarks a, b, k, l) (Fig. 3.1). Measurements were made on specimens by collecting X-Y coordinate data for relevant morphological features, and followed a three step-process as below. Firstly, fish were thawed and placed on acetate sheets, and body posture and fins were teased into a natural position. Secondly, each landmark was marked by piercing the acetate sheet with a dissecting needle. The twelve landmarks used in this study are illustrated in Figure 3.1. Measurements were made on the left side of the each specimen.

Additional data, such as eye diameter, head width, were also recorded and added in the truss data. A block of expanded polystyrene (2 cm) was placed beneath the acetate sheet to facilitate this step. Only undamaged fish were included in the analyses, and the sample sizes varied between 24 and 50 individuals. Thirdly, the acetate sheet was placed on a light box, and a camera, connected to a monitor, video and computer, was set at the top of light box, and the image was stored on the screen of the monitor to view interlandmark distances. The X-Y coordinate value (mm) for the positions of landmarks were scored and stored in Measurement TV program (Data Crunch Product) and transferred to a Lotus 1-2-3 spreadsheet file, and a macro written by L. Hauser was used to transform X-Y coordinate data into linear distances for subsequent analysis.

3.2.2 Data analysis

An important stage in the data preparation for morphometric analyses was to eliminate any size effect in the data set when comparing fish of different sizes. Variation should be attributable to body shape differences, and not related to the relative size of fish. In the present study, there were significant differences in size between the local samples (chapter 2). Therefore transformation of absolute measurements to size-independent shape variables was the first step of the analyses. Several transformation methods previously shown to be effective in removing such size-dependent variation were compared (Reist, 1985). The transformations were:

I) RATIO: $M_{adj}=M/SL$, ie. division by standard length.

II) LGRATIO: $M_{adj}=logM/logSL$ = the log of ratio

III)ALLOM1: $M_{adj}=logM-\beta_1(logSL-logSL_{mean})$

IV)ALLOM2: $M_{adj}=logM-\beta_2(logSL-logSL_{mean})$

Where:

M: original truss measurement

M_{adj}: size adjusted truss measurement

SL: standard length of fish

SL_{mean}: overall mean of standard length

β_1: coefficient of the overall linear regression of logM against logSL

β_2: average pooled within-sample coefficient of the regressions of log M against log SL

Base-10 logarithms was used for all variables.

The efficiency of size adjustment transformations was assessed by testing the significance of correlations between the transformed variables and the standard length. A significant correlation indicated an incomplete removal of size effects from the data.

Standard length (landmark distance between a and f, Fig. 3.1) was excluded from the analyses. The transformed data were standardised to 0 mean and a standard deviation of 1 and submitted to a principal components analysis (PCA) and a multiple-discriminant function analysis or canonical analysis (CA) using SPSSv6.1 (for Windows), and graphs were generated using SYSTAT (for windows). The transformed data were also used for other statistical analyses

(Analysis of Variance; ANOVA and Multivariate Analysis of Variance; MANOVA).

3.2.3 Multivariate analyses

Multivariate techniques simultaneously consider the variation in several characters and thereby assess the similarities between samples. PCA requires no *a priori* grouping of individuals but combines and summarises the variation associated with each of a number of measured variables into a smaller number of principal components (PC) which are a linear combination of the variables that describe the shape variations in the pooled sample. Correlations between original variables and the principal components (component loading) can be used to interpret the importance of individual variables in the description of the variation of the data set.

CA was used to discriminate the samples according to the variables. CA, requires *a priori* grouping of samples, calculates a function discriminating between samples of known identity and then reclassifies the individuals into the designated groups on the bases of this function. The percentage of correctly classified individuals gives a measure of the morphological distinctness of the samples.

Principal components and canonical analysis were used to produce graphs to visualise relationships among the individuals of groups: by plotting population centroids of 95% confidence ellipses of first two CFs and PCs The

measurements with high loadings in CA are between-sample diversity, and hence differ from those in PCA (which have total diversity). Each principal component (PC) contains the percentage of total variance of all variables. But in CA each function contains the percentage of the total between-groups variability. Therefore, CA was used to describe the pattern of phenotypic differentiation among samples.

Univariate analysis of variance (ANOVA) was used to compare the variation among samples for size-adjusted truss measurements. Post-Hoc multiple comparison tests was also performed to see the number of significant morphometric characters between pairs of samples. The number of significantly different measurements among groups is an additional indication of the degree of group separation. The effect of sex on the truss measurements was also tested using univariate statistics (ANOVA).

Multivariate analysis of variance (MANOVA) were performed to test the significance of differences among the samples in the data set.

3.3 Results

3.3.1 Efficiency of size adjusted methods

Both RATIO and LGRATIO transformed variables gave significant correlations with standard length (Table 3.1). Allometric variables (in this context, allometry refers to variation in shape that is related to variation in size; Thorpe, 1976) were adjusted according to their regression coefficient on the standard length. For the ALLOM1 method, no variables were significantly correlated with length (Table 3.1), hence this approach appeared to be most appropriate. However, CA and PCA could not effectively separate ALLOM1 transformed data (Fig 3.2). In contrast, there was significant correlation between allometric variables and standard length after treatment of ALLOM2 (Table 3.1) which apparently did not remove the effect of size from the data, but more than 83% of the fish were assigned to the correctly with ALLOM2 formula in CA. Also the plot of the first two discriminant functions and first two principal components showed a clear separate positioning of sample centroids in discriminant space. Similar observations were also reported by Thorpe (1976; Reist, 1985) who strongly advocated the use of a pooled within-sample regression coefficient (ALLOM2; taking the average regression slope of all population's regression slope) in the transformation, because the use of pooled regression coefficient (ALLOM1) (irrespective of geographic origin of samples; considering all populations as one population) in such circumstances may mask

genuine between sample variation. In other words the geographic variation may contaminate the allometric variation (Thorpe, 1976; Reist, 1985). Indeed the pattern of position of the samples in discriminant space as indicated by the loadings of the first two canonical functions and principal components suggest that size was removed effectively from the data with the ALLOM2 method. For example, in PCA plots (Fig. 3.3b), the larger Icelandic herring overlapped with the small Pacific herring rather than with the large Buchan (NSN1; 227 mm) and the Dogger Bank herring (NSC; 223 mm). Also in CA plots (Fig 3.3a), larger Icelandic herring (IC1, IC2; 254 mm and 259 mm mean length respectively) were much closer to the small Pacific (195 mm) and Celtic sea (191 mm) herring than they were to the larger Dogger Bank herring (NSC; 223 mm). In addition, Hauser (1996) reported that with ALLOM1, genuine shape differences among samples were eliminated. In contrast, data size-adjusted with ALLOM2 clearly separated the samples and the differences were not due to allometry. By plotting the first two PCs, smaller sized fish from one population overlapped with much larger fish from a different population (Hauser, 1996).

Therefore all morphometric measurements were adjusted by the ALLOM2 using the pooled within-sample regression coefficient due to its apparent better ability to retain stock specific shape variation in the data set, and were used for all analyses.

3.3.2 Univariate statistics

Univariate statistics (ANOVA) showed that all truss measurements were highly significantly different among samples (Table 3.2). In Post-Hoc tests, Baltic sample showed highest number of significantly different characters from other samples. However, there was no significant different morphometric charecters between Icelandic samples (IC1 and IC2), and between Buchan (NSN1) and Downs (NSD) herring samples (Table 3.3). Examination of the contribution of each variable to the first canonical function showed a high contribution from measurements KC, BJ, JD, AK and CJ (anterior part of body and body width) (Table 3.5). The second canonical function also showed a high loading of the same variables. Contribution of variables to the first and second principal components (Table 3.6) were also mostly from the measurements taken from the anterior part of fish (KC, JD, AK, CJ, BJ), indicating this region to be important in the description of population characteristics.

Testing the interaction (Univariate ANOVA) between variables and sex from 67 sex-recorded fish revealed that 25 out of 26 truss measurements did not differ significantly between sexes (Table 3.4), and thus the effect of sex on morphological variation was not considered further.

Pairwise comparisons (MANOVA) between the samples revealed highly significant inter-sample variation (Table 3.7). Only Buchan (NSN2) and Downs (NSD) herring samples were not significantly different from each other. 95% confidence ellipses of the temporal samples of Icelandic summer-spawners (IC1, IC2) collected at a similar location but in different years (1994 & 1995) were

overlapped on the same location of the chart, but when pairwise comparisons (MANOVA) were carried out they were significantly different from each other. In contrast, the confidence ellipses of Buchan (NSN2) and Downs herring (NSD) overlapped and did not significantly differ from each other in the pairwise comparison.

3.3.3 Multivariate analyses

All submitted variables (377) were used by multivariate analyses, and those fish without a full complement of variables were discarded. Sample sizes thus varied from 24 to 50 fish, where a sample size of 25 is considered to be appropriate for such approaches used (Reist, 1985), and thus can be considered robust.

The overall random assignment of individuals into their original population was high (84 %) (Table 3.8). The proportion of correctly classified Baltic samples to their original group was highest (94%), showing a clear separation from all others.

Plotting CF1 and CF2 explained a high percentage of between group variability and showed a clear between-sample differentiation (Fig 3.3a). The first CF accounted for 78 %, and the second accounted for 12 % of the between-group variability, explaining 90 % of the total between-group variability (Fig. 3.3a). All the samples except the Buchan (NSN2) and Downs (NSD) herring were distinct with the Baltic (BA), Icelandic (IC1 & IC2),

Dogger Bank herring (NSC) and also the Trondheimsfjord (NW4) herring, exhibiting the highest differentiation.

The percentage of total variance explained by the first two principal components was low (43 %), and plotting the first two principal components did not show a high separation of the samples (Fig 3.3b). Interestingly, the Icelandic summer-spawning (IC1 & IC2) are overlapped by the Pacific sample, while the Baltic sea (BA) sample is again separated clearly. The rest of the samples also looked distinct from each other, except Downs herring (NSD) which overlapped with the Trondheimsfjord (NW4) and Buchan herring (NSN2).

3.4 Discussion

The present morphometric study revealed evidence of highly significant morphometric heterogeneity among herring samples, with an overall high correct classification of individuals into their original sample. These morphological differences appeared not to be artefacts caused by either allometry or the statistical method used. CA and PCA revealed strong evidence of the success of the size adjustment of the original truss measurements (see Chapter 3.3.1).

Morphological differentiation may vary between the sexes in some fish species: Creech (1993) reported greater variation between two sandsmelt species in females than in males. In the present study, the limited number of sex-recorded fish did not allow a separate analysis of two sexes. However, testing the interaction between variables and sexes (ANOVA) from 67 sex recorded fish

revealed that 25 out of 26 truss measurements did not differ significantly between the sexes, demonstrating no marked effect of sex on the observed variation.

In this present study, findings reveal the potential power of the truss method for identification of phenotypic stocks in herring. An unbiased network of morphometric measurements over the whole body removes the need to find the types of characters and optimal number of characters for stock separation, and provides information over the entire fish form.

Results of both DFA and MANOVA suggests eight phenotypically distinct local samples varying in their degree of differentiation, though not necessarily with any clear geographic pattern. In the Norwegian Sea, in the CA and PCA the 95 % confidence ellipses of Icelandic samples (IC1 & IC2) collected at similar locations and in different years overlapped and were clearly distinct from other samples. Nevertheless plotting the first two canonical functions is a poor estimator of the statistical significance of morphological differentiation, as plotting one of the other canonical functions may still give statistically significant global separation of overlapped samples (the confidence ellipses). On the other hand, even overlapping samples exhibit significant differences from each other by other analyses (e.g. MANOVA), the extent of morphometric differentiation between them is much less than other samples. In the present study, although multivariate analysis of variance revealed statistically significant morphometric differences between the Icelandic samples, the detected temporal differences between these samples is small compared to spatial differences. Morphological and physiological discreteness of Icelandic-summer

spawners from Icelandic- and Norwegian-spring spawners were also previously reported (Fridriksson, 1944, 1958; Fridriksson & Aasen, 1952; Johansen, 1926; Liamin, 1959), suggesting persistent morphological differentiation. Furthermore, the detected weak temporal variation between the Icelandic samples may indicate the existence of environmental effects in different years on Icelandic herring, so underlining the strong influence of environmental factors on body shape.

Morphometric characters can show high plasticity in response to differences at environmental conditions such as food abundance and temperature. Therefore, temporal environmental differences in the habitat may cause the temporal morphometric variation. Alternatively, the detected temporal variation may be due also to seasonal variation though the samples were caught in February 1994 (winter) (IC1) and in November 1995 (autumn) (IC2) that would mean fish change shape seasonally.

The Trondheimsfjord herring sample also showed a clear separation from other samples. The morphometric differentiation of the Trondheimsfjord sample may also indicate local environmental effects of the fjord, though such differences should be considered in relation to the other methods employed. Indeed mean standard length of this sample showed considerable differences from the same or similar aged samples (Chapter 2, Fig. 2.2), and concordant marked genetic discreteness of the Trondheimsfjord population was also detected (Jørstad & Nævdal, 1981).

In the North Sea, Buchan herring (NSN2) showed statistically significant separation from Bank herring (NSC). They are also distinct in spawning time

and location, and meristic characters (ICES, 1956; Cushing, 1968; Cushing, 1981; Haegele & Schweigert, 1985). Downs herring (NSD) were morphologically very similar to the Buchan herring sample (NSN2) collected from the Northern North Sea, which may be attributable to possible inadvertent sampling of Downs herring in the northern North Sea. Indeed, the presence of Downs herring within the geographic area sampled has been previously reported (Wood, 1937; Burd & Cushing, 1962; Cushing, 1968). Furthermore, Celtic Sea sample (Dunmore) (CS) was highly isolated from the North Sea samples in the discriminant space, indicating very limited intermingling between North Sea and Celtic Sea spawning aggregations. In a previous study comprising meristic and otolith characters, the morphological discreteness of the Celtic Sea herring from North Sea spawning aggregations has also been reported (King, 1985).

The Baltic sea herring (BA) exhibited a marked separation from all others. This may be a result of their geographic isolation and specific environmental conditions of Baltic waters. Oyaveyer (1980) found significant morphometric differences between the spring and autumn spawning aggregations in Baltic Sea, which was, however, attributed to the adaptation of these aggregations to different ecological niches. The lack of genetic differentiation between these spawners has also been reported in later studies (Ryman *et al.*, 1984; Koskiniemi & Parmanne, 1991). Therefore the specific environmental conditions and closed geographic structure of the Baltic Sea may also be operating on morphometric differentiation of Baltic herring from the other populations sampled, through physical isolation and preventing, to some degree, intermingling from other populations. Alternatively, the difference in

type of gear (gillnets) for the Baltic fish may have contributed to the observed morphometric differentiation through catching a specific group of fish. However, a similar pattern of differentiation was detected with otoliths and genetic analyses (Chapter 4 & 7), therefore sampling bias is unlikely.

Interestingly the Pacific herring sample (PC), *Clupea pallasi* L., appeared to be close to the Celtic Sea sample (CS), and was not as differentiated as the Baltic herring sample (Fig. 3.3a). This finding supports the previously reported small morphological divergence between Atlantic and Pacific herring (Svetovidov, 1963). Therefore morphometric characters do not seem to be an effective tool to discriminating the two herring species. However these species in genetic analyses show genetic divergence at the species level (Grant, 1986; Jørstad *et al.*, 1994).

Both multivariate analyses indicated that morphometric differentiation between samples was largely located in the anterior region of herring, and from body depth measurements (Table 3.3 & 3.4). Body depth differences between samples were clearly visible, most notably for the Icelandic fish which had a deeper body. In contrast, Baltic herring appeared to have a shallow and fusiform body. The inter-population variation in body depth measurements may be attributed to swimming ability. Taylor & McPhail (1985) found morphological differences between salmon populations from inland and coastal rivers. Two forms were classified; a coastal form, with deep robust bodies, and an inland form, with shallower and more streamlined bodies. The characterisation of the groups was related to possible differences in adaptations to swimming (migrants) for prolonged periods. Inland populations, must cope with long and energetically

demanding migrations, thus selection should favour a more fusiform body shape that minimises energy expenditure (Taylor & McPhail, 1985). Several other studies have also reported the correlation between a fusiform body and either prolonged swimming performance or migration distance (Yevsin, 1977; Thomas & Dohahoo, 1977; Wood & Bain, 1995). Therefore the localised variation detected here may be attributable to possible morphological adaptation of stocks to prolonged swimming. The fusiform shape of Baltic herring may represent an adaptation to the closed structure of the Baltic Sea, and the Icelandic herring may represent an adaptation to coastal or pelagic waters. Analysis of further samples of respective populations are required to see whether such patterns are consistent.

The pattern of high inter-sample variation may indicate reproductive isolation among local populations which would confirm the genetic basis of observed morphometric differentiation among samples, though fish are known to exhibit a high component of environmentally-induced morphological variation (Allendorf *et al.*, 1987; Wimberger, 1992). Morphometric differences might reflect different adjustments by fish to their feeding environment, prey types and availability or other features associated with pre- or post settlement of fish. Some environmental factors such as temperature, salinity, food availability or prolonged swimming may, for example, determine the potential phenotypic discreteness of herring. Such effects may be especially relevant to the fjord and Baltic populations which have specific environmental conditions. Therefore, the reproductive isolation of the samples (especially the North and Celtic Sea samples) may not necessarily be absolute. The substantial morphometric

differentiation suggests that fish in each group spend their lives in separate regions, whether or not reproductive isolation is complete since morphology is especially dependent on development in early life-stages (Ryman et al., 1984; Cheverud, 1988). This is the important question for fisheries management, though the exploited populations sampled are apparently subjected to differential survival conditions. However, the management implications of the detected morphological discreteness of populations depends on the extent to which structuring persists over time. Consistent differences between fish collected at least in two sampling dates may indicate their temporal and spatial integrity. In this present study, repetitively collected Icelandic samples did indeed show temporal integrity which is also congruent with the present genetic study (Chapter 7). Therefore they strongly deserve treatment as a distinct stock in management programs.

The genetic bases of the morphometric discreteness was not examined here. Application of genetic markers is an effective method of examining the environmental component of phenotypic discreteness among geographic regions and facilitate the development of management recommendations.

Table 3.1. Correlation between standard length and each of the size adjusted measurements for different transformation methods.
The significance level are shown: ***P < 0.001; **P<0.01; *P<0.5.

Measurement	RATIO	LGRATIO	ALLOM1	ALLOM2
AB	-0.80***	0.95	0.0	-0.45***
AK	-0.71***	0.96***	0.003	-0.59***
AL	-0.69***	0.95***	0.004	-0.33***
BC	0.56***	0.99***	0.01	0.35***
BJ	0.52***	0.99***	0.020	0.99***
BK	-0.51***	0.98***	0.0	-0.16*
CD	-0.22***	0.92***	0.0	-0.04
CI	0.36***	0.99***	0.012	0.33***
CJ	0.27***	0.96***	0.0	0.57***
DE	0.29***	0.99***	0.01	0.23***
DH	0.33***	0.99***	0.0	0.38***
DI	0.44***	0.98***	0.0	0.41***
EG	-0.13**	0.95***	0.0	0.30***
EH	-0.15**	0.96***	0.02	0.41***
HG	-0.12*	0.87***	0.0	0.04
IE	-0.28***	0.94***	0.0	0.47***
IH	-0.22***	0.95***	0.0	0.24***
JD	0.26***	0.95***	0.0	0.56***
JI	0.47***	0.97***	0.01	0.27***
KC	0.53***	0.98***	0.0	0.98***
KJ	0.47***	0.98***	0.0	0.17**
LB	-0.79***	0.96***	0.0	-0.23***
LK	0.55***	0.91***	0.01	-0.59***
Eye diamater	-0.84***	0.84***	0.0	0.14***
Head width	-0.39***	0.89***	0.0	0.65***

Table 3.2. Analysis of variance (ANOVA) comparing ALLOM2 adjusted body truss measurements among all herring samples. The significance level are shown: ***P < 0.001

Measurement	Wilks' Lambda	F	P
AB	0.47999	44.1770	0.000***
AK	0.41728	56.9456	0.000***
AL	0.58549	28.8689	0.000***
BC	0.70431	17.1197	0.000***
BJ	0.10223	358.1167	0.000***
BK	0.82698	8.5315	0.000***
CD	0.88733	5.1779	0.000***
CI	0.69741	17.6925	0.000***
CJ	0.37135	69.0318	0.000***
DE	0.89366	4.8524	0.000***
DH	0.73725	14.5330	0.000***
DI	0.60808	26.2824	0.000***
EG	0.82048	8.9224	0.000***
EH	0.77578	11.7860	0.000***
HG	0.90966	4.0495	0.001***
IE	0.63058	23.8895	0.000***
IH	0.81017	9.5547	0.000***
JD	0.33724	80.1371	0.000***
JI	0.70989	16.6648	0.000***
KC	0.09150	404.8700	0.000***
KJ	0.87542	5.8030	0.000***
LB	0.70309	17.2203	0.000***
LK	0.51360	38.6183	0.000***
Head width	0.44061	51.7701	0.000***
Eye diameter	0.76622	12.4417	0.000***

Table 3.3. Post-Hoc multiple comparison tests of morphometric variables between pairs of populations. The values represent the number of significant variables observed out of 25 morphometric variables for corresponding populations.

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1	-									
IC2	-	-								
NW4	8	10	-							
BA	19	22	17	-						
NSN1	3	8	12	20	-					
NSN2	6	8	10	19	4	-				
NSC	14	14	17	19	8	8	-			
NSD	7	6	8	18	9	-	10	-		
CS	10	12	8	19	12	11	16	11	-	
PC	15	16	15	12	15	17	17	14	9	-

Table 3.4. Univariate analysis of variance (ANOVA) testing the interaction between measurements and sexes. The significance level are shown: * P<0.05.

Measurement	Wilks' Lambda	F	P
AB	0.99067	0.5936	0.4439
AK	0.96233	2.4661	0.1213
AL	0.98553	0.9253	0.3398
BC	0.99121	0.5584	0.4577
BJ	0.96176	2.505	0.1185
BK	0.95982	2.6371	0.1094
CD	0.99985	0.0097	0.9219
CI	0.99599	0.2537	0.6163
CJ	0.99992	0.0052	0.9429
DE	0.99332	0.4236	0.5175
DH	0.99969	0.0193	0.89
DI	0.99796	0.129	0.7207
EG	0.99901	0.0625	0.8034
EH	0.99163	0.5316	0.4686
HG	0.96178	2.5036	0.1186
IE	0.99191	0.5137	0.4762
IH	0.99574	0.2694	0.6055
JD	0.99996	0.0027	0.9589
JI	0.97914	1.3424	0.251
KC	0.96131	2.5358	0.1163
KJ	0.99972	0.0177	0.8946
LB	0.97891	1.3571	0.2484
LK	0.90821	6.3671	0.0142*
Eye diameter	0.99954	0.0288	0.8658
Head width	0.99998	0.0015	0.9693

Table 3.5. Contribution of each variable to the canonical functions.
*, correlation coefficient with large contribution to corresponding function.

Variables	Function 1	Function 2	Function 3	Function 4	Function 5
KC	-.84140*	0.0811	0.00266	0.05679	-0.18116
BJ	-.78352*	0.29701	0.02193	0.03001	-0.0836
JD	-0.30471	-.53835*	0.0034	-0.16664	0.15036
AB	0.20774	.42867*	-0.16547	-0.30633	-0.04872
AL	0.14487	.41243*	-0.10367	0.00627	0.32996
CJ	-0.29915	-.39969*	0.04337	-0.31257	0.32602
LB	0.09828	.34897*	-0.0066	-0.07025	0.06719
IE	-0.16265	0.04026	.50979*	-0.12304	-0.14185
IH	-0.06765	0.02446	.43522*	0.2022	-0.17598
CD	0.01228	0.03479	.31263*	-0.23948	0.25958
BC	-0.12581	-0.13364	-0.09594	.52388*	0.04576
EH	-0.13126	0.02614	0.07948	-.27274*	0.07702
CI	-0.12547	-0.1682	0.21533	-0.17891	.61157*
DI	-0.16953	-0.29545	-0.07906	-0.04458	.40153*
JI	-0.1115	-0.28304	0.08641	0.23634	.31498*
DH	-0.13709	-0.15882	-0.08457	0.13168	.24449*
KJ	-0.05499	-0.00794	-0.25369	0.07273	0.08616
BK	0.05366	0.06409	0.31433	-0.13646	0.36057
AK	0.27573	0.31071	0.19445	-0.17398	0.05318
LK	0.24286	0.13527	0.24944	-0.11191	-0.19072
EG	-0.08848	0.09881	0.22249	-0.25347	0.19984
HG	-0.01715	-0.08387	-0.09893	-0.28886	-0.23603
DE	-0.07587	-0.0589	-0.13196	-0.06526	0.16614
Eye diameter	-0.02665	0.3405	-0.0155	-0.04059	-0.13406
Head width	-0.26836	0.29949	-0.19446	-0.19926	0.15409

Table 3.6. Principal component (PC) loadings of PCA for morphometric characters of herring.

Variables	PC1	PC2	PC3	PC4	PC5
AK	-0.83285	0.34282	0.13748	0.0647	0.00528
AL	-0.5728	0.35425	-0.0803	-0.27254	0.17274
BC	0.40299	-0.39297	-0.25022	0.0384	0.01044
BJ	0.79083	0.12876	-0.30487	0.06978	0.19658
BK	-0.23224	0.5603	0.26884	0.31272	-0.03223
CD	-0.07983	0.31583	0.05824	0.63721	-0.18588
CI	0.56914	0.43751	0.53931	0.10796	-0.07857
CJ	0.82487	0.19965	0.24904	0.15483	-0.09699
DE	0.43159	0.23534	0.14496	-0.6036	0.14612
DH	0.60341	0.16276	0.38775	-0.31163	0.3999
DI	0.73838	0.20607	0.47963	-0.31134	-0.00046
EG	0.29676	0.48133	-0.20969	0.18051	-0.23037
EH	0.48353	0.38475	-0.36637	-0.18346	-0.56512
HG	0.14263	0.16559	-0.30319	-0.29579	-0.70608
IE	0.5049	0.27505	-0.44835	0.25175	0.00339
IH	0.20615	-0.03057	-0.16741	0.54879	0.52998
JD	0.83472	0.00293	0.21397	0.22953	-0.12143
JI	0.49549	-0.09825	0.47873	0.16368	-0.04606
KC	0.84727	0.06338	-0.27837	0.06022	0.17047
KJ	0.26519	-0.21313	0.08224	-0.18885	-0.00219
LB	-0.4438	0.57227	0.05182	-0.09761	0.17653
LK	-0.75598	0.2153	0.19561	0.18665	-0.07632
Head width	0.51963	0.32136	-0.4271	-0.04614	0.33038
Eye diameter	-0.0653	0.42803	-0.3738	-0.19678	0.39426

Table 3.7. Multivariate analysis of variance (MANOVA) between all local samples of herring for morphometric measurements. Significantly different group means; *P <0.05, **P<0.01,*** P<0.001, ns, not significant. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC).

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1	---									
IC2	***	---								
NW4	***	***	---							
BA	***	***	***	---						
NSN1	***	***	***	***	---					
NSN2	***	***	***	***	***	---				
NSC	***	***	***	***	***	*	---			
NSD	***	***	***	***	***	ns	***	---		
CS	***	***	***	***	***	***	*	*	---	
PC	***	***	***	***	**	*	**	***	***	---

Table 3.8. The summary of the classification results for morphometric characters. The diagonal numbers are the number of individuals classified correctly into their corresponding group. Overall 84 % of the grouped individuals were correctly classified. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC).

		Predicted group membership									
Sample	No of cases	NW4	IC1	NSC	NSN1	BA	NSD	NSN2	CS	IC2	PC
NW4	49	**45** **91.8%**	0 0.00%	0 0.00%	0 0.00%	1 2.0%	2 4.10%	0 0.00%	1 2.00%	0 .0%	0 0.00%
IC1	49	0 .0%	**32** **65.30%**	0 0.00%	6 12.20%	0 .0%	0 0.00%	0 0.00%	0 0.00%	11 22.4%	0 0.00%
NSC	24	0 .0%	0 0.00%	**23** **95.80%**	0 0.00%	0 .0%	0 0.00%	0 0.00%	0 0.00%	1 4.2%	0 0.00%
NSN1	28	0 .0%	3 10.70%	0 0.00%	**24** **85.70%**	0 .0%	0 0.00%	1 3.60%	0 0.00%	0 .0%	0 0.00%
BA	50	3 6.0%	0 0.00%	0 0.00%	0 0.00%	**47** **94.0%**	0 0.00%	0 0.00%	0 0.00%	0 .0%	0 0.00%
NSD	27	1 3.7%	0 0.00%	0 0.00%	2 7.40%	0 .0%	**18** **66.70%**	5 18.50%	1 3.70%	0 .0%	0 0.00%
NSN2	24	0 .0%	0 0.00%	0 0.00%	1 4.20%	0 .0%	5 20.80%	**16** **66.70%**	2 8.30%	0 .0%	0 0.00%
CS	50	4 8.0%	0 0.00%	0 0.00%	0 0.00%	0 .0%	2 4.00%	0 0.00%	**43** **86.00%**	0 .0%	1 2.00%
IC2	47	0 .0%	8 17.00%	0 0.00%	1 2.10%	0 .0%	1 2.10%	0 0.00%	0 0.00%	**37** **78.7%**	0 0.00%
PC	29	1 3.4%	0 0.00%	0 0.00%	0 0.00%	0 .0%	0 0.00%	0 0.00%	1 3.40%	0 .0%	**27** **93.10%**

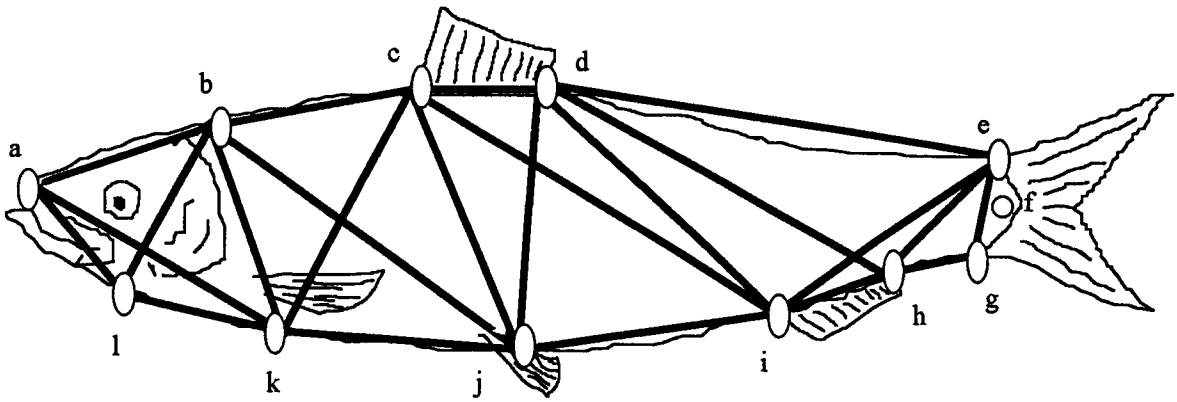


Figure 3.1. Locations of the 12 landmarks for constructing the truss network on fish illustrated as open circles and morphometric distance measures between circles as lines. Landmarks refer to (a) anterior tip of snout at upper jaw, (b) most posterior aspect of neurocranium (beginning of scaled nape), (c) origin of dorsal fin, (d) insertion of dorsal fin, (e) anterior attachment of dorsal membrane from caudal fin, (f) posterior end of vertebrae column, (g) anterior attachment of ventral membrane from caudal fin, (h) insertion of anal fin, (i) origin of anal fin, (j) insertion of pelvic fin, (k) insertion of pectoral fin, (l) posteriomost point of maxillary. For landmarks b, c, d, h, i, j, k, l, points were made at their respective positions at the closest point to the body on a line perpendicular to the horizontal axis of the fish.

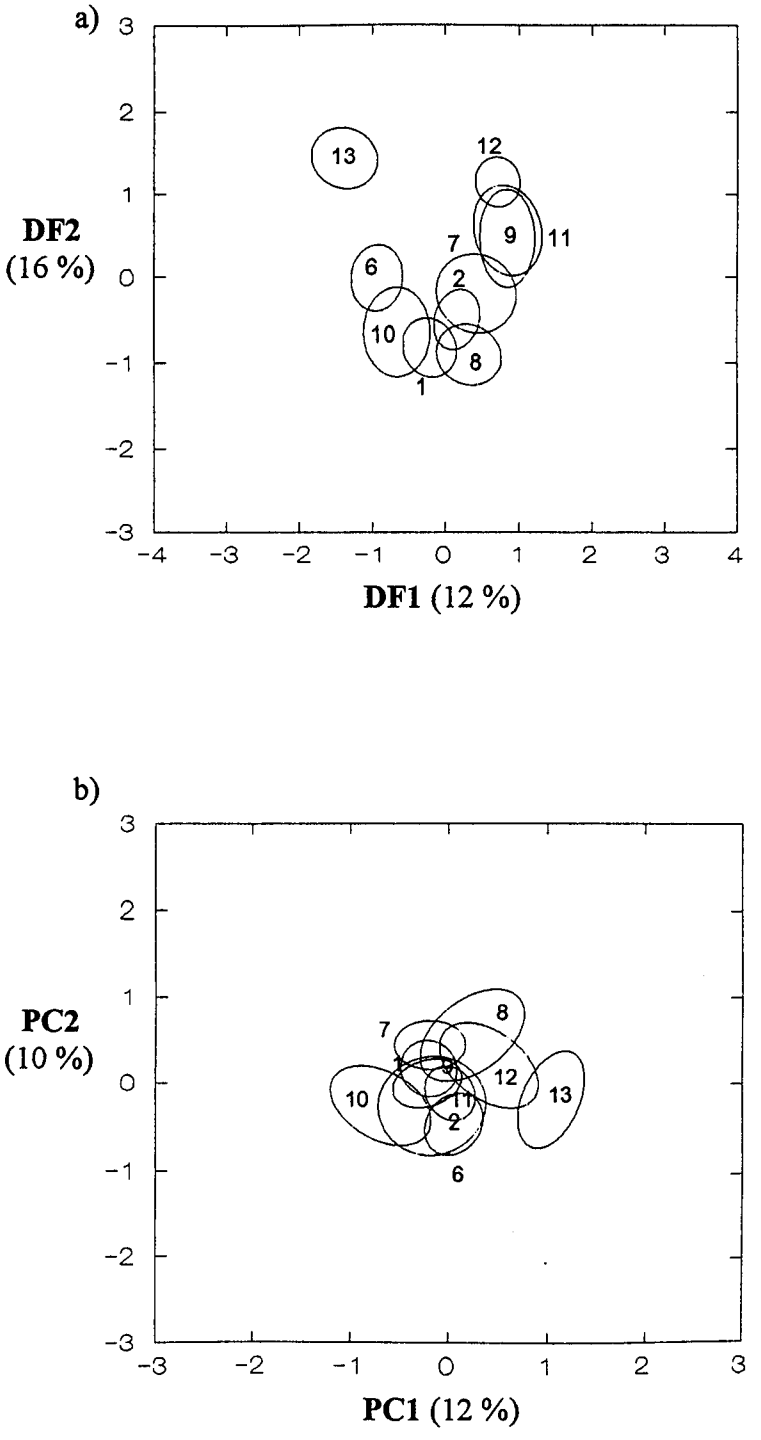


Figure 3.2. Sample centroids and 95 % confidence ellipses of CA (a) and PCA (b) scores using data transformed with ALLOM1. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1=1), Icelandic summer-spawners (second year) (IC2= 2), Trondheimsfjord (NW4= 6), Baltic herring (BA=7), Buchan herring (Northmost North Sea) (NSN1=8), Buchan herring (Northern North Sea) (NSN2= 9), Dogger herring (NSC= 10) Downs herring (NSD= 11), Dunmore (Celtic Sea) (CS= 12), Pacific herring (PC= 13).

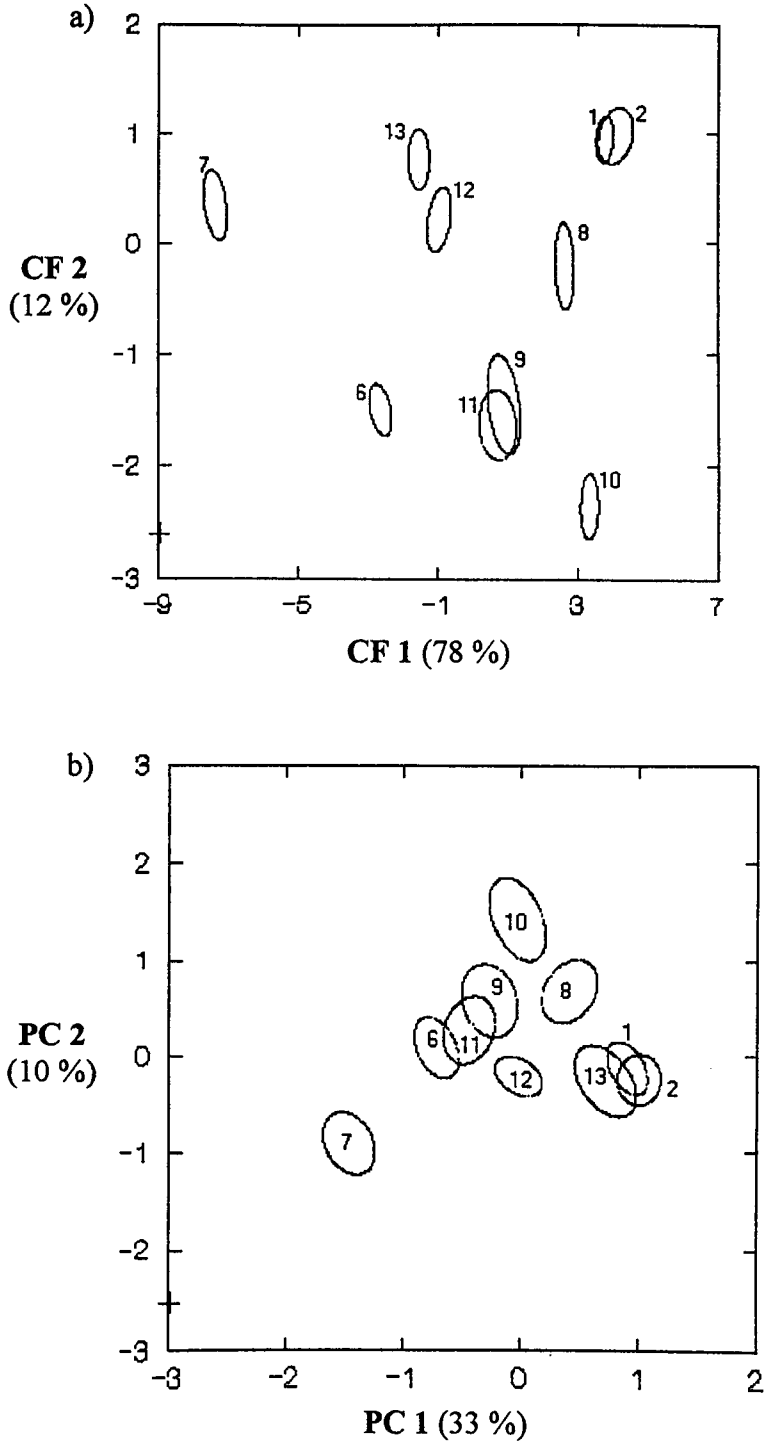


Figure 3.3. Sample centroids and 95 % confidence ellipses of CA (a) and PCA (b) scores using data transformed with ALLOM2. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1=1), Icelandic summer-spawners (second year) (IC2= 2), Trondheimsfjord (NW4= 6), Baltic herring (BA=7), Buchan herring (Northmost North Sea) (NSN1=8), Buchan herring (Northern North Sea) (NSN2= 9), Dogger herring (NSC= 10) Downs herring (NSD= 11), Dunmore (Celtic Sea) (CS= 12), Pacific herring (PC= 13).

CHAPTER 4

PHENOTYPIC VARIATION: OTOLITH SHAPE ANALYSES

4.1 Introduction

The study of calcified structures include both osseology (scales, otoliths, bones, calcified platelets, acellular structures, calcareous accretions) and asteology (bones with cellular structure) for stock identification (Ihssen *et al.*, 1981b). Osseometric studies (the measurement of calcified structures) have been commonly used to determine whether the shapes of these structures are specific for each population. Usually otoliths and scales have been mostly used to discriminate stocks (Chasselman *et al.*, 1981; Messieh, 1972; Scarnecchia & Wagner, 1980; Bird *et al.*, 1986; Messieh *et al.*, 1989; Friedland & Reddin, 1994) because other calcified structures have not revealed consistent differences among conspecific groups of fish (Gorshkov, 1979; Ihssen *et al*, 1981b). Although otoliths and scales give similar information, otoliths are generally used in shape analysis, because their structure is less variable, their growth is slower, and they are less influenced by environmental conditions (Casselman, 1978; Casselman *et al.*, 1981). For example, Casselman *et al.* (1981) examined lake whitefish (*Coregonus clupeaformis*) using morphometrics, otoliths, scales, and allozymic variation from the Ontario waters of Lake Huron. Whitefish were

divided into five stocks based on otolith data, whereas the other phenotypic techniques separated the same samples into just two groups. The otolith results were also more similar to tag-recapture data, suggesting that variation provided by the otoliths was the most effective overall discriminating tool.

Otolith shape is markedly species-specific and less variable in growth than fish body growth (Morrow, 1976; Gaemers, 1984). Otoliths grow throughout the life of the fish, and are metabolically inert, thus reducing the effect of short-term changes in fish conditions, such as starvation. Such factors can, however, confound body morphometrics. (Campana & Neilson, 1985; Casselman, 1987).

Despite the reduced impact of short-term environmental changes, stock identification studies based on otoliths have revealed the effects of the environmental differences among regions and presumed geographical separation among populations, giving rise to geographic variation in the shape or appearance of otoliths (Messieh, 1972; Neilson *et al.*, 1985; Dowson, 1991). Since otolith characteristics are related mainly to environmental conditions at early larval life, they provide a rare opportunity to study the reproductive interactions and intermingling among spawning aggregations (McQuinn, 1997). This is important from the perspective of fisheries management; where the relative independence of stocks is a critical component of stock assessment. In addition, there are otoliths studies (Maceina & Murphy, 1989; Williams, 1980; Castonguay *et al.*, 1991) which have been assumed to be based, at least in part, on genetic differences. Furthermore, since otoliths also provide information on age, growth of fish, racial origin, and to an extent, environmental history by

examining the number and spacing of growth rings (Messieh, 1972; Major *et al.*, 1972; Casselman *et al.* 1981; Martin, 1978; Jarvis *et al.*, 1978; Rowell, 1980), their use can provide biological information on stock structure and dynamics.

Characters such as outline, both length and weight, size and optical density of the nucleus, distance between nucleus and first annuli, and angles on the otolith have been used for population studies. Otoliths are usually collected for age and growth assessment, and require no preservation, their study is relatively cheap (Ihssen *et al.*, 1981b), facilitating their wide-scale collection and study as a current and archived resource of past populations.

There are several otolith shape analyses conducted to examine the population structure of Atlantic herring. In the Norwegian Sea, Einarsson (1951) observed differences in the otolith nucleus structure between Icelandic summer-spawners and Norwegian spring-spawners, in concordance with meristic, scale and physiological studies (Johansen, 1926; Fridriksson, 1944, 1958; Liamin, 1959). Also, Moksness & Fossum (1991) distinguished Norwegian spring-spawned herring larvae and North Sea autumn-spawned herring larvae by using otolith microstructure (distance from the nucleus to the hatch check), thus indicating otolith structuring within the Norwegian Sea and between Norwegian and North Sea spawning aggregations. King (1985) used both otolith shape and meristic characters to investigate herring spawning stocks around the west of the British Isles and in one Baltic Sea sample. He found a high degree of anatomical similarity among herring spawning groups, though the Celtic Sea sample (Dunmore) was significantly distinct from adjacent and neighbouring stocks. In a subsequent allozyme analysis (King *et al.*, 1987), the

homogeneity among the spawning aggregations including Celtic Sea sample has also been shown.

There has been an increasing number of studies using the truss network system to investigate morphometric variation among fish populations. However there has been no study using the truss method for the analysis of otolith shape. Other studies (e.g. Casselman *et al.*, 1981; Ihssen *et al.*, 1981a; Messieh, 1972; King, 1985; Messieh *et al.*, 1989) involving otolith shape use a limited number of characters (2-4) and bias on the coverage of otolith, thus reducing the discriminatory power of otolith shape variation. However employment of the truss system removes the need to find the types of characters for stock separation, and enables an unbiased coverage and more characters on the otolith shape, thus increasing its discriminatory ability in population and also species studies. This study constitutes a first attempt to use the truss method on otolith shape analysis of fish populations.

The purpose of this study is:

1) to examine stock structure using otolith shape for Northeast Atlantic herring populations.

2) to test the utility of truss network system on otolith shape analysis for stock identification.

4.2 Materials and Methods

4.2.1 Laboratory Procedure

The herring samples used in this study were collected from nine locations in the North east Atlantic and one sample from the Pacific Ocean as reported in chapter 2, section 2.

Sagittal otoliths were removed from the cranium of each herring and stored in envelopes. The left otolith was placed in a solution of glycerol and 0.5 % thymol to remove blood and debris before examination. If the left otolith could not be taken, or was damaged, the right was used.

The truss network system was used in this study as described in chapter 3, section 2. Six landmarks determining 11 distances were chosen on the otolith, and are illustrated in Figure 4.1. The otolith was placed on a binocular microscope at x12 magnification connected to a monitor and video, and the image was displayed on the screen of the monitor. The X-Y coordinate value (mm) for the positions of landmarks were scored on the monitor, and stored in a Measurement TV program (Data Crunch Product), and later transferred to a lotus 1-2-3 spreadsheet file. A macro written by L. Hauser was used to transform Cartesian coordinate data into linear distances for later analyses.

4.2.2 Multivariate Analyses

Significant correlations between the original otolith measurements and standard length of fish were observed, making it necessary to remove any size component in the data set and allow the detection of genuine shape differences among populations. The ALLOM2 formula described in chapter 3 was used to remove the size effect on the otolith variables for two reasons. First, the ALLOM2 was effective in removing the size effect on the morphometric variables, and second, it is more meaningful to use the same formula for all phenotypic markers to facilitate their direct comparison. ALLOM2 transformed data were standardised prior to a principal component analysis (PCA) and a canonical analysis (CA), the details of which has been given in chapter 3, section 2.

Population centroids with 95% confidence ellipses derived from the CA and PCA of morphological variation were used to produce graphs to visualise relationships among the individuals of groups. Univariate ANOVA was carried out for each parametric measurement to test for significant differences among sample means and to test the effect of sex on otolith measurements. Post-Hoc multiple comparison tests was also performed to find out the number of significant morphometric characters between pairs of samples. Multivariate analysis of variance (MANOVA) was performed to test the significance of differences among the samples in the data set.

4.3 Results

Univariate ANOVA revealed highly significant differences between groups from all parametric otolith measurements, showing that all variables were contributing to the differentiation of the populations (Table 4.1). Pacific and Baltic samples showed highest number of significantly different morphometric character from other samples, however, lowest number and no significant characters was observed between North Sea samples, and there was no any significantly different characters between Icelandic samples (IC1 & IC2) (Post-Hoc tests; Table 4.2). Testing the interaction between variables and sexes from 67-sex recorded fish showed that ten out of eleven otolith measurements did not differ significantly, demonstrating a negligible effect of sex on the observed variation (Table 4.3). It was therefore not considered necessary to remove this effect from the data set.

The first canonical function accounted for the largest amount of between-group variability (47 %), and the second and third accounted for 36 %, and 11 % respectively. CF1 and CF3 were plotted to describe the pattern of relationships among the samples (Fig. 4.2a). Plotting CF1 and CF3 produced three highly isolated Atlantic herring samples, Pacific sample and overlapped samples comprising North and Celtic Sea samples. The confidence ellipse of Baltic herring (BA) was clearly distinct from all other samples, as was the Pacific sample which was positioned at the extreme right of the chart. Icelandic summer-spawner samples (IC1 & IC2) clustered together in the uppermost side of the chart and also showed a clear separation from all other samples, thus

revealing temporal and spatial integrity. The Trondheimsfjord sample (NW4) was positioned at the top of the chart closer to the Icelandic and North Sea samples than the Baltic and Pacific herring. The North Sea samples (NSN1, NSN2, NSC, NSD) grouped with the Celtic Sea (CS) sample, with the Buchan herring (NSN1) being the most distinct sample in this group.

The PC1 and PC3 were plotted (Fig. 4.2b) to make a direct comparison with CA result, explaining a high percentage of the total variance (67 %) (PC2 accounted for 17 %). The pattern of separation of samples in the PCA plots was similar to the CA result. The contribution of each variable to the canonical functions and principal components are given in Table 4.4 and Table 4.5 respectively.

Pairwise comparisons (MANOVA) between samples revealed highly significant differences between most of the samples (Table 4.6). The only differences from CA was that the Celtic Sea sample was significantly different from Bank (NSC) and Downs herring (NSD), and the Bank herring also showed significant differences from the Buchan herring (NSN2). However the extent of differentiation was low compared to the magnitude of divergence of other samples.

In the CA the correct classification of individuals into their original populations was moderate with 56 % of individuals being classified into their correct *a priori* grouping (Table 4.7). Baltic and Pacific herring revealed the highest correct classification into their original samples, showing concordance with CFA and indicating high phenotypic differentiation of these samples.

4.4 Discussion

The present otolith study revealed highly significant phenotypic heterogeneity among most of the herring samples. The detected pattern of phenotypic discreteness among the samples suggests a direct relationship between the extent of phenotypic divergence and geographic distance, indicating that geographic distance is a limiting factor to migration among populations. The relatively larger phenotypic divergence between Baltic and either Icelandic or the Trondheimsfjord samples, and the high isolation of Pacific herring from the others, and also the similarity of the of North and Celtic Sea samples reflects the effect of isolation.

Icelandic summer-spawner samples (IC1 & IC2), collected at a similar location and in different years showed consistent temporal integrity and clear differences from other samples (CA & MANOVA). The detected temporal and spatial integrity of the Icelandic summer spawners is supported by the past previous phenotypic studies based on otolith nucleus structure (Einarsson, 1951), and other meristic, scale and physiological characters (Johansen, 1926; Fridriksson, 1944, 1958; Liamin, 1959). It is possible that the geographic isolation of this population may be an inhibiting factor to gene flow, thus causing their phenotypic differentiation, as shown also by morphometric, meristic and genetic differentiation (Chapter 3, 5, 7, 8). On the other hand, the detected temporal stability of differentiation may also suggest that otolith morphology does not respond to annual environmental variation (Friedland & Reddin, 1994), encouraging their use for stock identification studies.

Trondheimsfjord herring (NW4) (probably spring-spawner) also revealed a clear divergence from the other samples. The allozymic discreteness of this fjord population from other Atlantic herring populations has been previously reported (Jørstad & Nævdal, 1981). The observed high separation of this sample from the others suggests that there is limited or no mixing of Trondheimsfjord herring with other populations sampled, suggesting a self-recruiting structure of the Trondheimsfjord herring population in Norwegian waters. Furthermore, their geographic isolation coupled with fjord-specific environmental conditions such as low salinity and temperature may be governing the differentiation of the fjord herring.

The Baltic herring (spring-spawner) (BA) sample was most isolated from all other samples in the discriminant space. Based on morphological characters (mainly meristics) the Baltic herring has previously been classified as a different form of Atlantic herring, *Clupea harengus harengus n. membras* (Svetovidov, 1963). The geographic isolation of Baltic Sea may be the main factor in this differentiation. In addition, the specific topographical and hydrographical features of the Baltic Sea such as closed geographical structure, shallow water and low salinity may be acting as physical barriers and preventing migration of other populations into the Baltic Sea.

The samples from around the British Isles (NSN1, NSN2, NSC, NSD, CS) overlapped, or were close to each other. However in pairwise comparisons of the samples, a low degree of significant heterogeneity was detected between Downs herring (NSD) and Celtic Sea (CS) samples, and between Buchan herring and Dogger (NSC) herring. The detected significant differences between

these samples are small compared to the spatial differences (Fig. 4.2a). The overlapping distribution of these samples may be attributable to extensive migration in these waters. The spawning aggregations from the Downs, Dogger and Buchan have their own spawning time and space, but presumably mix on the feeding ground between the Dogger Bank and the Shetland Islands in the North Sea (Haegele & Schweigert, 1985; Cushing, 1981). Therefore the detected pattern may indicate sufficient mixing between these populations to prevent differentiation. Alternatively, the environmental conditions may not be sufficiently different between the regions to produce stock-specific otolith structuring even if there is limited migration between aggregations.

The Pacific sample (PC) revealed high discreteness from all other samples. Here the detected otolith variation between the two species suggest that the otolith shape is apparently an effective tool to identify different species of herring. Moreover, it is also in agreement to the correlation with geographic separation described.

The findings reported here demonstrate that the application of truss method on otolith shape provides a technique capable of detecting differences between populations. The result is similar to other studies, (Messieh *et al.*, 1989) where spring- , summer-, and autumn -spawning groups of Atlantic herring on the basis of otolith shape in the Southern Gulf of St. Lawrence were identified. Bird *et al.* (1986) found distinct differences between Atlantic and Alaskan herring using fourier series shape analysis. Similarly, Friedland & Reddin (1994) have also reported that using otolith shape was more effective to identify the North American and European origin of salmon (continent) than

only European origin of salmon (country). Alternatively, in this study, the best 6 landmarks were chosen on the otolith to produce the truss network which produced eleven otolith morphometric distances. Other studies involving otolith shape analysis usually use only 2-4 otolith morphometric characters (usually distances between a-b,, e-f, c-d (Fig.5.1), and between nucleus and first age ring; Casselman *et al.*, 1981; Ihssen *et al.*, 1981a; Messieh, 1972; King, 1985; Messieh *et al.*, 1989). The contributions of these characters to the fist CF and PC was moderate and small (Table 4.4 and Table 4.5). Thus employment of the truss network system facilitated the detection of variation over the entire otolith shape. In addition, otoliths are easy to preserve and store, and are rapidly processed, thus allowing the analysis of long-time series and extensive collection. Univariate statistics (ANOVA) revealed that all otolith measurements were highly significantly different between samples, confirming the high degree of inter-sample variance and information content of the landmarks employed. The major drawback of this technique is that it needs computer image processing equipment to perform the analysis though this is clearly less restrictive than the requirements for advanced molecular analysis. The particular usefulness of otolith shape analysis as a fisheries management tool is that it is capable of examining large numbers of samples in a short time. Also, it is effective in determining the origin of individuals composing a stock and improving the biological basis of management especially when they are used in conjunction with molecular genetic markers.

In the present study, repetitive sampling was achieved only in the Icelandic samples, revealing temporal and special integrity of the marked

variation, which is also supported by comparison with past previous phenotypic studies (Einarsson, 1951; Johansen, 1926; Fridriksson, 1944, 1958; Liamin, 1959). Thus the recognition of the Icelandic herring as a distinct management unit may be warranted. The detected phenotypic heterogeneity of the Trondheimsfjord herring provides evidence for their temporal and spatial integrity compared to a previous genetic study (Jørstad & Nævdal, 1981). Therefore, this fjord population should be considered separately in management decisions.

In summary, the pattern of phenotypic distinctness detected suggest a direct positive relationship between the extent of geographic isolation and phenotypic divergence. Due to the observed high phenotypic discreteness in relation to geography, the Icelandic summer-spawner, the Baltic and the Trondheimsfjord herring samples may be considered three self-contained stocks. Although the environmental factors may be governing the potential phenotypic discreteness of herring spawning aggregations, the detected pattern of differences at least show that there is some restriction to intermingling among populations. Therefore, from the management point of view, any depletion in one of these stock is unlikely to be compensated by immigration from other units, at least at a sufficiently rapid rate. The application of molecular techniques would provide a valuable approach for assessing the extent of genetic and environmental contributions to the observed phenotypic variation.

Table 4.1. Analysis of variance (ANOVA) comparing ALLOM2 adjusted otolith measurements among herring samples. The significance level are shown: **P <0.01, ***P < 0.001

Variable	Wilks' Lambda	F	P
AB	0.36497	68.4387	0***
AC	0.35465	71.5729	0***
AD	0.33841	76.8969	0***
BC	0.93679	2.6539	0.0055**
BD	0.91957	3.4403	0.0004***
CD	0.86182	6.3063	0***
CE	0.41619	55.1751	0***
CF	0.48705	41.4247	0***
DE	0.42315	53.6193	0***
DF	0.51228	37.4472	0***
EF	0.78815	10.5727	0***

Table 4.2. Post-Hoc multiple comparison tests of morphometric variables between pairs of populations. The values represent the number of significant variables observed out of 11otolith characters for corresponding populations.

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1										
IC2	-									
NW4	7	7								
BA	10	10	2							
NSN1	3	2	6	8						
NSN2	7	7	4	8	6					
NSC	6	6	7	8	4	3				
NSD	8	8	8	8	7	-	2			
CS	7	5	8	8	7	4	-	-		
PC	8	7	10	11	3	7	6	6	3	

Table 4.3. Univariate analysis of variance (ANOVA) testing the interaction between measurements and sexes. The significance level are shown: ** P<0.05.

Variable	Wilks' Lambda	F	P
AB	0.97386	1.4761	0.2296
AC	0.97662	1.3166	0.2562
AD	0.99081	0.0292	0.478
BC	0.99869	0.0723	0.7891
BD	0.95155	1.8946	0.100
CD	0.88269	7.3094	0.0091**
CE	0.96851	1.7881	0.1867
CF	0.96617	1.926	0.1708
DE	0.96249	2.1433	0.1489
DF	0.96454	2.0219	0.1607
EF	0.9992	0.0441	0.8344

Table 4.4. Contribution of each otolith variables to the canonical functions. *, denotes largest correlation between canonical variable and canonical function.

Variables	Function 1	Function 2	Function 3	Function 4	Function 5
AD	0.80779*	0.40546	0.18545	-0.02372	0.23095
AC	0.74371*	-0.46649	0.10781	0.33141	0.26975
AB	0.73375*	-0.44626	-0.03561	0.30153	0.28582
DE	0.63494*	-0.41085	-0.08315	0.26771	0.429
CE	0.62585*	-0.44793	-0.09557	0.33841	0.41661
CF	0.50751	-0.42411	-0.10458	0.60257*	0.16911
DF	0.50177	-0.37513	-0.07385	0.53823*	0.21343
BD	-0.11857	0.07909	-0.17141	0.32143*	0.20585
EF	0.20419	-0.12074	0.29051	-0.4011	0.66902*
BC	0.02721	0.00545	-0.32767	0.02427	0.07513
CD	-0.12073	0.20035	0.11441	0.28242	0.41668

Table 4.5. Principal component (PC) loadings of PCA for herring otolith variables

Variables	PC1	PC2	PC3	PC4
AB	0.97726	0.07028	0.04907	-0.03926
AC	0.9849	-0.01683	0.0582	-0.02612
AD	0.56705	0.02644	0.56546	0.07632
BC	0.13474	0.90571	-0.05788	-0.32778
BD	-0.03792	0.96517	-0.07439	0.07956
CD	-0.15996	0.24023	0.0067	0.95258
CE	0.98146	-0.04461	-0.05174	0.00866
CF	0.93075	-0.05005	-0.34222	0.00308
DE	0.97779	-0.03492	-0.0589	0.09556
DF	0.92389	-0.02821	-0.33914	0.12941
EF	0.39695	0.09185	0.80703	-0.01007

Table 4.6. Multivariate analysis of variance (MANOVA) between all local samples of herring for otolith measurements. Significantly different group means; *P <0.05, **P<0.01, P<0.001, ns, not significant. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC).

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1	---									
IC2	ns	---								
NW4	***	***	---							
BA	***	***	***	---						
NSN1	***	***	***	***	---					
NSN2	***	***	***	***	***	---				
NSC	***	***	***	***	ns	*	---			
NSD	***	***	***	***	***	ns	ns	---		
CS	***	***	***	***	***	ns	*	*	---	
PC	***	***	***	***	***	***	***	***	***	---

Table 4.7. The summary of the classification results for otolith measurements. The diagonal numbers are the number of individuals classified correctly into their corresponding group. Overall 56 % of the grouped individuals were correctly classified. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore (Celtic Sea) (CS), Pacific herring (PC).

		Predicted group membership									
Sample	Cases	NW4	IC1	NSC	NSN1	BA	NSD	NSN2	CS	IC2	PC
NW4	42	23 **54.80%**	0 0.00%	1 2.40%	0 0.00%	8 19.00%	1 2.40%	1 2.40%	0 0.00%	3 7.10%	5 11.90%
IC1	49	0 0.00%	26 **53.10%**	2 4.10%	4 8.20%	0 0.00%	0 0.00%	1 2.00%	1 2.00%	15 30.60%	0 0.00%
NSC	23	0 0.00%	2 8.70%	4 **17.40%**	5 21.70%	0 0.00%	3 13.00%	1 4.30%	6 26.10%	2 8.70%	0 0.00%
NSN1	28	0 0.00%	2 7.10%	1 3.60%	17 **60.70%**	0 0.00%	1 3.60%	0 0.00%	6 21.40%	1 3.60%	0 0.00%
BA	48	1 2.10%	0 0.00%	0 0.00%	0 0.00%	46 **95.80%**	1 2.10%	0 0.00%	0 0.00%	0 0.00%	0 0.00%
NSD	27	0 0.00%	0 0.00%	2 7.40%	1 3.70%	1 3.70%	13 **48.10%**	3 11.10%	5 18.50%	2 7.40%	0 0.00%
NSN2	24	0 0.00%	1 4.20%	0 0.00%	1 4.20%	3 12.50%	6 25.00%	4 **16.70%**	6 25.00%	3 12.50%	0 0.00%
CS	47	0 0.00%	2 4.30%	0 0.00%	6 12.80%	1 2.10%	6 12.80%	4 8.50%	27 **57.40%**	1 2.10%	0 0.00%
IC2	47	0 0.00%	16 34.00%	1 2.10%	3 6.40%	1 2.10%	1 2.10%	1 2.10%	4 8.50%	20 **42.60%**	0 0.00%
PC	29	0 0.00%	3 10.30%	0 0.00%	1 3.40%	0 0.00%	0 0.00%	0 0.00%	2 6.90%	0 0.00%	23 **79.30%**

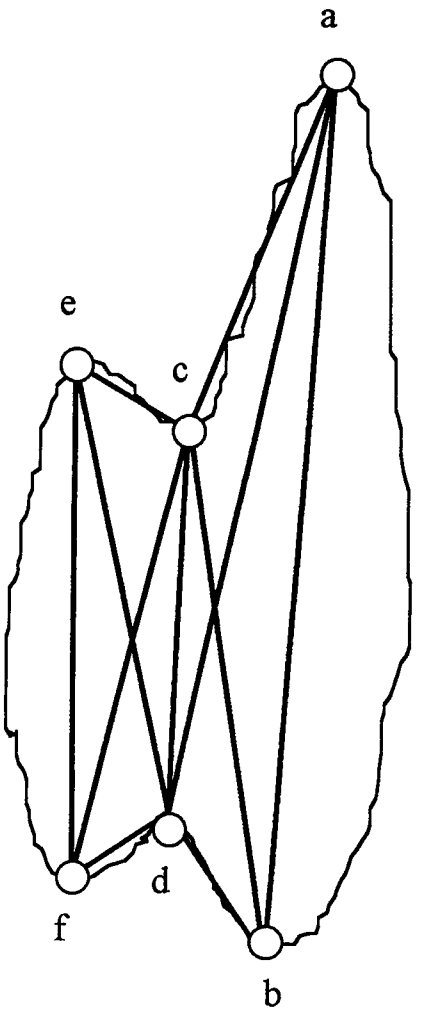


Figure 4.1. Location of landmarks for constructing the truss network on herring otolith are illustrated as open circles and the distance between circles as lines were measured. Landmarks refer to (a) rostrum, (b) postrostrum, (c) excisura major, (d) excisura minor, (e) antirostrum, (f) pararostrum.

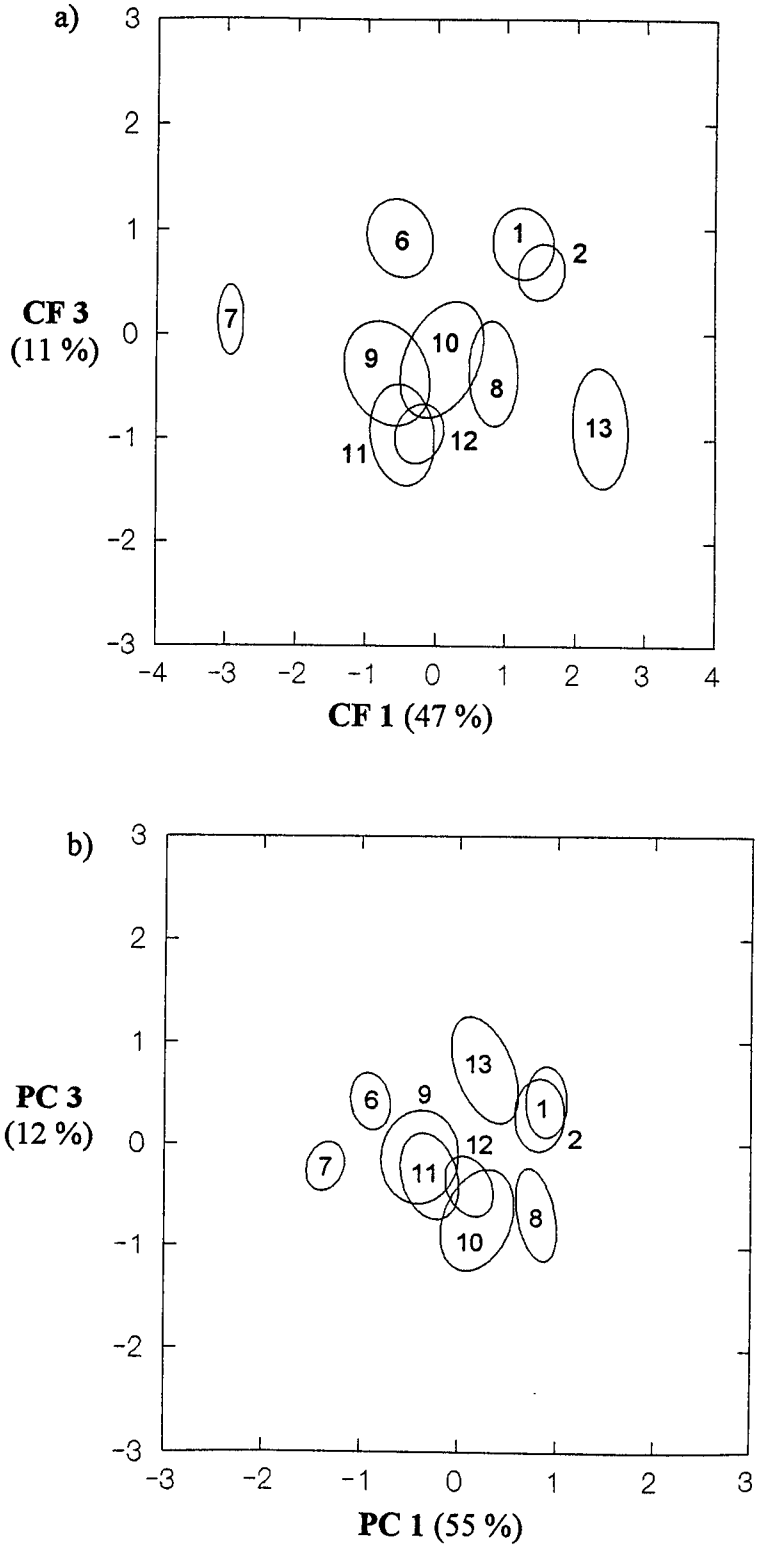


Figure 4.2. Sample centroids and 95 % confidence ellipses of CA (a) and PCA (b) scores. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1=1), Icelandic summer-spawners (second year) (IC2= 2), Trondheimsfjord (NW4= 6), Baltic herring (BA=7), Buchan herring (Northmost North Sea) (NSN1=8), Buchan herring (Northern North Sea) (NSN2= 9), Dogger herring (NSC= 10) Downs herring (NSD= 11), Dunmore (Celtic Sea) (CS= 12), Pacific herring (PC= 13).

CHAPTER 5

PHENOTYPIC VARIATION: MERISTIC ANALYSES

5.1 Introduction

Most marine fish populations occur on a broad geographic scale and are exposed to different environmental factors. The potential capacity of populations to adapt and evolve as independent biological entities in different environmental conditions is restricted by the exchange of individuals between populations. A sufficient degree of isolation may result in notable phenotypic and genetic differentiation among fish population within a species, which may be recognisable as a basis for separation and management of distinct populations. Meristic characters of fish have been conventionally used as a marker in fisheries biology for stock identification (Barlow, 1961; Martin & Olver, 1980; Ihssen *et al.*, 1981a; Mann & McCart, 1981; Bookstein *et al.*, 1982; Creech, 1992). Meristic analysis involves counts of discrete morphological features, for example, the number of fin rays, vertebrae, gill rakers, keeled scales, number of teeth and pyloric caeca. The number of fin rays, vertebrae number and gill rakers have been most commonly used for population studies. Differences in the number of meristic characters is attributed in terms of meristic differences among stocks.

In several fish species including herring it has been reported that meristic characters exhibit plasticity under the influence of environmental factors such as temperature, salinity, pH, and oxygen tension which modify the expression of the genes responsible for meristic characters (Dunham *et al.*, 1979; Balon, 1980; Todd *et al.*, 1981). For example, latitudinal changes in meristic characters (Gross, 1977; McGlade, 1981; Kanno, 1989b), and an inverse relationship between the average number of vertebrae and water temperature has been reported (e.g. Tester, 1936; Jean, 1967; Hulme, 1995). Thus the detection of meristic differences among populations may not be considered as evidence of genetic differences.

Despite the environmental contribution to meristic variation, they may provide information about the extent of intermingling of populations occupying different environments. Moreover there are several studies which report a genetic basis to meristic variation in various fish species (Barlow, 1961; Martin & Olver, 1980; Ihssen *et al.*, 1981a; Mann & McCart, 1981).

In some studies, morphometric and meristic data are treated together in the analyses to characterise different biological groups, though pooling both types of data in a single analysis is statistically suspect (Ihssen *et al.*, 1981a & 1981b; Bookstein *et al.*, 1982; Creech, 1992). The discrete nature of meristic characters renders their discriminatory ability possibly less than continues data (e.g. morphometric data), meaning that the two types of data should be analysed separately in multivariate analysis (Ihssen *et al.*, 1981a & 1981b). For example, Junquera & Perezgåndaras (1993) used 30 morphometric characters and 8 meristic characters to analyse the population diversity of anchovies (*Engraulis*

encrasicolus) in the Bay of Biscay. They found significant differences in morphometric characters between populations, though meristic characters were not significantly heterogeneous. The inverse is also seen, where for example, Shepherd (1991) investigated population structure in black sea bass (*Centropristis striata*) from north cape Hatteras and North Carolina using meristic and morphometric measurements. Significant differences were found between samples, and the meristic comparison provided greater separation between geographic areas than did morphometrics.

Numerous studies have been undertaken on meristic characters to unravel the taxonomic status of spawning groups of herring. In Norwegian waters, Icelandic summer-spawners exhibited significant differentiation from Icelandic spring-spawners on the basis of meristic characters (Johansen, 1926; Fridriksson, 1944, 1958) and from Norwegian spring-spawners (Johansen, 1927; Runnström, 1936). Parrish & Saville (1965) used meristic characters (vertebral counts and gill raker) with physiological, otolith nucleus and behavioural characters, and divided herring into two groups in the Northeast Atlantic; an 'Oceanic population', which was subdivided into five major stocks; an Norwegian winter-spring spawning stock, a Icelandic winter-spring spawning stock, a Icelandic summer spawning stock, a Scottish west coast winter-spring spawning stock, a southern Irish (Dunmore), winter-spawning stock. The 'Shelf population' was subdivided into six stock categories, a central and Northern North Sea (Bank) summer-autumn, a Southern North Sea eastern channel (Downs) winter-autumn, a north-eastern Kattegat summer-autumn, a Baltic

summer-autumn, a Scottish west coast (Minch) summer-autumn, and a Northern Irish Sea (Isle of Man) summer-autumn spawning stock.

Ryman *et al.* (1984) found significant meristic differences between Baltic spring-spawning samples and Kattegat or Skagerrrak spring spawning samples (southern west of Sweden), though the detected differences was not confirmed by allozyme data.

Almost all of the attempts in describing the population structure of Northeast Atlantic herring have used meristic and other morphological or physiological characters together. Therefore the ability of meristic characters taken alone in the identification of herring populations is not clear.

The aims of this study are:

1) to examine stock structure using meristic characters for Northeast Atlantic herring populations;

2) to test the ability of meristic characters for stock identification of herring.

5.2 Materials and Methods

5.2.1 Laboratory Procedure

Herring samples used in this study were collected from the nine local Atlantic and one Pacific herring populations as reported in chapter 2, section 2.

Six meristic characters were examined using the number of:

anal fin rays (AFR),

dorsal fin rays (DFR),

pelvic fin rays (PVFR),

pectoral fin rays (PFR),

gill rakers on the upper limb of the first gill arch (GRNU),

gill rakers on the lower limb of the first gill arch (GRND) under a biocular microscope. In the pectoral, dorsal and anal fin, all rays including rudimentary rays were counted; the last split rays originating from the same base were counted as one. All the data were transferred to a Lotus 1-2-3 spreadsheet.

5.2.2 Multivariate analysis

Correlation matrices were computed to assess the effect of size on all meristic counts. The significance of correlations was tested between the meristic counts and standard length. Significant correlation was not observed in DFR, however in other counts significant correlations were detected (Table 5.1). In order to remove any size component in the data set and obtain genuine differences among the samples, ALLOM2 was used as described in Chapter 3.2.2., to facilitate comparison with the otolith and morphometric data. In addition, CFA and PCA were also performed without transformation of meristic data in order to compare with transformed data analyses. The ALLOM2 transformed and untransformed data were standardised prior to a principal

component analysis (PCA) and a canonical analysis (CA), details of which have been given at Chapter 3.2.3. However, although the use of CA for meristic data (nonparametric character) has statistical constraints, it is commonly used for meristic data to describe population relationships. The population centroids with 95% confidence ellipses derived from first two PCs and CFs were plotted to examine the differences among samples.

Non-parametric statistics were used due to non-discrete structure of meristic characters. The Kruskal-Wallis *H* test was used to compare the variation among samples for individual meristic character. Post-Hoc multiple comparison tests was also performed to examine the number of significant morphometric characters between pairs of samples.

The effect of sex on meristic characters was also tested using the Kruskal-Wallis *H* test. Correlations between meristic characters and latitude, and between the first canonical function, principal component scores and latitude was also tested (Spearman correlation). Mann-Whitney *U* test was performed to test the significance of differences between pairs of samples.

5.3 Results

In the present study, 364 of the 377 submitted individuals were used by multivariate analyses, and 13 individuals were not used due to at least one missing discriminant variable in their row data, and thus the sample size varied between 22 to 49.

Analysis of data using Kruskal-Wallis H test showed significant differences among samples for all meristic counts (Table 5.2). Post-Hoc multiple comparison tests between pairs of samples revealed that most of the significantly different characters were from Pacific and Icelandic samples. Also there were 2 significantly different characters out of 6 between Icelandic samples (IC1 & IC2) (Table 5.3). Chi-square values were high, especially for gill raker numbers (Table 5.2), however, they were low for PVFR. Mode of meristic character of the samples are listed at Table 5.4.

Testing the interaction between the meristic counts and sexes from 67 sex-recorded fish showed that non of meristic characters was not significantly different for different sex types, demonstrating no effect of sex on the observed variation (Table 5.5).

Testing the correlation between meristic characters and latitude showed a significantly positive correlation for AFR, GRND, GRNU, PFR and negative correlation for PVFR (Fig. 5.1). Significantly different positive correlations were also observed between the first discriminant function, principal component scores and latitude (Fig. 5.2), indicating a possible environmental effect on the meristic characters and observed differences among samples.

Examination of loading on both the first discriminant function (Table 5.6) and principal component (Table 5.7) showed high loadings of GRNU, GRND and AFR. Hence these meristic counts can be considered as being the most discriminating characters. Loadings of untransformed meristic data to discriminant function and principal component was also given in Table 5.8 and Table 5.9, showing similar high loadings as described above.

Plotting CF1 and CF2 explained 88 % of the total between-group variability. Of this, 60 % was explained in the first (CF1) and 28 % in the second canonical function (CF2) (Fig. 5.3a). The 95% confidence ellipses of the Pacific (PC) and Icelandic summer-spawners (IC1 & IC2), and also of Trondheimsfjord (NW4) samples were highly isolated from the rest of samples, and from each other. The other samples overlapped, with hardly any separation from each other (Fig 5.3a). However a pairwise comparison (MANOVA) among samples revealed that most of the samples were highly significantly different from each other. Only some of the North Sea samples did not reveal significant differentiation (Table 5.10).

The first two principal components accounted for 51% of the total variance (Fig. 5.3b). When they were plotted (Fig. 5.3b), the population centroids (with 95% confidence ellipses) of Pacific herring (PC), Icelandic summer-spawner samples (IC1 & IC2) and also Trondheimsfjord herring were separated clearly from each other, and from all other samples, which grouped together in a cluster.

Ploting first two CFs and PCs generated from untransformed meristic data revealed same pattern of differentiation (Fig 5.4) as seen in transformed analyses of meristic data (Fig. 5.3).

In the discriminant function analysis the correct classification of the individuals into their original populations was weak, with a mean of 47 %, which varied between around 22 % (Downs herring sample) and 89 % (Pacific sample) (Table 5.11).

5.4 Discussion

In the present study, highly significant meristic variation among most of the herring samples was detected. The pattern of meristic differentiation among the samples apparently reflect their geographical proximity and latitude. Therefore, meristic structuring may be associated with latitude of the spawning grounds, and geographic distance is possibly an obstructer to gene flow or intermingling among populations, thus contributing to their phenotypic differentiation. Results from multivariate analyses suggest a clear meristic discreteness of the Icelandic-summer-spawners and Trondheimsfjord herring, and high meristic divergence between Pacific herring and Atlantic herring.

Interestingly, the temporally-separated Icelandic summer-spawning samples (IC1 & IC2) collected in the same location differed significantly from each other and from the rest of the samples. The detected spatial discreteness is in accordance with previous studies using (Johansen, 1926; Fridriksson, 1944,

1958), otoliths, scales and physiological traits (Einarsson, 1951; Liamin, 1959; Parrish & Saville, 1965). There is evidence that meristic counts may differ significantly between year classes, and that they are highly sensitive to environmental variations during the period of formation or early larval life (Parsons & Hodder, 1971; Fahy, 1983; Lindsey, 1988; Hulme, 1995). Variation in ecological conditions such as temperature, or the proportion of plankton in different years can cause temporal variation in the meristic characters of a population. For example, Berg & Grimaldi (1965) reported a significant difference in gill raker counts between year classes of bondella, *Coregonus sp.*, in Lake Maggiore, which was attributed to ecological conditions and growth rate in different years. Thus the observed temporal differences between the Icelandic summer-spawning samples (IC1 & IC2) may be attributed to a strong influence of environmental factors on meristic characters in different years, though temporal phenotypic stability of these samples (IC1 & IC2) were detected in morphometrics and otolith analyses (Chapter 3 & 4).

The Trondheimsfjord sample (NW4) also showed a clear separation in the multivariate analyses from the other samples. Allozymic discreteness of the Trondheimsfjord herring has also been detected (Jørstad *et al.*, 1986; Turan *et al.*, 1997). Fjord-specific environmental factors may be governing the phenotypic differentiation: low salinity and temperature, and high plankton density, and shallow water are the factors that differ most obviously in fjord waters, and there are numerous studies, showing the role that such factors can play in the meristic differentiation among populations (Parsons & Hodder, 1971; Lindsey, 1981 & 1988; Hulme, 1995).

The results of both the CA and PCA demonstrated that there is low meristic differentiation among the herring samples from around the British Isles compared to spatial differences detected with the other samples (Fig. 5.3a), even though most pairwise comparisons revealed statistically significant differences. King (1985) have also reported morphological meristic similarity among herring spawning aggregations from both side of the British Isles and the Baltic sea. Also genetic homogeneity of these aggregations has also been reported (King *et al.*, 1987; Jorstard *et al.*, 1991). However, low meristic differentiation detected here may indicate that there may be some restriction to migration among these aggregations.

The Pacific herring sample (PC) was most distinct from all others, and had the highest percentage of correctly classified individuals (over 89 %). The detected pattern suggests that meristic characters alone are an effective marker for distinguish different species of herring, in contrast to previously reported morphological similarity between Pacific herring and Atlantic herring (Svetovidov, 1963) using meristics, body dimensions and size at first maturity data.

In the present meristic analyses, all six meristic characters showed highly significant statistical differences among localities, the most obvious difference among samples being the number of gill rakers. Polymorphism in gill raker number has been attributed to genetic differences in herring (Krefft, 1958) and other fish species (Andreu, 1969; Lindsey, 1981 & 1988), and they have been reported to be less subjected to environmentally induced variation than other morphological characters (Lindsey, 1981 & 1988). However, in the present

study, most of the meristic characters revealed a significantly positive relationship with latitude (Fig. 5.1). Moreover, the detected significantly different positive correlations between the first discriminant function or principal component scores and latitude (Fig. 5.2), and high contribution of GRNU, GRND and AFR to the first canonical function and principal component (Table 5.6 & 5.7) indicate that the most discriminating meristic counts apparently are also the most environmentally induced characters among the samples. The effect of latitude on meristic structuring of the populations can also be inferred from the distribution of samples in both PCA and CA (Fig. 5.3a). For example, in the discriminant space, the Pacific herring are located at the lowest latitude (49°35'N), and plotted on the right land of the chart, and the Icelandic summer-spawners occur at the highest latitude (64°33') among the samples, and are positioned on the left land. Samples of intermediate latitude were plotted in the central chart area.

A similar relationship between meristic characters and latitude has also been found in Pacific herring. Kanno (1989b) reported that variation in upper and lower gill rakers of the spawning groups of Pacific herring was significantly related to latitude and water temperature, and there was no relation with the salinity, though the range of change in salinity in the environments in which populations have been placed was narrow. Hulme (1995) demonstrated that in Atlantic herring, vertebral counts are sensitive to temperature, with higher sea temperatures giving lower mean vertebral counts. Collectively, in the present study the principal causes of the meristic variation among herring populations seems to be related to the latitude and possibly water temperature.

Although the phenotypic variation observed may be environmentally-induced, it can provide useful knowledge in the stock structure analysis of exploited species for management decisions, especially if molecular markers fail to detect genetic variation among discrete spawning aggregations. Furthermore molecular markers are generally not available due to its expense and complexity in many developing countries, so, phenotypic markers can be practically used to partition phenotypically differentiated populations.

From the perspective of fishery management, the present meristic analyses revealed at least two identifiable management units: the Icelandic summer-spawners and Trondheimsfjord herring. In the case of persistence of the observed differentiation they would warrant separate management. The Icelandic samples did not reveal temporal integrity, nevertheless, the detected spatial integrity, which is also in agreement with other studies (Johansen, 1926; Fridriksson, 1944, 1958), prove their discreteness from other populations sampled, and indicate that they do not freely intermingle with the other populations. The Trondheimsfjord herring also exhibited temporal and spatial integrity by comparison with a previous genetic study (Jørstad & Nævdal, 1981), and therefore should be treated as a different management unit. Although the other samples especially, the Baltic herring, could not clearly be separated into unique biological entities in both the CA and PCA, significant differences were revealed by Mann-Whitney U tests among samples. Therefore the response of these spawning aggregations to exploitation may still be regionally independent.

In summary, the result of a large-scale study of meristic variation showed a high discriminatory ability of meristic characters to detect conspecific variation, and also for discriminating different species of herring. The present findings indicate a high degree of significant differentiation among samples, and the extent of separation among samples may be related to geographical proximity and latitude. The pattern of meristic distinctness detected among samples may be related to environmentally-induced morphological variation arising under the influence of environmental factors, especially temperature during the incubation period and early larval life. The detected meristic variation may also reflect genetic differentiation which can be examined using molecular genetic markers.

Table 5.1. Correlation (Spearman) between meristic characters and standard length. P denotes significance levels and are shown: ***P < 0.001; *P<0.05. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variable	Correlation	P
AFR	0.32	0.000
DFR	0.05	0.29
GRND	0.19	0.000
GRNU	0.3	0.000
PFR	0.28	0.000
PVFR	0.11	0.032

Table 5.2. Kruskal-Wallis *H* test comparing ALLOM2 adjusted meristic characters among herring samples. The significance level are shown: ***$P < 0.001$; *P<0.05. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variable	Chi-square	D.F	P
AFR	73.13	9	0***
DFR	130.97	9	0***
GRND	157.1	9	0***
GRNU	187.64	9	0***
PFR	64.8	9	0***
PVFR	19.51	9	0.0212*

Table 5.3. Post-Hoc multiple comparison tests of morphometric variables between pairs of populations. The values represent the number of significant variables observed out of 6 meristic characters for corresponding populations.

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1										
IC2	2									
NW4	3	1								
BA	2	3	3							
NSN1	1	2	-	-						
NSN2	1	2	2	-	-					
NSC	1	3	2	-	1	-				
NSD	4	3	1	2	-	-	1			
CS	2	3	3	1	1	-	1	-		
PC	4	4	3	4	3	3	3	2	3	

Table 5.4. Mode of meristic characters of herring. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays(DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Sample	AFR	DFR	GRND	GRNU	PFR	PVFR
IC1	17	19	22	49	18	9
IC2	18	18	22	47	18	9
NW4	17	18	21	45	17	9
BA	16	18	21	47	17	9
NSN1	17	18	22	45	17	9
NSN2	17	18	21	45	18	9
NSC	16	19	22	45	17	9
NSD	17	18	20	46	18	9
CS	16	18	20	45	17	9
PC	16	17	19	44	16	9

Table 5.5. Kruskal-Wallis *H* test for the interaction between meristic characters and sexes. P denotes significance level. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variable	Chi-square	D.F	P
AFR	0.78	1	0.3744
DFR	3.07	1	0.0797
GRND	0.55	1	0.4568
GRNU	1.49	1	0.2209
PFR	1.64	1	0.1991
PVFR	0.17	1	0.6715

Table 5.6. Contribution of each variables to the canonical functions.
* denotes largest correlation between canonical variable and discriminant function. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variables	Function 1	Function 2	Function 3	Function4	Function5
GRNU	0.70889*	0.1836	-0.58527	-0.02384	-0.00145
GRND	0.59972*	0.11953	0.37106	-0.40988	-0.56151
DFR	0.09706	0.73714*	0.3585	-0.04545	0.5581
PFR	0.30871	0.16014	0.30012	0.87827*	-0.13266
AFR	0.51328	-0.40789	0.38079	-0.02602	0.62555*
PVFR	-0.02931	-0.15973	0.2418	0.00225	0.03409

Table 5.7. Principal component (PC) loadings of PCA for meristic characters of herring.The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variables	PC1	PC2	PC3	PC4	PC5
AFR	0.61214	0.09693	-0.18677	0.57036	0.50039
DFR	0.33944	-0.68091	0.45331	-0.28741	0.36467
GRND	0.76407	0.22949	-0.17044	-0.29545	-0.0024
GRNU	0.75571	0.18591	-0.15592	-0.35399	-0.1417
PFR	0.57416	-0.35709	0.22328	0.41214	-0.5665
PVFR	0.10158	0.60031	0.79059	0.04964	0.04065

Table 5.8. Contribution of each untransformed meristic variables to the canonical functions. * denotes largest correlation between canonical variable and discriminant function. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variables	Function 1	Function 2	Function 3	Function4	Function5
GRNU	0.73533*	0.23357	0-.26082	-0.38471	0.39455
DFR	0.04684	0.76225*	0.30093	0.22981	0.18225
PFR	0.34468	0.14545	0.72619*	-0.33221	-0.46421
GRND	0.50742	0.20358	-0.09209	0.61374*	-0.11949
PVFR	-0.07261	-0.00936	0.58052	0.03066	0.71149*
AFR	0.51704	-0.37679	0.26037	0.38203	0.09134

Table 5.9. Principal component (PC) loadings of PCA using untransformed meristic data. The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the lower limb of the first gill arch (GRND), gill rakers on the upper limb of the first gill arch (GRNU).

Variables	PC1	PC2	PC3	PC4	PC5
AFR	0.62038	-0.26612	-0.04982	0.45772	0.57633
DFR	0.3204	0.6089	-0.62996	-0.29045	0.2107
GRND	0.74133	-0.18657	0.18849	-0.41269	-0.0114
GRNU	0.78042	-0.12463	0.19385	-0.25641	-0.1479
PFR	0.61277	0.27292	-0.14669	0.50871	-0.5072
PVFR	0.05079	0.75469	0.62236	0.0674	0.18939

Table 5.10. Mann-Whitney *U* test between all local samples of herring for meristic measurements.Significantly different group means: *P <0.05; **P<0.01; ***P<0.001; ns, not significant. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondhimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore herring (Celtic Sea) (CS), Pacific herring (PC).

Sample	IC1	IC2	NW4	BA	NSN1	NSN2	NSC	NSD	CS	PC
IC1	---									
IC2	***	---								
NW4	***	***	---							
BA	***	***	***	---						
NSN1	***	***	***	***	---					
NSN2	***	***	***	***	***	---				
NSC	***	***	***	***	ns	***	---			
NSD	***	***	***	***	*	ns	***	---		
CS	***	***	***	***	***	*	***	*	---	
PC	***	***	***	***	***	***	***	***	***	---

Table 5.11. The summary of the correct classification results for meristic characters. The diagonal numbers are the number of individuals classified correctly into their corresponding group. Overall 47 % of the grouped individuals were correctly classified. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Trondhimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN1), Buchan herring (Northern North Sea) (NSN2), Dogger herring (NSC), Downs herring (NSD), Dunmore herring (Celtic Sea) (CS), Pacific herring (PC).

		Predicted group membership									
Samples	No of individuals	NW4	IC1	NSC	NSN1	BA	NSD	NSN2	CS	IC2	PC
NW4	49	**23** **46.90%**	1 2.00%	1 2.00%	3 6.10%	2 4.10%	4 8.20%	3 6.10%	3 6.10%	9 18.40%	0 0.00%
IC1	49	1 2.00%	**25** **51.00%**	1 2.00%	1 2.00%	10 20.40%	0 0.00%	0 0.00%	1 2.00%	10 20.40%	0 0.00%
NSC	24	1 4.20%	1 4.20%	**11** **45.80%**	2 8.30%	5 20.80%	0 0.00%	2 8.30%	1 4.20%	0 0.00%	1 4.20%
NSN1	28	4 14.30%	0 0.00%	4 14.30%	**8** **28.60%**	2 7.10%	3 10.70%	3 10.70%	0 0.00%	0 0.00%	4 14.30%
BA	50	0 0.00%	9 18.00%	3 6.00%	3 6.00%	**24** **48.00%**	9 18.00%	0 0.00%	1 2.00%	0 0.00%	1 2.00%
NSD	23	3 13.00%	1 4.30%	2 8.70%	2 8.70%	1 4.30%	**5** **21.70%**	4 17.40%	4 17.40%	0 0.00%	1 4.30%
NSN2	22	2 9.10%	1 4.50%	3 13.60%	3 13.60%	1 4.50%	2 9.10%	**5** **22.70%**	5 22.70%	0 0.00%	0 0.00%
CS	44	2 4.50%	2 4.50%	5 11.40%	3 6.80%	5 11.40%	7 15.90%	2 4.50%	**18** **40.90%**	0 0.00%	0 0.00%
IC2	47	7 14.90%	10 21.30%	0 0.00%	0 0.00%	0 0.00%	2 4.30%	1 2.10%	0 0.00%	**27** **57.40%**	0 0.00%
PC	28	1 3.60%	0 0.00%	0 0.00%	0 0.00%	0 0.00%	1 3.60%	0 0.00%	1 3.60%	0 0.00%	**25** **89.30%**

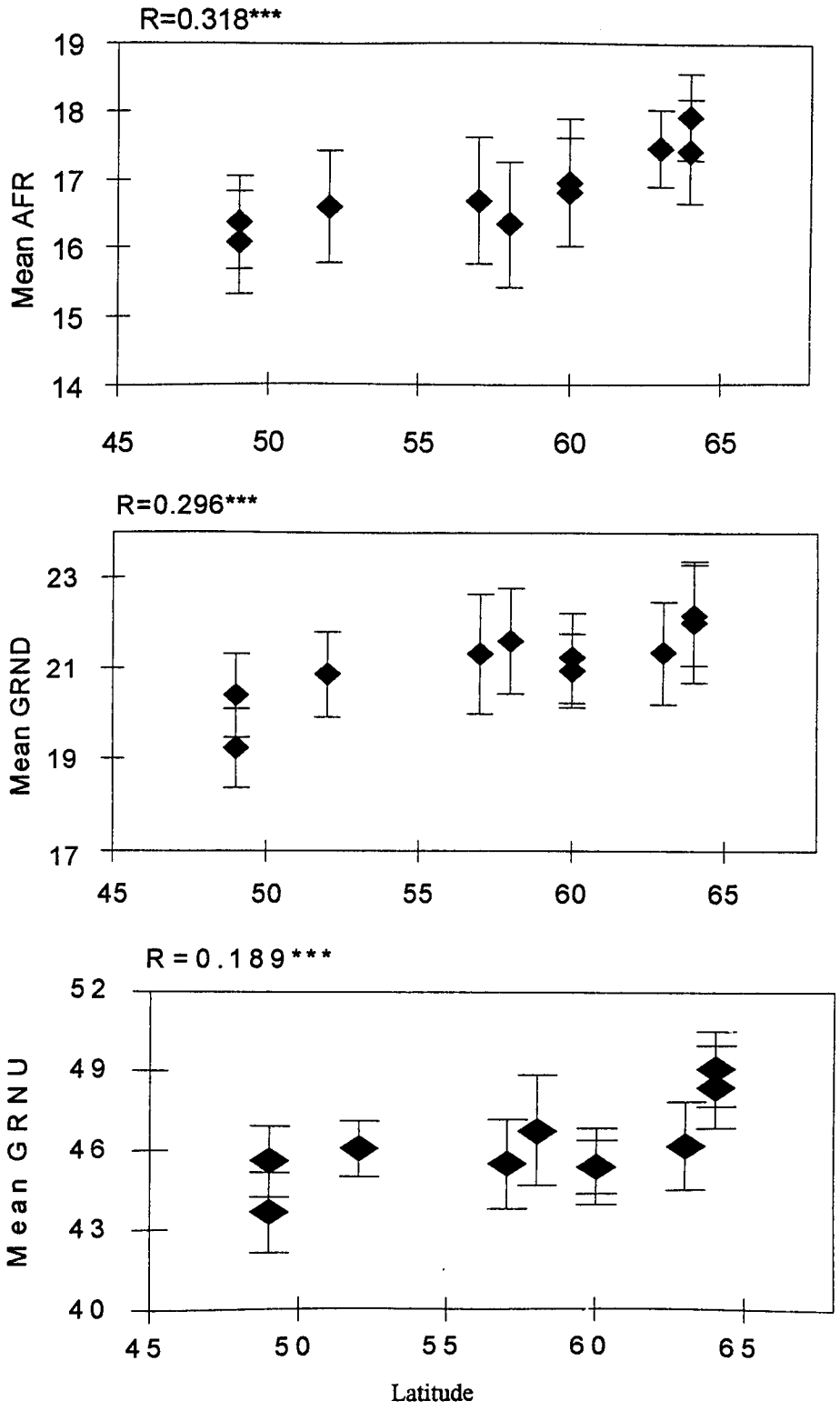
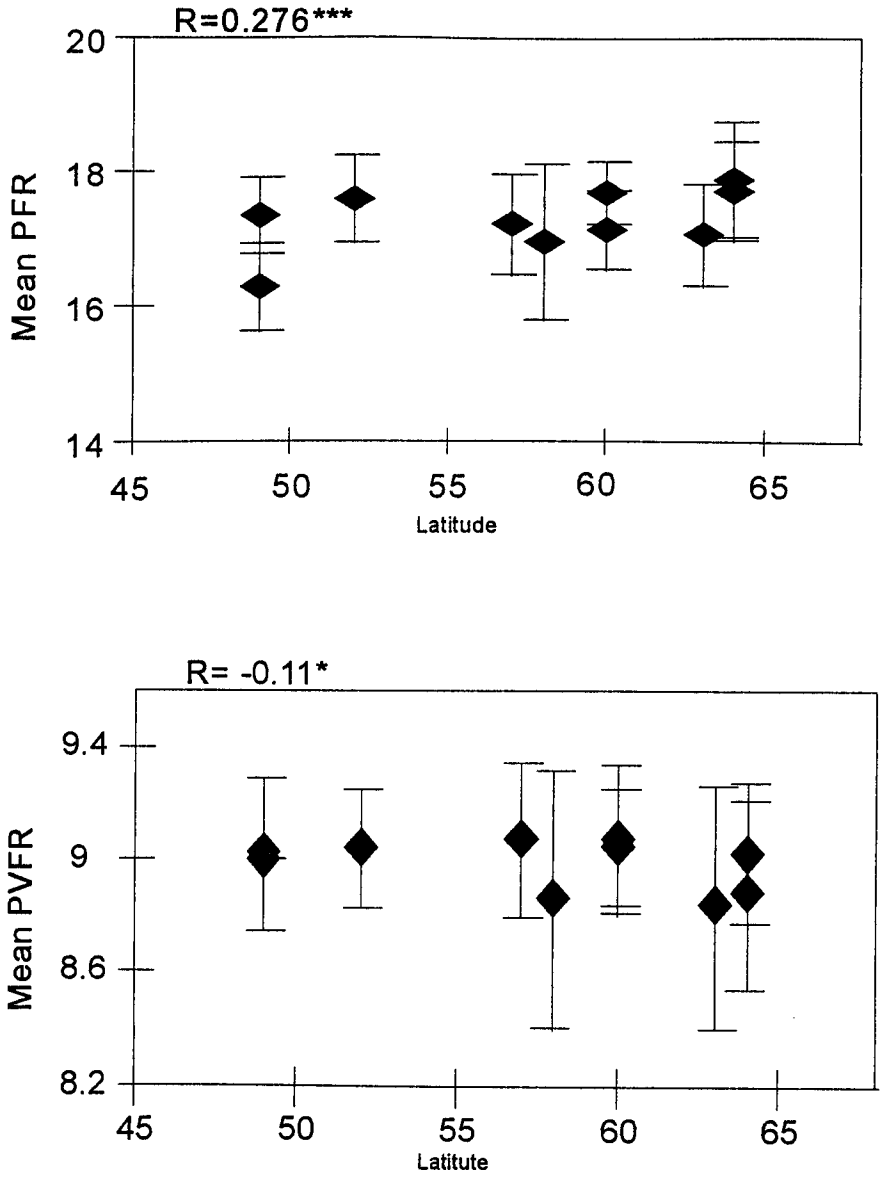


Figure 5.1. Relationship between meristic characters and latitude. R, correlation coefficient. ***, P<0.001; **, *P*<0.01; **P*<0.05 (the degree of significance of correlation coefficient). The abbreviations of meristic characters are: anal fin rays (AFR), dorsal fin rays (DFR), pelvic fin rays (PVFR), pectoral fin rays (PFR), gill rakers on the upper limb of the first gill arch (GRNU), gill rakers on the lower limb of the first gill arch (GRND).

Figure 5.1. continued.



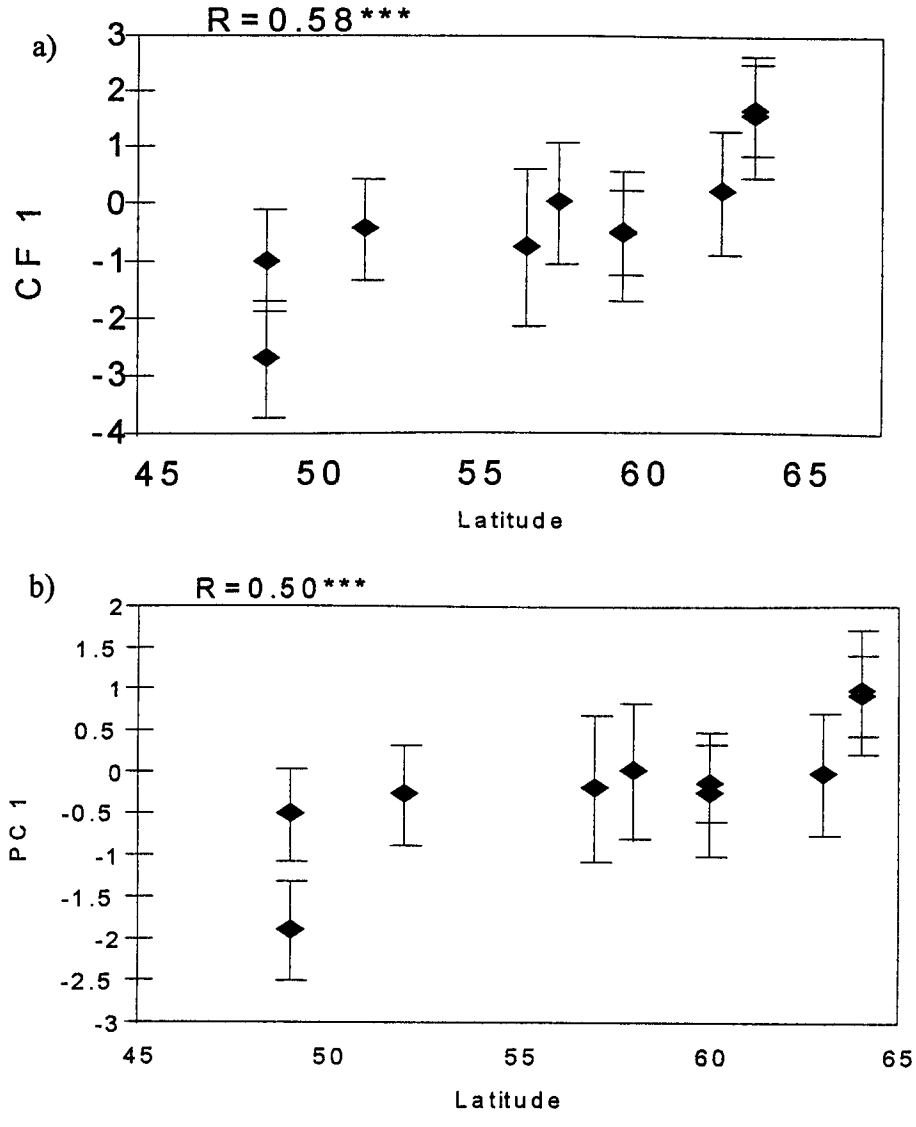


Figure 5.2. Relationship between first canonical function (CF1) (a), principal component (PC1) (b) scores of each sample and latitude for meristic characters. R, correlation coefficient. ***, $P<0.001$ (the degree of significance of correlation coefficient).

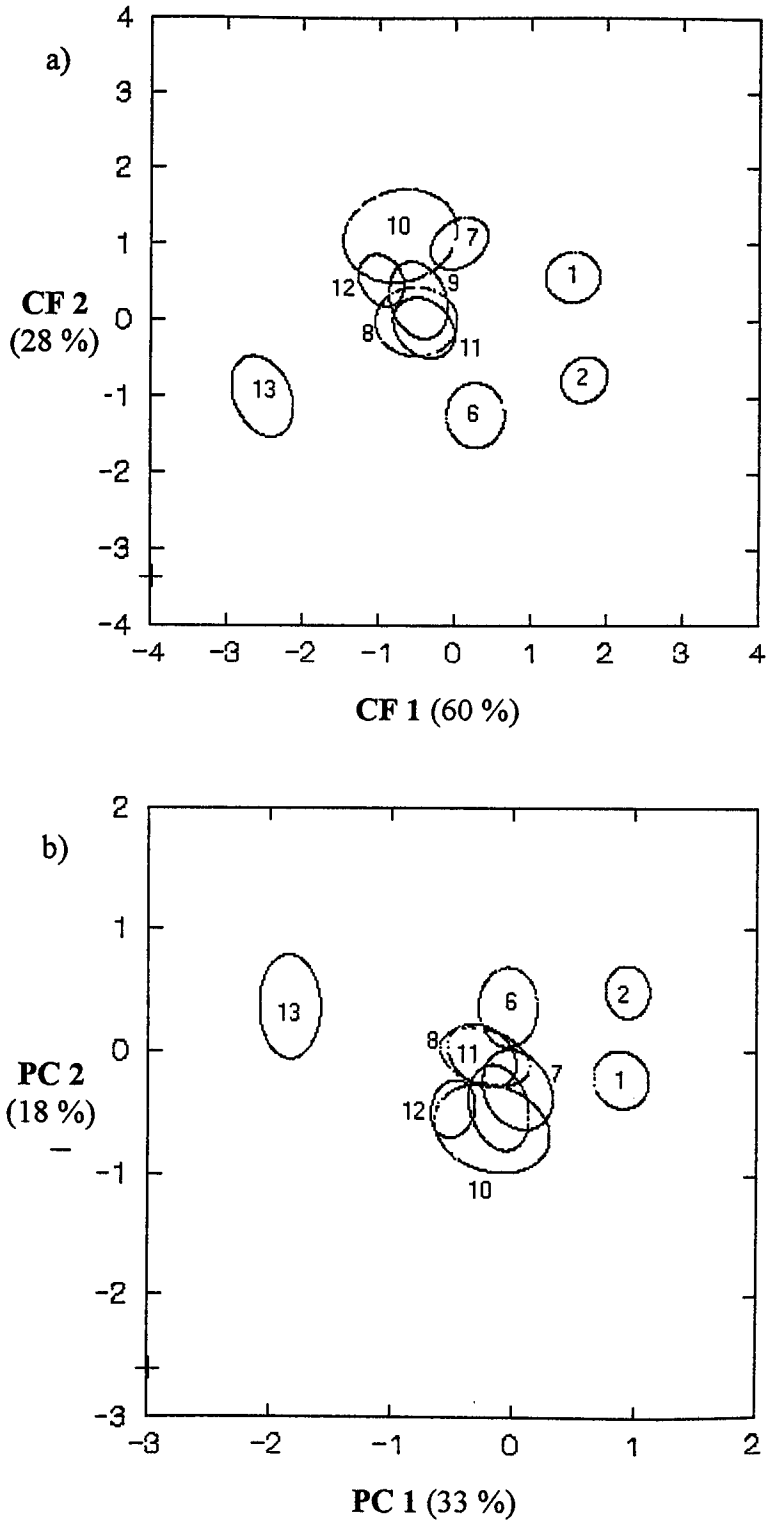


Figure 5.3. Sample centroids and 95 % confidence ellipses of CA (a) and PCA (b) scores. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1=1), Icelandic summer-spawners (second year) (IC2= 2), Trondheimsfjord (NW4= 6), Baltic herring (BA=7), Buchan herring (Northmost North Sea) (NSN1=8), Buchan herring(Northern North Sea) (NSN2= 9), Dogger herring (NSC= 10) Downs herring (NSD= 11), Dunmore (Celtic Sea) (CS= 12), Pacific herring (PC= 13).

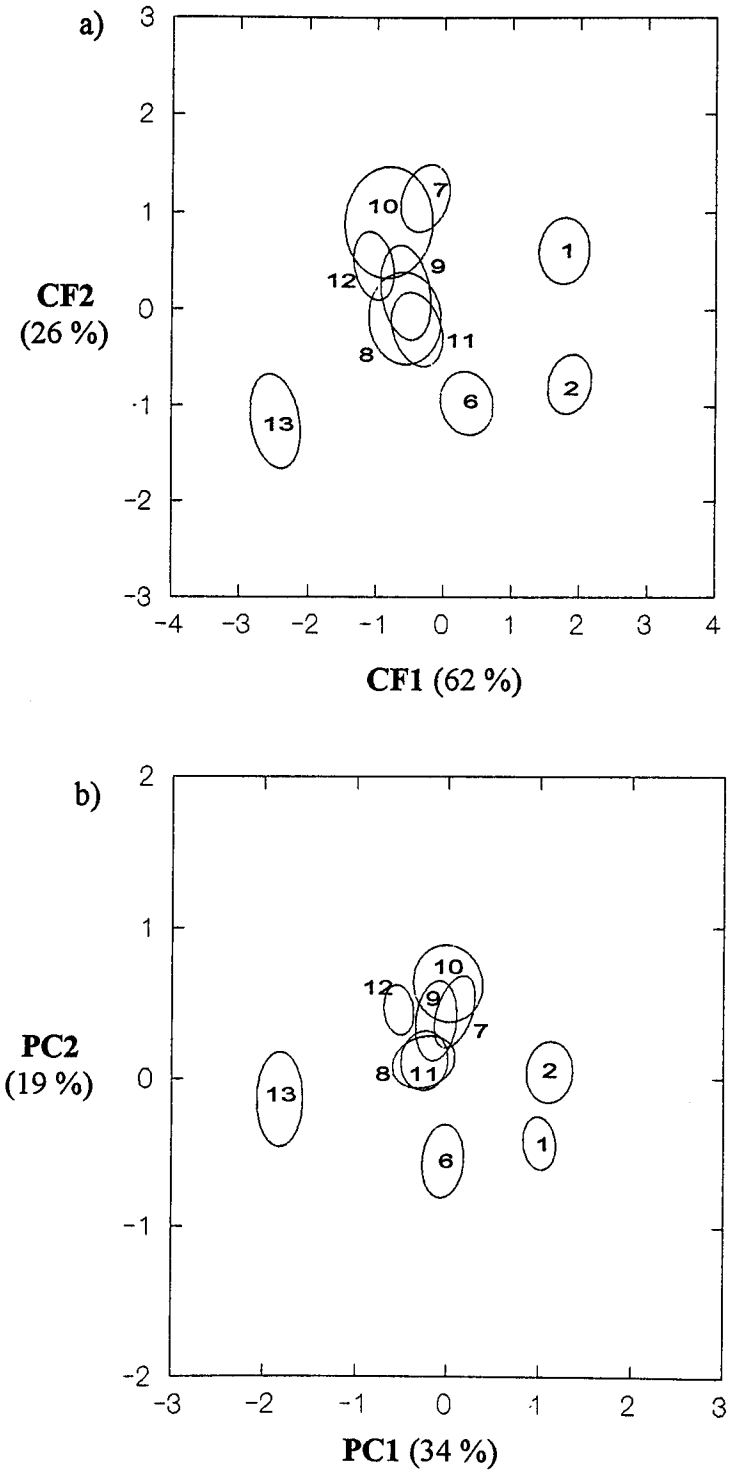


Figure 5.4. Sample centroids and 95 % confidence ellipses of CA (a) and PCA (b) scores using untransformed meristic data. Samples referred to in the text were Icelandic summer-spawners (first year) (IC1=1), Icelandic summer-spawners (second year) (IC2= 2), Trondheimsfjord (NW4= 6), Baltic herring (BA=7), Buchan herring (Northmost North Sea) (NSN1=8), Buchan herring(Northern North Sea) (NSN2= 9), Dogger herring (NSC= 10) Downs herring (NSD= 11), Dunmore (Celtic Sea) (CS= 12), Pacific herring (PC= 13).

CHAPTER 6

NUCLEAR DNA DIFFERENTIATION: EVIDENCE FROM ALLOZYME ELECTROPHORESIS

6.1 Introduction

Since the 1970s, the studies of allelic variation as revealed by electrophoresis has been the favoured method for exploring genetic variation in fish populations for the purpose of stock identification (Utter, 1991; Carvalho & Pitcher, 1994). A variable proportion of the phenotypic plasticity of fish in a population arises from environmental variation (Allendorf *et al.*, 1987), thus restricting the use of phenotypic markers and emphasising the necessity of genetic analysis in population studies. An analysis of genetic stock structure of a fish species can provide valuable information on the distribution of genetically unique stocks (Ryman & Utter, 1987), and thus identify definable management units (Carvalho & Hauser, 1994). Allozyme electrophoresis can be used either alone or in conjunction with phenotypic approaches such as morphometrics, meristics and parasite analysis to identify subpopulations that may be managed separately (Ward *et al.*, 1994a).

The lack of physical barriers to migration or gene flow in the marine environment compared with fresh waters generally results in relatively little

inter-population genetic variation, even on a broad geographic scale (Ward *et al.*, 1994b), restricting the use of allozymes in analysing closely related populations. Allozymes are particularly sensitive to low levels of gene flow (Ward & Grewe, 1994; Carvalho & Hauser, 1994), and a small but significant degree of gene flow is effectively indistinguishable from true panmixis: gene flow rates of 1%, 5%, 10%, and 50% would all probably mean that populations cannot be distinguished allozymically and appear panmictic, however, yet from a fisheries viewpoint, gene flow rates of 10% or less may justify treatment as separate stocks (Brown *et al.*, 1987). Nevertheless, there are many well documented allozyme studies which provide genetic evidence for stock discreteness of marine fishes (e.g. Richardson, 1983; Smith, 1990; Jorstard *et al.*, 1994; Bembo *et al.*, 1996a, 1996b; Edmands *et al.*, 1996)

Atlantic herring, *Clupea harengus* L., is widely distributed on both sides of the north Atlantic Ocean within each of which they have many spawning aggregations differing in spawning time and space (Svetovidov, 1963; Parrish & Saville, 1965; Haegele & Schweigert, 1985). The population structure of Atlantic herring is among the one of the most complex in marine teleosts. There have been numerous attempts to unravel the taxonomic status of such spawning groups around the British Isles, North Sea, Baltic Sea, Norwegian sea-waters and in the western part of the North Atlantic Ocean using a variety of phenotypic markers including morphological characters, time of spawning and migration behaviour (Svetovidov, 1963; Parrish & Saville, 1965; Fridriksson, 1944; Messieh, 1972; Ryman *et al.*, 1984; Haegele & Schweigert, 1985), all of which have shown varying degrees of geographic differentiation. Most genetic

attempts to define stock structure in herring are based on allozymes (Anderson *et al.*, 1981; Jørstad & Nævdal, 1981; Kornfield *et al.*, 1982; Ryman *et al.*, 1984; Jørstad & Pederson, 1986; Smith & Jamieson, 1986; King *et al.* 1987; Jørstad *et al.*, 1991; Koskiniemi & Parmanne, 1991), and have usually revealed genetic homogeneity over large geographic distances (Grant, 1984; Ryman *et al.*, 1984; King *et al.*, 1987; Jørstad *et al.*, 1991). Kornfield *et al.* (1982) detected a degree of allozymic variation between Atlantic herring spawning grounds in the Gulf of Maine, but it was not temporally stable.

The general lack of genetic structuring on a large geographic scale contrasts with marked localised genetic heterogeneity in herring collected from Norwegian fjords (Jørstad & Nævdal, 1983; Jørstad & Pederson, 1986; Jørstad *et al.*, 1991; Jørstad *et al.*, 1994). In allozyme studies, herring in the Balsfjord are most genetically distinct from the main group of Atlantic herring, and are almost fixed for different dominant alleles at polymorphic loci (e.g.; *LDH-2**, *IDHP-2**, *PGM-1**) (Jørstad & Pederson, 1986; Jørstad *et al.*, 1994). Surprisingly, a higher allozymic similarity of the Balsfjord herring to Pacific herring was reported and thus suggested these fjord herring to be treated as a sub-species (Jørstad *et al.*, 1994). Likewise in the morphological and allozyme analysis of herring in the White Sea revealed them to be more closely related to Pacific herring, *Clupea pallasi* L. (Soin, 1971; Truveller, 1979).

Pacific and Atlantic herring have been considered to be two different subspecies (Svetovidov, 1963) due to their small morphological divergence. Grant (1986) investigated the genetic divergence between Atlantic and Pacific herring using allozyme electrophoresis of 40 loci and concluded that the two

should be considered as separate species. The same conclusion was also given by Jørstad *et al.* (1994), though the genetic discreteness were based on only 6 polymorphic loci.

In the present project, the aim of the allozyme study is:

1) to investigate the genetic population structure of Northeast Atlantic herring populations and;

2) to investigate the genetic relationship between Norwegian fjord populations and Pacific herring, and to contribute to our understanding of the evolution of the different herring species in the Atlantic and Pacific Oceans.

6.2 Materials and methods.

6.2.1 Laboratory procedures

Details of sample collection, certain biological aspects of the samples and storage prior to electrophoretic analysis have been detailed earlier in chapter 2. As mentioned earlier, Buchan herring (NSN1) and Bank herring (NSC) samples were not used in the allozyme analysis due to poor storage condition.

Standard methods of horizontal starch gel electrophoresis (Harris & Hopkinson, 1976; Hillis & Moritz, 1990) were applied to screen allozymic variation between the samples. Two moulds composed of glass plates and 6 mm thick perspex frames (18x15 cm internal dimensions) were cleaned with alcohol and set prior to making gels. Starch gels were made using hydrolysed starch

(Connaught laboratories Ltd., Ontario, Canada) by mixing 54 g of starch in 440 (12%) ml of buffer (Table I. 1, Appendix I) in a round bottom flask. The starch solution was thoroughly mixed, and then heated on an electric heating mantel under continuous stirring. When the solution began to boil, the molten starch was removed from the heat, and degassed under vacuum for 30 seconds to remove air bubbles. The molten starch was then poured into two moulds and another glass plate was pressed on top of each mould to prevent evaporation and also to ensure a uniform thickness and a smooth upper gel surface. The gels were left at room temperature for a period of between 4 hours and two days, were then refrigerated at 4 °C for 30-45 minutes prior to the application of samples.

Muscle, eye and liver tissues were thawed, and approximately 0.3 g of the tissue was homogenised using a teflon homogenizer in 50 µL of 10 mM Tris-HCl, pH 7 (containing 5 mM dithiothereitol and 0.5 % polyvinylpyrrolidone-360, Kornfield *et al.*, 1982) and centrifuged at high speed (12,000 *g*) for 5 min to separate the extracted proteins from cellular debris. Filter paper (Whatman No. 3) inserts (6 mm x 3 mm) were soaked into the sample homogenate and were blotted onto paper tissue to remove excess liquid, and placed into the cut origin of the gel. The origin was cut along the short side of the gel into which 20-25 samples were placed. The location of the origin, relative to electrodes, was decided according to the enzyme and buffer system used, as some enzymes migrated anodally at low pH; the origin was thus cut near to middle of the gel. Ferritin stained inserts were used as standard to facilitate comparisons of mobility between gels and to monitor the progress of the run. In addition, the

front of the cut origin of the gel was traced with bromophenol blue to measure the rate of movement of the buffer front. For each locus the samples were also run on a single gel together in order to check relative mobilities.

The prepared gels were set into Shandon electrophoresis chambers containing appropriate pre-cooled buffer. Buffer saturated 'J cloths' were placed on the anodal and cathodal sides of the gel, which was covered with cling-film to avoid evaporation. An ice-tray was placed on the gel to reduce heating effects during electrophoresis, which was carried out in a refrigerator at 4 °C.

After electrophoresis, each gel was sliced horizontally to give three 2 mm slices. Only the sliced surfaces of the gel were stained to prevent loss of resolution. Stain recipes (Table I. 2, Appendix I) were used, some of them modified from Harris & Hopkinson (1976), Ferguson (1985), NOAA (1989), Hillis & Moritz (1990) and Piertney (1994). After appropriate staining for each locus, the gel moulds were left in the dark at room temperature for incubation until bands appeared. The banding patterns were recorded on a gel documentation system (Vilber-Lormat ltd, France).

Nomenclature for enzyme loci and allele designation followed the recommendations of Shaklee *et al.* (1990). Alleles were scored according to their mobility relative to the most commonly observed allele which was designated as 100. In cases of uncertainty of allelic identities, the respective samples were run in adjacent lanes for direct comparison.

6.2.2 Data analysis

Allele frequencies and measures of genetic variability were estimated by the BIOSYS-1 computer package (Release 1.7; Swofford & Selander, 1989), and deviations from Hardy-Weinberg equilibrium, and allozymic differentiation between populations were tested using Fisher's exact test (GENEPOPversion2, Raymond & Rousset, 1995). The level of genetic differentiation among populations was also measured by calculating the F_{ST}, and its significance was tested by Fisher's exact test (GENEPOPv2, Raymond & Rousset, 1995). Pairwise tests were performed to estimate allele frequencies differences between samples using the latter program.

Genetic distances between samples were estimated using Nei's D (1978), distance, also the F_{ST} value between pairs of samples was calculated using F-STAT (Goudet, 1996). The unweighted pair-group method with arithmetic averages (UPGMA) dendrogram was constructed using D to monitor phenetic relationships among the samples (PHYLIP, J. Felsenstein, 1993). Robustness of the UPGMA was analysed by bootstrapping (1000 random permutations of the original data) (Felsenstein, 1985) which resample the original data set.

In addition, multidimensional scaling analysis (MDS) was used to summarise the genetic distances between pairs of samples. MDS represents samples in multidimensional space and avoids the clustering of similar samples into groups as in tree constructing models. The location of samples on a chart is estimated from pairwise matrices of distances between pairs of samples. Co-ordinates were computed for each sample such that distances between samples

fit as closely as possible to the measured genetic distances between the respective samples. A measure of fit of the data into two dimensions is shown by s-stress factor, which varies between 0 and 1, value of 0 indicate perfect fit; 1 indicate complete lack of fit. RSQ (R^2) denotes the correlation between the estimated distances between samples on the chart and their genetic distances, and 1 indicate a perfect representation of the genetic distance matrix on the chart. The program SPSS version 6 for windows was performed for this statistical analysis.

6.3 Results

6.3.1 Allozyme polymorphism

In the enzyme screening programme, 50 enzymes (Table I. 3, Appendix I) were assayed with 5 buffer systems (Table I. 1, Appendix I). 17 enzymes encoding for 27 putative loci with sufficient activity and resolution, and were routinely screened in the population analysis (Table 6.1). The number of polymorphic loci was 15 using the 99% criterion (i.e. the frequency of the most common allele does not exceed 0.99) and 7 using 95% criterion.

6.3.2 Allele frequencies and genetic diversity

Allele frequency distributions of each local sample for polymorphic loci ($P_{0.99}$) are shown in Table 6.2. A large number of variant alleles was detected at *PGI-1** (9), *DHPI-2** (6), *GOT-2** (6) and *LDH-1** (5) loci.

Genetic diversity parameters (Table 6.3), based on all 28 loci, showed wide variation among populations. The percentage of polymorphic loci ranged from 7.4 to 25.9 using the 95% criterion, and ranged from 22.2 to 37 using the 99% criterion. The mean number of alleles per locus ranged from 1.3 in the pacific herring (PC) and the Downs herring (NSD) to 1.7 at the Norwegian spring spawner sample (NW2), with an average of 1.4. Mean observed heterozygosity ranged from 0.41 at Buchan herring (NSN2) to 0.66 at Trondheimsfjord (NW4) herring samples. The mean heterozygosity was higher in first year Icelandic sample (IC1) (0.60) than second year sample (IC2) (0.58).

6.3.3 Hardy-Weinberg tests

Genotypic frequencies were in Hardy-Weinberg equilibrium at the majority of polymorphic loci ($P > 0.05$) in populations, though 13 significant departures were detected out of 165 tests, representing 8 % of all tests. 5 % would be expected to be significant by chance alone (Table 6.2), and thus populations were characterised by having genotype frequency in accordance with Hardy-Weinberg expectations.

6.3.4 Genetic differentiation (F statistics)

Highly significant overall F_{ST} values were detected when all Atlantic samples were pooled (Table 6.4). Significant genetic differentiation was detected at five loci in Atlantic herring samples, with especially marked divergence at *LDH-1** due to the Trondheimsfjord sample and at *PGI-1** due to Norwegian spring-spawner sample (NW1). When the Pacific sample was included in the data set, highly significant F_{ST} values were detected, a fixed difference at *CK**, and a nearly fixed difference at *G6PDH** and *LDH** among the samples were apparent.

6.3.5 Differences in allele frequencies among samples

Fisher's exact test revealed overall highly significant allele frequency differences among populations, when the Pacific herring sample was included using 15 polymorphic loci, and excluded using 13 polymorphic loci (Tables 6.5; $P<0.001$).

In pairwise comparisons, the Trondheimsfjord (NW4) and Norwegian spring spawner (NW1) samples showed highly significant allele frequency differences from all other samples (Table 6.6). Particularly marked differences were found in the allele frequencies at *LDH-2** in the Trondheimsfjord sample and at *PGI-2** in the Norwegian spring spawner (NW1) sample when compared

with all other samples. Also the Icelandic summer-spawner samples (IC1, IC2) showed significant allele frequencies when compared with the Norwegian spring spawner (north-eastern coast of Norway, NW1) sample and the Trondheimsfjord (NW4) herring sample, and similarly the Baltic herring sample showed significant differences in allele frequencies at one or a few loci when compared with other samples, though there was no significant differences in pairwise comparisons when all loci were pooled.

Interestingly, a unique allele (*LDH-2*175*) occurring at appreciable frequencies (0.24) in the Trondheimsfjord sample was an identical and fixed allele in the Pacific sample, and a dominant allele at *PGI-1** in the Norwegian spring-spawner herring (NW1) was rare in other samples. For the Norwegian spring-spawner (NW1) sample, allele frequencies at the *PGI-1** locus were highly significantly different from all other samples, except the Trondheimsfjord sample. The Pacific herring sample showed highly significant differentiation with a number of highly significantly different loci.

6.3.6 Genetic distance

Estimates of Nei's D (1978) generated from the 15 polymorphic and 12 monomorphic loci examined in all individuals are shown in Table 6.7. Estimates of Nei's distance ranged from 0.0002 to 0.0134 within the Atlantic herring samples, and ranged from 0.1781 to 0.2189 between the Atlantic herring samples and the Pacific sample. The cluster analysis (UPGMA) on Nei's D

values clustered Atlantic samples and Pacific sample together below the 0.21 (Fig. 6.1a). The Norwegian spring-spawner sample was most distinct from the other Atlantic herring samples, with the Trondheimsfjord herring sample showing the next highest level of divergence. The strength of clustering for each pair of sample was also shown by the bootstrapped UPGMA tree (Fig. 6.1b). Pacific herring was bootstrapped 998 times on the same pattern out of 1000 tests. Within the Atlantic herring samples, both the Norwegian spring-spawner (NW1) and the Trondheimsfjord herring samples clustered over 500 out of 1000 tests, indicating strong differentiation from the other Atlantic herring samples, though differentiation of the Norwegian spring-spawners was stronger than the Trondheimsfjord herring.

In the MDS analysis, the Atlantic herring samples were grouped together (Fig. 6.2a) due to high genetic divergence of the Pacific herring. The Trondheimsfjord herring was closest to the Pacific herring among the Atlantic herring samples. The Norwegian spring-spawner (NW1) was also positioned far from Atlantic herring samples. Only the Atlantic herring samples were again used in MDS (Fig. 6.2b) to see the pattern of differentiation among them. Not surprisingly, the Norwegian spring-spawning sample (NW1) were clearly distinct positioned on the chart. Also the Trondheimsfjord and Baltic herring samples were plotted outlying from the other Atlantic samples.

6.4 Discussion

One important result of this present study is the discovery of an highly allozymically distinct oceanic Atlantic herring sample (NW1) in Northeast Norwegian waters (Barents Sea), in contrast to detected allozymic homogeneity among the other samples collected on a large geographic scale, including the Celtic sea, North sea, and Baltic sea. Studies based on the width of the scale annuli (Debarros & Holst, 1995) and otolith microstructure (Stenevik *et al.*, 1996) have also revealed substantial morphological differences between these two Norwegian spring-spawners, supporting a morphological concordance with the observed genetic differences. On the other hand, possible selection pressures arising from such factors as temperature or salinity may be operating on or contributing to allozymic differentiation of this population. Genetic divergence caused by limited gene flow tends to affect all loci simultaneously, whereas selectively induced divergence is typically observed at one or only a few loci (Clarke, 1975; Smith *et al.*, 1990). In the present study, the northern Norwegian spring-spawner sample (NW1), the dominant common allele *PGI-1**40 and a allele (*PGI-1** 0) occurring at appreciable frequencies (0.18) was rare or absent in other samples, resulting in highly significant divergence of the Norwegian spring-spawner sample from all others. However the detected genetic heterogeneity of this aggregation as revealed by mtDNA (Chapter 7), microsatellites (Chapter 8) and phenotypic (Debarros & Holst, 1995; Stenevik *et al.*, 1996) markers makes the possibility of selection weak. These northern herring may show a genetic similarity to the White Sea herring, and indeed may

even represent a migratory group of herring from White Sea. Repetitive sampling from the same location, and an extra sample from White Sea and other locations of Barents Sea would clarify the temporal and spatial basis of the detected genetic patterns.

In addition, an allozymically unique Trondheimsfjord herring (NW4) population was detected, supporting previous reports of genetic dissimilarity, though based only on one locus (*LDH-2**) (Jørstad & Nævdal, 1981). Furthermore, in research carried out at the Trondheim Biological Station (Norway) it was reported (Jarle Mork, *Personal Communication*) that there was one panmictic population within the fjord, which showed significant allele frequency differences from the Norwegian spring-spawning herring, supporting the self-contained status of the Trondheimsfjord herring.

Another important result is the similarity between the unique Trondheimsfjord sample (NW4) and the Pacific herring; the *LHD-2** 175 unique allele found in Trondheimsfjord sample was identical to the common allele at *LDH-2** in Pacific herring, and the lowest genetic distance between two samples was observed between the Trondheimsfjord and Pacific herring. Indeed, such allozymic similarity between Balsfjord herring and Pacific herring has been reported (Jørstad *et al.*, 1994). Such an observation is particularly interesting when considering the evolution of herring species in the Atlantic and Pacific Oceans. An allopatric speciation model for the evolution of the two species was given by Grant (1986) based on the geographic evolution of the Arctic-N Atlantic Basin. It is suggested that the opening of the Bering Strait allowed dispersion of ancestral herring into the Pacific Ocean for the first time during the

mid-Pleistocene period, 3.0-6.6 million yr. ago. The distribution of Atlantic and Pacific herring extended into the Arctic Ocean, except across Arctic Canada. Mayr (1982) suggested a "dumb-bell" form of allopatric speciation for the two species. In this hypothesis, a formerly continuous distribution of herring was divided in half by some barrier to migration that caused gradual cooling of Arctic Ocean leading to a subdivision of continuous distribution of ancestral herring across the warmer Pliocene Polar Ocean. There are, however, isolated populations of the Pacific herring located in the White and Kara Seas that are sympatric with migratory populations of Atlantic herring from Norway (Svetovidov, 1963). Close genetic affinities between the White Sea and Pacific herring has been reported with biochemical and immunological characters (Truveller, 1979). In the present study, the Trondheimsfjord herring sample showed greatest allozymic similarity to Pacific herring (Table 6.7 & 6.4, Fig. 6.2a), in a similar way to the previously reported (Jørstad *et al.*, 1994) allozymic similarity between the Balsfjord herring and Pacific herring. Therefore these fjord herring may be relict populations of ancestral herring, having retained to some degree their ancient genetic structure due to closed geographic structure of fjords and very limited gene flow.

Jørstad *et al.* (1983 & 1994) revealed a genetically distinct Balsfjord stock in Norwegian waters. In the present study, in contrast, it was found that herring collected from the Balsfjord were not genetically differentiated from the other Atlantic samples (except the Trondheimsfjord and the Norwegian-spring spawner (NW1) samples). Such apparently conflicting genetic data most likely arise from localised differences in the distribution of fjord and coastal stocks.

Jørstad & Pedersen (1986) suggested that during the non-reproductive phase, Norwegian spring spawning herring occur in the Balsfjord area in the upper water layers and leave the fjord before spawning. It is therefore likely, based on the timing (September), and sampling depth (15-20) of the collections that it was the spring-spawning population that was sampled.

The detected genetically distinct Trondheimsfjord sample was captured with different sampling gear, thus also representing a catch from deeper waters (30-35m), thereby raising the possibility of a similar difference in the distribution of distinct stocks with water depth. The genetic heterogeneity detected along the Norwegian coast may therefore arise from the existence of distinct deep water resident fjord populations with some degree of spatial segregation from coastal populations, though further comparisons in other fjords and at different depths are required. It is thus important when collecting from these waters to take account of localised migratory behaviour and depth distribution. Indeed, the fixed allelic differences at *LDH-2** between the resident fjord populations (Balsfjord and Trondheimsfjord) and Norwegian spring spawning population (Jørstad & Pedersen, 1986; Jørstad *et al.*, 1994) could serve as valuable markers to monitor seasonal variation in stock distribution.

Baltic herring samples did not show significant differences in pairwise comparisons (overall loci) from the other samples, though a number of loci exhibiting significant allele frequency differences at *GOT-2** (from Norwegian spring spawner; NW2), *MDH-3** (from Buchan herring; NSN2), and *LDH-2** (from Downs herring; NSD) (Table 6.8), indicating genetic differentiation of the Baltic herring.

Similarly, *PGI-1** and *GOT-2** showed significant allele frequencies differences between the Icelandic spring-spawner (IC1 & IC2) and Norwegian spring-spawner (NW2) samples, though there was no significant differences in pairwise comparisons overall loci. The detected consistent differences at *PGI-1** and *GOT-2** may suggest restricted gene flow between these spawners though the application of a more sensitive genetic marker would provide a more powerful test of population structure.

The F_{ST} tests revealed evidence for significant genetic differentiation among all Atlantic herring samples with an overall F_{ST} estimate of 0.04915 (Table 6.4). This suggests that there is restricted gene flow among spawning aggregations. Moreover the observed high genetic differentiation was due to several loci, *LDH-2**, *ME-2**, *GOT-2**, *PGI-1**, *SOD**, which may indicate the operation of factors other than selection such as restricted gene flow. In contrast, differentiation at a only few loci with very similar function may be due to selection rather than restricted gene flow (Lewontin & Krakauer, 1973; Slatkin, 1987).

Fisher's exact test (Table 6.5) revealed overall highly significant allele frequency differences among Atlantic herring populations with a number of significantly different loci, supporting the findings from the F_{ST} estimates. Pairwise comparisons between populations revealed that the source of divergence was due mainly to the genetically distinct Trondheimsfjord (NW4) and Norwegian spring-spawner samples (NW1) collected from the north-eastern coast of Norway (Barents Sea) (Table 6.6). Therefore these samples (NW1 & NW4) were excluded from the data set to examine the pattern of differences

among the other Atlantic herring samples. Again, overall highly significant differences were observed among populations (Tables 6.5; $P<0.001$), and allele frequencies differed significantly among the samples at 4 of 15 polymorphic loci at $P<0.05$ level, and for 1 of 15 polymorphic loci at $P<0.01$ (Table 6.5), thus indicating high allozymic differentiation among Atlantic herring populations.

Although only 13 significant departures were detected in 165 tests, the Hardy-Weinberg equilibrium test is very sensitive to homozygous genotypes, and only one or two homozygous genotypes may cause statistically significant departures. In the present analysis, departures were mainly due to the occurrence of only one or two homozygous individuals in a population at a locus, although these individuals were run twice on the gel to confirm their distinct mobility.

Heterozygosity is the most informative estimator of genetic variation in an outbreeding species, and for the teleosts ranges from 0.0005 to 0.180 (Winans, 1980). In this study, heterozygosity ranges from 0.041 to 0.66 with a mean of 0.054, which is more than the mean of 0.048 reported for 82 marine and freshwater fishes (Winans, 1980). In the present study a large number of alleles at low frequency were observed, which is an important feature of polymorphic loci in herring. The rate of random loss of low-frequency alleles, whether selectively neutral or not, is reduced by a large effective population size, and the existence of a large number may explain the occurrence of rare alleles in herring as stock numbers are generally very large (Anderson *et al.*, 1981).

In a review of fishes, Shaklee *et al.* (1982) reported that Nei's genetic distance (D) between nominal species of fishes ranged from only 0.025 to 0.60,

and averaged 0.30. Grant (1986) found that genetic distance based on 40 loci between Atlantic and Pacific herring was 0.27, and it was suggested that they are at the species level of evolutionary divergence. In this study, genetic divergence (~0.22) based on 27 loci between the two species was lower than the previous finding. However the observed lower genetic divergence is probably due mainly to a smaller number of loci analysed, that were fixed or nearly fixed (common-allele frequencies greater than 0.99) for the same allele in the two species in comparison to the previous study.

Management decisions for the commercially important and heavily exploited herring populations in the north-east Atlantic are currently based on demographic data on "stocks" identified by means of morphological and ecological characters (Cushing, 1975; Jakobsson, 1985). From a management viewpoint, the most important finding in the present study, is the discovery of the genetically differentiated Norwegian spring-spawning and Trondheimsfjord herring populations. The management implications of populations depends on whether marked variation persists over time. The Trondheimsfjord herring revealed its temporal and spatial integrity by comparison with a previous report by Jørstad & Nævdal (1981), and thus deserves recognition as a distinct unit. Consistent differentiation of the Norwegian spring-spawning herring (NW1) over at least one year may indicate its temporal and spatial integrity and thus would also require its consideration as an separate stock for management purposes.

Genetic differentiation among the Atlantic samples was indeed notably high (Table 6.4, 6.5, 6.6). The observed differentiation may be related to both

geographic separation of populations and environmental differences between populations. Indeed, the detected significant allozymic differences arose mainly from the geographically isolated Norwegian spring-spawner, Trondheimsfjord herring populations, and also the Baltic and Icelandic samples exhibited several loci with significant allele frequency differences. Therefore the findings suggest an absence of inter-population breeding for the Norwegian spring-spawner and Trondheimsfjord herring populations. In addition, limited gene flow (few migrants) may be leading to the detected non-significant differences for the Baltic and Icelandic samples, or differential environmental factors may be causing the observed differentiation at one or two loci in these samples. Ward & Grewe (1994) reported that, when using allozyme electrophoresis in stock discrimination, both low (but sufficient) and high levels of gene flow can lead to the conclusion that a single panmictic population is present. Therefore the detected nonsignificant level of genetic differentiation among samples does not necessarily mean that they represent a single genetic unit. Application of more sensitive genetic markers such as microsatellites may reveal the existence of greater significant genetic variation among such spawning aggregations.

Table 6.1. Enzymes used routinely, with their E.C. nomenclature, the buffer system used, number of loci (loci) number of polymorphic loci at the 0.99 level (poly), and the number of variant alleles (Allele).

Enzyme	Abrev	EC no	Buffer	Loci	Poly	Allele
Adenosine kinase	AK	2.7.4.3	CM	3		
Creatine kinase	CK	2.7.3.2	CM	1	1	2
Esterase	EST	3.1.1.1	CM	1		
Fumarate hydratase	FH	4.2.1.2	CM	2		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2.1.12	CM	1		
Glutamic-oxaloacetic transaminase	GOT	2.6.1.1	CM	2	1	6
Glycerol-3-phosphate dehydrogenase	G3PDH	1.1.1.8	TM	1	1	3
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	TM	1	1	2
Isocitrate dehydrogenase	IDHP	1.1.1.42	CM	2	1	6
Lactate dehydrogenase	LDH	1.1.1.27	CM	2	2	8
Malate dehydrogenase	MDH	1.1.1.37	CM	3	1	2
Malic enzyme	ME	1.1.1.40	CM	2	1	5
Phosphogluconate dehydrogenase	PGDH	1.1.1.44	CM	1	1	2
Phosphoglucose isomerase	PGI	5.3.1.9	CM	2	2	12
Phosphoglucose mutase	PGM	5.4.2.2	CM	1	1	3
Sorbitol dehydrogenase	SDH	1.1.1.14	TC	1	1	2
Superoxide dismutase	SOD	1.15.1.1	TC	1	1	2

Table 6.2. Allele frequencies at polymorphic loci in herring samples. Sample sizes were identical for monomorphic loci. * Locus with corresponding sample is not in Hardy Weinberg equilibrium and its statistical significance; *P<0.05, **P<0.01, ***P<0.001. n, sample size. Samples are identical defined in Table 6.

		Sample										
Locus	Allele	IC1	IC2	NW1	NW2	NW3	NW4	BA	NSN2	NSD	CS	PC
n		49	48	50	45*	50	50*	50*	25	26	50	30
LDH-1*	120	.000	.000	.000	.022	.000	.020	.000	.000	.000	.000	.000
	100	.949	.990	.970	.956	.970	.930	.960	.980	1.000	1.000	.000
	60	.000	.000	.030	.000	.030	.000	.020	.020	.000	.000	.000
	40	.041	.010	.000	.022	.000	.030	.020	.000	.000	.000	.000
	0	.010	.000	.000	.000	.000	.020	.000	.000	.000	.000	1.000
n		49	48	50	45	50	50	50	25	26	50	30
LDH-2*	175	.000	.000	.000	.000	.000	.240	.000	.000	.000	.000	1.000
	100	.939	.948	.950	.922	.950	.750	.980	1.000	.904	.950	.000
	50	.061	.052	.050	.078	.050	.010	.020	.000	.096	.050	.000
n		49	48	50	45	50	50	50	25	26	50*	30
MDH-3*	100	.480	.427	.460	.511	.460	.390	.490	.380	.442	.490	.067
	65	.520	.573	.540	.489	.540	.610	.510	.620	.558	.510	.933

Table 6.2. Continued.

Locus	Allele	IC1	IC2	NW1	NW2	NW3	NW4	BA	NSN2	NSD	CS	PC
n		47	48	50	46	50	50***	50**	25	26	50	30
ME-2*	180	.000	.000	.000	.000	.000	.000	.020	.000	.000	.000	.000
	130	.000	.000	.000	.000	.000	.000	.020	.000	.000	.000	.000
	100	.989	1.000	1.000	1.000	1.000	.930	.960	1.000	1.000	.990	.933
	53	.000	.000	.000	.000	.000	.070	.000	.000	.000	.010	.033
	20	.011	.000	.000	.000	.000	.000	.000	.000	.000	.000	.033
n		47***	48	50	46*	50	50	50	25	26	50	30
G6PDH*	100	.957	1.000	1.000	.978	1.000	1.000	1.000	1.000	1.000	1.000	.000
	87	.043	.000	.000	.022	.000	.000	.000	.000	.000	.000	1.000
n		47	48	50	46	50	50	50*	25	26	50	30
G3PDH*	300	.000	.000	.000	.000	.000	.000	.000	.020	.000	.000	.000
	200	.000	.000	.000	.000	.020	.000	.020	.000	.019	.000	.000
	100	1.000	1.000	1.000	1.000	.980	1.000	.980	.980	.981	1.000	1.000
n		49	48	50	46	50	50	50	25	26	50	30
CK*	115	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	1.000
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.000

Table 6.12. Continued

Locus	Allele	IC1	IC2	NW1	NW2	NW3	NW4	BA	NSN2	NSD	CS	PC
n		49	48	50	45	50	50	50	25	26	50	30
GOT-2*	130	.000	.000	.000	.011	.000	.000	.000	.000	.000	.000	.000
	122	.000	.000	.000	.011	.000	.000	.000	.000	.000	.000	.000
	100	1.000	1.000	1.000	.956	.990	.990	1.000	.960	1.000	1.000	1.000
	78	.000	.000	.000	.011	.010	.010	.000	.020	.000	.000	.000
	65	.000	.000	.000	.000	.000	.000	.000	.020	.000	.000	.000
	55	.000	.000	.000	.011	.000	.000	.000	.000	.000	.000	.000
n		47	48	50	46	50	50	50	25	26	50	30
IDHP-2*	200	.000	.000	.000	.011	.000	.000	.000	.020	.000	.000	.000
	150	.011	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
	133	.021	.010	.000	.011	.010	.020	.020	.000	.000	.020	.000
	116	.000	.010	.010	.011	.000	.050	.020	.020	.000	.000	.700
	100	.947	.979	.990	.967	.990	.930	.960	.960	1.000	.980	.300
	83	.021	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
n		47	48	50	46	50	50	50	25	26	50	30
PGI-1*	250	.011	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
	200	.138	.083	.000	.087	.050	.250	.070	.160	.058	.080	.000
	150	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.283
	100	.606	.667	.100	.641	.670	.560	.650	.640	.673	.660	.717
	70	.000	.000	.040	.000	.020	.000	.010	.000	.000	.000	.000
	40	.085	.083	.600	.196	.110	.110	.100	.140	.058	.140	.000
	10	.138	.125	.080	.076	.130	.060	.150	.060	.192	.100	.000
	0	.021	.042	.180	.000	.020	.020	.010	.000	.000	.010	.000
	-45	.000	.000	.000	.000	.000	.000	.010	.000	.019	.010	.000

Table 6.12. Continued

Locus	Allele	IC1	IC2	NW1	NW2	NW3	NW4	BA	NSN2	NSD	CS	PC
n		47	48	50	46	50	50	50	25	26	50	30
PGI-2*	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.983
	50	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.017
n		47	48	50	46	50	50	50	25	26	50	30
PGM*	150	.011	.021	.010	.011	.000	.000	.000	.000	.000	.000	.000
	100	.957	.979	.970	.957	.960	.920	.960	1.000	.981	.970	.767
	50	.032	.000	.020	.033	.040	.080	.040	.000	.019	.030	.233
n		50	48	50	46*	50	50*	50	25	26	50	30
SDH*	157	.000	.000	.000	.022	.000	.020	.000	.000	.000	.000	.000
	100	1.000	1.000	1.000	.978	1.000	.980	1.000	1.000	1.000	1.000	1.000
n		50	48	50	46*	50	50	50	25	26	50	30
6PGDH*	110	.000	.000	.000	.022	.000	.000	.000	.000	.000	.000	.017
	100	1.000	1.000	1.000	.978	1.000	1.000	1.000	1.000	1.000	1.000	.983
n		50	48	50	46	50	50	50***	25	26	50	30
SOD*	160	.000	.000	.000	.000	.000	.000	.040	.000	.038	.000	.000
	100	1.000	1.000	1.000	1.000	1.000	1.000	.960	1.000	.962	1.000	1.000

Table 6.3. Measures of genetic diversity observed in herring samples, with means and standard deviations (in brackets) for each sample. * *: polymorphic loci 0.95 criterion, * polymorphic loci 0.99 criterion

Sample	Mean sample size per locus	Mean no. of alleles per locus	% of loci polymorphic *	% of loci polymorphic **	Mean heterozygosity	
					Direct-count	H-W expected
IC1	48.9	1.6	29.6	18.5	.060	.059
IC2	48.0	1.4	22.2	11.1	.048	.045
NW1	50.0	1.4	22.2	11.1	.050	.049
NW2	45.7	1.7	37	11.1	.065	.061
NW3	50.0	1.4	29.6	11.1	.053	.050
NW4	50.0	1.6	33.3	25.9	.066	.077
BA	50.0	1.6	33.3	7.4	.049	.056
NSN2	25.0	1.4	22.2	7.4	.041	.047
NSD	26.0	1.3	22.2	11.1	.046	.050
CS	50.0	1.4	22.2	11.1	.056	.046
PC	30.0	1.3	25.9	18.5	.048	.057
Mean		1.464 (0.136)	28.12 (6.19)	12.58 (5.57)	0.054 (0.008)	0.054 (0.009)

Table 6.4. F_{ST} values among herring samples. Significance of F_{ST} is indicated: *: P<0.05, **: P<0.01, ***: P<0.001.

Locus	F_{ST} (excluding Pacific sample)	F_{ST} (including Pacific sample)
*LDH-1**	+0.00365	+0.68709***
*LDH-2**	+0.07640***	+0.51544***
*MDH-3**	-0.00305	+0.03697***
*ME-2**	+0.02496**	+0.02384**
*G6PDH**	+0.00930	+0.91190***
*G3PDH**	+0.00072	+0.00050
*CK**	-	+1.00000***
*GOT-2**	+0.00878*	+0.00865*
*IDHP-2**	+0.00372	+0.42129***
*PGI-1**	+0.10511***	+0.11335***
*PGI-2**	-	+0.00566
*PGM**	+0.00113	+0.05182***
*SDH**	-0.00435	-0.00493
*6PGDH**	-0.00084	-0.00071
*SOD**	+0.01828*	+0.01782*
Total	+0.04915***	+0.31174***

Table 6.5. Tests of population heterogeneity using Fisher's exact test from all polymorphic loci. *P* represents significance level (*: P<0.05, **: P<0.01, ***: P<0.001); P1 denotes overall samples; P2, excluding Pacific sample (PC); P3, excluding Pacific, Trondheimsfjord (NW4) and Norwegian spring-spawning herring (NW1).

	P-value		
Locus	*P* 1	*P* 2	*P* 3
*LDH-1**	0.00000***	0.00870**	0.03970*
*LDH-2**	0.00000***	0.00000***	0.23154
*MDH-3**	0.00000***	0.76252	0.77050
*ME-2**	0.00000***	0.00000***	0.21512
*G6PDH**	0.00000***	0.00590**	0.01534*
*G3PDH**	0.04704*	0.05300	0.10164
*CK**	0.00000***	-	-
*GOT-2**	0.23442	0.21262	0.12184
*IDHP-2**	0.00000***	0.22324	0.70196
*PGI-1**	0.00000***	0.00000***	0.34586
*PGI-2**	0.16346	-	-
*PGM**	0.00000***	0.23348	0.52548
*SDH**	0.11130	0.08264	0.03994*
*6PGDH**	0.05668	0.02090*	0.04790*
*SOD**	0.00012***	0.00044***	0.00144**
X^2	Infinity	Infinity	59.83
Df	30	26	26
P	Highly significant	Highly significant	0.0002***

Table 6.6. Pairwise comparisons of allozyme frequencies among all herring samples. The overall significance levels are shown: ***, P<0.001; **, P<0.01; P<0.05. Significantly different loci are presented with a corresponding arabic number in the brackets; 1 (LDH-1), 2 (LDH-2), *3 (MDH-2), 3 (MDH-3), 4 (ME-2), 5 (G5PDH), 6 (CK), 7 (GOT-2), 8 (IDPH-2), 9 (PGI-1), 10 (PGM).* Samples referred to in the text were Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

Sample	IC1	IC2	NW1	NW2	NW3	NW4	BA	NSN2	NSD	CS	PC
IC1		—	1*, 8*, 9***	7*, 9*	1*	2***, 4*, 8*	—	—	—	—	1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
IC2	ns		9***	7*, 9*	—	2***, 4*, 9*	—	—	—	—	1***, 2***, 3***, 4*, 5***, 6***, 8***, 9***, 10***
NW1	***	***		1*, 7*, 9*	9***	1*, 2***	9***	9***	9***	9***	1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
NW2	ns	ns	***		1*	2***, 4*, 9*	7*	—	—	1*, 7*	1***, 2***, 3***, 4*, 5***, 6***, 8***, 9***, 10***
NW3	ns	ns	***	ns		1*, 2***, 8* 4* 9***	—	—	—	—	1***, 2***, 3***, 4*, 5***, 6***, 8***, 9***, 10***
NW4	***	***	***	***	***		2***, 4**, 9**	2***, 11*	2***, 9*	1*, 2***, 9*	1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
BA	ns	ns	***	ns	ns	***		3*	2*		1***, 2***, 3***, 4***, 5* 6***, 8***, 9***, 10***
NSN2	ns	ns	***	ns	ns	***	ns		—	—	1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
NSD	ns	ns	***	ns	ns	***	ns	ns		—	1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
CS	ns	ns	***	ns	ns	***	ns	ns	ns		1***, 2***, 3***, 5***, 6***, 8***, 9***, 10***
PC	***	***	***	***	***	***	***	***	***	***	

Table 6.7. Measures of genetic differentiation between pairs of samples: Nei's genetic distance (below diagonal) and FST values estimated by FSTAT (above diagonal). The significance of F_{ST} are shown; ***P<0.001. Samples are identical defined in Table 6.

Sample	NW4	IC1	NW2	NW3	BA	CS	IC2	NW1	NSN2	NSD	PC
NW4	-	0.0298***	0.0373***	0.0432***	0.0417***	0.0434***	0.0401***	0.1577***	0.0284***	0.0368***	0.6848***
IC1	0.0030	-	0.0006	-0.0017	-0.0035	-0.0013	-0.0027	0.1589***	0.0016	-0.0036	0.7414***
NW2	0.0036	0.0006	-	0.0009	0.0011	-0.0030	0.0063	0.1376***	0.0080	0.0058	0.7411***
NW3	0.0037	0.0005	0.0005	-	-0.0059	-0.0054	-0.0058	0.1765***	0.0021	-0.0075	0.7672***
BA	0.0055	0.0013	0.0013	0.0013	-	-0.0043	-0.0023	0.1676***	0.0028	-0.0066	0.7531***
CS	0.0035	0.0004	0.0003	0.0002	0.0010	-	-0.0029	0.1740***	0.0044	-0.0015	0.7762***
IC2	0.0033	0.0004	0.0008	0.0002	0.0017	0.0003	-	0.1890***	-0.0028	-0.0079	0.7752***
NW1	0.0134	0.0114	0.0101	0.0117	0.0129	0.0111	0.0122	-	0.1723***	0.1940***	0.7811***
NSN2	0.0032	0.0010	0.0012	0.0009	0.0027	0.0009	0.0006	0.0115	-	0.0060	0.7658***
NSD	0.0039	0.0007	0.0011	0.0004	0.0017	0.0006	0.0003	0.0134	0.0014	-	0.7621***
PC	0.1781	0.1962	0.2000	0.2010	0.2082	0.2031	0.2001	0.2189	0.2004	0.2009	-

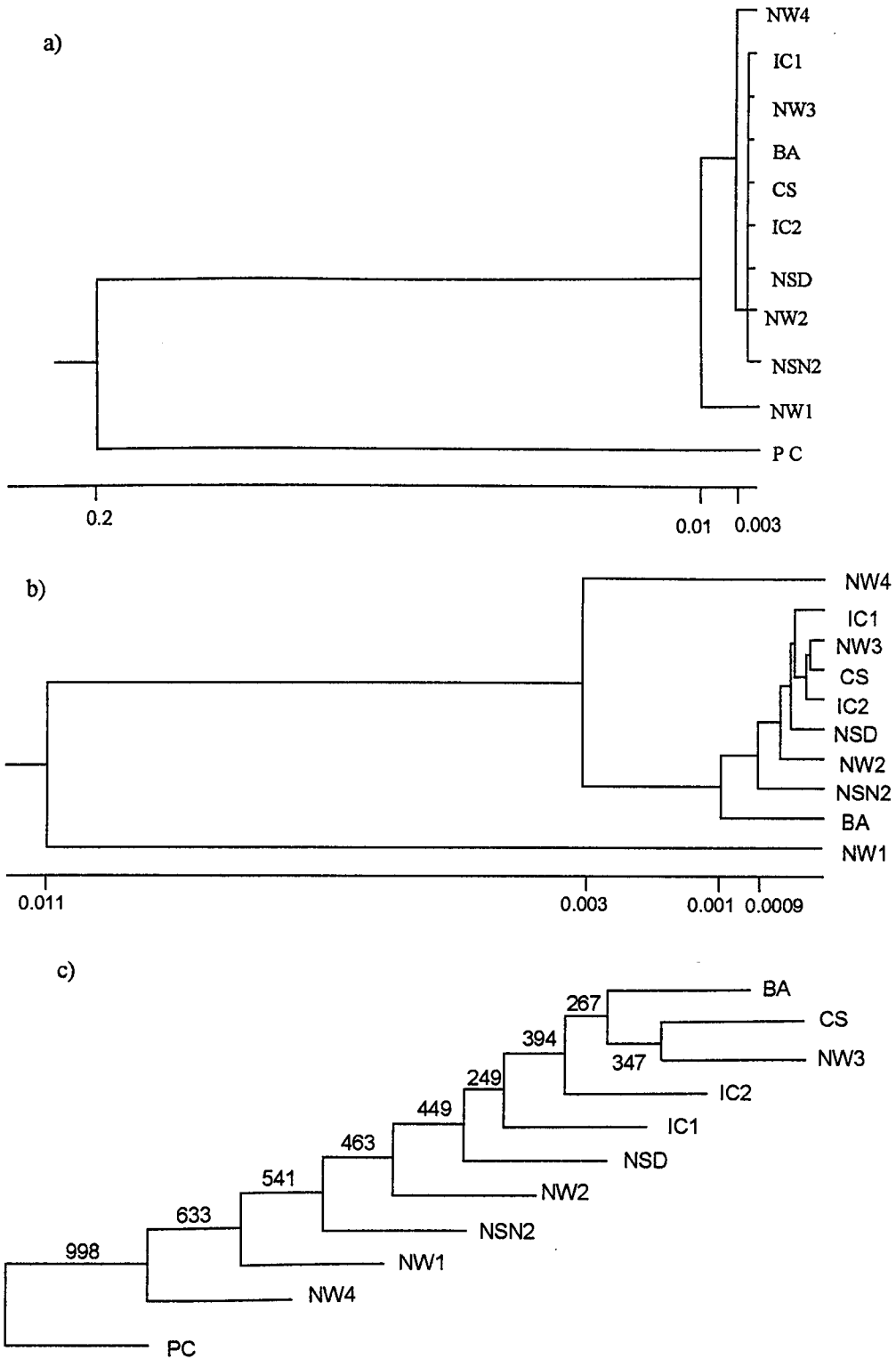


Figure 6.1. a) UPGMA tree of Nei's (1978) distances among samples based on allozyme frequencies overall loci. b) Bootstrapped UPGMA dendrogram, based on Nei's (1978) distances, showing the relationship between the samples. Number on the branches of forks indicates the number of times (out of 1000 iterations) that the group outside of the fork has been occurred.

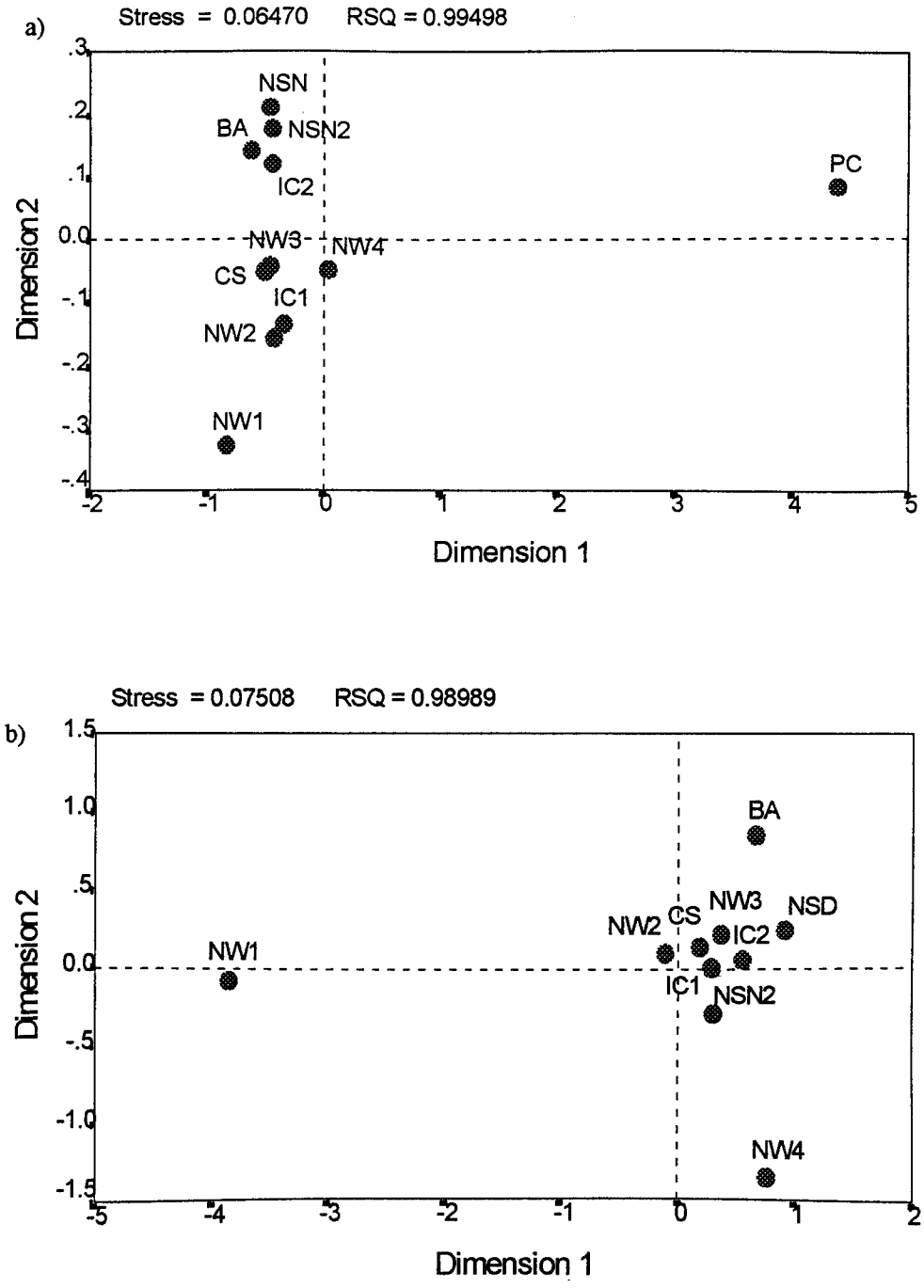


Figure 6.2. Multidimensional scaling analysis of allozyme allele frequency data for overall loci, including (a) and excluding (b) Pacific herring.

CHAPTER 7

MITOCHONDRIAL DNA DIFFERENTIATION: FRAGMENT ANALYSIS OF PCR-AMPLIFIED MTDNA

7.1 Introduction

For over the past decade, mitochondrial DNA (mtDNA) analysis has been increasingly adopted by fisheries geneticists as a marker for population genetic studies due to its favoured features. Its maternal inheritance and haploidy reduce the effective population size for mtDNA to one quarter of that for nuclear DNA (Nei & Tajima, 1981, Birky *et al.*, 1989), thus producing a greater differentiation between recently isolated gene pools (populations). Moreover, mtDNA accumulates mutations up to 5-10 times more rapidly than single copy nuclear DNA (Perler *et al.* 1980; Brown *et al.*, 1982; Ferris & Berg, 1987) due to a higher frequency of point and length mutations (Brown *et al.*, 1982; Cann *et al.*, 1984; Wilson *et al.*, 1985; Kornfield, 1991; Meyer, 1993). Furthermore allozymes are phenotypic expression of the underlying genetic code that many mutations at the first and third codon positions will not be detected when they do not alter the amino acid sequences of the resulting protein product (Lewontin, 1974; Moritz *et al.*, 1987). MtDNA analysis in contrast, represents a direct analyses of the genetic code at all positions.

Employment of mtDNA analyses has often revealed finer population structuring than allozyme analysis. For example a major genetic discontinuity was detected with mtDNA (Saunders *et al.*, 1986) analysis in populations of the horseshoe crab off Florida, but the divergence was not detected using allozymes (Selandar *et al.*, 1970). Similar observations were reported in many other studies (Ward *et al.*, 1989; Reeb & Avise, 1990; Hansen & Loeschcke, 1996; Smolenski *et al.*, 1993). Nonetheless, the reverse has also been demonstrated (Ward & Grewe, 1994; Ward *et al.*, 1994a; Turan *et al.*, 1997). Although there are some disadvantages associated with allozyme studies, the ability to monitor several independent loci is an advantage of allozyme and nDNA techniques since mtDNA, acts effectively as a single locus.

There are several published studies which investigated mtDNA variation in marine clupeid fish which include American shad, *Alosa sapidissima*, (Bentzen *et al.*, 1988; Nolan *et al.*, 1991; Chapman *et al.*, 1994), menhaden, *Brevoortia tyrannus*, and, *Brevoortia patronus*, (Bowen & Avise, 1990), sardines, *Sardinella aurita*, (Tringali & Wilson, 1993), anchovies, *Engraulis encrasicolus*, (Bembo *et al.* 1995), and other marine species including cod, *Gadus morhua* (Dahle, 1991; Pepin & Carr, 1993; Carr, 1995), whitefish, *Coregonus lavaretus* (Hartley, 1995), shortfin mako, *Isurus oxyrinchus* (Heist *et al.*, 1995), demonstrating varying degrees of significant conspecific differentiation within each species.

The application of DNA technology is of high priority for studies on stock discrimination in herring where allozyme analysis has usually failed to reveal genetic heterogeneity over large geographic distances. However, only

limited mtDNA (Kornfield & Bogdanowicz, 1987; Dahle & Erikson, 1990; Stephenson & Kornfield, 1990; Jørstad *et al.*, 1994) data are available for stock discrimination in herring. Kornfield & Bogdanowicz (1987) did not find any stock-specific mtDNA variation using restriction fragment length polymorphism (RFLP) analysis between samples collected from the Gulf of Maine and St. Lawrence. Similarly, Dahle & Erikson (1990) investigated the genetic relationships between autumn and spring spawners of herring in the North Sea and Baltic Sea using RFLP analysis, revealing no evidence of genetic differentiation among samples. In contrast, Jørstad *et al.* (1994) revealed significant genetic differentiation with mtDNA RFLP analysis among Balsfjord, Norwegian spring-spawners and Pacific herring.

Until recently, mtDNA studies required large tissue samples and time-consuming laboratory protocols to extract and isolate mtDNA. Amplification of selected regions by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) has made examination of the mtDNA variation considerably easier and faster. Researchers now apply the technique to investigate mtDNA regions of particular interest. Since different regions of the mtDNA evolve at different rates, certain regions of the mtDNA have been targeted for species identification, or for the detection of intraspecific variation and stock markers (Chow *et al.*, 1993; Cronin *et al.*, 1993; Chapman *et al.*, 1994; Bembo *et al.*, 1995). The D-loop in particular has been targeted for population studies, and although its rate of evolution is two to five times higher than mitochondrial protein-coding genes (Aquadro & Greeenberg, 1983; Meyer, 1993), low level of variability have been observed in may fish (Nielsen *et al.*, 1994; Park *et al.*, 1993). For example,

Bernatchez *et al.* (1992) found no variation in brown trout from the Atlantic basin by sequencing of D-loop, but extensive variation among Atlantic brown trout using the ND1, 5 and 6 and 16SRNA genes (Hall & Nawrocki, 1995; Hynes *et al.*, 1996) with an RFLP approach.

The mitochondrial genome of animals contains 13 protein coding genes, including the seven genes of the NADH dehydrogenase complex (ND genes) which code for enzymes subunits that play a vital role in cell respiration (Meyer, 1993). Therefore these genes can be subjected to selection for certain haplotypes and potentially leads to differentiation of populations under different environmental conditions. The ND genes have been targeted in a number of recent studies involving salmonids (Cronin *et al.*, 1993; Hall, 1992; Park *et al.*, 1993; O'Connell *et al.*, 1995) and clupeids (Bembo *et al.*, 1995; Hauser *et al.*, 1995), usually revealing sufficient variation to provide useful genetic markers. Indeed, clupeids have so far shown extraordinarily high levels of genetic variability in the ND 5/6 genes.

In the present study, mtDNA RFLP analysis was used:

1) to investigate the genetic population structure of Northeast Atlantic herring populations as revealed by ND genes (ND3/4 and ND5/6) of mtDNA;

2) to compare results obtained from mtDNA regions (ND3/4 and ND5/6) using the same samples;

3) to explore the degree of genetic divergence at ND3/4 and ND5/6 genes between Atlantic and Pacific herring.

7.2 Materials and methods

Sampling, biological characters of samples and storage prior to extraction were described in chapter 2. In the present study, 11 of the samples were used for the analysis of ND5/6 genes, and 6 of the 11 samples were used in analysing ND3/4 genes (Chapter 2, Table 2.2).

For DNA extraction, the protocol by Taggart *et al.* (1992) was followed with a modification. A piece of muscle tissue approximately 5x3x3 mm was added to 300 µl extraction buffer (0.1 M Tris, 0.01M EDTA, 0.1 M $NaCl_2$, 2 % SDS, pH 8.0, made up weekly) in an eppendorf tube. Thereafter 25 µl proteinase K stock solution (Boehringer, 10 mg/ml) was added, and the suspension was mixed by inverting it several times and leaving overnight at 37 °C or for 2 hours at 55 °C to disrupt cell walls and digest proteins. 330 µl phenol (pH 8.0 equilibrated) was added, then shaken vigorously for 20 seconds, and placed on a rotary mixer for 10 minutes. The samples were spun in a microcentrifuge for 3 minutes. The upper aqueous layer was transferred to a new tube using a wide bore pipette tip to avoid shearing of the DNA and the lower organic phase (phenol) discarded. An equal volume of phenol/chloroform (1:1) was added and the samples mixed and rotated as described previously. 330 µl chloroform-isoamylalcohol (24:1) was added, and the samples were rotated and centrifuged again, and the aqueous layer transferred to a new tube. 1 ml ice-cold absolute ethanol was added to precipitate the DNA, and tubes were

inverted several times and spun for 5 minutes in a microcentrifuge to pellet the precipitated DNA. The ethanol was pipetted off, 1ml 70% ethanol was added, and the samples were placed on a rotary mixer for 1 hour to wash the pellet DNA and dissolve all remaining salts which may inhibit the enzyme activity during PCR. The ethanol was removed again, and the samples were air-dried for 20 minutes in a desiccator. The DNA was resuspended in 100 µl TE buffer (1.3 M Tris, 1 mM EDTA, pH 8.0), and stored at 4 °C. The quality of DNA was examined on 0.8% agarose gels (containing ethidium bromide).

Universal vertebrate primer sequences supplied by Appligene Ltd. were employed to amplify a 2.5 Kb region coding for the ND5/6 genes and a 2.4 Kb region coding for the ND3/4 genes of the NADH dehydrogenase complex modified from Cronin *et al.* (1993). The primer sequences were:

ND5/6

A: 5'- AAT AGT TTA TCC AGT TGG TCT TAG -3' 24 mer

B: 5'- TTA CAA CGA TGG TTT TTC ATA GTC A -3 ' 25 mer

ND3/4

A: 5'- TAA (C/T)TA GTA CAG (C/T)TG ACT TCC AA -3' 23 mer

B: 5'- TTT TGG TTC CTA AGA CCA A(C/T)G GAT -3' 24 mer

The PCR reaction cocktail was set up in a 500 µl eppendorf tube containing 50 µl reaction for each sample (Table II. 1, Appendix II). *Taq* polymerase was initially supplied by Applied Biotechnologies, and later by Bioline Ltd. (U.K.)

using the respective buffers supplied. Reactions were overlaid with two drops of autoclaved mineral oil (Sigma) to avoid evaporation. PCR was carried out using an Omnigene Thermocycler (Hybaid), using the cycle and temperature conditions shown in Table II. 2 Appendix II.

The PCR product was checked on a mini gel to ensure it contained no non-specific products and sufficient yield. 3 µl PCR product was restricted with one of 6 endonucleases recognising four base sequences: *Alu* I, *Cfo* I, *Hae* III, *Hinf* I, *Msp* I, *Rsa* I. The fragments of the restricted DNA samples were separated on 6% polyacrylamide gels, together with a pGM marker (Promega). A standard silver nitrate staining protocol was followed to visualise the DNA fragments: the gels were washed twice for 5 minutes in buffer A (10% ethanol, 0.5% acetic acid), then after washing with distilled water the gels gently agitated for 10 min in buffer B (0.1 % $AgNO_3$, Sigma) and washed again with distilled water. The gels were left in buffer C (1.5% NaOH, 0.1% $NaBH_4$, 0.15% formaldehyde) and agitated to reduce silver nitrate for 15 minutes until the bands appeared. Thereafter the gels were left in distilled water for 5-10 minutes and sealed in plastic bags.

7.2.1 Data analysis

Fragment sizes were estimated from their mobilities relative to a standard pGM DNA-marker (Promega) using the BIOGENE gel documentation package (Vilber-Lormat, France) and DNA-FRAG version 3.03. For each enzyme, the

various restriction fragment patterns were named alphabetically, and the resultant composite haplotype was determined for each individual fish.

Fragment data were used to measure genetic distances among haplotypes (Nei & Li, 1979, Nei, 1987). Haplotype diversities (the level of variety of haplotypes analogous to heterozygosity in allozyme) were calculated using Nei's unbiased estimate (Nei, 1987), using composite haplotypes. Nucleotide diversities (the level of variety at nucleotide sequence of mtDNA genes) were calculated by the method of Nei & Tajima (1981) and Nei (1987), using haplotype frequencies and number of nucleotide substitutions per site between haplotypes estimated by fragment data. Nucleotide divergence (the degree of differentiation at nucleotide sequence of mtDNA) between populations was calculated according to Nei & Tajima (1981) and Nei (1987) (REAP version 4.0, McElroy *et al.*, 1992). The significance of geographic heterogeneity in haplotype distribution was tested using a Monte Carlo chi-squared (x^2, Roff & Bentzen 1989; REAP version 4.0, McElroy *et al.*, 1992) with 1000 randomisations of the data set.

An Analysis of Molecular Variance (AMOVA) program package (WINAMOVA version 1.53, Excoffier *et al.*, 1992) was used to estimate population subdivision, the resulting population statistics are equivalent to the F_{ST} analysis for allozymes (based on mere haplotype frequency). The significance of the F_{ST} values was tested by 1000 random permutations of the original data.

Multidimensional scaling (MDS) was used to summarise the pairwise genetic divergence data derived from haplotype frequencies for each mtDNA region among samples (see Chapter 6). Genetic distance data were also analysed

with clustering algorithms (UPGMA) using PHYLIP version 3.1 (Felsenstein, 1993).

7.3 RESULTS

7.3.1 ND 3/4 Genes

All six restriction enzymes (*Alu* I, *Cfo* I, *Hae* III, *Hinf* I, *Msp* I, *Rsa* I) produced polymorphic fragment patterns and led to the discrimination of 61 ND3/4 haplotypes among 280 fish (250 Atlantic and 30 Pacific herring) (Table 7.1). Of these, 22 haplotypes were observed more than once, and the remaining 39 were unique. Whereas 5 haplotypes were found in all five Atlantic herring samples, the Pacific sample did not share any haplotypes with the Atlantic samples. Restriction fragment size estimates of each haplotype for each enzyme are given in Table II. 3 Appendix II. The average size of the fragment amplified by PCR of ND3/4 genes was 2410 nucleotides (± 60), thus about 8.86 % of the amplified fragment or 1.33 % of the whole mtDNA genome was examined.

Haplotype diversity (Table 7.2) within Atlantic herring samples ranged from 0.8763 in the Icelandic sample (IC1) to 0.9188 in the Trondheimsfjord sample, with an average of 0.8924. The Pacific sample revealed lower levels of haplotype diversity (0.4927). Within-sample nucleotide diversity (Table 7.3) was similar for the Atlantic samples, and ranged from 0.004930 in the Icelandic sample (IC1) to 0.005984 in the Baltic sample with an average of 0.004702, and

in the Pacific sample was relatively low (0.001494). Nucleotide diversity between pairs of samples is also given in Table 7.4.

Nucleotide divergence between Atlantic herring samples was small (Table 7.4), and the highest value was between the Icelandic summer-spawners (IC1) and the Baltic herring sample (0.000232), with the lowest value (0.000013) was between the Trondheimsfjord (NW4) and Baltic herring. The highest nucleotide divergence between the Pacific herring and Atlantic herring samples was shown by the Icelandic summer-spawner sample (0.018318).

Highly significant overall F_{ST} values were detected at the ND3/4 region among the five Atlantic herring samples (Table 7.5). The amount of total genetic variance among populations was 0.083, including the Pacific sample, and 0.013, excluding the Pacific herring. In pairwise comparisons of F_{ST} (Table 7.6), the Baltic herring sample was significantly different from the Celtic sea and Icelandic summer-spawner (IC1) samples. Significant differentiation was also detected between Celtic sea and Icelandic summer-spawner (IC1) samples.

Monte Carlo χ^2 analysis of geographic heterogeneity revealed overall highly significant heterogeneity in haplotype frequencies among the Atlantic herring samples ($P<0.001$). When individual samples were compared (Table 7.7), the Pacific herring sample showed highly significant genetic differences from all the Atlantic herring samples ($P<0.001$). Furthermore, the Icelandic herring sample (IC1) exhibited varying degrees of significant geographic heterogeneity from all the other samples. Significant differences in haplotype frequency were also observed between the Baltic and Celtic sea samples

($P<0.001$) and between the Baltic and Norwegian spring spawner (NW1) samples ($P<0.05$).

Constructing a dendrogram using genetic divergence derived from ND3/4, ND5/6, and combination of ND3/4 and ND5/6 regions was not sufficiently informative (Fig. 7.1a, 7.1b, 7.1c) to display the genetic relationship among the Atlantic herring samples due to high genetic divergence of Pacific sample. Therefore the Pacific sample was extracted from the analyses (Fig.7.2a, 72b, 7.2c). To examine this further the MDS analysis was performed which avoids the clustering of similar samples into groups. In the MDS, the Icelandic and Baltic samples were most divergent from the other Atlantic samples (Fig. 7.3a). Moreover the Pacific herring were clearly most distinct.

7.3.2 ND5/6

All the six restriction enzymes produced polymorphic fragment patterns (Table II. 4, Appendix II), revealing 163 ND5/6 composite haplotypes in 474 fish, composed of 11 Atlantic herring samples and 1 Pacific herring sample (Table 7.8). The number of unique haplotypes was high (116), and 47 haplotypes were observed more than once. Again, there were no shared haplotypes between Atlantic and Pacific herring samples. The average size of surveyed ND5/6 genes was 2515 nucleotides ($\pm$ 70), therefore 7.46 % of the amplified fragment or 1.17 % of the whole mtDNA genome was examined.

The mean within-sample haplotype diversity (Table 7.2) was high (0.8883) within Atlantic herring, and was relatively low in the Pacific though the

lowest level of haplotype diversity (0.8090) was in the Pacific and the highest (0.9384) was in the Norwegian spring-spawners (NW2). Nucleotide diversity within samples was high in comparison to ND3/4 region, ranging from 0.004634 in the Icelandic sample (IC2) to 0.011484 in Baltic sample, with an average of 0.008114, with a similar value of 0.006261 in Pacific herring (Table 7.3).

Low levels of nucleotide divergence between pairs of the Atlantic herring samples were observed (Table 7.9) but nucleotide divergence between the Pacific and the Atlantic herring samples was high (ranging from 0.031278 to 0.034862). The highest value of 0.034862 was between the Celtic sea and the Pacific samples.

The overall F_{ST} value was not significantly different in the ND5/6 region among all Atlantic herring samples, but was highly significant when the Pacific sample was included (Table 7.5). Furthermore, in pairwise comparison of F_{ST}, the only significant genetic differentiation was observed between the Baltic and the Icelandic summer-spawner herring (IC1) samples (Table 7.10). The Pacific herring sample was highly significant in all pairwise comparisons.

There was no overall significant geographic heterogeneity in haplotype frequencies at ND5/6 region when all 10 Atlantic herring samples were included in the Monte Carlo χ^2 analysis. Out of 45 pairwise comparisons between all Atlantic herring samples, only 9 were significantly different (Table 7.11). In the pairwise comparisons, the Baltic herring revealed significant genetic differences from all samples, except the Celtic sea (CS), Buchan herring (NSN2) and Norwegian spring-spawner (NW1) samples. Norwegian spring-spawners (NW1) also showed significant differences in haplotype frequency from the Icelandic

(IC1 & IC2) and Trondheimsfjord (NW4) samples. The Pacific sample showed highly significantly different haplotype frequency differences from all the Atlantic herring samples.

In the MDS analysis, the position of the samples within the graph (Fig. 7.3b) suggest that the Norwegian spring-spawners (NW2 & NW4) were notably divergent from each other, and from the remaining Atlantic herring samples. Not surprisingly, the Pacific sample was most distinct from the Atlantic herring samples.

7.3.3 Combination of ND3/4 and ND5/6

In order to increase the proportion of mtDNA analysed, the haplotypes generated from the ND3/4 and ND5/6 regions were combined. Doing this increased the number of haplotypes (177 haplotypes in 280 fish, including Pacific herring) (Table 7.12), and the number of unique haplotypes (148 haplotypes encountered only once). Only one haplotype was observed in all five Atlantic herring samples, however there was no shared haplotypes between the Atlantic and Pacific samples. By combining the ND3/4 and ND5/6 genes, 16.32 % of the amplified fragment or 2.5 % of the whole mtDNA genome was surveyed.

A high degree of within-sample haplotype diversity was observed in the Atlantic herring samples (Table 7.2). The lowest value (0.9382) was in the Baltic herring, the highest value (0.9826) in the Celtic Sea sample, and with a value of 0.9130 in the Pacific sample. The average excluding the Pacific sample value was also very high (0.9701). Within sample nucleotide diversity (Table 7.3) was

low in the Pacific herring (0.003629), but, was moderate in the Atlantic samples. Here it ranged from 0.006072 in Norwegian spring-spawner sample (NW1) to 0.007452 in the Baltic herring with an average value of 0.006780.

Low levels of nucleotide divergence were detected between the Atlantic samples varied between 0.000096 to 0.00180. (Table 7.13). The highest values of nucleotide divergence between the Atlantic and Pacific herring was shown by the Norwegian spring spawner sample (NW1, 0.025317).

A significant overall F_{ST} value was detected in the combined ND3/4 and ND5/6 regions for the Atlantic herring samples (Table 7.5). The amount of total genetic variance among populations was, 0.014 including Pacific sample, and 0.005 excluding Pacific herring sample. In a pairwise comparison of F_{ST} (Table 7.14), the Baltic herring sample was significantly different from the Celtic sea and Icelandic summer-spawner (IC1) samples. Significant differentiation was also detected between the Icelandic summer-spawner (IC1) and Norwegian spring-spawner (NW1) samples. Highly significant differences was observed in all pairwise comparisons of the Pacific herring sample.

The Monte Carlo χ^2 analysis using all the five Atlantic herring samples exhibited no overall significant geographic heterogeneity in haplotype frequency (P=0.1010). In pairwise comparisons (Table 7.15), significant differences between the samples was lower than was in ND3/4 region, and out of 10 pairwise comparison between the Atlantic herring samples, 4 were significantly different. The Icelandic sample (IC1) revealed significant haplotype frequency differences from all other samples except the Celtic Sea sample. Also, there was a significant differences between the Baltic and the Celtic Sea samples.

In the MDS analysis (Fig. 7.3c), The Baltic and Norwegian spring-spawning (NW4) samples were divergent from the other samples. The Pacific sample was most distinct from the Atlantic samples.

7.4 Discussion

7.4.1 Levels of variability

Different levels of variation were detected in each region and combination of regions of mtDNA. In common with other studies on clupeids (Carvalho *et al.*, 1994; Bembo *et al.*, 1995; Hauser, 1996), high genetic variability was detected in herring at the ND5/6 region. The ND3/4 region also showed high levels of genetic variability, but less than ND5/6. The combination of the two region revealed extremely high levels of haplotype diversity. Comparing the results of mtDNA studies carried out by different investigators is complicated by differences in the number of restriction enzymes and mtDNA region employed. Similar levels of variation using a set of six enzymes and the same primers as in the current study for the ND5/6 region was detected in the freshwater clupeid, *Limnothrissa miodon* (144 haplotypes in 531 fish; haplotype diversity 0.905; Hauser, 1996), the marine anchovy, *Engraulis encrasicolus* (53 haplotypes in 140 fish; haplotype diversity 0.88; Bembo *et al.*, 1995) and for ND3/4 regions, in the marine sardine, *Sardina pilchardus* (41 haplotypes in 104 fish; haplotype diversity 0.76; Carvalho *et al.*, 1994). Therefore the high level of

variation seen in the ND genes may be typical for the Clupeiformes. These high values contrast with the salmonids; intermediate levels of variation in brown trout, *Salmo trutta* (19 haplotypes in 219 fish using seven enzymes on ND5/6 region; Hall, 1992), in chum salmon, *O. keta* (9 haplotypes in 50 fish using 9 enzymes on combination of ND5/6 and ND3/4 regions; Cronin *et al.* 1993), Chinook salmon, *Oncorhynchus tshawytscha* (7 haplotypes in 57 fish using 7 enzymes on combination of ND1, ND5/6 and D-loop; Cronin *et al.* 1993). The higher rate of evolution of the genes of the NADH dehydrogenase complex compared to other mtDNA genes has been previously reported (Meyer, 1993; Chapman *et al.*, 1994), which may lead to high estimates of intra-specific sequence divergence. Hansen & Loeschcke (1996) found no variable restriction sites in the amplified D-loop region of Danish brown trout, *Salmo trutta*, while in the ND-1 and ND5/6 region revealed 13 haplotypes. Similar observation was also reported in different studies (Hall & Nawrocki, 1995; Hynes *et al.*, 1996).

Interestingly, although similar levels of haplotype diversity were detected at the ND5/6 region in both Atlantic and Pacific herring, haplotype diversity at the ND3/4 region in Pacific herring was approximately half that of Atlantic herring. The discordance in the pattern of haplotype diversity may suggest that evolutionary divergence of the ND 3/4 and ND5/6 genes has proceeded at different rates among these species. On the other hand, there is evidence for selection acting on these genes of mtDNA (Ballard & Kreitman, 1995), and thus haplotype diversity may not always result from a stable neutral distribution. Therefore selection may be a factor for the different levels of haplotype diversity.

7.4.2 Genetic differentiation

Despite the detected high levels of haplotype diversity within the samples, interpopulation comparisons of Atlantic herring at ND3/4 and ND5/6 regions revealed low levels of mtDNA differentiation. Combinations of the two region (ND3/4/5/6), which make it possible to examine a higher proportion of mtDNA, also revealed low levels of mtDNA differentiation. This is apparently due to the low number of common haplotypes which reduces the statistical power for detecting differentiation. For example, when testing for significant population differentiation, the presence of unique haplotypes in a population has a very little impact on the overall result that has also been reported by Hauser (1996). Therefore the result mainly depends on the common haplotypes. Moreover, the detected high levels of polymorphism at these mtDNA regions may suggest that sample sizes of 50 individuals may be inadequate for an effective population comparison, and should thereby be increased. A higher number of individuals increase the number of common haplotypes making the statistical test more powerful.

By analysing the same 250 individuals, the observed average nucleotide divergence of ND5/6 (0.000225) was much lower than in the ND 3/4 regions (0.006105), indicating a higher level of genetic differentiation at the ND3/4 regions among Atlantic herring populations. Each mtDNA protein-coding gene has its own particular rate of evolution that depends on factors such as functional constraints on the gene product and nucleotide base compositional

biases (Meyer, 1993). Alternatively, the detected higher nucleotide divergence of ND3/4 may be a chance result caused by the typically low precision of nucleotide divergence estimates (Lynch & Crease, 1990). Only six restriction enzymes were employed for the ND 3/4 and ND 5/6 regions, surveying only 8.86 % and 7.46 % of the genes respectively. Such a low proportion of the genes examined generally results in large standard errors of estimates (Lynch & Crease, 1990). Moreover, increasing the number of enzymes to enable the examination of a higher proportion of genes also results in a large number of unique haplotypes; the number of enzymes employed in the present study was limited to six.

Although there was a lower level of genetic divergence detected at the ND5/6 regions among the Atlantic herring samples, the degree of genetic divergence between the Atlantic and Pacific herring samples was much higher in the ND5/6 (highest value 0.034862) than in ND3/4 (highest value 0.018269). A similar differences is also seen at the F_{ST} analysis. The differences in the degree of divergence of ND3/4 and ND5/6 genes between the two species may be related to historical events they have experienced. According to an allopatric speciation model given by Grant (1986), Pacific herring entered the Pacific basin for the first time at the opening of the Bering Strait in the mid-Pliocene. Therefore Pacific herring may have undergone changes due to selection or stochastic event arising from adaptation to new environmental conditions. Alternatively, there may have been a differential effect of environmental factors on ND3/4 and ND5/6 genes. Selection due to environmental factors may, for example, have been operating particularly on ND5/6, thus causing more

differentiation in the ND5/6 gene between the two species. However, all genes on the mtDNA genome are linked (Meyer, 1993), and therefore selection should effect all loci.

It is more likely that large differences in the levels of haplotype diversity between samples may statistically reduce the level of nucleotide divergence between the samples (Lorenz Hauser, *personal communication*). Thus, the low levels of genetic divergence detected in the ND3/4 region between the two species, may be the result of the low level of haplotype diversity in Pacific herring sample and a high haplotype diversity in Atlantic herring samples. Furthermore, as mentioned previously, a small percentage of the PCR-amplified ND3/4 and ND5/6 genes was examined; other portions of the amplified genes may demonstrate a different pattern.

Monte Carlo pairwise comparisons of haplotype frequencies revealed significant differences between samples, depending on the mtDNA regions examined. Collectively, the data revealed genetic discreteness of the Icelandic, Baltic and Norwegian spring-spawner (NW1) herring.

The pairwise comparison of both ND3/4 and ND5/6 haplotype frequencies between the Atlantic samples revealed that the Baltic herring sample is a genetically discrete population as it differs significantly from most of the other populations sampled. This is in contrast with other allozyme (Ryman *et al.*, 1984; King *et al.*, 1987; Jørstad *et al.*, 1991) and mtDNA (using whole mtDNA; Dahle & Eriksen, 1990) studies which did not provide evidence of genetic differentiation between the Baltic and the other Atlantic herring populations. It is possible that this differentiation may be caused by natural selection. Although

there is no information on the type of selection that may occur, low salinity, higher temperature, and large scale changes in food intensity (Aneer, 1985) in the Baltic sea may be major factors. For example, Aneer (1985) found that the eutrophication of the Baltic Sea can cause differential mortality in eggs as survival depends on the species of the algae present at the spawning site. Furthermore, it is reasonable to assume that the detected genetic differentiation of the Baltic herring may be associated with the hydrographical and topographical features of the Baltic Sea, which may restricting gene flow between the Baltic Sea and the Atlantic Ocean. It could also be that the Baltic population has experienced population bottlenecks and continuing isolation due to ecological conditions, although no genetic evidence for a population bottleneck, such as reduced genetic variation, was found in the present study.

Icelandic summer-spawner herring also revealed its clear genetic heterogeneity from most of the other Atlantic herring samples. Indeed, Icelandic summer-spawners have their own spawning time and place (Parrish & Saville, 1965; Haegele & Schweigert, 1985), and there is evidence (Fridriksson, 1944, 1958; Fridriksson & Aasen, 1952; Johansen, 1926; Liamin, 1959; chapter 3, 4, 5) for their morphological and physiological discreteness. In addition to the observed significant differences of Icelandic sample (IC1) in the ND3/4 region, there was consistent temporal and spatial genetic discreteness of the Icelandic samples (IC1 & IC2) in the ND5/6 region from the Trondheimsfjord and the Baltic samples. This evidence supports the existence of restricted or an absence of gene flow among these populations.

The Norwegian spring-spawners (NW1) collected from the Northern-west coast of Norway also showed significant differences in the ND3/4 region from the Baltic and Icelandic herring (IC1), and in the ND5/6 region from the Icelandic (IC1 & IC2) and Trondheimsfjord herring, supporting the genetic differentiation detected by allozymes (Chapter 6) and microsatellites (Chapter 8). Morphological discreteness of the northern Norwegian spring-spawners has also been reported (Debarros & Holst, 1995; Stenevik *et al.*, 1996). This may indicate a self-recruiting structure of this aggregation (Chapter 6).

ND3/4 and ND5/6 regions were treated as one unit in the analyses in order to investigate the effect of fragment size on the pattern of population differentiation and to allow examination of ~4.9 Kb of ~16 Kb total mtDNA. The pairwise comparison of haplotype frequency of samples using the combined data reduced the overall significance level, and caused a loss of the previously detected significant differences in pairwise comparisons. Thus, not surprisingly, the overall genetic heterogeneity in haplotype frequency was not significant (P=0.1010), compared with separate analysis of ND3/4 (P= 0.000) and ND5/6 (P= 0.042) regions. This may be due to the larger number of unique haplotypes that reduced the power of the statistical tests, suggesting that the examination of whole mtDNA by RFLP can camouflage the existent genetic differentiation at a specific region of mtDNA. In other studies involving salmonids (Hall, 1992; Cronin *et al.*, 1993, Hansen & Loeschcke, 1996) due to the detection of a very low number of haplotypes at mtDNA regions, usually two mtDNA regions are combined to increase the number of haplotypes, and to examine a higher proportion of total mtDNA, thus allowing detection of differences between

populations. For example, Cronin *et al.* (1993) investigated mtDNA differentiation in chinook salmon, *Oncorhynchus tshawytscha*, using RFLP analysis, and found only 9 haplotypes in 50 fish using 9 enzymes for the combined ND3/4 and ND5/6 regions. However, due to the abundance of haplotypes at ND regions in clupeids (Bembo et al., 1995; Hauser, 1996) the combination of mtDNA regions was shown not to be a good choice for detecting population differentiation in herring or perhaps in clupeids showing similarly high haplotype diversity.

In restriction analysis of whole mtDNA, a large number of fragments (between 300-500 bp) are not observed on agarose gel using an ethidium bromide stain (e.g. Kornfield & Bogdanowicz, 1987; Dahle & Erikson, 1990; Murdoch & Hebert, 1994). Thus, genetic information associated in the missed-fragments (between 300-500) were not observed. However the analysis of individual mtDNA genes and the use of polyacrylamide gels with silver staining permits the detection of fragments as small as 40 bp. Therefore, different restriction sites are screened with the two methods. Furthermore, with the latter method, genes of mtDNA are individually searched. In the first method (using whole mtDNA), mtDNA is considered as one unit and scanned randomly, therefore some significant differentiation in one gene of mtDNA may not be monitored. Indeed, in previous mtDNA studies (Kornfield & Bogdanowicz, 1987; Stephenson & Kornfield, 1990; Dahle & Erikson, 1990) using whole mtDNA on Atlantic herring, genetic homogeneity among Atlantic herring populations was revealed. Nonetheless in this study, individual analysis of mtDNA regions by the PCR appears to have overcome the problem of the low

level of genetic differentiation among discrete spawning aggregations of Atlantic herring. This indicates the importance of not only the approach (whole mtDNA versus specific genes), but also the use of more than a single region.

With the availability of 'universal' primers a suitable choice can be made based on identifying the most differentiated region for population studies through studying the level of variation at several mtDNA regions. Moreover, RFLP analysis of PCR-amplified mtDNA regions allow the rapid screening of a large number of fish with minute tissue quantity, not possible with whole mtDNA studies.

In summary, the present data provide one of the few cases of general geographic differentiation in a marine teleost (Ward & Grewe, 1994). The result of the mtDNA analysis indicates that there are at least three genetically different herring populations in Northeast Atlantic (IC, BA, NW1). However, from the perspective of management, it is not sufficient to merely describe genetic differentiation among populations, since it is the persistence of spatial and temporal stability of detected differentiation that is required for management decisions. The spatial and temporal genetic stability of the Icelandic summer-spawners (IC1 & IC2) suggests that they have to be treated as a genetically distinct stock in management programs in Northeast Atlantic. Marked genetic heterogeneity of the Baltic herring sample(BA) from the most of the Atlantic herring samples indicates their self-recruiting structure and genetic uniqueness. The Norwegian spring spawner (NW1) also appeared to be genetically differentiated from at least some of the other Atlantic herring samples,

suggesting a degree of reproductive isolation from other stocks in the Northeast Atlantic.

However, the difference between the ND3/4 and ND5/6 genes in the ability to detect population differentiation may suggest that other mtDNA regions may also be useful for the population identification of Atlantic herring, and further such studies may be valuable.

Table 7.1. Composite haplotypes derived from ND3/4 regions of mtDNA, and their frequency across all samples. Enzymes used to construct composite haplotypes were (left to right): *Alu*-I, *Cfo*-I, *Hae*-III, *Hinf*-I, *Msp*-I, *Rsa*-I. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

		Sample					
Haplotype	Restriction morphs	PC	NW1	BA	CS	NW4	IC1
1	FAGNCD	21	0	0	0	0	0
2	GAGNCD	2	0	0	0	0	0
3	IAGNCD	1	0	0	0	0	0
4	FBGNCD	1	0	0	0	0	0
5	FAGNCB	4	0	0	0	0	0
6	FAGACD	1	0	0	0	0	0
7	ABAABA	0	0	5	0	3	0
8	AAAHBA	0	0	0	1	1	0
9	AAAAAA	0	3	3	4	3	8
10	AAABBA	0	3	0	0	1	2
11	BAAABA	0	0	0	0	1	0
12	ABAADA	0	0	0	0	1	0
13	AAAABA	0	9	8	5	8	6
14	AAAAAB	0	7	10	11	8	13
15	AAAABB	0	3	0	1	2	4
16	AAAEBA	0	0	0	2	1	1
17	AAAAEA	0	2	0	0	2	0
18	AAAAEB	0	6	12	4	7	1
19	ACAAAA	0	0	0	0	1	0
20	ADAAAA	0	0	0	1	1	0
21	AAAABE	0	0	0	0	1	0
22	AAAIBA	0	2	1	1	3	1
23	AAAAAE	0	1	0	0	1	0
24	AAANDD	0	0	0	0	1	0
25	DAAHAB	0	0	0	0	1	0
26	AAAADA	0	3	0	11	2	0
27	ABAABE	0	0	0	0	1	0
28	AADAAB	0	1	1	0	0	0
29	AADEBA	0	0	1	0	0	0
30	ABAAAA	0	0	1	0	0	1
31	AAEAEB	0	0	1	0	0	0
32	CAAABA	0	0	1	0	0	0
33	AAAADB	0	2	2	0	0	1
34	AABADA	0	0	1	0	0	0
35	AAAKBA	0	0	1	0	0	2
36	ACCDED	0	0	1	0	0	0
37	ADAABA	0	0	1	0	0	0
38	ABAAEA	0	0	0	1	0	0
39	AAAIAA	0	0	0	1	0	0
40	AAAADC	0	0	0	1	0	0

Table 7.1. Continued.

Haplotype	Restriction morphs	PC	NW1	BA	CS	NW4	IC1
41	AABAAA	0	1	0	1	0	0
42	AAAMEA	0	0	0	1	0	0
43	AAAEAB	0	0	0	1	0	0
44	AAAFBB	0	0	0	1	0	0
45	AAADBC	0	0	0	1	0	0
46	CAAADA	0	0	0	1	0	0
47	AAEMAB	0	0	0	0	0	1
48	AAAEDB	0	0	0	0	0	1
49	AAFABA	0	0	0	0	0	1
50	AADAAA	0	0	0	0	0	1
51	AAADDA	0	0	0	0	0	1
52	AAFAEA	0	0	0	0	0	1
53	AAAEAA	0	0	0	0	0	0
54	ADAAAB	0	0	0	0	0	1
55	CAAADE	0	0	0	0	0	1
56	AAHEAB	0	1	0	0	0	0
57	BAAAAB	0	2	0	0	0	0
58	CAAAAB	0	1	0	0	0	0
59	EAAAAB	0	1	0	0	0	0
60	EAAADB	0	1	0	0	0	0
61	EAAIAB	0	1	0	0	0	0
Total		30	50	50	50	50	50

Table 7.2. Levels of mtDNA variation at mtDNA regions within herring samples measured as haplotype diversity. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

	MtDNA region		
Sample	ND5/6	ND3/4	ND3/4/5/6
IC1	0.9212	0.8763	0.981
IC2	0.8351	-	-
NW1	0.9018	0.9180	0.9794
NW2	0.9384	-	-
NW3	0.9026	-	-
NW4	0.9083	0.9188	0.9737
BA	0.8404	0.8663	0.9382
CS	0.9366	0.8824	0.9826
NSN2	0.8359	-	-
NSD	0.8627	-	-
Mean	**0.8883 (0.0411)**	**0.8924 (0.0245)**	**0.9710 (0.0186)**
PC	0.8090	0.4927	0.9130

Table 7.3. Levels of mtDNA variation at the ND regions within herring samples measured as nucleotide diversity. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

	MtDNA region		
Sample	ND5/6	ND3/4	ND3/4/5/6
IC1	0.008431	0.004930	0.006541
IC2	0.004634	-	-
NW1	0.008398	0.005444	0.007348
NW2	0.009198	-	-
NW3	0.007267	-	-
NW4	0.009826	0.005228	0.006072
BA	0.011484	0.005984	0.007452
CS	0.008143	0.005133	0.006485
NSN2	0.006550	-	-
NSD	0.007203	-	-
Mean	**0.008114 (0.00188)**	**0.005344 (0.0004)**	**0.006780 (0.00060)**
PC	0.006261	0.001494	0.003629

Table 7.4. Nucleotide divergence (below diagonal) and diversity (above diagonal) estimated from fragment data of ND3/4 genes of mtDNA between samples. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	IC1	NW4	NW1	BA	CS	PC
IC1	-	0.005327	0.005101	0.005689	0.005124	0.021530
NW4	0.000140	-	0.005383	0.005727	0.005431	0.021738
NW1	0.000022	0.000047	-	0.005637	0.005218	0.021121
BA	0.000232	0.000013	0.000031	-	0.005753	0.021871
CS	0.000093	0.000142	0.000038	0.000195	-	0.021460
PC	0.018318	0.018269	0.017760	0.018132	0.018147	-

Table 7.5. Overall F_{ST} values among the herring samples with their significance level (* P<0.05, ** P<0.01, *** P<0.001). # the samples used in ND3/4 analysis were used for comparison.

MtDNA region	F_{ST} (including Pacific)	F_{ST} (excluding Pacific)
ND3/4	0.083***	0.013**
ND5/6#	0.036***	0.004
ND5/6	0.022***	0.003
ND3/4/5/6	0.014***	0.005**

Table 7.6. F_{ST} values of ND3/4 mtDNA regions between pairs of samples with their significance level (*P<0.05, ***P<0.001). Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	PC	NW4	BA	CS	NW1	IC1
PC	--					
NW4	**0.2690*** **	--				
BA	**0.2959*** **	-0.0036	--			
CS	**0.2875*** **	0.0120	**0.0353***	--		
NW1	**0.2685*** **	-0.0106	0.0070	0.0124	--	
IC1	**0.2916*** **	0.0124	**0.0308***	**0.0236***	0.0120	--

Table 7.7. Pairwise comparisons of ND3/4 haplotype frequencies among herring samples. The overall significance levels are shown: ***, P<0.001; **, P<0.01;* P<0.05. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	IC1	NW4	NW1	BA	CS	PC
IC1	-					
NW4	*					
NW1	*	ns				
BA	**	ns	*			
CS	**	ns	ns	***		
PC	***	***	***	***	***	

Table 7.8. Composite haplotypes derived from ND5/6 region of mtDNA, and their frequency across all samples. Enzymes used to construct composite haplotypes were (left to right): *Alu*-I, *Cfo*-I, Hae-III, *Hinf*-I, *Msp*-I, *Rsa*-I. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

		Sample										
Haplotype	Restriction morphs	IC1	IC2	NW1	NW2	NW3	NW4	BA	CS	NSN2	NSD	PC
1	AAAAAA	13	18	11	10	13	14	16	11	8	9	0
2	AAABAA	0	0	0	0	0	1	0	0	0	1	0
3	BAABBA	0	0	0	0	0	1	0	0	0	0	0
4	CABCAB	0	0	0	0	0	1	0	0	0	0	0
5	AAADAA	0	2	1	2	0	2	0	2	0	0	0
6	AAACAA	1	1	0	0	0	2	0	2	0	1	0
7	CAAAAA	0	1	1	0	1	1	0	1	1	0	0
8	AACAAA	0	0	1	0	0	1	0	0	0	0	0
9	ABCAAA	0	0	0	0	0	1	0	0	0	0	0
10	MADICA	0	0	0	0	0	1	0	0	0	0	0
11	ECADDA	0	0	0	0	0	1	0	0	0	0	0
12	ACAFAA	0	0	0	0	0	1	0	0	0	0	0
13	GBEDDA	0	0	0	0	0	1	0	0	0	0	0
14	ABCDDA	1	0	6	2	1	4	9	3	2	0	0
15	BAAAAA	2	7	9	3	8	1	8	4	6	3	0
16	ECAAAA	0	0	0	1	0	2	0	0	0	0	0
17	AAAAFA	0	0	0	1	1	1	0	0	0	0	0
18	GAAAAA	1	1	0	0	1	2	0	1	0	0	0
19	AAAAAC	0	0	0	0	1	1	0	1	0	0	0
20	CAAAAC	0	0	0	0	0	1	0	0	0	0	0
21	BAACAA	0	2	0	0	0	1	0	1	0	0	0
22	CAAAEA	0	0	0	0	0	1	0	0	0	0	0
23	HBFLGD	0	0	0	0	0	1	0	0	0	0	0
24	ADAAAA	0	0	0	1	0	1	0	0	0	0	0
25	AAGAAA	0	0	0	0	0	1	0	0	0	0	0
26	ACAAAA	0	0	1	0	0	1	1	0	1	0	0
27	GBCDDA	0	0	0	0	0	1	0	0	0	0	0
28	IBCDDA	0	0	0	0	0	1	0	0	0	0	0
29	CBCDKA	0	0	0	0	0	1	0	0	0	0	0
30	AAACAC	0	0	0	0	0	1	0	0	0	0	0
31	AAAGEA	1	0	0	0	0	0	0	0	0	0	0
32	LAAAAA	1	0	0	0	0	0	0	0	0	0	0
33	AAAGAA	1	0	0	0	0	0	0	0	0	0	0
34	BAGAAA	1	0	0	0	0	0	0	0	0	0	0
35	JAAAAA	1	0	0	0	0	0	0	0	0	0	0
36	CABAAA	1	0	0	0	0	0	0	0	0	0	0
37	AAAHAA	1	0	0	0	0	0	0	1	0	0	0
38	BAABAA	2	0	0	0	0	0	0	0	0	0	0
39	AAHAAA	1	1	0	2	1	0	1	0	0	1	0
40	CAAIAA	1	0	0	0	0	0	0	0	0	0	0

Table 7.8. Continued.

Haplotype	Restriction morphs	IC1	IC2	NW1	NW2	NW3	NW4	BA	CS	NSN2	NSD	PC
41	ABCJAA	3	0	0	0	0	0	2	0	0	0	0
42	AADCAA	1	0	0	0	0	0	0	0	0	0	0
43	ABADAA	1	0	0	0	0	0	0	0	0	0	0
44	AAIAIA	1	0	0	0	0	0	0	0	0	0	0
45	KAAAAA	1	0	0	0	0	0	0	0	0	0	0
46	BAJAAA	1	0	1	1	0	0	0	0	0	0	0
47	GAAACA	1	0	0	0	0	0	0	0	0	0	0
48	CAACAA	1	0	0	0	0	0	0	0	0	0	0
49	ABACAB	1	0	0	0	0	0	0	0	0	0	0
50	AAACAB	2	1	1	1	1	0	0	0	0	0	0
51	BBKHAA	1	0	0	0	0	0	0	0	0	0	0
52	MBAAAA	1	0	0	0	0	0	0	0	0	0	0
53	AAAAAB	1	0	1	0	0	0	0	0	0	0	0
54	NAACAA	1	0	0	0	0	0	0	0	0	0	0
55	AAAAAD	1	0	0	0	1	0	0	0	0	0	0
56	AEAAAA	2	0	0	0	0	0	0	1	0	0	0
57	AAAAIA	1	1	0	0	0	0	0	0	0	0	0
58	BAAGAA	0	0	0	1	0	0	0	0	0	0	0
59	ACACAA	0	0	0	1	0	0	0	0	0	0	0
60	ABDAAA	0	0	0	1	0	0	0	0	0	0	0
61	AACDDA	0	0	0	1	0	0	0	0	0	0	0
62	AAACJA	0	0	0	1	0	0	0	0	0	0	0
63	CAAAAB	0	0	0	1	0	0	0	0	0	0	0
64	AAAFAA	0	0	0	1	0	0	0	0	0	0	0
65	AFLDDA	0	0	0	1	0	0	0	0	0	0	0
66	AAAAAE	0	0	0	1	0	0	0	0	0	0	0
67	BAADAA	0	0	0	2	0	0	0	0	0	1	0
68	CAABAA	0	0	1	1	0	0	0	0	0	1	0
69	AAMAAA	0	0	0	1	0	0	0	0	0	0	0
70	AAADDA	0	0	0	1	0	0	0	0	0	0	0
71	AANDAA	0	0	0	1	0	0	0	1	0	0	0
72	AANAFA	0	0	0	1	0	0	0	0	0	0	0
73	BBAAAA	0	0	0	2	0	0	0	0	1	1	0
74	AGAAKA	0	0	0	1	0	0	0	0	0	0	0
75	AAHAAF	0	0	0	1	1	0	0	0	0	0	0
76	ABCKAA	0	0	0	1	0	0	0	0	0	0	0
77	FBCDDG	0	0	0	1	0	0	0	0	0	0	0
78	TAAAAA	0	0	0	0	1	0	0	1	0	0	0
79	AABAAA	0	0	0	0	1	0	0	0	0	0	0
80	AAAMEA	0	0	0	0	1	0	0	0	0	0	0
81	AHADAA	0	0	0	0	1	0	0	0	0	0	0
82	AAACEI	0	0	0	0	1	0	0	0	0	0	0
83	DAAAAA	0	0	0	0	2	0	0	1	0	1	0
84	CBABAA	0	1	0	0	1	0	0	0	0	0	0
85	AAJAAA	0	0	2	0	1	0	0	0	0	0	0
86	AAAJAA	0	0	0	0	1	0	0	0	0	0	0

Table 7.8. Continued.

Haplotype	Restriction morphs	IC1	IC2	NW1	NW2	NW3	NW4	BA	CS	NSN2	NSD	PC
87	SAAAAA	0	1	0	0	1	0	0	0	0	0	0
88	BAAAAF	0	0	0	0	1	0	0	0	0	0	0
89	OAAAAA	0	0	0	0	1	0	0	0	0	0	0
90	EAAAAA	0	1	0	0	2	0	0	1	0	0	0
91	ABAAAA	0	1	0	0	1	0	1	1	1	0	0
92	OAAAAF	0	0	0	0	1	0	0	0	0	0	0
93	PAAAAA	0	0	0	0	1	0	0	0	0	0	0
94	RAJAAA	0	0	0	0	1	0	0	0	0	0	0
95	FBCDDA	0	1	0	0	1	0	0	0	0	0	0
96	AADAAA	0	0	1	0	0	0	1	1	0	0	0
97	HBCDDA	0	0	0	0	0	0	1	0	0	0	0
98	AAAAEA	0	0	0	0	0	0	1	0	0	0	0
99	HACAAA	0	0	0	0	0	0	1	0	0	0	0
100	BJAAAA	0	0	0	0	0	0	1	0	0	0	0
101	BAAALA	0	0	0	0	0	0	1	0	0	0	0
102	BAAAAB	0	0	0	0	0	0	1	0	0	0	0
103	AAHDAA	0	0	0	0	0	0	1	0	0	0	0
104	BAIAAA	0	0	0	0	0	0	1	0	0	0	0
105	CAJAAA	0	0	0	0	0	0	1	0	0	0	0
106	VKPSNJ	0	0	0	0	0	0	1	0	0	0	0
107	ABTAAA	0	0	0	0	0	0	1	0	0	0	0
108	AHUALA	0	0	0	0	0	0	0	1	0	0	0
109	AAHCAA	0	0	2	0	0	0	0	1	0	0	0
110	ABCDMA	0	0	0	0	0	0	0	1	0	0	0
111	GANAAA	0	0	0	0	0	0	0	1	0	0	0
112	OAARAA	0	0	0	0	0	0	0	1	0	0	0
113	AAOAAA	0	0	0	0	0	0	0	1	0	0	0
114	ABCADA	0	0	0	0	0	0	0	1	0	0	0
115	AAAIPA	0	0	0	0	0	0	0	1	0	0	0
116	AAACRA	0	0	0	0	0	0	0	1	0	0	0
117	BAAARA	0	0	0	0	0	0	0	1	0	0	0
118	BAJHAA	0	0	0	0	0	0	0	1	0	0	0
119	SADAAA	0	0	0	0	0	0	0	1	0	0	0
120	OAAPAA	0	0	0	0	0	0	0	1	0	0	0
121	OAHAAF	0	0	0	0	0	0	0	1	0	0	0
122	AIAAAA	0	0	0	0	0	0	0	1	0	0	0
123	ABOAAG	0	0	0	0	0	0	0	1	0	0	0
124	WAAASA	0	1	0	0	0	0	0	0	0	0	0
125	CARAAB	0	1	0	0	0	0	0	0	0	0	0
126	GAADAA	0	1	0	0	0	0	0	0	0	0	0
127	AASCAB	0	1	0	0	0	0	0	0	0	0	0
128	BAAAFA	0	1	0	0	0	0	0	0	0	0	0
129	ABJAAA	0	1	0	0	0	0	0	0	0	0	0
130	AAACCA	0	1	0	0	0	0	0	0	0	0	0
131	AAAOAH	0	1	0	0	0	0	0	0	0	0	0
132	BCAAAA	0	0	0	0	0	0	0	0	1	0	0

Table 7.8. Continued.

Haplotype	Restriction morphs	IC1	IC2	NW1	NW2	NW3	NW4	BA	CS	NSN2	NSD	PC
133	AANAAA	0	0	2	0	0	0	0	0	1	0	0
134	AARAAA	0	0	0	0	0	0	0	0	1	0	0
135	UAAAAA	0	0	0	0	0	0	0	0	1	0	0
136	AAALAB	0	0	0	0	0	0	0	0	1	0	0
137	BAAATA	0	0	0	0	0	0	0	0	0	1	0
138	AFCDDA	0	0	0	0	0	0	0	0	0	1	0
139	BBJAAA	0	0	0	0	0	0	0	0	0	1	0
140	ABABAA	0	0	0	0	0	0	0	0	0	1	0
141	DBVDDA	0	0	0	0	0	0	0	0	0	1	0
142	HAAHAA	0	0	0	0	0	0	0	0	0	1	0
143	AAANAA	0	0	0	0	0	0	0	0	0	1	0
144	EAARAA	0	0	1	0	0	0	0	0	0	0	0
145	ABGDDA	0	0	1	0	0	0	0	0	0	0	0
146	BAAIAA	0	0	1	0	0	0	0	0	0	0	0
147	GAACAA	0	0	1	0	0	0	0	0	0	0	0
148	EAAFAA	0	0	1	0	0	0	0	0	0	0	0
149	GACDDA	0	0	1	0	0	0	0	0	0	0	0
150	GAARAA	0	0	1	0	0	0	0	0	0	0	0
151	AATAAB	0	0	1	0	0	0	0	0	0	0	0
152	AAJAFA	0	0	1	0	0	0	0	0	0	0	0
153	HLYTGD	0	0	0	0	0	0	0	0	0	0	12
154	HLYUGD	0	0	0	0	0	0	0	0	0	0	3
155	ALZUVD	0	0	0	0	0	0	0	0	0	0	2
156	YLYVYD	0	0	0	0	0	0	0	0	0	0	3
157	ZLYUGD	0	0	0	0	0	0	0	0	0	0	3
158	HMYZGD	0	0	0	0	0	0	0	0	0	0	2
159	XLYUGD	0	0	0	0	0	0	0	0	0	0	1
160	QLYZGD	0	0	0	0	0	0	0	0	0	0	1
161	HLYVYD	0	0	0	0	0	0	0	0	0	0	1
162	YLYTYD	0	0	0	0	0	0	0	0	0	0	1
163	HLXUGD	0	0	0	0	0	0	0	0	0	0	1
Total		50	48	50	46	50	50	50	50	25	26	30

Table 7.9. Nucleotide divergence (below diagonal) and diversity (above diagonal) estimated from fragment data of ND5/6 genes of mtDNA between samples. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

Sample	IC1	IC2	NW4	NW2	NW3	NW1	BA	CS	NSN2	NSD	PC
IC1	-	0.005916	0.008954	0.008474	0.006798	0.008528	0.010073	0.008272	0.007062	0.007062	0.040737
IC2	0.000037	-	0.007676	0.007557	0.00657	0.011534	0.009232	0.010197	0.009638	0.009469	0.039234
NW4	0.000238	0.000447	-	0.009447	0.008170	0.008555	0.010526	0.008676	0.008443	0.008610	0.039954
NW2	0.000161	0.000036	0.000023	-	0.007600	0.008722	0.010962	0.009230	0.009008	0.008687	0.040870
NW3	0.000036	-0.00049	0.000298	0.000129	-	0.007752	0.010044	0.008362	0.007906	0.007713	0.041046
NW1	0.000114	0.00009	0.000016	0.000016	0.000012	-	0.009858	0.008242	0.011551	0.011565	0.041796
BA	0.000115	-0.000024	0.000048	0.000103	0.000220	-0.000083	-	0.009803	0.009301	0.009233	0.043079
CS	-0.000016	0.00016	0.000053	-0.000086	0.000081	-0.000029	-0.000010	-	0.010413	0.010162	0.042065
NSN2	0.000225	-0.000144	0.000256	0.000026	-0.000041	-0.000024	-0.000086	0.000190	-	0.009608	0.038680
NSD	-0.000062	-0.000216	0.000096	-0.000129	0.000219	0.000087	-0.000058	0.000036	0.000208	-	0.039375
PC	0.034044	0.031278	0.031911	0.033114	0.034282	0.034467	0.034206	0.034862	0.030597	0.031384	-

Table 7.10. F_{ST} values of ND5/6 mtDNA regions between pairs of samples with their significance level (*P<0.05, ***P<0.001). Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic summer-spawners (second year) (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

Sample	NW4	IC1	NW2	NW3	BA	CS	IC2	NSN2	NSD	NW1	PC
NW4											
IC1	-0.0010										
NW2	-0.0024	-0.0020									
NW3	0.0062	0.0026	-0.0003								
BA	0.0102	**0.0157***	0.0138	0.0072							
CS	-0.0054	-0.0025	-0.0056	-0.0041	0.0085						
IC2	0.0076	0.0076	0.0105	-0.0033	0.0109	0.0064					
NSN2	0.0127	0.0140	0.0083	-0.0090	-0.0094	0.0036	-0.0062				
NSD	-0.0007	-0.0021	-0.0039	-0.0078	0.0067	-0.0015	-0.0117	-0.0078			
NW1	0.0097	0.0116	0.0034	-0.0016	-0.0018	-0.0010	0.0135	-0.0104	0.0072		
PC	**0.1276*****	**0.1209*****	**0.1119*****	**0.1306*****	**0.1633*****	**0.1129*****	**0.1660*****	**0.1622*****	**0.1489*****	**0.1310*****	

Table 7.11. Pairwise comparisons of ND5/6 haplotype frequencies among herring samples. The overall significance levels are shown: ***, P<0.001; **, P<0.01; P<0.04. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Icelandic (IC2), Norwegian spring spawners (Northeast Norway coast) (NW1), Norwegian spring spawners (central Norwegian sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Baltic herring (BA), Buchan herring (Northmost North Sea) (NSN2), Downs herring (NSD), Celtic Sea (CS), Pacific herring (PC).

	IC1	IC2	NW4	NW2	NW3	NW1	BA	CS	NSN2	NSD	PC
IC1	-										
IC2	ns	-									
NW4	ns	ns	-								
NW2	ns	ns	ns	-							
NW3	ns	ns	ns	ns	-						
NW1	*	*	*	ns	ns	-					
BA	**	*	**	*	*	ns	-				
CS	ns	ns	ns	ns	ns	ns	ns	-			
NSN2	ns	ns	ns	ns	ns	ns	ns	ns	-		
NSD	ns	ns	ns	ns	ns	ns	*	ns	ns	-	
PC	***	***	***	***	***	***	***	***	***	***	-

Table 7.12. Composite haplotypes derived from ND3/4/5/6 region of mtDNA, and their frequency across all samples. Enzymes used to construct composite haplotypes for each ND3/4 and ND5/6 regionswere (left to right): *Alu*-I, *Cfo*-I, *Hae*-III, *Hinf*-I, Msp-I, Rsa-I. Samples referred to in thetext were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners(NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

		Sample					
Haplotype	Restriction morphs	NW1	BA	CS	IC1	NW4	PC
1	AAAAAAABAABA	0	4	0	0	3	0
2	AAABAAAAAHBA	0	0	0	0	1	0
3	BAABBAAAAAAA	0	0	0	0	1	0
4	CABCABAAABBA	0	0	0	0	1	0
5	AAADAABAAABA	0	0	0	0	1	0
6	AAACAAABAADA	0	0	0	0	1	0
7	CAAAAAAAAAAA	0	0	1	0	1	0
8	AACAAAAAAABA	1	0	0	0	1	0
9	ABCAAAAAAABA	0	0	0	0	1	0
10	MADICAAAAAAB	0	0	0	0	1	0
11	ECADDAAAAABB	0	0	0	0	1	0
12	ACAFAAAAAABA	0	0	0	0	1	0
13	AAAAAAAAAEBA	0	0	1	1	1	0
14	AAADAAAAAABA	1	0	1	0	1	0
15	AAAAAAAAAABB	0	0	0	0	1	0
16	GBEDDAAAAAEA	0	0	0	0	1	0
17	ABCDDAAAAAEB	4	9	2	0	4	0
18	BAAAAAAAAAAB	5	5	3	0	1	0
19	ECAAAAAAAABA	0	0	0	0	2	0
20	AAAAFAAAAAEA	0	0	0	0	1	0
21	AAAAAAACAAAA	0	0	0	0	1	0
22	AAAAAAADAAAA	0	0	0	0	1	0
23	AAAAAAAAAAAB	2	2	2	4	3	0
24	GAAAAAAAAABA	0	0	0	0	1	0
25	AAAAAAAAAABE	0	0	0	0	1	0
26	AAAAACAAAAAB	0	0	0	0	1	0
27	CAAAACAAAAAA	0	0	0	0	1	0
28	GAAAAAAAAIBA	0	0	0	1	1	0
29	BAACAAAAAAAB	0	0	1	0	1	0
30	CAAAEAAAAAAE	0	0	0	0	1	0
31	AAAAAAAAAIBA	2	1	0	0	1	0
32	HBFLGDAAANDD	0	0	0	0	1	0
33	AAAAAADAAHAB	0	0	0	0	1	0
34	ADAAAAAAAAAB	0	0	0	0	1	0
35	AAGAAAAAAADA	0	0	0	0	1	0
36	ACAAAAAAAABA	1	1	0	0	1	0
37	GBCDDAAAAAEB	0	0	0	0	1	0
38	AAACAAAAAIBA	0	0	1	0	1	0
39	IBCDDAAAAAEB	0	0	0	0	1	0
40	CBCDKAAAAAEB	0	0	0	0	1	0
41	AAAAAAABAABE	0	0	0	0	1	0
42	AAACACAAAADA	0	0	0	0	1	0

Table 7.12. Continued.

Haplotype	Restriction morphs	NW1	BA	CS	IC1	NW4	PC
43	AAAGEAAAAAAA	0	0	0	1	0	0
44	LAAAAAAAAAAA	0	0	0	1	0	0
45	BAAAAAAAAAAA	1	0	0	1	0	0
46	AAAAAAAAEMAB	0	0	0	1	0	0
47	AAAGAAAAAEDB	0	0	0	1	0	0
48	BAGAAAAAAAAB	0	0	0	1	0	0
49	AAAAAAAAFABA	0	0	0	1	0	0
50	JAAAAAAAAAAA	0	0	0	1	0	0
51	CABAAAAADAAA	0	0	0	1	0	0
52	AAAHAAAAAABA	0	0	0	1	0	0
53	AAAAAAAAAABA	2	5	2	3	0	0
54	BAABAAAAAABB	0	0	0	1	0	0
55	AAHAAAAAADDA	0	0	0	1	0	0
56	CAAIAAAAFAEA	0	0	0	1	0	0
57	ABCJAAAAAAEB	0	1	0	1	0	0
58	AADCAAAAAAAB	0	0	0	1	0	0
59	ABCDDAAAAAAB	0	0	0	1	0	0
60	ABADAAAAAAAB	0	0	0	1	0	0
61	ABCJAAAAAAAB	0	0	0	2	0	0
62	AAAAAAAAAAAA	0	2	1	2	0	0
63	AAIAIAAAAEAA	0	0	0	1	0	0
64	KAAAAAAAAABB	0	0	0	1	0	0
65	BAJAAAADAAAB	0	0	0	1	0	0
66	GAAACAABAAAA	0	0	0	1	0	0
67	CAACAAAAAAAA	0	0	0	1	0	0
68	BAABAAAAAAAB	0	0	0	1	0	0
69	AAACAAAAAABB	0	0	0	1	0	0
70	ABACABAAAKBA	0	0	0	1	0	0
71	BAAAAACAAADE	0	0	0	1	0	0
72	AAACABAAABBA	1	0	0	1	0	0
73	BBKHAAAAAADB	0	0	0	1	0	0
74	MBAAAAAAAAAB	0	0	0	1	0	0
75	AAAAABAAAAAA	0	0	0	1	0	0
76	NAACAAAAABBA	0	0	0	1	0	0
77	AAAAADAAAKBA	0	0	0	1	0	0
78	AAAAAAAAAEAA	0	0	0	1	0	0
79	AEAAAAAAAAAB	0	0	0	1	0	0
80	AEAAAAAAAABA	0	0	0	1	0	0
81	AAAAIAAAAABB	0	0	0	1	0	0
82	AAACABAAAABA	0	0	0	1	0	0
83	BAAAAAAADAAB	0	1	0	0	0	0
84	AADAAAAAAABA	0	1	0	0	0	0
85	AAAAAAAADEBA	0	1	0	0	0	0
86	AAHAAAABAAAA	0	1	0	0	0	0
87	BAAAAAAAAAEB	0	1	0	0	0	0
88	HBCDDAAAEAEB	0	1	0	0	0	0

Table 7.12. Continued.

Haplotype	Restriction morphs	NW1	BA	CS	IC1	NW4	PC
89	AAAAEAABAABA	0	1	0	0	0	0
90	HACAAACAAABA	0	1	0	0	0	0
91	BJAAAAAAAADB	0	1	0	0	0	0
92	BAAALAAAAAAB	0	1	0	0	0	0
93	AAAAAAAABADA	0	1	0	0	0	0
94	BAAAABAAAABA	0	1	0	0	0	0
95	BAAAAAAAAKBA	0	1	0	0	0	0
96	ABAAAAAAAAAB	0	1	0	0	0	0
97	AAHDAAAAAAEB	0	1	0	0	0	0
98	BAIAAAAAAAAB	0	1	0	0	0	0
99	ABCJAAAAAADB	0	1	0	0	0	0
100	CAJAAAAAAAAA	0	1	0	0	0	0
101	VKPSNJACCDED	0	1	0	0	0	0
102	ABTAAAADAABA	0	1	0	0	0	0
103	AAAHAAAAAADA	0	0	1	0	0	0
104	AHUALAAAAADA	0	0	1	0	0	0
105	AAAAAAABAAEA	0	0	1	0	0	0
106	AEAAAAAAAAAA	0	0	1	0	0	0
107	AAHCAAAAAIAA	0	0	1	0	0	0
108	AAAAAAAAAADA	2	0	3	0	0	0
109	ABCDMAAAAAEB	0	0	1	0	0	0
110	TAAAAAAAAADC	0	0	1	0	0	0
111	GANAAAAABAAA	0	0	1	0	0	0
112	OAARAAAAAAAB	0	0	1	0	0	0
113	EAAAAAAAAAAB	0	0	1	0	0	0
114	AADAAAAAAADA	1	0	1	0	0	0
115	AAOAAAAAAMEA	0	0	1	0	0	0
116	ABCADAAAAAEB	0	0	1	0	0	0
117	AAAIPAAAAAAA	0	0	1	0	0	0
118	AAAAAAAAAEAB	0	0	1	0	0	0
119	AAACRAADAAAA	0	0	1	0	0	0
120	BAAAAAAAAFBB	0	0	1	0	0	0
121	ABAAAAAAAADA	0	0	1	0	0	0
122	AAAAACAAAABA	0	0	1	0	0	0
123	BAAARAAAAAAB	0	0	1	0	0	0
124	BAJHAAAAAAAB	0	0	1	0	0	0
125	AAADAAAAAEBA	0	0	1	0	0	0
126	GAAAAAAAAHBA	0	0	1	0	0	0
127	SADAAAAAAADA	0	0	1	0	0	0
128	OAAPAAAAAADA	0	0	1	0	0	0
129	ABCDDAAAAABB	1	0	1	0	0	0
130	OAHAAFAAAADA	0	0	1	0	0	0
131	DAAAAAAAADBC	0	0	1	0	0	0
132	AIAAAACAAADA	0	0	1	0	0	0
133	ABOAAGAAAAAB	0	0	1	0	0	0
134	AANDAAAAAABA	0	0	1	0	0	0

Table 7.12. Continued.

Haplotype	Restriction morphs	NW1	BA	CS	IC1	NW4	PC
135	AAACAAAAAADA	0	0	1	0	0	0
136	AAAAABAAAABA	1	0	0	0	0	0
137	EAARAAAAABBA	1	0	0	0	0	0
138	ABGDDAAAAAEB	1	0	0	0	0	0
139	BAAIAAAAAABB	1	0	0	0	0	0
140	AAJAAAAAAABA	1	0	0	0	0	0
141	GAACAAAAABBA	1	0	0	0	0	0
142	AAAAAAAADAAB	1	0	0	0	0	0
143	ABCDDAAAHEAB	1	0	0	0	0	0
144	EAAFAAAAAAEA	1	0	0	0	0	0
145	CAAAAAAAAAAE	1	0	0	0	0	0
146	BAAAAAAAAABB	1	0	0	0	0	0
147	GACDDAAAAAEB	1	0	0	0	0	0
148	BAJAAAAAAADB	1	0	0	0	0	0
149	GAARAAAAAADB	1	0	0	0	0	0
150	BAAAAABAAAAB	1	0	0	0	0	0
151	AAAAAAAAAAEA	1	0	0	0	0	0
152	AANAAAAAAABA	1	0	0	0	0	0
153	AAAAAACAAAAB	1	0	0	0	0	0
154	AAJAAABAAAAB	1	0	0	0	0	0
155	AAHCAAAAAAAA	1	0	0	0	0	0
156	AATAABEAAAAB	1	0	0	0	0	0
157	AAJAFAEAAADB	1	0	0	0	0	0
158	CAABAAAAAAAA	1	0	0	0	0	0
159	BAAAAAEAAIAB	1	0	0	0	0	0
160	AAHCAAAAAABA	1	0	0	0	0	0
161	AANAAAAABAAA	1	0	0	0	0	0
162	HLYTGDFAGNCD	0	0	0	0	0	7
163	HLYUGDGAGNCD	0	0	0	0	0	2
164	ALZUVDFAGNCD	0	0	0	0	0	2
165	HLYTGDIAGNCD	0	0	0	0	0	1
166	YLYVYDFAGNCD	0	0	0	0	0	3
167	HLYTGDFBGNCD	0	0	0	0	0	1
168	HLYUGDFAGNCD	0	0	0	0	0	1
169	ZLYUGDFAGNCD	0	0	0	0	0	2
170	ZLYUGDFAGNCB	0	0	0	0	0	1
171	HLYTGDFAGNCB	0	0	0	0	0	3
172	HMYZGDFAGNCD	0	0	0	0	0	2
173	XLYUGDFAGACD	0	0	0	0	0	1
174	QLYZGDFAGNCD	0	0	0	0	0	1
175	HLYVYDFAGNCD	0	0	0	0	0	1
176	YLYTYDFAGNCD	0	0	0	0	0	1
177	HLXUGDFAGNCD	0	0	0	0	0	1
Total		50	50	50	50	50	30

Table 7.13. Nucleotide divergence (below diagonal) and diversity (above diagonal) estimated from fragment data of ND3/4/5/6 genes of mtDNA between samples.Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

	IC1	NW4	NW1	BA	CS	PC
IC1	-	0.006809	0.006504	0.007048	0.006737	0.029886
NW4	0.000180	-	0.006859	0.007966	0.007021	0.029634
NW1	0.000085	0.000028	-	0.007054	0.006210	0.030168
BA	0.000052	0.000145	0.000159	-	0.008265	0.030758
CS	0.000045	0.000146	-0.000096	0.000152	-	0.030340
PC	0.025117	0.024145	0.025317	0.024325	0.025218	-

Table 7.14. F_{ST} values of ND3/45/6 mtDNA regions between pairs of samples with their significance level (*P<0.05, ***P<0.001). Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	NW4	BA	CS	IC1	NW1	PC
NW4	--					
BA	0.0072	--				
CS	0.0006	**0.0108***	--			
IC1	0.0042	**0.0196*****	0.0018	--		
NW1	0.0007	0.0032	-0.0038	**0.0063***	--	
PC	**0.0402*****	**0.0615*****	**0.0390*****	**0.0394*****	**0.0431*****	--

Table 7.15. Pairwise comparisons of ND3/4/5/6 haplotype frequencies among herring samples. The overall significance levels are shown: ***, P<0.001; **, P<0.01; P<0.04. Samples referred to in the text were: Icelandic summer-spawners (first year) (IC1), Norwegian spring spawners (NW1), Baltic herring (BA), Celtic Sea (CS), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	IC1	NW4	NW1	BA	CS	PC
IC1	-					
NW4	*	-				
NW1	*	ns	-			
BA	**	ns	ns	-		
CS	ns	ns	ns	*	-	
PC	***	***	***	***	***	-

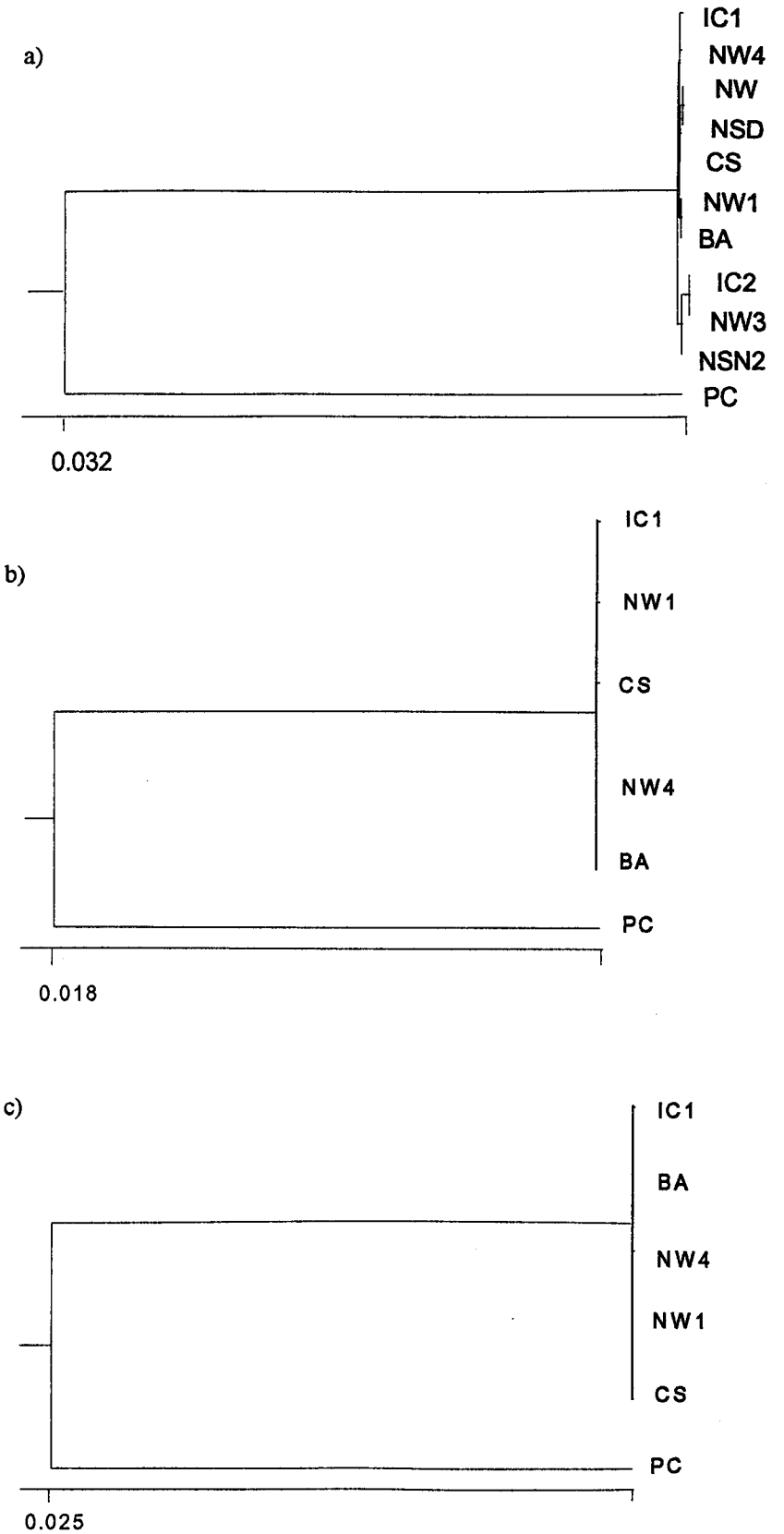


Figure 7.1. ND5/6 (a), ND3/4 (b) and combination of ND3/4 and ND5/6 (c) regions of mtDNA phenogram based on the distance matrix resulting from the estimation of interpopulation nucleotide divergence. For location of the samples see Chapter 2, Figure 2.1.

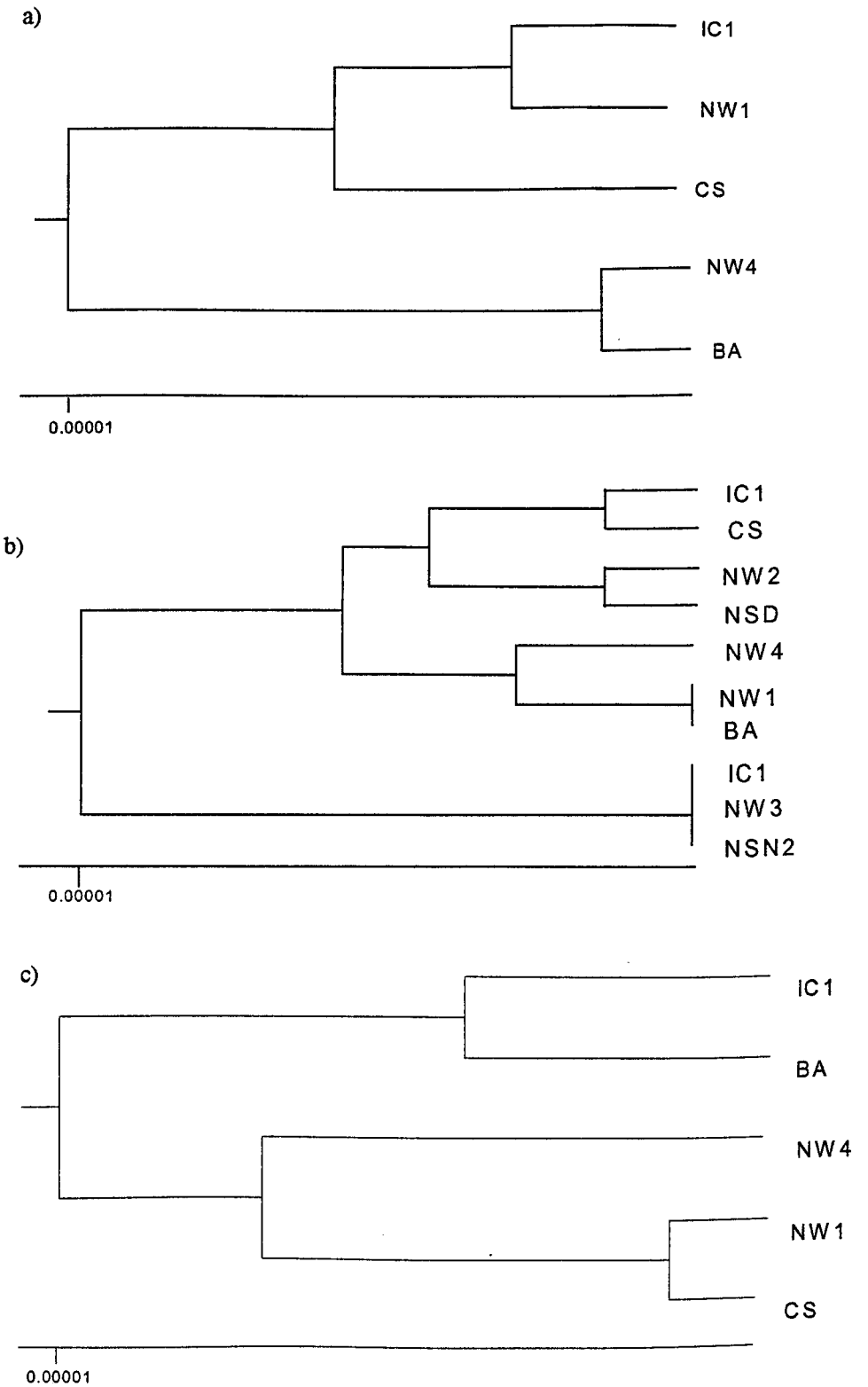


Figure 7.2. ND3/4(a), ND5/6(b) and combination of ND3/4 and ND5/6 (c) regions of mtDNA phenogram based on the distance matrix resulting from the estimation of interpopulation nucleotide divergence (Excluding Pacific sample). For location of the samples see Chapter 2, Figure 2.1.

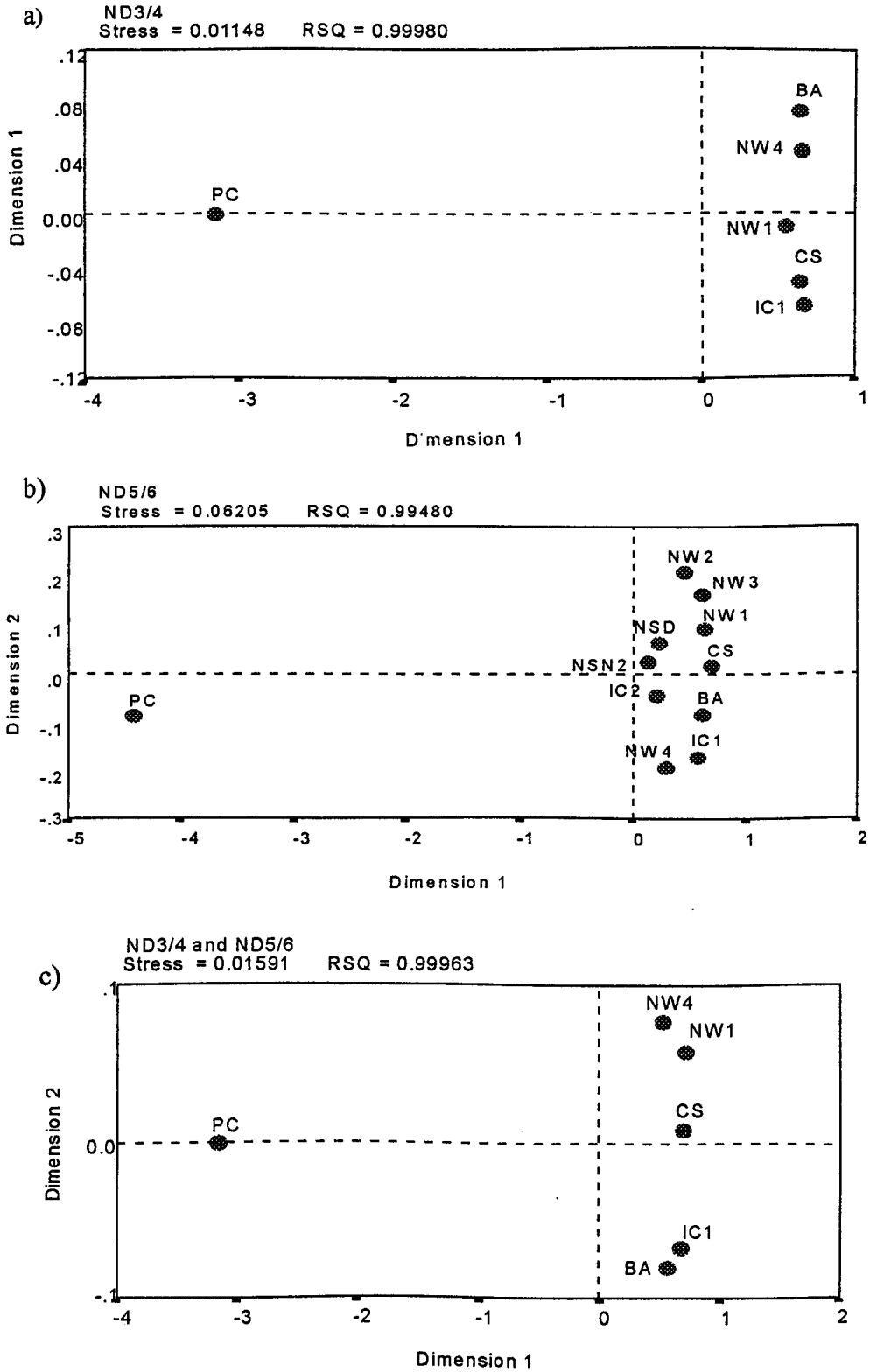


Figure 7.3. Multi dimensional scaling analysis of haplotype frequency data for ND3/4 (a), ND5/6 (b) and combination of ND3/4 ND5/6 (c) regions of mtDNA among herring samples. For location of the samples see Chapter 2, Figure 2.1.

CHAPTER 8

NUCLEAR DNA DIFFERENTIATION: MICROSATELLITES

8.1 Introduction

The specific features of marine species such as high dispersal ability, high mobility, large population size as well as a scarcity of clear geographical barriers in the marine environment, have led to certain doubts concerning the power of the genetic approaches to discriminate stocks or elucidate population structure (Graves *et al.*, 1984; Smith *et al.*, 1990; Hedgecock, 1994; Palumbi, 1994). Indeed, allozymes and mitochondrial-DNA-based genetic studies on marine pelagic and demersal fishes have usually revealed lower levels of genetic subdivision when compared with freshwater fishes (Gyllensten, 1985; Hedgecock, 1994; Ward *et al.*, 1994b). Therefore attention is increasingly concentrated on the development of more polymorphic markers to improve prospects for studies of population structure.

Recently the assay of variable number of tandem repeat (VNTR) loci has been introduced as a potentially powerful tool for studies of genetic variation within and among fish populations (Taggart & Fergusson, 1990a & 1990b; Wrigth & Bentzen, 1994; García de Leon *et al.*, 1997). Initial studies focused on the use of minisatellite DNA loci for population differentiation (Gilbert *et al.*,

1990; Bentzen *et al.*, 1991), however, more recently, microsatellite DNA has become the focus for hypervariable single locus markers of population studies (Goff *et al.*, 1992; Slettan *et al.*, 1993; Estoup *et al.*, 1993; O'Connell *et al.*, 1996; Bentzen *et al.*, 1996; García de Leon *et al.*, 1997).

Microsatellite loci consist of tandemly repeated short core sequences of one to five nucleotides, flanked by regions of non-repetitive DNA (Beckmann & Weber, 1992), that often show a high level of polymorphism mostly due to length variations in the number of the tandemly repeated core sequences (Litt & Lùty, 1989; Tautz, 1989; Weber & May, 1989). Using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) microsatellite loci can be amplified from a minute amount of DNA sample, which speeds up the processing of large sample numbers required for population surveys.

Microsatellite loci characteristically exhibit high levels of length mutation, resulting in extensive allelic variation and high levels of heterozygosity ranging from 59% to 90 % (Taggart & Fergusson, 1990b; Wright, 1993; Brooker *et al.*, 1994; Bentzen *et al.*, 1996), thus making them especially attractive in stock identification of marine fishes often showing low levels of variation using allozymes or even mtDNA (Bentzen *et al.*, 1991; Wright, 1993; Wright & Bentzen, 1994). For example, Bentzen *et al.* (1996) found significant microsatellite heterogeneity among Atlantic cod populations in contrast to surveys of allozymes (Pogros, 1995) and mtDNA (Smith *et al.*, 1989; Carr & Marshall, 1991) variation. Highly significant inter-population heterogeneity was detected among brook charr populations from five lakes in La Mauricie national park by Angers *et al.* (1995). Significant genetic structuring was revealed by

microsatellite investigation among Pacific herring populations from the Gulf of Alaska and the Bering Sea using microsatellites (O'Connell *et al.*, 1996).

Since selection is expected to operate on different loci in a heterogeneous fashion it is difficult to determine whether gene flow or selection is sustaining either homo- or heterozygosity between populations, though it is not possible to ensure selective neutrality for any genetic markers (Ferguson & Mason, 1981). However microsatellites are believed to be neutral for selection or at least the strength of selection on microsatellites is thought to be weak (Jarne & Lagoda, 1996), thus providing an effective estimative of gene flow.

Atlantic herring exhibits a mosaic of spawning aggregations throughout the North Atlantic Ocean. Most spawning aggregations are thought to represent separate stocks, and this hypothesis has been supported by morphological characters, and spawning time and spawning location data (Svetovidov, 1963; Parrish & Saville, 1965; Haegele & Schweigert, 1985; Smith & Jamieson, 1986). Most of the genetic studies based on allozymes have shown genetic uniformity between the Northwest and Northeast Atlantic (Jørstad *et al.*, 1991), as well as within the Northeast Atlantic (Anderson *et al.*, 1981; Jørstad & Nævdal, 1981; Kornfield *et al.*, 1982; Ryman *et al.*, 1984; Jørstad & Pederson, 1986; King *et al.* 1987). There are a few mtDNA studies showing genetic homogeneity or a low level of genetic differentiation over large geographic distances, supporting the proposed complex stock structure of Atlantic herring (Kornfield & Bagdanowicz, 1987; Stephenson & Kornfield, 1990; Dahle & Eriksen, 1990).

In contrast to the genetic homogeneity seen in studies of large geographic regions, discrete localised allozymic differentiation has been detected

in Norwegian fjord populations (Jørstad & Nævdal, 1981; Jørstad & Nævdal, 1983; Jørstad *et al.*, 1994; Chapter 6). Most notably the Balsfjord and Trondheimsfjord herring are very different from those of other areas and also from each other (Jørstad *et al.*, 1994; Jørstad & Nævdal, 1981; Chapter 6). In addition, the indication of allozymic differentiation between Icelandic summer-spawners and Norwegian-spring spawners has also been reported (Turan *et al.*, 1997).

The limited data available show the degree of genetic divergence between Atlantic and Pacific herring (Grant, 1986; Jørstad *et al.*, 1994; Domanico *et al.*, 1996), and indicate that the degree of divergence is enough to consider them as constituting a different species.

In the present context, the aim of the microsatellite analysis is :

1) to study the utility of PCR-based microsatellite analysis to examine herring stock structure;

2) to investigate the genetic structure of herring populations in the Norwegian sea;

3) to explore the level of genetic divergence at microsatellite loci between Atlantic and Pacific herring.

8.2 Materials and Methods

8.2.1 Laboratory procedure

The herring samples used in this study were collected from the Norwegian Sea and Barents Sea, comprising Icelandic summer-spawners (IC1), and Norwegian spring-spawners (NW1 & NW2), two Norwegian fjord samples (NW3 & NW4) and also one Pacific sample (PC) (see Chapter 2, Fig 2.1).

In the polymerase chain reaction (PCR), the same extracted DNA used in mtDNA analysis (Chapter 7) was used to amplify four loci using primers developed by O'Connell *et al.* (1996) for Pacific herring, *Clupea pallasi* L. The primer sequences were:

Locus *Cha17*

5'- GAG ACT TAC TCT CAT CGT CC -3' 20 mer

5'- GCA CAG TAG ATT GGT TCC AC -3' 20 mer

Locus *Cha20*

5'- GTG CTA ATA GCG GCT GCT G -3' 19 mer

5'- TTG TGG CTT TGC TAA GTG AG -3' 20 mer

Locus *Cha123*

5'- GGG ACG ACC AGG AGT G -3' 16 mer

Locus 5'- AAA TAT AGT TTT ATG ATT GGC T -3' 22 mer

Locus *Cha63*

5'- TGC CTG CTG AAG ACT TCC -3' 18 mer

5'- CCC CTA AAT GTG TTC TTT TAG C -3' 22 mer

One primer from each pair was labelled with a Cy5 fluorescent dye group, which allows detection and sizing of alleles on an *ALFexpress* automated DNA sequencer (Pharmacia Biotech, U.K.). PCR reactions were performed in a 10 µl reaction volume containing: 0.05 µl units of *Taq* polymerase supplied by Bioline Ltd (U.K), 1µl 10x reaction buffer supplied with the *Taq*, 1 µl Tween (1 %), 0.8 µl dNTP (2 mM stock solution), 0.2 µl $MgCl_2$ (50 mM stock solution), 0.5 µl each primer (10 µM stock solution) and 1 µl (50-100 ng) of extracted sample µMW DNA (Table III. 1, Appendix III). Thermal cycling was performed in an Omnigene Thermocycler (Hybaid) with the temperature and cycle profile given in Table III. 2 Appendix III. 5 µl PCR product was checked (for non-specific products and sufficient yield) on an 0.8% agarose minigel containing 5 µl ethidium bromide. 4 µl stop mix (Pharmacia Biotech) and 0.5 µl each of internal sizing standard (67 pb and 259 pb) sequence (VanOppen *et al.*, 1997) were added to 1 µl of each PCR product, and the sample mixture was denatured at 90 °C for 5 minutes. 2 µl of the sample mixture was loaded into each lane on the ALFexpress DNA sequencer. 6 % denaturing gels (National Diagnothisis, Sequagel XR) in 0.6 x TBE buffer were used for electrophoresis at 55 °C, at 1900 volt, 85 mA current, 44 W power. 40 individual sample can be load at each run, and the same gel can be re-loaded 3-4 times. When samples pass through

the gel a laser read (or detect) the primers, sizing standards and alleles according to their size or molecular weight, and monitor them as a pick on computer screen (Fig. 8.1a, 8.1b, 8.1c). Data collection and sizing of alleles were estimated using the Fragment ManagerV1.2 software (Pharmacia Biotech, U.K.). the time of run (Fig. 8.1a and 8.1b) is considered to estimate size of alleles. For example, the size of the standards is given to the Fragment Manager which convert the time to base pairs according to their running time and estimate allele sizes on the basis of the size of standards.

8.2.2 Data analysis

Recent publications have shown that the analysis of population structure using microsatellites depends on the application of the appropriate mutational model (Zhivotovski & Feldman, 1995; Goldstein *et al.*, 1995; Garza *et al.*, 1995; Bentzen *et al.*, 1996; García de Leon *et al.*, 1997). Two models of mutation have been proposed for the study of microsatellites (Estoup *et al.*, 1995): first, the infinite allele model (IAM) in which each mutation creates a new allele at a given rate (u); second, the stepwise mutation model (SMM) in which mutations add or subtract (with equal probability u) a single unit to/from the current allele, therefore most mutations involve the gain or loss of only one or two repeat units (Shriver *et al.*, 1993; DiRienzo *et al.*, 1994). Insufficient data exist at present to confirm which mutation model microsatellite loci most closely follow. Therefore,

in the present study both types of approach were followed using different statistical software packages, taking into account both of the mutation models.

The level of genetic differentiation among populations was calculated with two methods; first, R_{ST} (Slatkin, 1995) based on the SMM of mutation using the computer program RST-CALC (Goodman, 1996), and F-statistics (Weir & Cockerman, 1984) based on the IAM of mutation using F-STAT (Goudet, 1995) and GENEPOPv2 (Raymond & Rousset, 1995). Genotype frequencies in each population at each locus were tested for conformity to Hardy-Weinberg equilibrium using Fisher's Exact test. Differences in microsatellite allele frequencies between samples were assessed using Fisher's exact test (GENEPOPv2, Raymond & Rousset, 1995).

Multidimensional scaling analysis (MDS) was used to summarise F_{ST} and R_{ST} distances derived from allele frequency data over all 4 microsatellite loci among samples. F_{ST} and R_{ST} distance data were also analysed with clustering algorithms (UPGMA; unweighted pair group with mathematical average) using PHYLIP version 3.1 (Felsenstein, 1993).

8.3 Results

All four microsatellites loci were highly polymorphic in all samples with many different alleles exhibiting high levels of heterozygosity (Table 8.1). The total number of alleles per locus varied between 32 at *Cha17*, and 49 at *Cha123*, and the observed heterozygosity within each sample ranged from 70% to 96%. Allele frequencies and distribution at the *Cha17, Cha20, Cha123, Cha63* loci for all samples are given in Table 8.2 and Fig. 8.2 respectively.

Genotypic frequencies were in Hardy-Weinberg equilibrium at the majority of polymorphic loci ($P > 0.05$) in samples, though some significant departures (7 in 24 tests; all representing deficity of heterozygotes) were detected (Table 8.2), but none of these at the *Cha20* locus.

Pairwise comparisons of all loci with Fisher's exact test between samples showed highly significant differences ($P<0.001$) in allele frequency between all samples (Table 8.3). Especially the Trondheimsfjord (NW4) and Norwegian spring-spawner (NW1) samples showed highly significant differences at the highest number of loci from the other Atlantic samples. The Pacific herring sample also revealed highly significant differences from all the Atlantic herring samples.

The level of genetic differentiation among populations sampled was highly significant as revealed by both R_{ST} and F_{ST} values (Table 8.4). Thus the amount of genetic subdivision among Atlantic samples ranged from 0.011 at *Cha17* to 0.046 at *Cha63* with the F_{ST} analysis, and from 0.030 at *Cha17* to 0.52 at *Cha63* with R_{ST} analysis. In pairwise comparisons of both R_{ST} and F_{ST}

values (Table 8.5), the R_{ST} value was highest between Icelandic summer-spawning (IC1) and the Norwegian spring-spawning sample (NW1) collected from north-eastern coast of Norway. However, the lowest values were detected between Icelandic and Norwegian spring-spawning (NW2) samples (0.0350), and between the Norwegian spring-spawning (NW2) and Balsfjord (NW3) (0.0354). On the other hand, F_{ST} values were highest between Icelandic summer-spawner (IC1) and Norwegian spring-spawner (NW2) samples, and lowest in concordance with the R_{ST} values between the Norwegian spring-spawning (NW2) and Balsfjord (NW3) samples. When the Pacific sample was excluded in both analyses, significant levels of differentiation were detected.

The differences in allele frequency among the samples were also summarised by the MDS and UPGMA (Fig. 8.3a and 8.4a). The MDS of F_{ST} and R_{ST} values showed the Norwegian spring-spawning (NW1) and Trondheimsfjord (NW4) samples to be clearly most divergent from all other Atlantic and Pacific herring samples with respect to allele frequency. In the MDS of R_{ST}, NW1 was more closely positioned with NW4 than was in MDS of F_{ST}, and these samples seem considerably more divergent than Pacific herring from the other Atlantic herring samples. In the MDS of R_{ST}, first the Norwegian spring-spawning (NW1) and second Trondheimsfjord herring samples were highly divergent from the other samples. Icelandic and Norwegian spring-spawning sample (NW2) were close to each other with a higher divergent of Balsfjord herring. Pacific sample was positioned in the middle of the chart. The MDS of F_{ST} revealed the similar pattern with a higher divergence of the Pacific herring than Trondheimsfjord herring sample.

An isolation with distance analysis was undertaken by comparing pairwise values of R_{ST} and F_{ST} with geographical distance between localities. This relationship was not significant between populations ($P > 0.05$; Mantal test), which suggests that geographic distance is not a factor in determining the extent of the differentiation of the populations sampled.

The UPGMA dendrogram showed samples to be clustered in a similar way to the pattern shown by the MDS analyses: NW1 was the most divergent sample from the all other samples, though with F_{ST} and R_{ST} values, and the Pacific herring was clustered more closely to NW1 than to NW4 with F_{ST} values.

8.4 Discussion

This study provides the first report of a microsatellite analysis in Atlantic herring. The preliminary investigation revealed that microsatellite loci were highly polymorphic in herring, having 32-49 alleles per locus, and with an expected heterozygosity ranging between 0.76 to 0.96, with a mean of 0.88. Such high heterozygosity is expected for microsatellite loci, given their high mutation rates, enhancing their discriminatory potential in population studies. Microsatellite surveys on marine species, in contrast to anadromous and freshwater species, are still rare. In comparison, the same level of variation using the same primers have been detected in marine species: Pacific herring, *Clupea pallasi* (expected heterozygosity 88 %; O'Connell *et al.*, 1996); and a similar level of variation: Atlantic cod, *Gadus morhua* (expected heterozygosity 85%;

Brooker *et al.*, 1994); and lower level of variation: in anadromous species, Atlantic salmon, *Salmo salar* (expected heterozygosity 35%; Tessier *et al.*, 1995); and rainbow trout, *Oncorhynchus mykiss* (expected heterozygosity 25%; Presa & Guyomard, 1996); and euryhaline species, European sea bass, *Dicentrarchus labrax* (expected heterozygosity 79 %; García de Leon *et al.*, 1997). A review of these studies also indicates that the detected high level of polymorphism at microsatellite loci provides a marker system capable of detecting differences among closely related populations.

In the pairwise comparisons, highly significant allele frequency differences at microsatellite loci were detected among all samples. The detected pattern of genetic differentiation revealed by all the distance measures (Fisher's exact test, F_{ST} and R_{ST}) indicates that each population sampled possesses specific genetic identity.

The detected significant allele frequency differences of Icelandic summer-spawners supports observed genetic differentiation at allozyme loci (Chapter 6), and mtDNA (Chapter 7), also in morphological charecters (Chapter 3, 4, 5).

Interestingly in the present study, the Norwegian spring-spawner sample (NW1) collected from the north-eastern coast of Norway showed highly significant genetic differences from the other Norwegian spring-spawner sample (NW2), and from all the other samples. Indeed, differences in morphological characters of herring between northern and southern regions of the Norwegian Sea have been reported (Debarros & Holst, 1995; Stenevik *et al.*, 1996). Here, the detected genetic differences within the Norwegian spring-spawners may

indicate ongoing genetic sub-structuring between coastal Norwegian waters, as well as between Norwegian fjord populations as reported in previous studies (Jørstad & Nævdal, 1981; Jørstad *et al.*, 1994; Chapter 6). Therefore there may be a self-recruiting herring population in the northern Norwegian Sea, and selection as a cause of the observed differentiation is a weak possibility though the other molecular genetic (Chapter 6 & 7) and phenotypic (Debarros & Holst, 1995; Stenevik *et al.*, 1996) markers revealed the genetic and phenotypic heterogeneity of this population. Additional repetitive sampling from this location would clarify the temporal genetic distinctness of patterns detected.

The microsatellite diversity of Balsfjord sample revealed highly significantly differences at least at three loci from all the other samples with the exception of the Norwegian spring-spawner sample (central Norwegian sea; NW2), which revealed only one significantly different locus from the Balsfjord (NW3) sample. However, allozyme and mtDNA markers on the same samples did not show significant genetic differences of the Balsfjord sample (Chapter 6 & 7) in contrast to previous allozyme and mtDNA studies (Jørstad & Nævdal, 1981; Jørstad *et al.*, 1994). Therefore there might be a possible sampling of Norwegian spring-spawners and a few Balsfjord herring in the fjord at the present study. Norwegian spring-spawners migrate to Balsfjord for feeding during the non-reproductive phase and occur in the upper water layers and leave the fjord before spawning (Jørstad & Nævdal, 1981: Jørstad & Pedersen, 1986; Jørstad *et al.*, 1994). The timing (September), and sampling depth (15-20 m) raises the possibility of the sampling only a few Balsfjord herring in the fjord. On the other hand, the findings also suggest that microsatellites are sensitive enough

to detect allele frequency differences caused by the mixing of few Balsfjord herring.

The Trondheimsfjord sample revealed high genetic differences from all the other samples, reinforcing the findings observed from allozymic studies (Jørstad & Nævdal, 1981: Turan *et al.*, 1997). The Trondheimsfjord sample was collected using different sampling gear, and in deeper waters (30-35m) within the fjord, supporting the existence of distinct stocks in deeper waters. It is therefore important to consider localised migratory behaviour and depth distribution when collecting from these waters which underline the importance of assessing the variability within a side as well as between sites.

A strong pattern of population subdivision, reflected in the significantly high overall F_{ST} and R_{ST} estimates, indicates a restricted or absent gene flow among populations. In both analyses, a strong inter-population differentiation at the *Cha63*, locus and low inter-population differentiation at *Cha17* locus were detected. However when the Pacific sample was excluded from the data set, an overall higher level of differentiation in R_{ST} value and a very similar level of subdivision in the F_{ST} values were observed, indicating a higher level of differentiation at microsatellite loci within the Atlantic herring than between the Atlantic and Pacific herring. Nevertheless, the pairwise comparisons (Table 8.5 & 8.7) revealed that the source of high F_{ST} and R_{ST} at microsatellite loci is caused mainly by the Norwegian spring-spawner sample (NW1) sample. Also the level of genetic differentiation of the Trondheimsfjord herring (R_{ST}) was also higher than between the two herring species. Greater genetic differentiation at microsatellite loci within species compared to between species might be

observed. Such a genetic pattern may be caused by the effect of population size, bottleneck, reduced inter-breeding, or selection in one population causing extreme genetic differentiation at one or several loci. Such genetic divergence may, in the short term, be greater than that expected under the mutation/drift equilibrium condition, which is thought to determine the average level of differentiation seen between two separate species. Microsatellites have also been reported to be poor markers for phylogenetic inferences due to detected smaller genetic differences between species than observed with other genetic markers (Garza *et al.*, 1995; Jarne & Lagoda, 1996). The origin of the unexpected low differentiation between species is still controversial. Low differentiation at microsatellite loci between species may be attributed to the mutation process (Zhivotovski & Feldman, 1995; Jarne & Lagoda, 1996), which is biased towards larger alleles, and increases with allelic size. Small alleles tend to increase in size while large alleles tend to decrease in size. Alternatively, microsatellites may be under selective pressure eliminating larger alleles (Garza *et al.*, 1995). Therefore, the expected large alleles between species on an evolutionary scale may be eliminated through mutations or selection which cause detection of low differences among species (Jarne & Lagoda, 1996). There has been no microsatellite study to date involving two fish species with which, to compare the present findings. However, the present results support the studies above (involving mammals and turtles) that microsatellites are not a good choice for fish species identification, however a powerful molecular tool for population identification.

The observed pattern of differentiation among samples may reflect the geographical separation of populations sampled. For example, if the differentiation among populations is determined by the limited dispersal of individuals, the proportion of among-population allele frequency variation should increase with increasing geographic distance between localities. Comparing the pairwise values of R_{ST} and F_{ST} with geographic distance (Mantel test) showed that differentiation by distance is not a significant factor across the geographic range. This suggests that geographic distance is not an contributory factor to the detected population heterogeneity among these stocks. The effect of geographic distance on the levels of among population differentiation is evident in allozymes (e.g. Mork *et al.*, 1985). In Atlantic cod the genetic distances between populations were significantly correlated with geographic distance, so geographic distance was an inhibiting factor to gene flow (Mork *et al.*, 1985). Therefore, the present findings may suggest that topographic and hydrographic factors may be more important in structuring genetic variation among these populations. For example, characteristic environmental conditions of each population in terms of adaptation such as low temperatures in northern Norwegian coasts, and closed geographic structure of fjords in terms of restricting gene flow may be the reason of the observed differentiation among herring populations.

So far, microsatellite studies have revealed no consensus on which test statistics best discriminated between the populations sampled. In the present study, a variety of analyses were used to reach the above conclusions. Fisher's Exact test seems to be a sensitive indicator of genetic differentiation using

Monte Carlo methods, and yielded similar results to the R_{ST} and F_{ST} analyses. Unlike F_{ST}, R_{ST} incorporates information about the size of microsatellite alleles, thus the inclusion of allele size is expected to make R_{ST} more informative (Slatkin, 1995). Nonetheless, estimates of R_{ST} in pairwise comparisons can be relatively large when sample sizes differ, in contrast to F_{ST}, which is less effected by unequal sample sizes (Bentzen *et al.*, 1996). In the present study, the only unique sample (30 individuals) is the Pacific herring sample which showed a notably different pattern of differentiation in F_{ST} estimates, as revealed by pairwise comparison of the samples (Table 8.7; Fig. 8.3a, 8.3b; Fig. 8.4a, 8.4b). Therefore, for the analysis of R_{ST}, it seems that it may be important to equalise sample sizes (Bentzen *et al.*, 1996). Moreover, from the remaining samples it is difficult to conclude which is the best model, though they each revealed the same pattern of differentiation. However combined use of the models provide a better understanding of population interactions in the present microsatellite data.

The detected departures from Hardy-Weinberg equilibrium expectation can arise from several factors including, mis-scoring of alleles, null alleles, inbreeding, assortative mating, selection against heterozygotes, or some combinations of these factors. Two microsatellite alleles may have the same size or even the same sequence (Homoplasy) which are not the product of a single mutation event, as required by the stepwise mutation model (Slatkin, 1995; Goldstein *et al.*, 1995). This can cause scoring errors and lead to artificial excesses of homozygotes. Even rare mis-scoring of heterozygotes as homozygotes on a gel, though having one or two homozygotes more than expected, can cause significant deviations from Hardy-Weinberg equilibrium

without significantly effecting the overall allele frequencies in the data set (P. W. Shaw, *personal Communication*). Null alleles that are not amplified or weakly amplified also cause an excess of homozygotes to be detected. There has been an increasing number of studies reporting null alleles at microsatellite loci (e.g. Callen *et al.*, 1993; Allen *et al.*, 1995; Pemberton *et al.*, 1995). The null alleles may be due to PCR failures for large alleles (>150bp) (large allele drop out) or changes in primer site. In the present study this may not be the case since large alleles are abundant in the data set. The inbreeding and assortative mating effect is thought to be weak for marine fish, since during spawning, large number of females and males concentrate in mid-water, thereby increasing the chance of panmixis. Microsatellites are believed to be selectively neutral, however Slatkin (1995) has demonstrated that a microsatellite linked to a selected locus will exhibit a smaller degree of genetic variability. In the present data, the *Cha123* and *Cha63* loci showed the majority of the deviations from Hardy-Weinberg equilibrium, and were also highly variable with the largest number of total alleles at the *Cha123* locus. Therefore, the detected high number of alleles at microsatellite loci may eliminate selection as a possible effect.

In consequence, no single hypothesis could conclude unambiguously the detected excess of homozygotes alone. However a combination of miss-scoring and null alleles could the most likely explanation.

Management of herring in the Northeast Atlantic is currently based on demographic data related to the morphological and ecological discreteness of stocks (Cushing, 1975; Jakobsson, 1985) and because genetic markers have usually revealed genetic homogeneity among geographically isolated spawning

aggregations (Anderson *et al.*, 1981; Jørstad & Nævdal, 1981; Kornfield *et al.*, 1982; Ryman *et al.*, 1984; Jørstad & Pederson, 1986; King *et al.* 1987; Kornfield & Bagdanowicz, 1987; Dahle & Eriksen, 1990; Jørstad *et al.*, 1991; Koskiniemi & Parmanne, 1991). The findings in this study reveal genetic heterogeneity among all samples. The highly significant F_{ST} and R_{ST} values indicate the absence or restriction of gene flow among the populations sampled. Therefore, depletion of any one of these populations may not be recovered by recruitment from the others. The management implications of such population differentiation depends on its temporal and spatial integrity. Consistent genetic differences seen in at least two sampling events for presumptive populations would support their temporal and spatial integrity. Thus the marked genetic heterogeneity of the Balsfjord and Trondheimsfjord populations provides evidence for their temporal and spatial integrity compared to the previous reports (Jørstad & Nævdal, 1981; Jørstad *et al.*, 1994; Chapter 6), and thus strengthens their status as distinct management units.

In summary, the data obtained from the genetic analysis of 4 polymorphic microsatellite loci of herring clearly demonstrates the existence of genetically differentiated populations of Northeast Atlantic herring, demonstrating their usefulness for detecting genetic differentiation in a highly mobile, pelagic teleost. Moreover, the microsatellite technique provides a new perspective on past estimates of the low levels of genetic differentiation detected using allozyme and mtDNA data.

Table 8.1. Observed microsatellite alleles, allele number, size range and observed (H_{obs}) and expected (H_{exp}) heterozygosities for 4 microsatellite loci in each herring sample and in loci in each herring sample and in pooledsamples. Samples referred to in the text were: Icelandic summer spawners (first year) (IC1), Norwegian spring-spawner (Northeast coast of Norway) (NW1), Norwegian spring-spawner (central Norwegian Sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Pacific herring (PC).

	Sample						
Locus	IC1	NW1	NW2	NW3	NW4	All	PC
Cha17							
No. of alleles	22	22	21	25	22	**32**	21
Allele size	104-152	102-164	104-148	102-168	100-170	**100-170**	108-166
H_{obs}	0.90	0.94	0.93	0.94	0.88	**0.92**	0.93
H_{exp}	0.91	0.93	0.94	0.91	0.87	**0.91**	0.94
Cha20							
No. of alleles	28	13	27	26	27	**43**	15
Allele size	116-202	150-180	116-198	120-204	120-206	**116-206**	132-204
H_{obs}	0.74	0.86	0.96	0.88	0.84	**0.85**	0.77
H_{exp}	0.79	0.87	0.94	0.93	0.89	**0.88**	0.91
Cha123							
No. of alleles	16	17	22	17	32	**49**	18
Allele size	110-144	104-186	116-214	114-152	118-206	**104-214**	120-192
H_{obs}	0.74	0.74	0.78	0.88	0.86	**0.80**	0.90
H_{exp}	0.84	0.86	0.76	0.83	0.96	**0.85**	0.93
Cha63							
No. of alleles	15	27	17	13	16	**32**	22
Allele size	144-172	112-174	118-174	142-178	134-170	**112-178**	122-178
H_{obs}	0.70	0.72	0.91	0.74	0.78	**0.77**	0.86
H_{exp}	0.77	0.92	0.92	0.87	0.88	**0.87**	0.93
Total H_{obs}	0.77	0.82	0.90	0.86	0.84	**0.84**	0.86
Total H_{exp}	0.82	0.89	0.89	0.88	0.90	**0.88**	0.93

Table 8.2. Allele frequencies at polymorphic microsatellite loci of herring samples. * Locus with corresponding sample is not in Hardy Weinberg equilibrium and its statistical significance;*P<0.05,**P<0.01, ***P<0.001. n, sample size. Samples referred to in the text were: Icelandic summer spawner (first year) (IC1), Norwegian spring-spawner (Northeast coast of Norway) (NW1), Norwegian spring-spawner (central Norwegian Sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Pacific herring (PC).

	Sample					
Allele	IC1	NW1	NW2	NW3	NW4	PC
Locus *Cha17*						
n	48 *	50	46	48	47	30
100	0.0	0.0	0.0	0.0	1.1	0.0
102	0.0	1.0	0.0	2.1	2.1	0.0
104	3.1	0.0	1.1	3.1	0.0	0.0
106	3.1	2.0	4.3	0.0	3.2	0.0
108	6.3	8.0	2.2	1.0	3.2	5.0
110	5.2	6.0	2.2	4.2	11.7	0.0
112	9.4	3.0	9.8	5.2	12.8	1.7
114	5.2	3.0	4.3	6.3	8.5	1.7
116	2.1	4.0	8.7	4.2	7.4	6.7
118	3.1	7.0	7.6	8.3	16.0	8.3
120	9.4	3.0	10.9	14.6	8.5	5.0
122	3.1	13.0	6.5	6.3	4.3	6.7
124	7.3	14.0	9.8	5.2	4.3	5.0
126	4.2	12.0	6.5	7.3	2.1	3.3
128	8.3	6.0	7.6	3.1	3.2	8.3
130	10.4	3.0	4.3	5.2	2.1	1.7
132	4.2	6.0	5.4	0.0	2.1	1.7
134	2.1	1.0	1.1	5.2	1.1	18.3
136	4.2	1.0	2.2	7.3	1.1	8.3
138	4.2	3.0	2.2	3.1	2.1	6.7
140	2.1	1.0	1.1	1.0	1.1	3.3
142	1.0	0.0	1.1	1.0	0.0	0.0
144	1.0	1.0	0.0	0.0	0.0	1.7
146	0.0	0.0	0.0	2.1	0.0	1.7
148	0.0	1.0	1.1	0.0	1.1	1.7
150	0.0	0.0	0.0	1.0	0.0	0.0
152	1.0	0.0	0.0	1.0	0.0	0.0
154	0.0	0.0	0.0	0.0	0.0	1.7
164	0.0	1.0	0.0	1.0	0.0	0.0
166	0.0	0.0	0.0	0.0	0.0	1.7
168	0.0	0.0	0.0	1.0	0.0	0.0
170	0.0	0.0	0.0	0.0	1.1	0.0

Table 8.2. Continued.

Allele	IC1	NW1	NW2	NW3	NW4	PC
Locus *Cha20*						
n	42	50	46	50	47	30
116	1.2	0.0	1.1	0.0	0.0	0.0
120	0.0	0.0	0.0	1.0	1.1	0.0
124	2.4	0.0	0.0	3.0	0.0	0.0
126	0.0	0.0	4.3	6.0	7.4	0.0
128	3.6	0.0	3.3	4.0	2.1	0.0
130	9.5	0.0	6.5	3.0	3.2	0.0
132	2.4	0.0	6.5	7.0	3.2	1.7
134	2.4	0.0	15.2	16.0	16.0	5.0
136	15.5	0.0	7.6	11.0	8.5	6.7
138	6.0	0.0	6.5	12.0	10.6	8.3
140	8.3	0.0	8.7	2.0	4.3	10.0
142	6.0	0.0	6.5	1.0	3.2	20.0
144	11.9	0.0	0.0	5.0	4.3	13.3
146	2.4	0.0	2.2	2.0	3.2	6.7
148	1.2	0.0	5.4	2.0	3.2	11.7
150	3.6	1.0	1.1	6.0	1.1	5.0
152	1.2	5.0	2.2	1.0	2.1	5.0
154	3.6	9.0	3.3	4.0	3.2	0.0
156	0.0	21.0	3.3	0.0	5.3	0.0
158	1.2	21.0	0.0	1.0	1.1	0.0
160	0.0	11.0	4.3	0.0	0.0	0.0
162	2.4	12.0	0.0	1.0	1.1	1.7
164	0.0	9.0	0.0	1.0	1.1	0.0
166	0.0	5.0	1.1	0.0	0.0	0.0
168	0.0	2.0	2.2	1.0	2.1	0.0
170	0.0	2.0	1.1	3.0	0.0	0.0
172	1.2	0.0	0.0	1.0	2.1	0.0
174	0.0	1.0	1.1	0.0	4.3	0.0
176	2.4	0.0	0.0	0.0	1.1	0.0
178	2.4	0.0	0.0	0.0	0.0	0.0
180	0.0	1.0	1.1	2.0	0.0	0.0
182	2.4	0.0	1.1	0.0	0.0	0.0
184	1.2	0.0	0.0	0.0	0.0	0.0
186	1.2	0.0	0.0	0.0	0.0	1.7
188	0.0	0.0	1.1	2.0	0.0	0.0
190	1.2	0.0	1.1	0.0	2.1	1.7
192	0.0	0.0	1.1	0.0	0.0	0.0
194	1.2	0.0	0.0	0.0	0.0	0.0
198	0.0	0.0	1.1	1.0	0.0	0.0
202	1.2	0.0	0.0	0.0	0.0	0.0
204	0.0	0.0	0.0	1.0	2.1	1.7
206	0.0	0.0	0.0	0.0	1.1	0.0

Table 8.2. Continued.

Allele	IC1	NW1	NW2	NW3	NW4	PC
Locus *Cha123*						
n	46	48 ***	39 *	49	50 ***	30
104	0.0	3.1	0.0	0.0	0.0	0.0
106	0.0	2.1	0.0	0.0	0.0	0.0
110	1.1	6.3	0.0	0.0	0.0	0.0
112	2.2	1.0	0.0	0.0	0.0	0.0
114	6.5	9.4	0.0	1.0	0.0	0.0
116	1.1	10.4	1.3	1.0	0.0	0.0
118	0.0	16.7	0.0	0.0	1.0	0.0
120	4.3	6.3	1.3	1.0	0.0	8.3
122	6.5	18.8	3.8	4.1	1.0	6.7
124	9.8	13.5	10.3	7.1	12.0	1.7
126	14.1	1.0	16.7	18.4	5.0	5.0
128	13.0	3.1	14.1	19.4	8.0	3.3
130	8.7	0.0	6.4	7.1	1.0	6.7
132	12.0	2.1	19.2	26.5	5.0	0.0
134	13.0	3.1	6.4	3.1	4.0	1.7
136	2.2	0.0	1.3	4.1	1.0	3.3
138	3.3	0.0	1.3	0.0	0.0	10.0
140	0.0	1.0	1.3	1.0	0.0	11.7
142	1.1	0.0	1.3	1.0	0.0	13.3
144	1.1	0.0	0.0	0.0	0.0	6.7
146	0.0	0.0	0.0	1.0	0.0	5.0
148	0.0	0.0	0.0	1.0	0.0	0.0
150	0.0	0.0	0.0	1.0	3.0	1.7
152	0.0	0.0	0.0	2.0	2.0	0.0
154	0.0	0.0	0.0	0.0	2.0	10.0
156	0.0	0.0	0.0	0.0	2.0	1.7
158	0.0	0.0	1.3	0.0	4.0	0.0
160	0.0	0.0	3.8	0.0	2.0	1.7
164	0.0	0.0	1.3	0.0	5.0	0.0
166	0.0	0.0	0.0	0.0	1.0	0.0
168	0.0	0.0	1.3	0.0	6.0	0.0
170	0.0	0.0	1.3	0.0	5.0	0.0
172	0.0	0.0	0.0	0.0	4.0	0.0
174	0.0	0.0	0.0	0.0	1.0	0.0
176	0.0	0.0	0.0	0.0	1.0	0.0
178	0.0	0.0	0.0	0.0	1.0	0.0
180	0.0	1.0	1.3	0.0	0.0	0.0
182	0.0	0.0	1.3	0.0	1.0	0.0
184	0.0	0.0	2.6	0.0	5.0	0.0
186	0.0	1.0	0.0	0.0	1.0	0.0
188	0.0	0.0	0.0	0.0	2.0	0.0
190	0.0	0.0	0.0	0.0	3.0	0.0
192	0.0	0.0	0.0	0.0	3.0	1.7
198	0.0	0.0	0.0	0.0	1.0	0.0
200	0.0	0.0	0.0	0.0	4.0	0.0
206	0.0	0.0	0.0	0.0	3.0	0.0
214	0.0	0.0	1.3	0.0	0.0	0.0

Table 8.2. Continued.

Allele	IC1	NW1	NW2	NW3	NW4	PC
Locus *Cha63*						
n	43	50 *	46 **	50	48 **	30
112	0.0	1.0	0.0	0.0	0.0	0.0
116	0.0	3.0	0.0	0.0	0.0	0.0
118	0.0	3.0	1.1	0.0	0.0	0.0
120	0.0	2.0	0.0	0.0	0.0	0.0
122	0.0	17.0	0.0	0.0	0.0	15.0
124	0.0	9.0	0.0	0.0	0.0	0.0
126	0.0	8.0	0.0	0.0	0.0	1.7
128	0.0	19.0	0.0	0.0	0.0	3.3
130	0.0	4.0	0.0	0.0	0.0	5.0
132	0.0	2.0	0.0	0.0	0.0	3.3
134	0.0	4.0	0.0	0.0	5.2	5.0
136	0.0	1.0	0.0	0.0	3.1	3.3
138	0.0	2.0	0.0	0.0	1.0	5.0
140	0.0	0.0	0.0	0.0	15.6	0.0
142	0.0	1.0	2.2	4.0	13.5	3.3
144	1.2	2.0	3.3	5.0	9.4	8.3
146	4.7	4.0	6.5	20.0	7.3	10.0
148	3.5	1.0	10.9	22.0	4.2	1.7
150	2.3	3.0	14.1	13.0	9.4	1.7
152	9.3	0.0	13.0	11.0	6.3	1.7
154	14.0	1.0	12.0	8.0	7.3	6.7
156	12.8	1.0	3.3	8.0	7.3	8.3
158	15.1	5.0	3.3	2.0	4.2	6.7
160	16.3	1.0	5.4	0.0	3.1	3.3
162	7.0	1.0	6.5	2.0	0.0	0.0
164	5.8	0.0	5.4	1.0	0.0	1.7
166	4.7	1.0	4.3	1.0	0.0	1.7
168	1.2	2.0	5.4	0.0	1.0	0.0
170	1.2	0.0	1.1	2.0	2.1	0.0
172	1.2	1.0	0.0	0.0	0.0	1.7
174	0.0	1.0	2.2	0.0	0.0	0.0
178	0.0	0.0	0.0	1.0	0.0	1.7

Table 8.3. Pairwise comparisons of microsatellite allele frequencies using all loci among all herring samples using Fisher's Exact test, and the overall significance levels (belove diagonal) are shown: ***P<0.001. Significantly different loci are presented with corresponding arabic number (above diagonal) in the brackets; 1 (*Cha17*), 2 (*Cha20*), 3 (*Cha123*), 4 (*Cha63*). Samples referred to in the text were: Icelandic summer spawner (first year) (IC1), Norwegian spring-spawner (Northeast coast of Norway) (NW1), Norwegian spring-spawner (central Norwegian Sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	IC1	NW4	NW2	NW3	NW1	PC
IC1	-	1*, 2***, 3***, 4***	2***, 4***	2***, 3***, 4***	1*, 2***, 3***, 4***	1***, 2***, 3***, 4***
NW4	***	-	3***, 4***	1***, 3***, 4***	1***, 2***, 3***, 4***	1***, 2***, 3***, 4***
NW2	***	***	-	4***	2***, 3***, 4***	1***, 2***, 3***, 4***
NW3	***	***	***	-	1***, 2***, 3***, 4***	2***, 3***, 4***
NW1	***	***	***	***	-	1***, 2***, 3***, 4***
PC	***	***	***	***	***	-

Table 8.4. F_{ST} and R_{ST} values among herring samples for each microsatellite locus. Statistical significance of F_{ST} and R_{ST} are indicated: ***P<0.001.

Locus	F_{IS}	F_{ST}	F_{ST} (excluding Pacific)	R_{ST}	R_{ST} (excluding Pacific)
Cha 117	-0.008	0.011***	0.008***	0.070	0.030
Cha 120	0.049	0.04***	0.039***	0.147	0.159
Cha 123	0.059	0.042***	0.038***	0.416	0.432
Cha 163	0.117	0.046***	0.051***	0.436	0.520
Total		0.035***	0.034***	0.302***	0.325***

Table 8.5. Measurement of genetic differentiation between pairs of the samples: unbiased R_{ST} values estimated by RST-CALC (below diagonal) and F_{ST} values estimated by FSTAT (above diagonal). Statistical significance of F_{ST} and R_{ST} are indicated: ***P<0.001. Samples referred to in the text were: Icelandic summer spawner (first year) (IC1), Norwegian spring-spawner (Northeast coast of Norway) (NW1), Norwegian spring-spawner (central Norwegian Sea) (NW2), Balsfjord herring (NW3), Trondheimsfjord herring (NW4), Pacific herring (PC).

Sample	IC1	NW4	NW2	NW3	NW1	PC
IC1	-	0.0237***	0.0086***	0.0225***	0.0553***	0.0267***
NW4	0.2941***	-	0.0124***	0.0230***	0.0521***	0.0301***
NW2	0.0350**	0.1586***	-	0.0042	0.0537***	0.0319***
NW3	0.0539**	0.2374***	0.0354**	-	0.0716***	0.0425***
NW1	0.4610***	0.4373***	0.4348***	0.3800***	-	0.0520***
PC	0.2351***	0.1911***	0.1566***	0.1020***	0.2937***	-

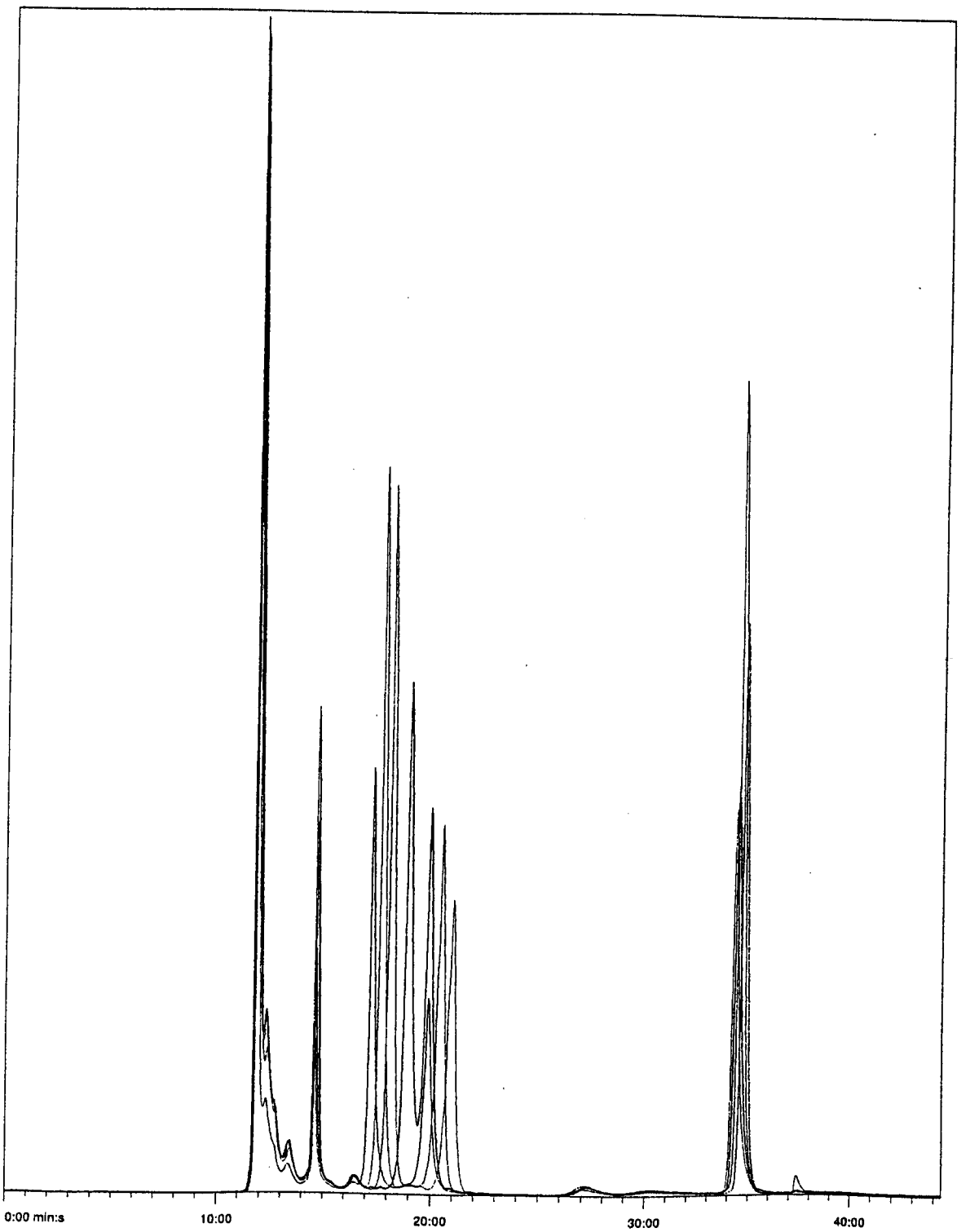


Figure 8.1a. The picks of primers, size standards and alleles read for four individual sample.

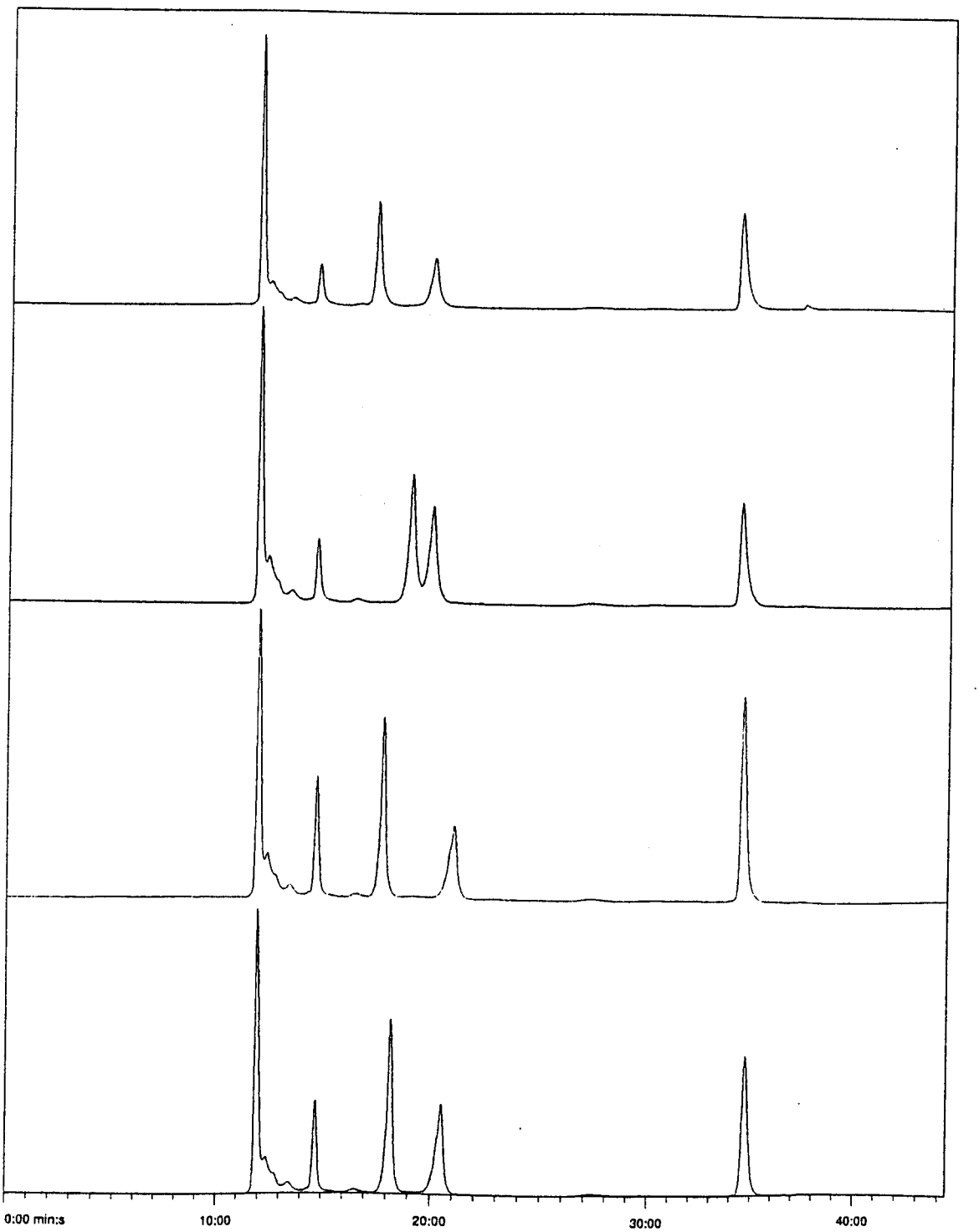


Figure 8.1b. Individual view of samples in the gel. Each lane represent a different sample. Primers, size standards and alleles pass through the gel according to their size and shown as time. Therefore primers, size standard, alleles and standard are distributed in each lane respectively (see Fig. 8.1c).

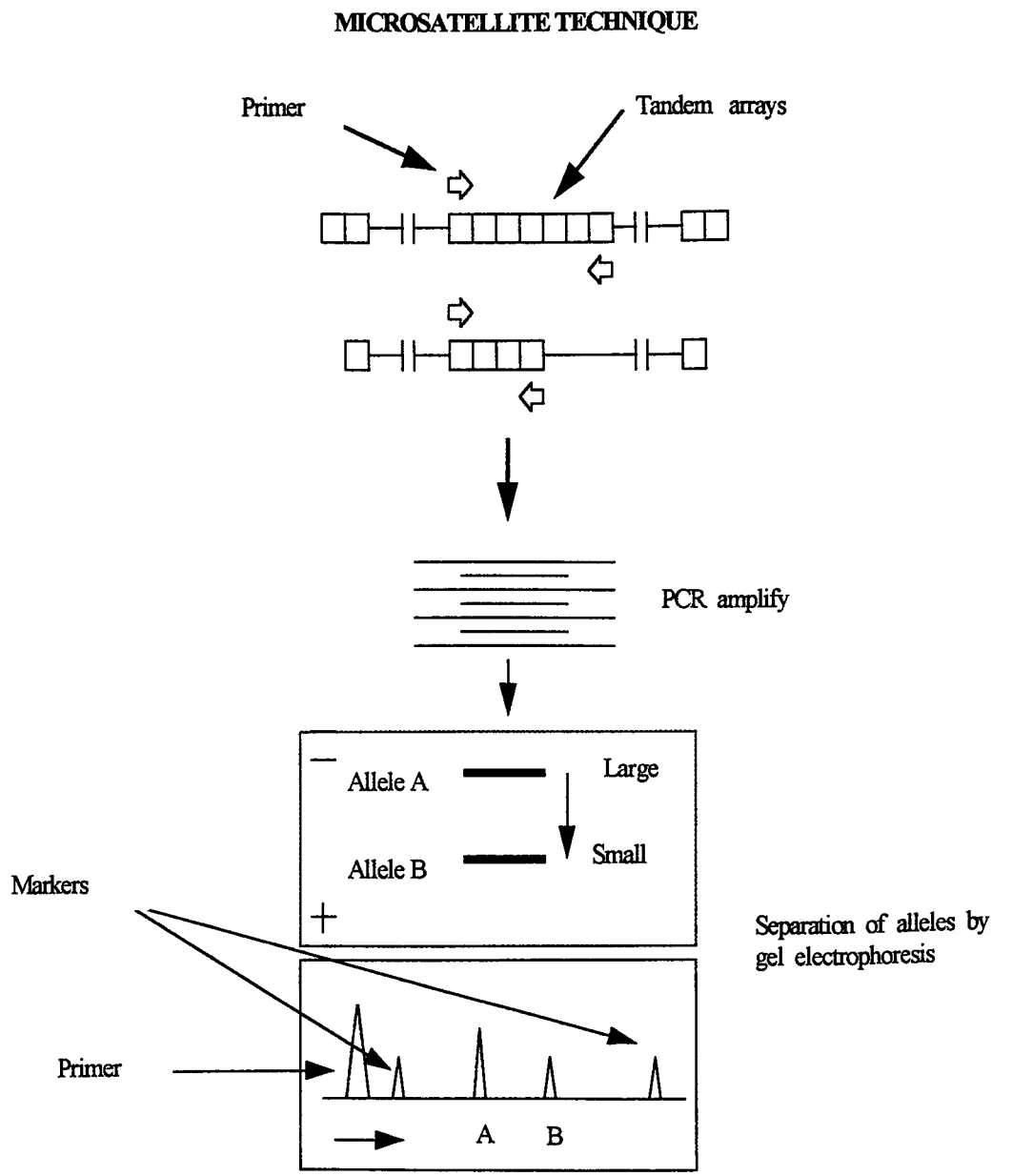


Figure 8.1c. Schematic illustration of microsatellite protocol from PCR to scoring alleles on gel.

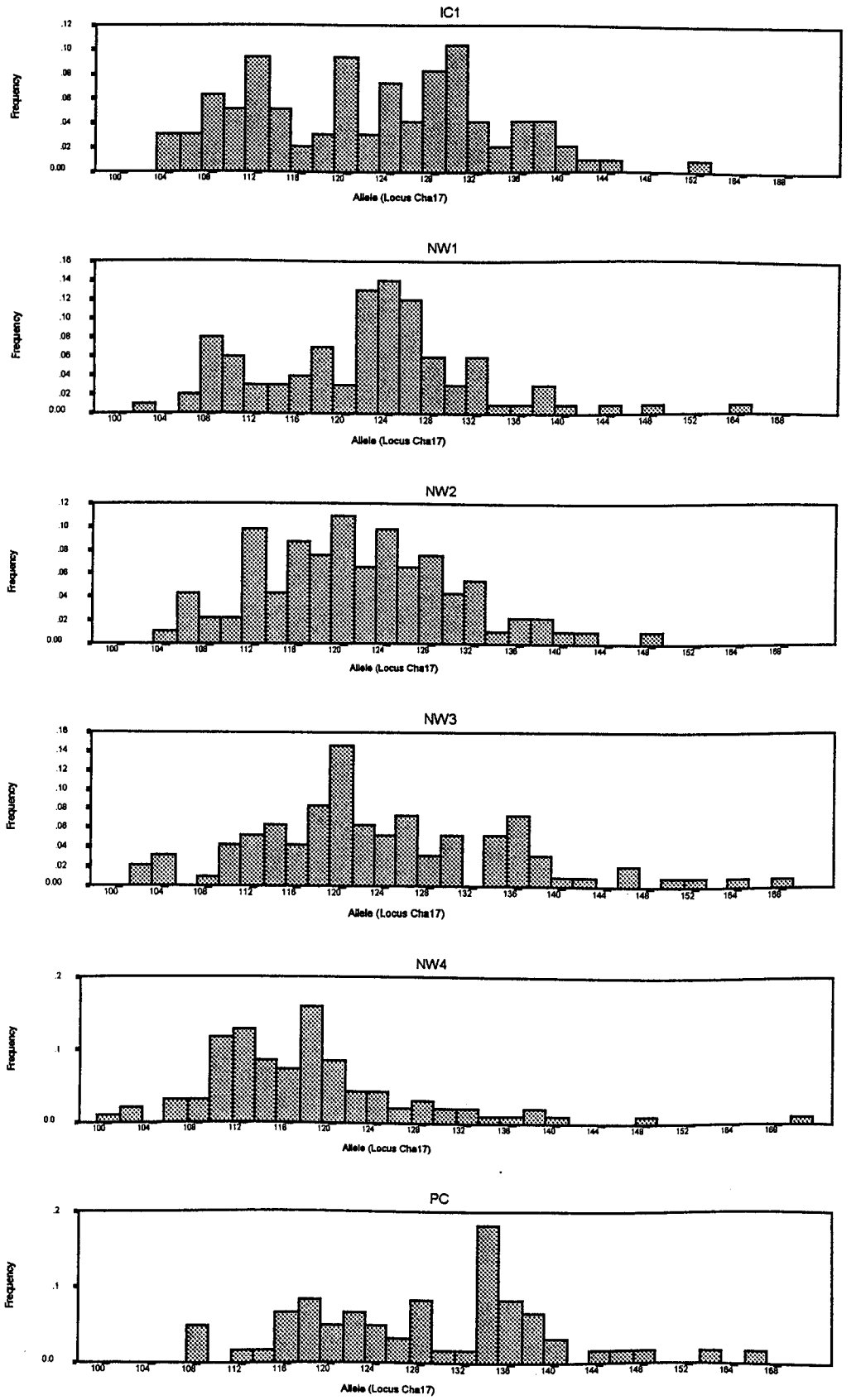


Figure 8.2. Frequency distribution of four microsatellite loci in herring samples. For abbreviation of samples (at the top of charts) and sampling locations see Chapter 2, Figure 2.1.

Figure 8.2. Continued.

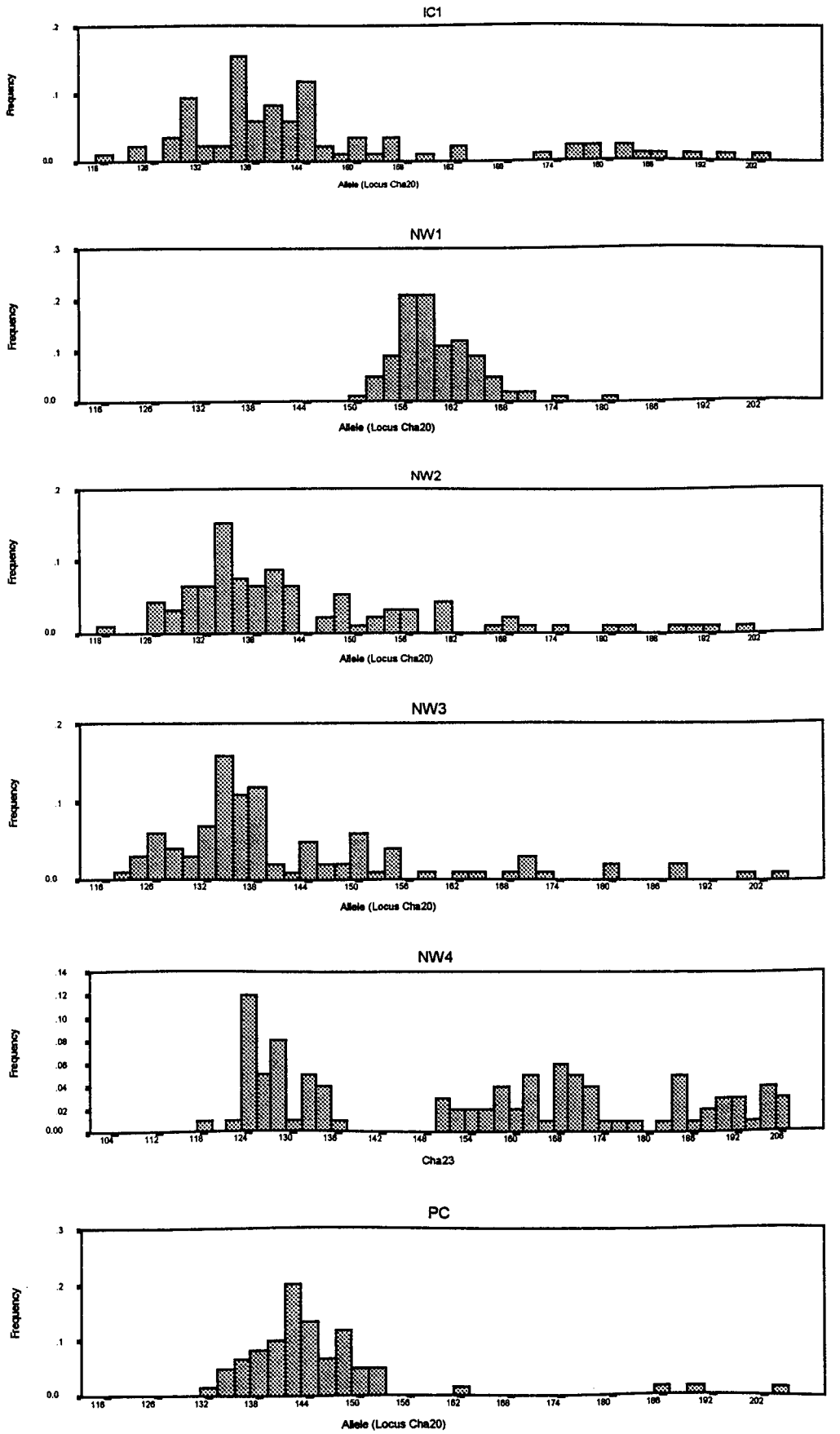


Figure 8.2. Continued.

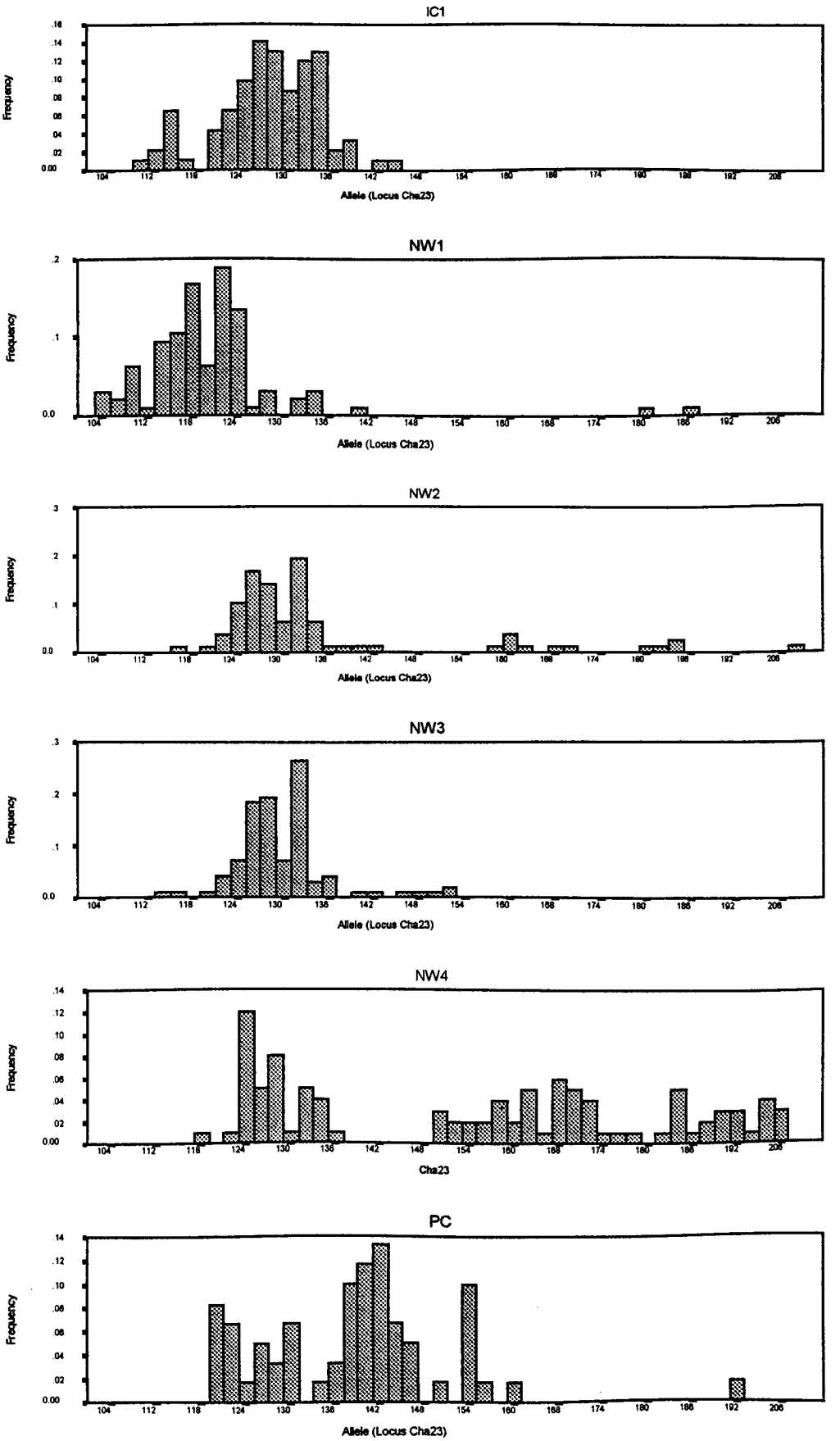
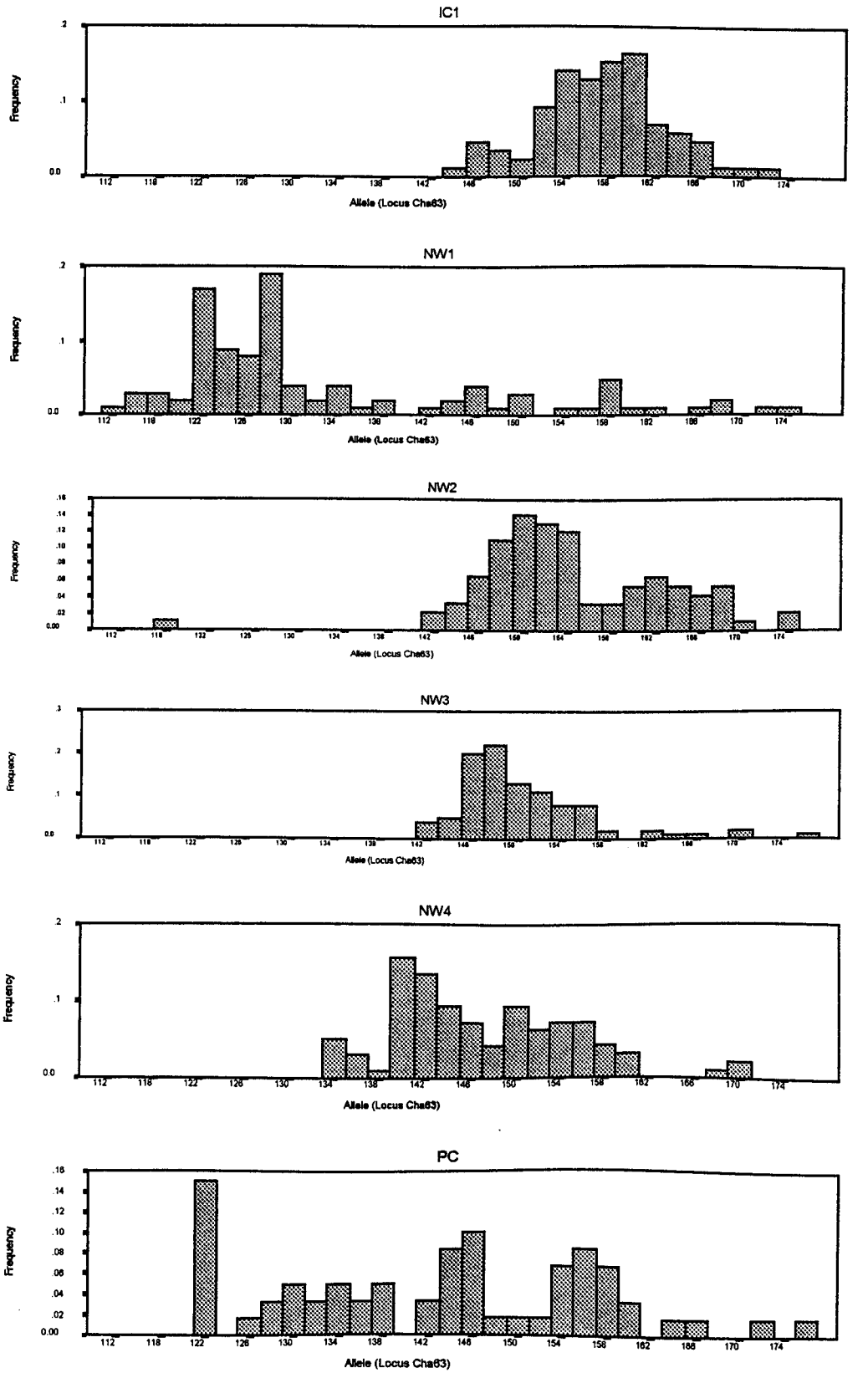


Figure 8.2. Continued.



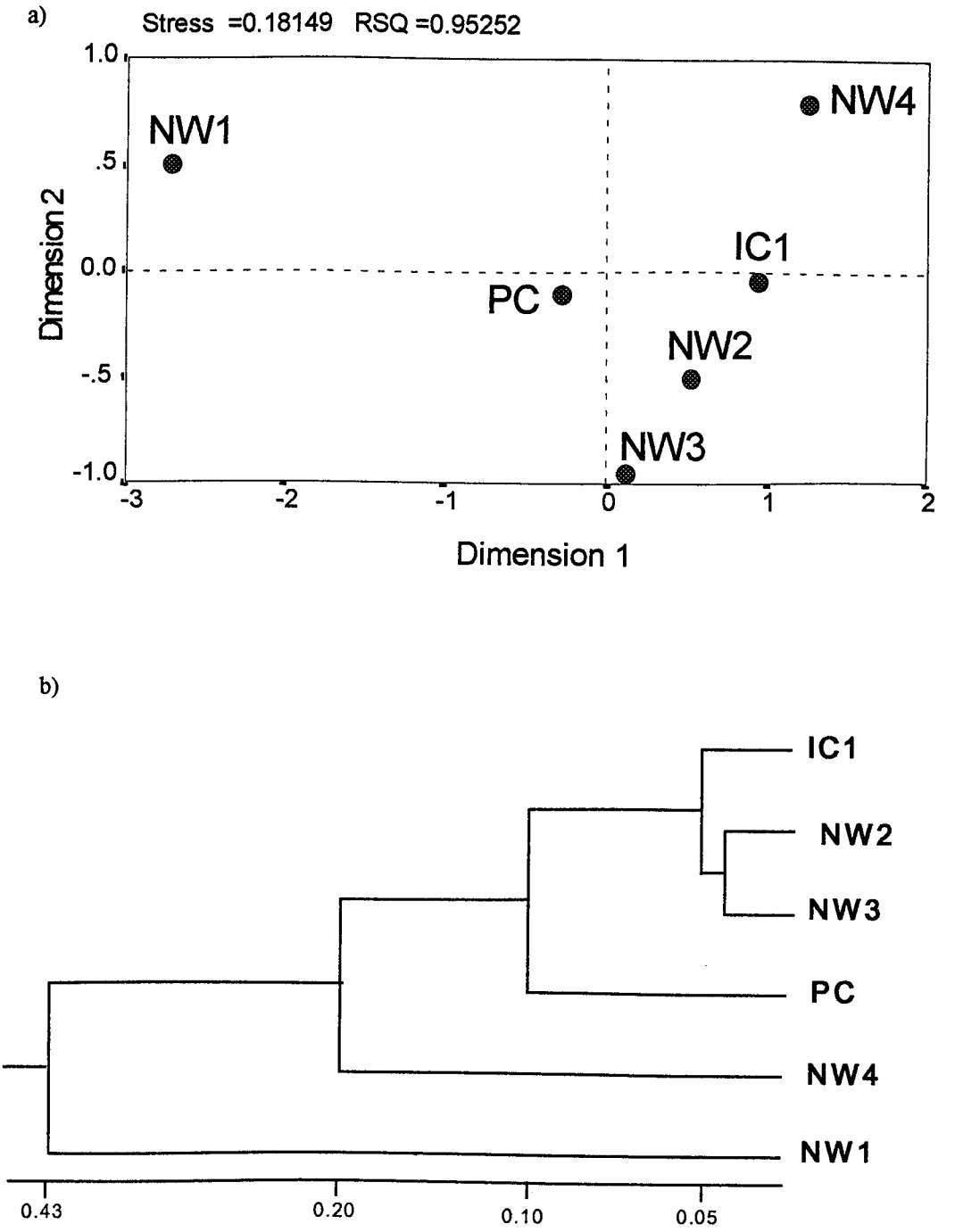


Fig. 8.3. Multidimensional scaling plots of pairwise R_{ST} (a) values, and phenogram (b) of herring samples using UPGMA cluster analysis of pairwise R_{ST} values based on microsatellite data. For sampling locations see Chapter 2, Figure 1.

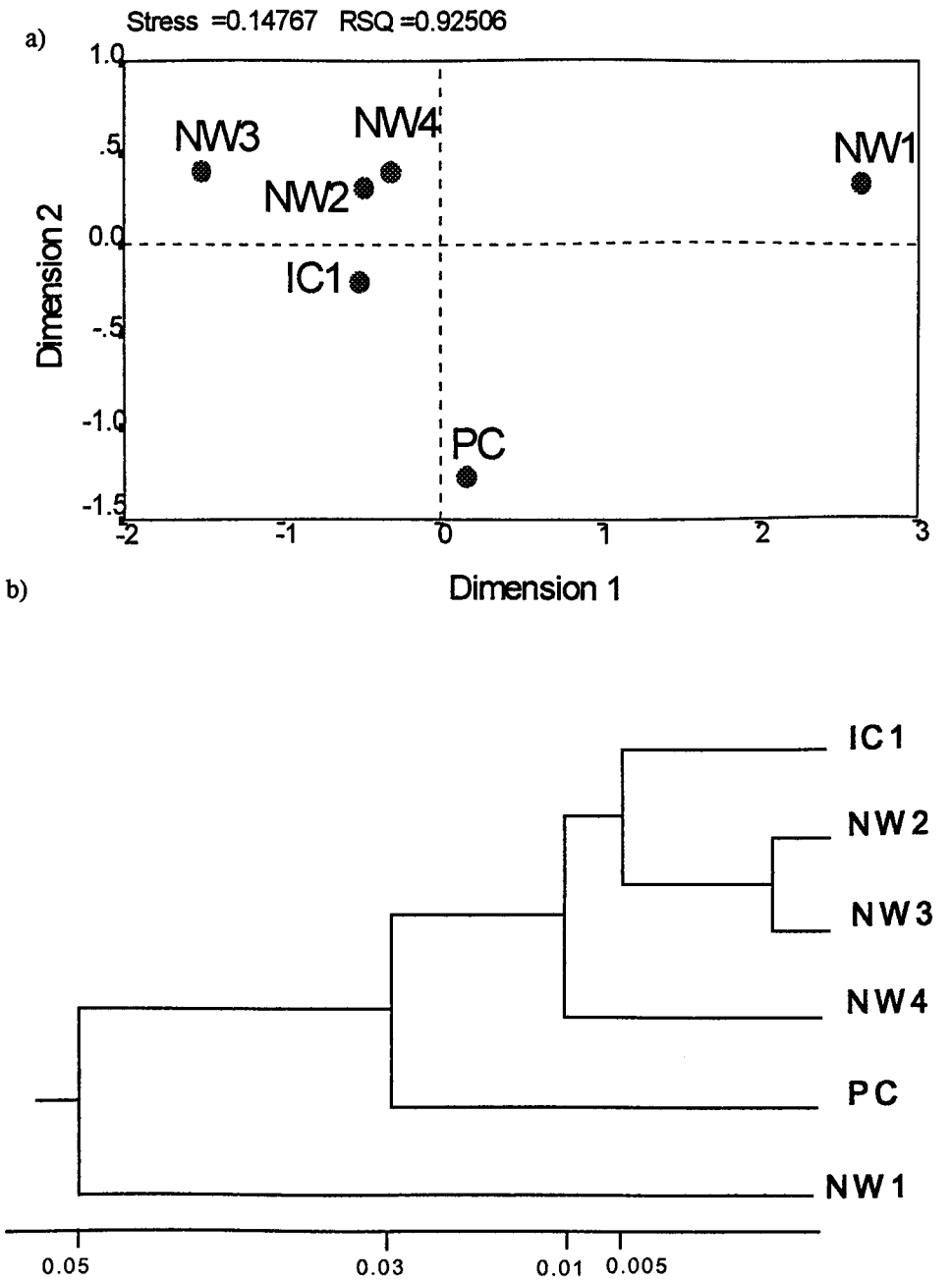


Fig. 8.4. Multidimensional scaling plots of pairwise F_{ST} (a) values, and phenogram (b) of herring samples using UPGMA cluster analysis of pairwise F_{ST} values based on microsatellite data. For sampling locations see Chapter 2, Figure 1.

CHAPTER 9

GENERAL DISCUSSION

9.1 Population structure of Northeast Atlantic herring

9.1.1 Accuracy of sampling program

One of the most important decisions by fisheries geneticists and managers is the accuracy of sample size for estimating population statistics: which sample size is feasible, most efficient and also provides the most information? In the present study, the findings indicate that the adequacy of a particular sample size depends critically on the marker employed. As sample size of 50 individuals seems to be sufficient for the phenotypic markers to separate herring populations on the basis of their morphological differentiation. A sample size of 25 individuals has previously been reported to be representatively large for morphological analyses (Reist, 1985).

One of the major limitations of applying allozymes to herring is usually the high incidence of low-frequency alleles at polymorphic loci, which do not effectively contribute to the detection of differences among samples. For example, in pairwise comparisons the occurrence of a large number of low-frequency alleles at *GOT** and *IDHP** did not appear to be very effective in producing significant differentiation between populations. Therefore an increase in sample size may also increase the frequency of low occurring alleles at a given

locus, which thus may magnify allele frequency differences between samples. The sample size of 50 for allozyme analysis should thereby be increased to more than 100 individuals in herring. Increasing sample size does not seem to be a problem since allozyme electrophoresis is very amenable to rapid and large sample size investigations.

Clupeids show a high level of genetic variation at the ND genes (Carvalho *et al.*, 1994; Bembo *et al.*, 1995; Hauser, 1996) which contrasts with low levels of mtDNA differentiation among populations. The low genetic differentiation is due mainly to the detection of a high number of unique haplotypes, and low number of common haplotypes. In testing the statistical significance of differences between populations, the presence of unique haplotypes in a sample has little statistical effect on the overall result which has also been reported by Hauser (1996). Therefore the number of common haplotypes is the main factor which contributes to differentiation. As a result, the detected high levels of haplotype diversity at ND regions suggests that sample sizes of 50 individuals may be inadequate for an effective population comparison, and should thereby be increased. Because a higher number of individuals increases the number of common haplotypes, it may enhance the power of the statistical test and a larger sample gives a better quantification of variability and thus the opportunity to detect significant differences.

The detected high number of moderate-frequency alleles at microsatellite loci provide a new perspective on past estimate of observed the low level of differentiation associated with other molecular markers among herring populations. Therefore the sample size of 50 seems to be sufficient to detect

differentiation at the four microsatellite loci used here. Indeed, the adequacy of sample size appears to depend on the species studied and microsatellite loci employed. For example, Morris *et al.* (1996) used approximately 307 fish from each population of rainbow trout, *Oncorhynchus mykiss*, and the average number of alleles per locus was 9. On the other hand, García de Leon *et al.* (1995) used approximately 24 individuals from each population of sea bass, *Dicentrarchus labrax*, with an average number of alleles 8. However in Atlantic cod, *Gadus morhua*, the average number of alleles was 41 from a mean sample size of 54 (Bentzen *et al.*, 1996).

Although a high number of haplotypes has also been observed in ND genes the distribution of haplotypes usually reveals one central peak (common haplotypes) and a smooth curve (unique haplotypes). Therefore differences in the number of common haplotypes between populations determine the levels of differentiation between populations. In contrast, allelic distribution at microsatellite loci generally show a number of peaks (Chapter 8, Fig. 8.1; Roy et al., 1994; Bentzen *et al.*, 1996; García et al., 1997) which may increase the statistical power in detecting differences between populations (P. W. Shaw, *personal Communication*).

An important aim in the present project was to obtain a representative sampling of spawning aggregations throughout the Northeast Atlantic. The sampling from various locations comprising the Celtic Sea, North Sea, Baltic Sea, Norwegian Sea, Barents Sea represented a sufficiently broad geographic range to examine phenotypic and genetic differentiation among Northeast Atlantic herring populations.

It is important to undertake temporal sampling at similar locations to understand the persistence of discontinuities of morphological and genetic characters, especially when analysing the stock structure of commercially important, highly mobile pelagic fishes. Kornfield *et al.* (1982) found significant spatial heterogeneity between spring and fall spawning populations of herring in the Gulf of Maine and Gulf of St. Lawrence, though, the detected heterogeneity was not temporally stable. Bembo *et al.* (1996a) identified two genetically distinct anchovy stocks, *Engraulis encrasicolus,* in Adriatic waters based on the temporal persistence of the detected differentiation. In the present study, temporal integrity of differences between samples could not be analysed, except in the case of the Icelandic summer-spawner herring, though the majority of studies carried out on commercially important species are similarly constrained by the availability of samples collected from commercial trawls.

An important point in stock structure analysis is that biological information should be collected from each population sampled, such as fecundity, age classes, distribution of spawning individuals, and appearance of fish (e.g. colour). In the present study, the collection of information on standard length, age, spawning condition, sex, and sampling gear facilitated the interpretation at both the phenotypic and molecular data.

Sampling time, spawning condition and location are important components in population studies to facilitate interpretation of data, though stocks may be discreet during spawning, and mix at other times of the year. In the present study, some of the samples were collected from their spawning

locations and indeed, some fish were recorded in spawning conditions (Chapter 2, Table 2.2).

In population studies it is important to use a combination of markers, though, some of which may be responding to local environmental variation such as morphology. In addition, a number of other markers such as molecular markers should ideally be used, and as far as possible in estimating molecular variation, it is helpful to examine different regions of DNA. In the present study a variety of phenotypic and molecular markers were used to assess the population structure of Atlantic herring.

9.1.2 Comparison of molecular and phenotypic markers

All the phenotypic and molecular markers, with the exception of allozymes were in agreement in describing the morphological and genetic discreteness of the Icelandic summer-spawners from main group of Atlantic herring. In addition, temporal stability of the significant spatial differentiation of the Icelandic sample was also revealed by morphometric, otolith and mtDNA analyses. However, significant temporal variation between years (1994 and 1995) was detected in meristic analysis which may suggest that meristic characters are more sensitive to environmental modifications than morphometrics, otoliths, and also genetics.

Highly significant morphological and genetic differentiation of the Trondheimsfjord herring was revealed by all the phenotypic and molecular

markers, with the exception of the mtDNA data. Interestingly, the patterns of genetic differentiation between the nuclear and mtDNA data were not congruent. There is increasing evidence that differentiation at the nuclear DNA level may not be shown in mitochondrial genes (Ferguson *et al.*, 1991; Ward & Grewe, 1994; Ward *et al.*, 1994a), though there remain many cases to the converse (Ward *et al.*, 1989; Reeb & Avise, 1990; Hansen & Loeschcke, 1996). Several factors may affect the relative magnitude of variability at the nuclear and mitochondrial DNA levels, including, patterns and extent of gene flow, selective constraints, genetic drift and differential mutation rates. Allozymes are generally held to provide better markers for response to selection because most genes affecting fitness traits are likely to be nuclear, and any variation in nuclear DNA may be in linkage disequilibrium with genes controlling fitness traits (Ward & Grewe, 1994). Therefore the detected significant allozymic divergence may arise from selection pressures in the fjord environment. Especially the *LHD-2** 175 unique allele and the predominance of the *PGI-1**200 allele in the Trondheimsfjord herring strengthens support for the operation of natural selection. Also there is evidence for selection at an *LDH* locus in brown trout (Henry & Ferguson, 1985), and the predominance of the *LDH-5**90 allele in sea trout populations has been attributed to a selective advantage of this allele over the ancient *LDH-5**100 allele (Ferguson, 1989). Extensive allelic variation and high levels of heterozygosity at microsatellite loci usually provide better markers for stock discrimination than mtDNA (Bentzen *et al.*, 1991; Wright, 1993; Wright & Bentzen, 1994). Therefore the detected significant microsatellite DNA structuring, relative to mtDNA structuring, is perhaps not surprising.

All phenotypic markers and mtDNA analyses revealed the phenotypic and genetic discreteness of Baltic herring from other Atlantic herring populations sampled, though meristic characters were relatively less effective in separating this sample from other Atlantic herring samples. In contrast to other genetic data, allozyme results did not indicate the genetic distinction of the Baltic herring. Here, the specific advantages of mt DNA compared to allozymes (Chapter 7) may be the reason of the detected differentiation of Baltic herring. Collectively, the present morphometric and genetic markers suggest that the Baltic herring are phenotypically and genetically differentiated population in Northeast Atlantic.

All of the molecular markers revealed a clear genetic discreteness of Norwegian spring-spawners (NW1) from all other Atlantic herring populations sampled. Microsatellites seem to be the most effective marker to discriminate this population, and also allozymes revealed greater genetic differentiation than mtDNA. Since all the molecular markers were in agreement on the genetic divergence of this population, it is highly likely that there is a restricted or an absence of gene flow from other populations. However, the greater allozymic differentiation may arise from some contribution of natural selection in the allozymic differentiation, especially since mtDNA genes are held generally to be more neutral than allozymes (Ward & Grewe, 1994; Rand *et al.*, 1994). In this sample, the dominant allele *PGI-1**40 and a allele (*PGI-1**0) occurring at appreciable frequencies (0.18) were rare in other samples and caused the significant allozymic differentiation of this sample. Such genetic characteristics, where only one or a few loci are differentiated, may be characteristics of

response to selection (Clarke, 1975; Smith *et al.*, 1990), though the maintenance of such divergence is likely to be indicative of restricted gene flow.

Significant genetic differentiation of the Balsfjord and Norwegian spring spawner (NW2) samples were revealed by only the microsatellite analysis data which shows the high discriminatory ability of microsatellite analysis in detecting allele frequency differences between populations.

No genetic differentiation was detected among the other Atlantic herring samples comprising North Sea and Celtic Sea samples using allozymes and mtDNA analysis in accordance with otolith and meristic multivariate analyses. However morphometric analysis exhibited significant differences among these samples. This may suggest that morphometric characters may be more induced by environmental conditions which increase their discriminatory ability even on a small geographic scale. Alternatively, there may be genetic basis of the detected morphometric differences among the North Sea and Celtic samples which could not be revealed due to over-sensitivity of molecular markers to gene flow.

It seems that there is genetical control of morphological and anatomical differentiation detected among herring populations. The genetical control is especially high for the Icelandic, Trondheimsfjord and Baltic herring. For example, the standard length of Trondheimsfjord herring demonstrated significant differences for the same or similar age classes of other populations (Chapter 2). Higher body depth in Icelandic samples, in contrast, a clear shallow body form in Baltic herring was also observed (Chapter 2). This anatomic differences were also supported by morphometric and otolith analyses (Chapter 3, 4) and proved genetic basis by molecular markers (Chapter 6, 7, 8).

All phenotypic and molecular markers were effective in discriminating the Atlantic and Pacific herring. Meristic characters, and to a lesser extent, otoliths, were the most discriminating among phenotypic markers. Allozymes and mtDNA analysis were efficient for species distinction, though mtDNA showed a different discriminatory ability depending on the genes employed. Microsatellites assayed appear to be a relatively weak tool for species identification in line with previous observations on their phylogenetic information content (Garza *et al.*, 1995; Jarne & Lagoda, 1996), but a good choice for population identification.

In summary, different patterns of differentiation among herring populations were exhibited by the molecular markers: Microsatellites appeared to be most powerful in discriminating populations among the molecular markers employed. Secondly PCR-amplified mtDNA was also effective in differentiating among populations. The extent of allozymic differentiation is likely to be moderately influenced by its occasional selective responses. Phenotypic markers usually revealed concordance with molecular markers. Otolith analysis was most concordant with the mtDNA analysis, showing a similar pattern of differentiation within and between herring species. Morphometric analysis appeared to be more sensitive in detecting differences among populations, though the statistical test procedures applicable to morphological characters have higher statistical power than tests of allele frequency differences and meristic characters (Ihssen *et al.*, 1981b; Lewontin, 1984). Meristic analysis seems to be more effective in discriminating different species than among conspecific populations.

9.1.3 The proposed model for population structuring of herring

Data from the phenotypic and molecular markers revealed a significant degree of stock separation of herring in the Northeast Atlantic. In accordance to previous data on morphological and physiological characters (Fridriksson, 1944, 1958; Johansen, 1926; Liamin, 1959), the present genetic and phenotypic data strictly confirm the existence of a separate Icelandic stock.

Clear phenotypic and genetic discreteness of the Baltic herring was also demonstrated in contrast to previous allozyme (Ryman *et al.*, 1984; King *et al.*, 1987; Jørstad *et al.*, 1991) and mtDNA (using whole mtDNA; Dahle & Eriksen, 1990) and morphological (King, 1985) studies.

Present genetic and phenotypic data agree with a previous genetic study (Jørstad & Nævdal, 1981) that Trondheimsfjord herring are highly genetically and phenotypically distinct from other Atlantic herring populations sampled. Balsfjord herring also revealed its discreteness by microsatellite analysis from other populations in accordance with previous studies (Jørstad & Nævdal, 1981; Jørstad & Pedersan, 1986; Jørstad et al., 1994).

The genetic data concerning the three molecular markers revealed a high genetic discreteness of the Norwegian spring-spawner herring (NW1) off the northern Norwegian coast. In addition, differences in morphological characters of Norwegian-spring spawners between northern and southern Norwegian Sea have recently been reported (Debarros & Holst, 1995; Stenevik *et al.*, 1996) and suggested that there are two reproductively separated spawning groups in northern and southern Norwegian Sea. On the other hand the detected

genetically-unique herring may represent a migratory group of herring from White Sea. Indeed morphological and genetic similarity has been detected (disc-gel electrophoresis) between White Sea herring and Pacific herring (Soin, 1971; Truveller, 1979). A more thorough understanding of the apparent genetic distinction of the Norwegian spring-spawner requires further insights based on additional repetitive samples from the same locations and also from the White Sea.

9.2 The utility of mtDNA and nuclear DNA markers

The simultaneous examination of three molecular markers on population structure of Atlantic herring provided a first opportunity to compare their utility, and potential to understand population interactions of more fully than using a single marker.

Allozyme analysis generally revealed a number of low-frequency alleles allowing the determination of weak differences in allele frequencies among populations, which fits the previously exhibited pattern of low variation at the protein loci in Atlantic herring (Ryman *et al.*, 1984; Smith & Jamieson, 1986; King *et al.*, 1987; Koskiniemi & Parmanne, 1991). Significant differentiation in allozymes between samples are usually based on the assumption of selective neutrality, though allele frequency differences are known be respond to selection associated with contrasting environments rather than mutation, gene flow and random genetic drift (Utter, 1991; Powers *et al.*, 1991). In the present data, the detected greater significant allozymic differentiation (at *PGI**) than mtDNA (ND

5/6 region) in Norwegian spring-spawner sample (NW1), and high allozymic differentiation at *LDH** in Trondheimsfjord sample, and the genetic homogeneity of this sample revealed by mtDNA may provide some indication that selection may be contributing at least in part to the detected significant differentiation of these samples. Moreover, although one of the major limitations of molecular markers in stock identification is probably their high sensitivity to gene flow, allozymes are especially sensitive to low levels. With sample sizes of 100 or less, gene flow rates of 1% and 50% between populations are often not allozymically distinguishable from each other and appear panmictic (Ward & Grewe, 1994; Carvalho & Hauser, 1994). However, molecular markers are only useful to fishery managers if they detect differentiation, and gene flow rates of 10% or less may justify treatment as separate stocks (Brown *et al.*, 1987).

The PCR-based mtDNA analysis was more effective in discriminating populations than allozymes. This can be explained by such factors as the differential effects of patterns and extent of gene flow, genetic drift and differential mutation rates on the magnitude of variability at the nuclear and mitochondrial DNA levels. The maternally inherited and haploid nature of mtDNA may cause the detection of greater genetic heterogeneity among samples. Similar observation has also been reported by Hansen & Loeschcke (1996) that allozymes were not able to discriminate among Danish brown trout, *Salmo trutta* L., but mtDNA involving ND1 and ND5/6 region were able to distinguish three main groups. However sample sizes of 50 seems to be insufficient due to the small number of common haplotypes which reduce the power of the statistical analysis. Therefore increasing the sample size may also

enhance the strength of mtDNA analysis in stock discrimination. In addition, the utility of mtDNA analysis seems to be dependent on the mtDNA regions employed. ND3/4 and ND5/6 protein-coding mtDNA genes revealed considerable differentiation among herring populations. Likewise the other genes of mtDNA such as D-loop and ND1 may provide a better marker for detecting variation between discrete spawning aggregations of herring.

Microsatellite analysis proved to be most useful for detecting genetic differentiation among populations. The high number of alleles and high heterozygosity clearly enhances their discrimination of conspecific populations, which has also been reported involving different species (Tessier *et al.*, 1995; Bentzen *et al.*, 1996; O'Connell *et al.*, 1996; Presa & Guyomard, 1996; García de Leon *et al.*, 1997). Although high genetic diversity was detected with the four loci, microsatellite analysis could be improved by including new loci and by using specific microsatellite primers for Atlantic herring.

A similar pattern among the molecular markers has also been observed in Atlantic cod, *Gadus morhua* (e.g. Mork *et al.*, 1985): allozymes revealed genetic homogeneity among discrete spawning populations occurring over the North Atlantic Ocean, and mtDNA (e.g. Dahle, 1991; using whole mtDNA) could separate the Atlantic cod into two populations: Arctic and coastal cod, but high genetic differentiation has been detected among the Atlantic cod populations by microsatellites even on a small geographic range (Bentzen *et al.*, 1996; Ruzzante *et al.*, 1996). Tessier *et al.* (1995) has also simultaneously used molecular markers (allozymes, mtDNA and microsatellites) on the same samples of Atlantic salmon, *Salmo salar*, and suggested the use of a combination of

mtDNA and microsatellites. Allozymes were again the most limited marker due to the low polymorphism detected.

In summary, allozymes proved to be of rather limited value in detecting allelic differences between populations due both to the low-frequency alleles and their high sensitivity to gene flow with the sample size employed. The mtDNA analysis was more effective than allozymes in discriminating herring populations, and it is apparently important to use more than one region of mtDNA to improve its potential in detecting differences among populations. Microsatellites were the most powerful marker in detecting allele frequency differences among conspecific populations, but not necessarily between species. Microsatellite and mtDNA methods also have the advantages of requiring only minute and non-destructive amounts of tissue which can be stored indefinitely in ethanol. Given the advantages and disadvantages of the three methods, it appears that microsatellites offer the most informative tool in examining the genetic structure of Atlantic herring populations within their usual range of population differentiation.

9.3 Fishery implications and the stock concept

A common aim of fishery managers is to ensure the sustainability of resources to maximise economic returns to the fishery. When two pioneering fishery biologists, F. Heincke and J. Hjort identified the local self-recruiting stock, as opposed to the typological species, it was realised that species should

be managed at subspecies level (Sinclair, 1988). The desire to identify such stocks initiated the development of variety of phenotypic markers for stock structure analysis. However the application of such markers is complicated by the high plasticity of phenotypic characters to environmental modifications, therefore phenotypic differences may often not have a genetic basis. Such difficulties could be overcome by using molecular markers which initiated the search to find the perfect molecular stock marker. However such markers have generally shown low levels of genetic differentiation between previously described morphologically discrete marine populations (Ryman *et al.*, 1984; Pepin & Carr, 1993; Brown *et al.*, 1987). The detected low levels of genetic differentiation is due apparently to absence of physical barriers in marine environment and high mobility of many pelagic teleosts (Gyllensten, 1985; Hedgecock, 1994; Ward & Grewe, 1994). In addition, molecular markers are generally over-sensitive to gene flow though relatively low levels of exchange between stock, negligible from a management perspective, may be sufficient to provide genetic homogeneity (Ward & Grewe, 1994; Carvalho & Hauser, 1994).

Unfortunately in marine species (e.g. Carvalho *et al.*, 1994) perhaps due to socio-economic factors (Chapter 1) genetic structure of populations are usually not considered in management programs. However molecular genetic markers have been widely applied in the management of anadromous salmonids, perhaps because of their high commercial and social value (Utter & Ryman, 1993). In particular, fishery managers have applied genetic markers if the origin of individual fish cannot be readily identified on the bases of their morphology (Lavery & Shaklee, 1991; Campton *et al*, 1992; Utter & Ryman, 1993). Fishery

managers are also interested in the relative contribution of the various local stocks to the catch in mixed-stock fisheries to identify either weaker or more productive stocks in order to achieve the sustainable yield (Utter, 1991; Utter & Ryman, 1993).

Management of herring has been based on demographic data such as recruitment levels, meristic counts, morphometric characters, fecundity (ICES, 1956; Cushing, 1975; Jakobsson, 1985), perhaps, since genetic markers have not conclusively demonstrated genetic differentiation among discrete spawning aggregation on a large geographic scale (IFREMER / MAFF, 1993).

In view of this, the present data provide one of few cases of consistent spatial and temporal differentiation in marine fishes. Detected significant spatial genetic heterogeneity of Icelandic, Norwegian spring-spawners (NW1), Trondheimsfjord, Balsfjord and Baltic herring suggests that limited, or an absence of gene flow is occurring between these and other Atlantic herring populations. Selection as a cause for this differentiation seems unlikely, as significant differences among samples were revealed by at least two independent molecular markers, with the exception of Baltic herring and Balsfjord herring with concordant patterns also shown by phenotypic markers. The discrimination of such spatial heterogeneity would be valuable from management perspective only if temporal persistence of the detected pattern was observed. The marked strong spatial and temporal phenotypic and genetic stability of the Icelandic summer-spawners (IC1 & IC2) based on morphometric, otolith, mtDNA suggests that they have to be treated as a genetically distinct stock in management programs from the other Northeast Atlantic herring populations, as

suggested from morphological studies (Fridriksson, 1944, 1958; Johansen, 1926; Liamin, 1959). Moreover, the observed genetic discreteness of the Trondheimsfjord herring population, and the Balsfjord herring provides indirect evidence of their temporal and spatial integrity when compared with previous studies (Jørstad & Nævdal, 1981; Jørstad *et al.*, 1994), thus strengthening their existence as distinct management units. Continued monitoring of the temporal stability of the detected spatial differentiation from other populations sampled is an obvious research priority.

Although the morphological differences were detected among North Sea and between North Sea and Celtic Sea spawning aggregations, the lack of genetic differentiation does not confirm the absence of any population substructuring because of the high sensitivity of molecular markers to gene flow. Further study on the stock structure of these aggregations should concentrate on microsatellite markers.

Since genetically distinct stocks may differ in their rates of recruitment and mortality, effective conservation should be based on measures which take account of any such genetic and demographic variation to optimise economic returns without compromising natural perpetuation. Thus, fishery managers should be aware of the detected genetic heterogeneity among herring populations in the Northeast Atlantic since it is highly likely that these populations react independently to exploitation, and any depletion in one of these stock is unlikely to be compensated by immigration from other units. Therefore future management protocols should include independent catch regulations at least for the Icelandic, Trondheimsfjord and Balsfjord herring due

to the temporal stability of their variation. Furthermore, documenting the distribution and abundance of these stocks is necessary to management programs for stock boundaries. Therefore stock assessments should be estimated at least for the Icelandic, Trondheimsfjord and Balsfjord stocks separately.

9.4 The priorities in fishery genetics and future studies

9.4.1 Importance of using more than one marker

In the present study using more than one data set to describe population structure of Atlantic herring demonstrated clearly the potential to describe population interactions more fully than would be possible using a single marker set. Each phenotypic and molecular character can be effected by different factors, and thus they may reveal different patterns of differentiation among populations. For example, starvation due to low food density may effect morphometric differentiation, however, latitude differences may effect meristic differentiation of populations within phenotypic markers, and nuclear DNA and mtDNA can also be differentially effected by demographic events. Therefore it is valuable to use more than one marker, and a synergistic combination of phenotypic, such as otolith, and molecular, such as microsatellite, markers to the study of stock structure provide for the most powerful analysis.

9.4.2 Conservation genetics

Management of populations based only on phenotypic structure or life history characters would be ineffective to ensure sustainability of stocks. In this respect, fisheries managers should deal with both demographic and genetic changes. The differential effects of microevolutionary forces (mutation, natural selection, genetic drift, and migration) is likely to produce differentiation among populations within a species. When substantial divergence exists among geographic groups of populations, alleles become restricted to particular regions but are common where they occur. Conservation programs should be concerned with the conservation of alleles and maintain as much genetic variation within and between populations as possible. Loss of an allele represents a permanent decrease in genetic diversity. Once an allele is lost it can be recovered only by mutation, the probability of which is very low. The loss of alleles therefore permanently reduces the ability of populations to make adaptive responses to altered environmental conditions, for example, can reduce their resistance to disease (Allendorf, 1986). Furthermore, progressive directional changes in allele frequencies may modify life-history traits, especially where the nature of harvesting is intense or selective (Turner, 1977, Smith *et al.*, 1991; Carvalho & Hauser, 1992; Smith, 1994).

However, fisheries managers are usually concerned with short-time scale in terms of the amount of fish caught, and the consequent economic benefits of that catch. Nevertheless fishery geneticists are concerned with long-term scales in terms of the long-term adaptability of stocks in order to predict future

availability. Fishery managers usually wrongly assume that genetic changes can occur over an evolutionary scale; genetic change can occur within a few years due to strong selection pressures and genetic drift (Kirkpatrick, 1991; Smith, 1991). Therefore populations are more likely to undergo severe population fluctuations or eventual extinction (Nelson & Soulé, 1987; Witte *et al.*, 1992). In the view of this, there must be an active bridge (dialogue) between fishery managers and geneticists. From a practical point of view, fishery geneticists should be realistic, since conserving every small genetic unit of a species within very short distances is not practicable, and would do little to bring consensus between fishery managers and geneticists. Therefore it should be decided what is the minimum action required to maintain the economic and biological viability to conserve fish populations, making it important to identify key populations for conservation.

9.4.3 Biological and sampling information

Obtaining information on the biological characteristics of fish is a important task to take into account in sampling programmes. Age, size and colour of fish, gear, depth of sampling, migration pattern provide important background information, and differences in these characters may confound the detected pattern of differentiation. These characters can also be used in conjunction with molecular data for interpreting the spatial distribution of populations, providing data on the correspondence between genotypic and

phenotypic divergence. Standard length of some samples showed significant differences for the same or similar age classes between populations sampled which was also in accordance with high genetic differentiation of these samples (Chapter 2, 6, 8). Moreover the occurrence of Balsfjord herring in deep water and Norwegian spring spawners in upper waters within the Balsfjord indicates the importance of noting water depth in a sampling programme (Chapter 6; Jørstad & Pedersan, 1986; Jørstad *et al.*, 1994).

9.4.4 The Scope for future study

The first future research priority would be monitoring the temporal stability of the detected spatial differentiation through repeated sampling. Such an approach may also reveal information on the population dynamics and migration patterns of genetically discrete populations in relation to size and reproductive status. These data would not only provide information on the stability of spatial pattern, but may also provide valuable insights into the origin of the divergence observed.

From the view point of molecular markers, microsatellites seem to be effective in detecting allele frequency differences between populations. Therefore it would be valuable to carry out further microsatellite studies on other Atlantic herring populations which could not be recognised using allozymes and mtDNA markers. Therefore the number of microsatellite loci should be increased by developing new set of primers specific to Atlantic herring.

The present study and previous studies (Jørstad & Nævdal, 1981; Jørstad & Pederson, 1986; Jørstad *et al.*, 1991; Jørstad *et al.*, 1994) indicated that there is microgeographic phenotypic and genetic structuring of herring within Norwegian fjords. Thus new studies should be directed on these fjord populations through a detailed knowledge of the genetic relationships among fish in these fjords, and between the fjord and main group of Atlantic herring is needed for fishery management and future conservation. In addition, the present data suggest that herring from Barents Sea and White Sea should also be analysed to clarify the status of the detected genetically-unique Norwegian spring-spawner (NW1). Such information can also facilitate the understanding of the evolution of different herring species in the Atlantic and Pacific Oceans.

Although it was not generally possible here, it is important to analyse spawning and non-spawning individuals at separate times from each location or spawning aggregation. This would facilitate the interpretation of population interactions, elucidating whether there is intermingling from any other populations during the spawning and non-spawning phase. The lack of differentiation detected among non-spawning fish may, for example, indicate the intermingling of other populations during only the non-reproductive phase, if spawning individuals were shown to be genetically discrete. Such comparisons would be valuable.

Different age classes of herring from each location should also be analysed, and may provide useful information on whether environmental factors are influencing allele frequencies of a population in different years.

9.5 Concluding remarks

The current investigation on the genetic and phenotypic population structure of Atlantic herring provides some important conclusions. First there is phenotypic and genetic differentiation observed among herring populations which is important not only for the management of fisheries, but also for the maintenance of the genetic variability. Second, simultaneous application of a variety of markers improves the understanding of population structure. Accordingly, otoliths followed by morphometric analyses provided agreement with molecular markers, and the use of both nuclear and mtDNA markers is advised. Third, studies should not be undertaken independent of biological data, and they should be used together for interpreting the spatial distribution of populations. Fourth, population structuring should be demonstrated to persist over time before any realistic conclusion on population genetic structuring can be attained, though logistic constraints normally imposed on the sampling of geographically wide-separated marine teleosts render this a difficult task.

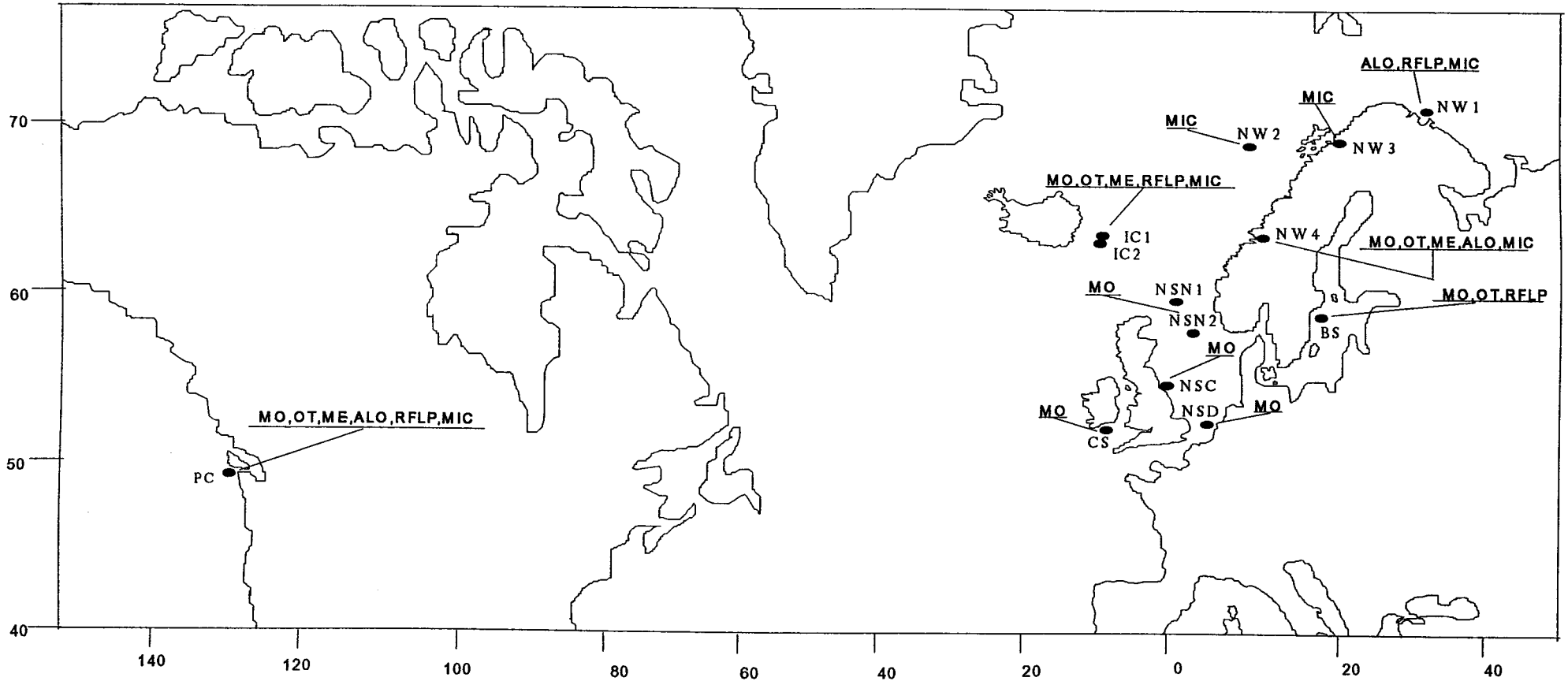


Figure 9.1. Schematic illustration of detected phenotypic revealed by morphometric (MO), otolith (OT), meristic (ME) and genetic revealed by allozymes (ALO), mtDNA (RFLP), microsatellites (MIC) discreteness of herring populations.

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APPENDIX I: ALLOZYME ELECTROPHORESIS

Table I.1: Buffer systems used for screening resolvable allozymes in herring. For routine screening only CM 6.2, TM 7.4 and TC 8.0 were used.

Buffer	Electrode (g/l)	Gel (g/l)	Current & duration of run
Citrate morpholine (pH 6.2) CM 6.2	8.4 citric acid adjust to pH 6.2 with N-(3-aminopropyl)-morpholine	Dilute 1:20	35 mA / gel 8 h
Tris citrate (pH 8.0) TC 8.0	30.3 g Tris 11.98 g citric acid adjust to pH 8.0 with HCl	Dilute 1:25	35 mA / gel 8 h
Tris maleic (pH 7.4) TM 7.4	12.1 g Tris 11.6 g maleic acid 3.7 g EDTA 4.05 g $MgCl_2$	Dilute 1:10	15 mA / gel 17 h
Ridgeway's	18.6 g boric acid 2.5 g lithium hydroxide PH 8.1	3.6 g Tris 1.05 g citric acid 53 ml electrode buffer pH 8.5	30 mA / gel 18 h
mod. Ridgeway's (pH 6.8) TCB 8.6	18.6 g boric acid 4.2 g lithium hydroxide	9.2 g Tris 1.05 g citric acid 53 ml electrode buffer	30 mA / gel 18 h

Table I. 2: Stain recipes used for routine screening of herring samples. Recipes are modified from Harris & Hopkinson (1976), Ferguson (1985), Murphy *et al.* (1996) and Piertney (1994). With the exception of AAT and EST-D, all stains also contained 1 ml 10 mg/ml MTT (tetrazolium salt), 1ml 10 mg/ml PMS (phenazine methosulfate) and 25 ml of 2% agar solution.

Enzyme	EC no.	Running buffer	Ingredients	Stain buffer	Linking enzymes
GOT	2.6.1.1	CM	150 mg Ketoglutaric acid 200 mg L-Aspartic acid 10 mg Pyridoxal-5-phosphate 500 ml 1M NaOH (to adjust to pH 8) *leave for 30 min* 100 mg Fast blue BB in 15 ml H_2O	15ml 0.1 M Tris-HCl pH 8	30 u Hexokinase 10 u G6PDH
AK	2.7.4.3	CM	1 gr Glucose 50 mg ADP 20 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	10 u Hexokinase 20 u G6PDH
CK	2.7.3.2	CM	40 mg Phosphocreatine 30 mg Glucose 15 mg ADP 8 mg NADP		30 u Hexokinase 10 u G6PDH
EST	3.1.1.1	CM	1 mg 4-Methyl-umbelliferyl acetate *dissolve in 5 ml acetone*	50 ml 0.1 M Na_2PO_4 pH 6.5	
FH	4.2.1.2	CM	400 mg Fumaric acid 40 mg Na Pyruvic acid 60 mg NAD	15ml 0.1 M Tris-HCl pH 8	60 u MDH

Table I. 2: continued.

Enzyme	EC no.	Running buffer	Ingredients	Stain buffer	Linking enzymes
G3PDH	1.1.1.8	TM	200 mg α-Glycerophosphate 15 mg NAD	15ml 0.1 M Tris-HCl pH 8	
G6PDH	1.1.1.49	TM	200 mg Glucose-6-phosphate 40 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	
GAPDH	1.2.1.12	CM	10 mg Arsenic acid 150 mg Fructose-1,6-diphosphate	15ml 0.1 M Tris-HCl pH 8	20 u Aldolase
IDHP	1.1.1.42	CM	150 mg Isocitric acid 8 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	
LDH	1.1.1.27	CM	400 mg DL-Lactic acid 60 mg NAD 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	
MDH	1.1.1.37	CM	250 mg DL-Malic acid 60 mg NAD	15ml 0.1 M Tris-HCl pH 8	
ME	1.1.1.40	CM	250 mg DL-Malic acid 8 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	
PGDH	1.1.1.44	CM	40 mg Phosphogluconic acid 20 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	
PGI	5.3.1.9	CM	40 mg Fructose-6-phosphate 8 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	15 u G6PDH
PGM	5.4.2.2	CM	80 mg Glucose-1-phosphate 10 mg NADP 1 ml 1M $MgCl_2$	15ml 0.1 M Tris-HCl pH 8	15 u G6PDH
SDH	1.1.1.14	TC	125 mg D-Sorbitol 10 mg NAD	15ml 0.1 M Tris-HCl pH 8	
SOD	1.15.1.1	TC	1 ml 1M $MgCl_2$ 10 mg NADP	15ml 0.1 M Tris-HCl pH 8	

Table I. 3: Enzyme systems used in the intial screening. Legend: A: activity; R: resolution; 1 excellent, 2 good, 3 moderate, 4 bad, 5 insufficient.

Enzyme	Abbrev.	EC number	A	R
Aconitase hydratase	ACOH	4.2.1.3	5	
Acid phosphatase	ACP	3.1.3.2	3	2
Adenosine deaminase	ADA	3.5.4.4	5	
Alcohol dehydrogenase	ADH	1.1.1.1	5	
Adenylate kinase	AK	2.7.4.3	1	2
Alanine aminotransferase	ALAT	2.6.1.2	5	
Aldehyde dehydrogenase	ALDH	1.2.1.5	5	
Aldehyde oxidase	AO	1.2.3.1	4	3
Alkaline phosphatase	AP	3.1.3.1	5	
Carbonate hydratase	CAR	4.2.1.1	3	2
Creatine kinase	CK	2.7.3.2	1	2
Diaphorase	DIA	1.6.4.3	5	
Enolase	ENO	4.2.1.11	5	
Esterase-D	EST-D	3.1.1.1	1	2
Fructose biphosphate aldolase	FBA	4.1.2.13	3	2
Formaldehyde dehydrogenase	FDH	1.2.1.1	5	
Fructose 1,6 diphosphatase	FDP	3.1.3.11	5	
Fumarate hydratase	FH	4.2.1.2	1	1
Fructokinase	FK	2.7.1.4	4	2
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	1	2
Glycerol-3-phosphate dehydrogenase	G3PDH	1.1.1.8	1	2
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	2	2
Glyceraldehyde-3-phosphate dehydrog.	GAPDH	1.2.1.12	1	2
Guanine deaminase	GDA	3.5.4.3	4	3
Glucose dehydrogenase	GDH	1.1.1.47	5	
Glutamate pyruvate transaminase	GPT	2.6.1.2	5	
Glutamate dehydrogenase	GTDH	1.4.1.2	5	
Glutamate dehydrogenase NADP	GTDH-P	1.4.1.4	5	
(S)-2-Hydroxy-acid phosphatase	HAOX	1.1.3.15	5	
ß-N-Acetylglucosaminidase	HEX	3.2.1.30	5	
Hexokinase	HK	2.7.1.1	5	
Isocitrate dehydrogenase	IDH	1.1.1.42	1	1
Lactate dehydrogenase	LDH	1.1.1.27	1	1
Malate dehydrogenase	MDH	1.1.1.37	1	2
Malic enzyme	ME	1.1.1.40	2	2
Mannose phosphate isomerase	MPI	5.3.1.8	3	4
Purine-nucleoside phosphorylase	PNP	2.4.2.1	5	
Octanol dehydrogenase	ODH	1.1.1.73	4	3
Peptidase	PEP	3.4.-.-	4	4
Phosphogluconate dehydrogenase	PGDH	1.1.1.44	1	2
Phosphoglucose isomerase	PGI	5.3.1.9	1	1
Phosphoglycerate kinase	PGK	2.7.2.3	5	
Phosphoglucose mutase	PGM	5.4.2.2	1	2
Pyruvate kinase	PK	2.7.1.4	4	
Sorbitol dehydrogenase	SDH	1.1.1.14	1	2
Shikimate dehydrogenase	SKDH	1.1.1.25	5	
Superoxidase dismutase	SOD	1.15.1.1	1	2
Succinate dehydrogenase	SUDH	1.3.99.1	5	
Xanthine dehydrogenase	XDH	1.2.1.37	5	
Xanthine oxidase	XO	1.2.3.2	4	2

APPENDIX II: MTDNA ANALYSES

Table II.1. Ingredients for 10 µl PCR reaction used to amplify the ND3/4 and ND5/6 genes of *Clupea harengus* and *Clupea pallasi*. The PCR buffer contained 15 mM $MgCl_2$.

	Stock	µl / 50 µl reaction	final concentration
PCR buffer	10 x	5	1 x
dNTP	2 mM	5	200 µlM
$MgCl_2$	25 µlM	2	2.5 mM
primer	5 u/ µl	1 each	0.5 µlM
***Taq* polymerase**		0.2 (=1 unit)	1 unit
template DNA		0.5	
sterile, filtered water		36.3	

Table II. 2. Temperature cycle used to amplify the ND3/4 and ND5/6 genes of *Clupea harengus* and *Clupea pallasi*. ': minutes, '': seconds.

Temperature (°C)	Time	Function	Cycle
95	5'	initial denaturation	1
49	1'30''	annealing of primers	┐
72	1'30''	extension	25
94	30''	denaturing	┘
49	1'30''	annealing	1
72	30''	final extension	1

Table II. 3. Restriction fragment length estimates (in number of base pairs, pb) of ND5/6 regions of mtDNA for each of six restriction enzymes (*Alu*-I, *Cfo*-I, *Hae*-III, *Hinf*-I, *Msp*-I, *Rsa*-I). Upper case letters denote haplotypes. 1, denotes the presence of a fragment; 0, denotes the absence of a fragment.

Alu-I

bp	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	R	S	T	U	V	W	Y	Z	X	Q
1281	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
900	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
741	1	1	0	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	1	0	1	0	0	0	0
640	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
525	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	0	1	1	1
440	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
435	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
430	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
370	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1
340	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
333	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0
328	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
313	1	0	1	0	0	0	1	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	1	1	1	0
303	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
298	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
295	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	0	1	0	0	1
290	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
274	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1
224	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
215	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
170	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
151	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0
140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
138	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
75	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
58	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
45	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table II.3. Continued.

Hae-I

bp	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	R	S	T	U	V	Y	Z	X
553	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	0	1	1	1	1
530	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
521	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	0	0	0	1	1	1	0	0	0
485	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
470	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
437	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
389	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
385	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
380	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
375	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
365	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
360	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
290	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
277	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
255	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
247	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
240	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
238	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
222	1	0	1	1	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1
210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
203	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
190	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
180	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
173	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	0
154	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
133	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
113	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
108	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
100	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
90	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
51	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
46	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
40	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1

Table II.3. Continued.

Hinf-I

bp	A	B	C	D	F	G	H	I	J	L	K	M	N	O	P	R	S	T	V	U	Z
1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
920	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
910	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
869	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	0
750	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
625	1	0	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	1
525	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
520	0	0	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0
470	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	1	0	0	1	1
430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
410	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
399	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
377	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
360	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
350	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
345	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
336	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
280	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
170	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0
160	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
125	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
105	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
98	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	0
66	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
52	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1

Table II.3. Continued.

Msp-I

bp	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	R	S	T	Y	V
1043	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1
907	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
880	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
801	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0
617	1	1	1	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	0	0
600	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
590	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
554	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	1
550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
540	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
520	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
400	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
390	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0
375	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
360	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
355	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
350	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
343	1	0	1	0	0	1	0	1	1	1	0	1	1	0	0	0	1	1	0	0
315	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
293	1	1	0	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	0	0
290	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	1	0	1	0	0
285	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
245	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
240	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
200	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
165	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
82	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1	1	0	1	0	0
65	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
55	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0
46	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	0	0

Table II.3. Continued.

Cfo-I

bp	A	B	C	D	E	F	G	H	I	J	K	L	M
1700	0	0	0	0	0	0	0	0	0	0	1	0	0
1198	0	0	1	0	0	0	0	0	1	0	0	0	0
1008	0	1	0	0	0	0	0	0	0	0	0	1	1
993	1	1	0	0	1	1	0	1	0	1	0	1	1
753	1	0	1	1	1	0	0	1	1	0	0	0	0
743	0	0	0	1	0	0	0	0	0	0	0	0	0
680	0	0	0	0	0	0	1	0	0	0	0	0	0
577	0	0	0	0	0	0	1	0	0	0	0	0	0
560	0	0	0	0	0	0	0	0	0	1	0	0	0
400	0	0	0	0	0	0	0	0	0	1	0	0	0
370	0	0	0	0	0	0	1	0	0	0	0	0	0
365	0	0	0	0	0	1	0	0	0	0	1	0	0
360	0	0	0	0	0	1	0	0	0	0	0	0	0
320	0	0	0	0	0	0	1	0	0	0	0	0	0
318	0	0	0	0	0	0	1	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	1	1
270	0	0	0	0	0	0	0	1	0	0	0	0	0
259	1	1	1	1	1	0	0	1	1	1	0	0	0
239	1	0	0	1	1	0	0	0	0	0	0	0	0
215	0	0	0	0	0	1	0	0	0	0	0	0	0
210	1	1	1	1	0	1	0	1	1	1	1	1	0
205	0	0	0	0	0	0	0	0	0	0	1	0	0
195	0	0	0	1	0	0	0	0	0	0	0	0	0
188	0	0	0	0	1	0	0	0	0	0	0	0	0
185	0	0	0	0	0	0	0	0	0	0	0	0	1
95	0	0	0	0	0	0	1	0	1	0	0	0	0
63	1	1	1	1	1	0	0	1	1	0	0	0	0
52	1	1	1	1	1	1	1	1	1	1	1	1	1

Rsa-I

bp	A	B	C	D	E	F	G	I	H	J
1100	0	0	0	0	1	0	0	0	0	0
985	1	1	1	1	0	1	0	1	1	0
820	0	0	0	0	0	0	0	0	0	1
620	0	0	0	1	0	0	0	0	0	0
517	0	1	0	0	0	0	1	0	0	1
474	1	0	0	1	0	1	1	1	1	0
455	0	0	0	0	0	0	0	1	0	0
445	0	0	0	0	0	0	0	0	0	1
440	0	0	0	0	0	0	0	0	0	1
363	1	1	1	0	1	0	1	0	0	0
330	0	0	1	0	0	0	0	0	0	0
315	1	1	1	0	1	1	1	1	1	0
290	0	0	0	0	0	1	0	0	0	0
275	0	0	0	0	0	0	0	0	0	1
225	0	0	0	0	0	0	0	0	1	0
165	0	0	0	0	1	0	0	0	0	0
150	0	0	1	0	0	0	0	0	0	0
140	0	0	0	0	0	0	0	0	1	0
120	0	0	0	0	1	0	0	0	0	0
97	1	1	1	1	1	1	1	1	1	0
93	1	1	1	1	1	1	1	0	1	0
82	0	0	0	0	0	1	0	0	0	0
63	1	1	1	1	1	1	1	1	1	0
53	1	1	1	1	1	1	1	1	1	1

Table II. 4. Restriction fragment length estimates (in number of base pairs, pb) of ND3/4 regions of mtDNA for each of six restriction enzymes (*Alu*-I, *Cfo*-I, *Hae*-III, *Hinf*-I, *Msp*-I, *Rsa*-I). Upper case letters denote haplotypes 1, denotes the presence of a fragment, 0 denotes the absence of a fragment

Alu-I

bp	A	B	C	D	E	F	G	I
1039	0	1	0	0	0	0	0	0
844	0	0	0	0	0	1	1	1
835	0	0	0	1	0	0	0	0
790	0	0	1	0	0	0	0	0
770	1	0	0	1	1	0	0	0
748	0	0	0	0	0	1	1	0
483	1	1	1	0	0	0	0	0
395	0	0	0	0	0	0	0	1
362	1	1	1	1	1	1	1	1
351	1	1	1	0	1	0	0	0
320	0	0	0	0	0	0	0	1
270	0	0	0	0	1	0	0	0
250	1	0	0	1	1	1	0	1
230	0	0	1	0	0	0	0	0
210	0	0	0	0	1	0	1	0
190	1	1	1	1	1	1	1	1
60	0	0	0	0	0	0	1	0
46	1	1	1	1	1	1	1	1

Cfo-1

bp	A	B	C	D
2017	1	0	0	0
1420	0	1	0	0
1020	0	0	0	1
950	0	0	1	0
920	0	0	0	1
630	0	0	1	0
590	0	1	0	0
450	0	0	1	0
356	1	1	0	1
340	0	0	1	0
40	1	1	1	1

Hae-III

bp	A	B	C	D	E	F	G	H
550	0	0	1	0	0	0	0	0
462	0	0	0	0	0	1	0	0
398	1	1	0	1	1	1	1	1
397	0	0	0	0	0	0	0	1
385	0	0	1	0	0	0	0	0
252	1	1	1	1	1	0	1	1
244	0	0	0	1	0	0	0	0
236	1	1	1	1	1	1	1	1
230	1	1	1	1	1	1	1	1
230	0	0	0	0	1	0	0	0
230	0	0	0	0	1	0	0	0
220	1	1	0	1	1	0	1	0
210	0	1	0	0	0	0	0	0
190	1	1	0	1	0	1	1	0
155	0	0	1	0	0	0	0	0
150	1	1	0	0	0	1	1	1
136	1	0	1	1	1	1	1	1
130	0	0	0	0	0	0	1	0
122	1	1	0	1	1	1	1	1
120	1	1	0	1	0	1	0	1
96	0	0	1	0	0	0	0	0
94	1	1	1	0	1	1	1	1
91	1	0	1	1	1	1	1	1
53	1	1	1	1	1	1	0	1
50	0	0	0	0	0	0	1	0
47	1	1	1	1	1	1	1	1
42	1	1	1	1	1	1	1	1

Table II.4. Continued

Hinf-I

bp	A	B	D	E	F	H	I	K	M	N
1250	0	0	1	0	0	0	0	0	0	0
1115	0	0	0	0	0	0	0	0	1	0
800	0	0	0	0	1	0	0	0	0	0
780	0	0	0	0	0	0	0	0	0	1
710	1	0	0	0	1	1	1	0	0	0
525	0	0	0	1	0	0	0	0	0	0
410	0	1	0	0	0	0	0	0	0	0
405	1	1	0	1	1	1	0	1	0	1
400	1	1	0	1	0	1	1	1	1	1
395	1	1	1	1	0	0	0	1	1	0
300	0	0	0	0	0	1	0	0	0	0
290	0	1	0	0	0	0	0	1	0	1
250	0	0	0	0	0	0	1	1	0	0
190	0	0	1	1	0	0	0	0	0	0
175	0	0	0	0	0	0	1	1	0	0
153	1	1	1	1	1	1	1	1	1	1
114	1	1	1	1	1	1	1	1	1	1
103	1	1	1	1	1	1	1	1	1	1
98	0	0	0	0	0	1	0	0	0	0
85	0	0	1	0	0	0	0	0	0	0
70	1	1	1	1	1	1	1	1	1	1
47	1	1	1	1	1	1	1	1	1	1

Msp-I

bp	A	B	C	D	E
1000	1	0	1	1	0
730	0	1	0	0	1
490	1	1	1	1	1
270	0	1	1	0	1
260	1	1	1	1	1
214	0	0	1	0	0
200	1	1	1	1	1
167	1	1	1	1	1
165	1	1	0	1	1
120	0	0	0	1	1
75	1	1	0	0	0
47	1	1	1	1	1
45	1	1	0	0	0

Rsa-I

bp	A	B	C	D	E
967	0	1	1	1	0
900	0	0	0	1	0
850	0	0	0	0	1
705	1	0	0	0	1
575	0	0	1	0	0
520	1	1	0	0	0
362	1	1	1	1	1
342	1	1	1	0	0
255	1	0	0	0	1
166	1	1	1	1	1
58	1	1	0	1	1

APPENDIX III: MICROSATELLITES

Table III. 1. Ingredients for 10 µl PCR reaction used to amplify the Cha17, Cha20, Cha123 and Cha63 genes of *Clupea harengus* and *Clupea pallasi*. The PCR buffer contained 15 mM $MgCl_2$ and Tween.

	Stock	µl / 10 µl reaction	Final concentration
PCR buffer	10 x	1	
dNTP	2 mM	0.8	
Tween		1	
$MgCl_2$	50 mM	0.2	
Primer	10 µM	0.5 each	
Taq polymerase		0.05	
Template DNA		1	
Sterile, filtered water		4.97	

Table III. 2. Temperature cycle used to amplify the Cha17, Cha20, Cha23 and Cha63 genes of *Clupea harengus* and *Clupea pallasi*. ': minutes, '': seconds. #: 56 °C was used for Cha17, *: 25 cycles were used for Cha17.

Temperature (°C)	Time	Function	Cycle
95	1'	initial denaturation	1
94	20		
57#	20''	annealing of primers	5
72	20''	extension	
90	20''	denaturing	
56	20''	annealing	30*
72	20''	extension	