APPLICATION OF MOLECULAR MARKERS IN POPULATION AND CONSERVATION GENETICS, WITH SPECIAL EMPHASIS ON FISHES

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Preface

This thesis represents the major part of my research conducted at the Danish Institute for Fisheries Research (DIFRES), Department of Inland Fisheries from 1994 to 2002. Let me state right away that the term "my research" needs some clarification. The work presented here is really the result of the combined effort by several people. As is the case nowadays for many scientific disciplines, molecular population genetics research is so labour-intensive and requires so much different expertise that it clearly calls for group work. Fortunately, I have been gifted by many very good collaborators, both at DIFRES and elsewhere.

First of all, I want to thank my very good colleagues in the DIFRES population genetics group for excellent collaboration over the years in a stimulating and often very entertaining environment. Einar Eg Nielsen has been my closest collaborator, always helpful and ready for a good discussion which has led to many new ideas and wild plans. Einar has a well-developed sense of criticism, and if an idea, a proposal or a manuscript has survived Einar's sharp mind and tongue, then it is unlikely that even the meanest reviewer will be able to come up with strong objections. Karen-Lise Mensberg was employed at DIFRES at the same time as myself, in 1994, and has been of incredible help ever since. Besides being an extremely competent technician she has always been a good support, totally committed to the work and eager to try out new methods and techniques. Daniel Ruzzante, now at Dalhousie University in Halifax, Canada, worked in the group from 1998-2002. Daniel is something of a "statistics cowboy", who shoots at everything that moves, and it has been very inspiring for me to collaborate with him and discuss data analysis and evolutionary biology. Dorte Bekkevold has been an important team player and contributed to the work of this thesis, first as a PhD student and later as a scientist in the group. Besides a sharp mind she has also contributed with important new angles, especially from the perspective of behavioral ecology. Believe it or not, Karen-Lise is not the only "super-technician" in the group. Dorte Meldrup and Tina Brandt Andersen have been with us since 1997 and 2001, respectively, and their skills and enthusiasm have made them invaluable members of the group. Together with Karen-Lise they are also messy students' worst nightmare, thereby providing a good balance between a creative environment and some degree of order in the laboratory. I also want to thank a number of other persons who have worked in or been associated with our group over the years: Lise-Lotte W. Andersen, the postdocs (Jens Carlsson and Carmen Bouza), PhD students (Hanne Jørgensen and Jakob Hemmer Hansen) and Masters students (Torben Meldgaard Madsen, Peter Høtbjerg Nielsen, Birgitte Jakobsen, Peter Foged Larsen, Siri Østergaard, Nina Poulsen and Niels G. Fritzner).

I also want to thank a number of non-population geneticist colleagues from the DIFRES Department of Inland Fisheries for good and inspiring collaboration and discussions. Søren Berg and Lene Jacobsen have been important collaborators in the work on whitefish and otters, respectively. Anders Koed, Kim Aarestrup, Christian Skov, Finn Sivebæk and Niels Jepsen have all contributed to establishing a lively and creative environment with many good and sometimes even heated discussions. Stig Pedersen and Jes Dolby have always been helpful in fixing computer problems. I also want to thank Peter Geertz Hansen for many discussions on stocking and fisheries management, which have all convinced me of the necessity of studying the impact of stocking activity on wild fish populations. Also thanks to everybody else at the DIFRES Department of Inland Fisheries, both for technical assistance and for making this a nice place to work. A special thanks goes to the Research Director of the Department of Inland Fisheries, Gorm Rasmussen, and the Director of DIFRES, Niels Axel Nielsen, for always being supportive of the population genetics work and (mis?)understanding its potential value.

I want to give a special thanks to Volker Loeschcke, Dept. of Ecology and Genetics, University of Aarhus. Volker was my PhD supervisor and has continued to be an important collaborator, especially in relation to common supervision of students. He has furthermore always been supportive and interested in our work in the DIFRES population genetics group and ready to give good advice.

Cino Pertoldi, National Environmental Research Institute, took the leading force in the work on otter historical samples and I want to thank him for a very stimulating and entertaining collaboration. Also thanks to Vibeke Simonsen, National Environmental Research Institute, for collaboration on some of the work on brown trout and on an ongoing project on Indian major carps.

Ellen Kenchington, Bedford Institute of Oceanography, Department of Fisheries and Oceans, Canada, is thanked for good collaboration on a review paper about assignment tests and for many good discussions in the ICES Genetics Working Group.

A number of persons have provided invaluable help by collecting samples for the studies. I am particularly grateful to Mogens Thomassen and Karup Å Sammenslutningen for collecting trout samples from the Karup River over several years and for making detailed information available on their stocking activities. Also, Søren Larsen and Gert Holdensgaard, Dansk Center for Vildlaks, have been very helpful in collecting trout and Atlantic salmon samples.

I also want to thank many good colleagues from abroad, too many to mention, for good and stimulating discussions, particularly within the framework of the "TROUTCONCERT" EU concerted action and within the ICES Genetics Working Group, and a lot of reviewers and editors for their valuable comments and suggestions on the papers included in the thesis.

Very warm thanks goes to my parents, Bente and Ebbe, and my brother, Kenneth, for all their encouragement and support over the years and for always showing a genuine interest in the weird work I have been doing. Finally, but foremost, I want to thank Anne-Merete for all her endless love, support and, not least, patience, and our two children, Frederikke and Andreas, for being such lovely kids and for always giving me a true perspective of what really matters in life. I am sure they can turn this thesis into a lot of nice paper fliers and paper hats.

Silkeborg, 4 February 2003

Michael M. Hansen

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Papers included in the thesis

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Paper 2. Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1996). Genetic structure of European populations of Atlantic salmon (*Salmo salar* L.) inferred from RFLP analysis of PCR amplified mitochondrial DNA. *Heredity*, **77**, 351-358.

Paper 3. Hansen, M.M., Nielsen, E.E. & Mensberg, K.-L.D (1997). The problem of sampling families rather than populations: Relatedness among individuals in samples of juvenile brown trout (*Salmo trutta* L.). *Molecular Ecology*, **6**, 469-474.

Paper 4. Hansen, M.M., Mensberg, K.-L. D., Rasmussen, G. & Simonsen, V. (1997). Genetic variation within and among Danish brown trout (*Salmo trutta* L.) hatchery strains, assessed by PCR-RFLP analysis of mitochondrial DNA segments. *Aquaculture*, **153**, 15-29.

Paper 5. Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1997). Analysis of microsatellite DNA from old scale samples of Atlantic salmon: A comparison of genetic composition over sixty years. *Molecular Ecology*, **6**, 487-492.

Paper 6. Hansen, M.M. & Mensberg, K.-L. D. (1998). Genetic differentiation and relationship between genetic and geographical distance in Danish sea trout (*Salmo trutta* L.) populations. *Heredity*, **81**, 493-504.

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Paper 8. Hansen, M.M., Mensberg, K.-L. D. & Berg, S. (1999). Postglacial recolonisation patterns and genetic relationships among whitefish (*Coregonus sp.*) populations in Denmark, inferred from mitochondrial DNA and microsatellite markers. *Molecular Ecology*, **8**, 239-252.

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Paper 17. Nielsen, E.E., Hansen, M.M., Schmidt, C., Meldrup, D. & Grønkjær, P. (2001). Population of origin of Atlantic cod. *Nature*, **413**, 272.

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Paper 19. Pertoldi, C., Hansen, M.M., Loeschcke, V., Madsen, A.B., Jacobsen, L. & Baagoe, H. (2001). Genetic consequences of population decline in European otter (*Lutra lutra*): An assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proceedings of the Royal Society of London, Series B Biological Sciences*, **268**, 1775-1781.

Paper 20. Ruzzante, D.E., Hansen, M.M. & Meldrup, D. (2001). Distribution of individual inbreeding coefficients, relatedness and influence of stocking on native anadromous brown trout (*Salmo trutta*) population structure. *Molecular Ecology*, **10**, 2107-2128.

Paper 21. Bekkevold, D., Hansen, M.M. & Loeschcke, V. (2002). Male reproductive competition in spawning aggregations of cod (*Gadus morhua*, L.). *Molecular Ecology*, **11**, 91-102.

Paper 22. Hansen, M.M. (2002). Estimating the long-term effects of stocking domesticated trout into wild brown trout (*Salmo trutta*) populations: An approach using microsatellite DNA analysis of historical and contemporary samples. *Molecular Ecology*, **11**, 1003-1015.

Paper 23. Hansen, M.M., Ruzzante, D.E., Nielsen, E.E., Bekkevold, D. & Mensberg, K.-L.D. (2002). Long-term effective population sizes, temporal stability of genetic composition and potential for local adaptation in anadromous brown trout (*Salmo trutta*) populations. *Molecular Ecology*, **11**, 2523–2535.

Application of molecular markers in population and conservation genetics, with special emphasis on fishes

1. Introduction

The unifying concept of the papers included in this thesis is "molecular population genetics", or more specifically the application of molecular markers for studying the genetic structure of populations. Population genetics in itself can be defined as the science of how genetic variation is distributed among species, populations and individuals, and fundamentally it is concerned with how the evolutionary forces of mutation, selection, random genetic drift and migration affect the distribution of genetic variability. As such, it is often difficult to make a sharp distinction between population genetics and the more broadly defined discipline of evolutionary biology. Population genetics is one of the theoretically most well-founded disciplines among the biological sciences, starting with the classical works by Fisher, Wright, Haldane and others in the 1920s – 1930s describing the fundamental relationships between the evolutionary forces (e.g., Fisher, 1930; Haldane, 1930; Wright, 1931).

Whereas the possibilities for empirical studies of variation at single loci were initially scarce, the research opportunities were drastically improved with the advent of allozyme electrophoresis in the 1960s and has since the 1980s been boosted with the ever accelerating developments in molecular biology, which have supplied numerous new techniques and genetic markers suitable for population genetics studies. At the same time, population genetics theory has continued to evolve and has in many cases led to novel approaches and methods for analysing empirical data, which are currently revolutionising population genetic data analysis (e.g., Luikart & England, 1999). Many of these approaches are based on coalescence theory, where popularly speaking, one is "looking back in time" to assess the gene genealogy, which contains important information about the history of populations, such as population declines or expansions. Also, the developments in computer technology have made it feasible to develop highly sophisticated analyses based on Markov Chain Monte Carlo numerical resampling techniques for estimating the probability distributions of various parameters of interest (see Luikart & England, 1999 and Beaumont, 2001 for recent reviews). All together, the developments in molecular biology and statistical population genetics have resulted in unprecedented opportunities for empirical population genetics studies. It is now possible to do research that one could only dream about, say, fifteen years ago, such as assigning individuals to populations, detecting population bottlenecks and analysing the genetic composition of historical populations based on old archival samples.

My own work in molecular population genetics has aimed at using molecular tools to obtain information on the genetic population structure and population history of primarily fish species (with the exception of one mammal), which are all subject to direct human exploitation or more indirect human disturbance. Thus, the research has involved basic scientific aspects, such as describing and understanding the genetic structure of populations, but I have also been interested in studying how human activity has affected the genetic composition of populations by causing population declines or by causing mixing of divergent gene pools due to stocking of exogenous fish. On the following pages I first give a short introduction to the species studied and the different kinds of molecular markers applied. Next, I describe the results we have obtained within the different research themes and put the results into a broader context. Finally, I sum up the general conclusions reached through the work.

2. The species studied

Fishes have many virtues making them excellent objects for population genetics studies. In particular, they often have the convenient property of exhibiting discrete populations, a feature assumed in many models of genetic population structure. Also, from a conservation genetics point of view fishes can be regarded as good model organisms. This is not least the case in studies of the impact of human mediated gene flow and transplantations among populations, where stocking activities and, more recently, escapes of farmed domesticated fish have been ongoing in salmonid fishes for several decades.

The research of this thesis has focused on four fish species, i.e. brown trout, Atlantic salmon, whitefish and cod, and one mammal, the European otter. Below, I give a brief introduction to the biology of these species.

2.1 Brown trout (*Salmo trutta* L.)

The brown trout belongs to the family of salmonid fishes, *Salmonidae*. It is naturally distributed in Europe and western Asia, and the southern limit of its range of distribution is the Atlas Mountains in North Africa. However, the species has also been extensively introduced worldwide and is now found in such diverse locations as USA and Canada, Argentina and New Zealand (Elliott, 1994). The species is of only minor importance for commercial fishing. However, it is of tremendous importance as a target for angling and is therefore of considerable socioeconomic importance in many European countries.

Fig. 1. Anadromous brown trout (sea trout), caught by my colleague Einar.

The species is basically omnivorous. Smaller trout prey mostly on invertebrates, whereas larger trout often become piscivorous. Brown trout spawn in autumn and winter, usually in running water, though it may also spawn in lakes. The female excavates a spawning redd in the gravel for her eggs, which are subsequently fertilised by one or more males. Following this, the redd is again covered with gravel. The larvae hatch in the spring. The juveniles spend one or more years in the nursery area before they initiate one of several possible types of life-history. Basically, they can either become resident or migratory. If they become resident they spend the rest of their life in the natal river, whereas if they become migratory they migrate to other foraging areas, either within the same river system, into a lake or into the sea (anadromous trout or sea trout). Anadromy (and in some cases also lake-migratory life history types) involves smoltification. This can be regarded as a type of metamorphosis, where the fish starts to migrate out of the nursery area, adapts physiologically to life in a marine environment, and adopts a silverish colour. In Denmark, smoltification typically takes place at 1 to 3 years of age (Rasmussen, 1986), but this may vary considerably among regions and, presumably, climatic conditions (Elliott, 1994). Brown trout and most other salmonids exhibit a strong homing instinct (e.g., Stabell, 1984). Most fish return to their natal river to spawn and only a few percent stray to different rivers (see Altuhov *et al.*, 2000 for an overview of straying rates in different salmonids). One life-history form that deserves special mentioning is mature male parr, i.e. males that become sexually mature at a very young age (1-2 years) and at lengths down to 10-15 cm. This is a phenomenon that is observed not only in brown trout, but also in Atlantic salmon and other salmonids (e.g. Fleming, 1996).

The possible genetic basis of life-history variation (residency versus anadromy) in brown trout has been subject to several studies (e.g., Skaala & Nævdal, 1989; Hindar *et al.*, 1991a; Ferguson *et al.*, 1995; Pettersson *et al.*, 2001) and remains a controversial issue. Hindar *et al.* (1991a) undertook a detailed study of this problem and were unable to detect genetic differentiation between coexisting anadromous and resident trout, whereas genetic differentiation was observed between land-locked and accessible populations from the same drainages. It is also worth noting that the sex ratio has been found to differ between resident and anadromous trout, with 75-100% of resident trout being males, whereas there is a surplus of females among anadromous trout (Jonsson, 1982; Rasmussen, 1986). However, these results do not imply that there is not a genetic component to life-history variation within a population; this question must still be regarded as unresolved. It does imply, though, that co-existing resident and anadromous trout are normally (but not necessarily always) part of the same population.

Brown trout does not only exhibit considerable ecological variability, it also exhibits strong morphological variation, both within and among populations. This has previously resulted in the identification of more than one hundred different species of brown trout. Although the trend is now to regard brown trout as one highly variable species (reviewed by Ferguson, 1989) some taxonomists still prefer to regard it as a species complex rather than one single species (e.g., Kottelat, 1997).

One aspect of brown trout biology that has been subject to particular interest is the phenomenon of sympatric reproductively isolated populations. The first study describing the occurrence of sympatric populations of brown trout was by Ryman *et al.* (1979), who discovered a significant heterozygote deficit at the *LDH-A1** allozyme locus in brown trout in the Swedish Lake Bunnarsjöarna. This heterozygote deficit was shown to be due to a Wahlund effect caused by the existence of two sympatric but non-interbreeding populations. Later, Ferguson and coworkers described a remarkable set of three coexisting trout morphs in the Irish Lough Melvin, called sonaghen, gillaroo and ferox (e.g., Ferguson & Mason, 1981; Ferguson & Taggart, 1991; McVeigh *et al.*, 1995). Extensive studies of this system have shown that the three morphs exhibit striking genetic and ecological differences, the latter encompassing feeding preferences and use of distinct spawning locations. The currently held view is that the morphs have diverged allopatrically and may represent descendants from different postglacial recolonisation events. Examples like this, along with the presence of at least five phylogeographical lineages within brown trout (see below) challenge the view that brown trout consist of just one species (e.g. Ferguson & Taggart, 1991). On the other hand, there are several examples showing that when distinct phylogeographical lineages of brown trout are brought into contact as a result of stocking, they still do interbreed and produce fertile offspring, thus not satisfying the species criterion assumed in the biological species concept (Mayr, 1963). Personally, I think one has to reconcile with the fact that the taxonomy of brown trout is a complex situation that will always remain debatable whether or not a "monospecies" or "polyspecies" approach to taxonomy is taken. Unfortunately, however, legislation in most European countries only recognizes species as the unit of conservation. It is a big challenge throughout Europe to convince authorities that species are only conserved by conserving populations, and that any single population is unlikely to contain all the genetic variability and evolutionary potential of the whole species. The Evolutionary Significant Unit (ESU) concept used in the USA (Waples, 1991a) represents an approach aimed at conserving population units that represent an important part of the evolutionary legacy of the species. Application of the ESU concept in Europe would greatly

improve conservation not only of brown trout but also other species exhibiting a similar complicated genetic population structure (Laikre, 1999).

Whereas the taxonomy of brown trout as such is still somewhat controversial, studies undertaken during the past decade have shed new light on the presence of distinct evolutionary lineages. A now classical study by Bernatchez *et al.* (1992), based on sequencing of the mitochondrial DNA d-loop, led to the identification of five major phylogeographical lineages, with one of them, the so-called Atlantic lineage, being the only lineage present in the previously glaciated northern Europe, whereas the other lineages are distributed in Central and Southern Europe. Later studies (e.g., Bernatchez & Osinov, 1995; Weiss *et al.*, 2000; Bernatchez, 2001; Antunes *et al.*, 2002; Duguid, 2002) have added new important pieces to the puzzle, but have not fundamentally challenged the original suggestion by Bernatchez *et al.* (1992) of five major lineages. Within the Atlantic lineage it has also been suggested that postglacial recolonisation of Northern Europe has occurred from more than one refuge. This suggestion was originally based on the distribution of alleles at the lactate dehydrogenase *LDH-C1** allozyme locus (Hamilton *et al.*, 1989; see also Garcia-Marin *et al.*, 1999). A later study based on a large mitochondrial DNA data set provides support for a postglacial recolonisation scenario involving two-three separate glacial refuges (Bernatchez, 2001). Attempts to link variation at the *LDH-C1** locus with a specific mitochondrial DNA lineage were initially unsuccessful (Hynes *et al.*, 1996), but a recent study does find some concordance of *LDH-C1** variation and distinct mitochondrial DNA lineages (Duguid, 2002).

As outlined above brown trout has been the target of several studies attempting to resolve the distribution and origin of the major phylogeographical lineages. The work of this thesis has also briefly touched on phylogeography of brown trout (/6/), but it has been primarily centred at two different issues. First, it has been of interest to elucidate the genetic population structure of a species exhibiting strong homing and discrete populations (/1/; /3/; /6/; /20/; /23/). Second, brown trout is subject to significant stocking activities, both in Denmark and elsewhere in Europe. It has been of interest to obtain knowledge of the actual genetic consequences of stocking domesticated trout into wild trout populations, not only to improve management and conservation of the species *per se*, but also to gain experiences of general relevance to conservation biology (/4/; /12/; /13/; /14/; /15/; /20/; /22/).

2.2 Atlantic salmon (*Salmo salar* L.)

Atlantic salmon is closely related to brown trout, and in fact the two species are able to hybridise under natural conditions (e.g., Verspoor & Hammar, 1991). The species is distributed in the Atlantic region of Europe, from Spain to the Barents Sea region of Russia, and in eastern North America. Phylogeographical studies have shown that the species consists of three major lineages: North American Atlantic salmon, European Atlantic salmon and Baltic salmon (Ståhl, 1987; Nilsson *et al.*, 2001). However, recent studies have suggested that this general picture may in some cases be more complicated than initially suggested, with salmon in the Baltic Sea region possibly consisting of two distinct lineages derived from two independent recolonisation events (Koljonen *et al.*, 1999; Nilsson *et al.*, 2001).

In comparison to brown trout Atlantic salmon displays less diversity in life-history variation. Except for examples of landlocked populations most Atlantic salmon smoltify and migrate to the sea. Salmon from North America and the Atlantic region of Europe undertake very long feeding migrations to the sea around Greenland, whereas Baltic salmon remain in the Baltic Sea (Mills,

1991). However, similar to brown trout and several other salmonids the life-history form mature male parr is observed in most, if not all populations.

Atlantic salmon is of very high economical importance, both as a popular game fish, as a target for commercial fishing and for rearing in aquaculture. In fact, salmon farming is one of the most important industries in Norway and Scotland, though at the same time causing severe problems for wild salmon populations due to ecological and genetic interactions between escaped farmed salmon and their wild conspecifics (Heggberget *et al*., 1993). Atlantic salmon has declined drastically throughout most of its distributional range due to overfishing and habitat destruction (Mills, 1991), and in Europe protection of the species is highly prioritised in conventions and policies such as the Berne Convention (protection of endangered and vulnerable European species) and the EU Habitat Directive.

In the context of this thesis, most emphasis has been on investigating the spatiotemporal genetic structure of the species, based on analysis of historical and contemporary samples. This has been done in order to assess if the genetic structure of populations is indeed stable over longer time spans, thereby allowing for local adaptations (/10/), but also to determine whether or not there are still indigenous Atlantic salmon populations left in Denmark (/5/; /18/).

2.3 Whitefish (*Coregonus sp.*)

Coregonid fishes, whitefish, also belong to the family *Salmonidae*, though some taxonomists place them in their own family, *Coregonidae* (Kottelat, 1997). Whitefish are widely distributed in temperate and subarctic regions of the northern hemisphere, where they in particular inhabit lakes, but also rivers. Some populations are anadromous, though mostly in connection to brackish water bodies.

The most prominent feature of whitefish in the context of evolutionary biology is that they often exhibit considerable morphological variation, e.g. “dwarf” versus “normal” forms (e.g., Vuorinen *et al.*, 1993) and “high gill raker number” forms versus “low gill raker number” forms (Bernatchez *et al.*, 1996; reviewed by Himberg & Lehtonen, 1995). Furthermore, these forms often occur sympatrically, and considerable effort has been put into investigating the origin and genetic distinctiveness of coexisting morphs. Apparently, there is no simple single mechanism involved. In some cases, divergence appears to have occurred sympatrically, whereas in other cases it is suggested to be the result of secondary contact between lineages that have diverged in parapatry (e.g., Bernatchez & Dodson, 1990; Pigeon *et al.*, 1997; Douglas *et al.*, 1999; Turgeon *et al.*, 1999).

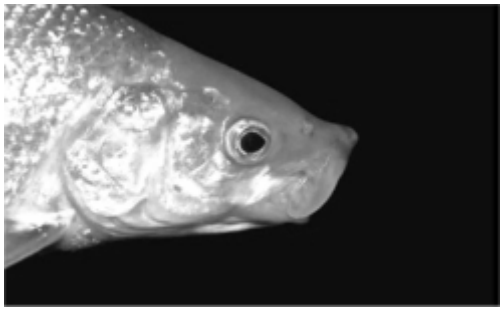

Fig. 2. North Sea houting. Note the elongated upper jaw (photo by Finn Sivebæk).

The tremendous morphological variability has caused severe taxonomical problems. On one hand, some taxonomists argue that there are just a few polytypic species present, whereas others argue in favour of numerous species (Himberg & Lehtonen, 1995; Kottelat, 1997). It adds to the problems that some of the previous distinctions between species do not reflect the distribution of distinct phylogeographical lineages. Thus, a distinction has been made between the European whitefish (*Coregonus lavaretus*) and the North American lake whitefish (*Coregonus clupeaformis*), mainly on the basis of the continent of origin of the populations. However,

mtDNA analyses show that one lineage corresponding to *lavaretus* is distributed also in North America and, overall, several distinct lineages are present within the two defined "species" (reviewed in Bernatchez, 1995).

The focus on whitefish in this thesis has been on the Danish populations of lake whitefish, where a distinction has been made between "normal" whitefish (*Coregonus lavaretus*) and the highly endagered North Sea houting (classified as *Coregonus oxyrhynchus*), which is a "high gill raker number form" and also differs morphologically from other whitefish of the North Sea area by exhibiting an elongated upper jaw (Grøn, 1987). Also, it is anadromous, and in contrast to most other whitefish it is capable of living at high salinities (Grøn, 1987). Our research has aimed at resolving possible postglacial recolonisation routes of Danish whitefish and assessing the status of North Sea houting relative to "normal" whitefish (/8/). Do they represent recently diverged forms, or have they been derived from different postglacial recolonisation events?

2.4 Atlantic cod (*Gadus morhua* L.)

The Atlantic cod is a marine fish belonging to the family *Gadidae*. It is omnivorous and preys both on invertebrates and fish. The species is widely distributed in the North Atlantic Ocean, ranging from North America to Europe, including the Baltic Sea. It is of tremendous importance for commercial fisheries but, unfortunately, it is so heavily exploited that the stocks are in some regions close to a collapse. A collapse of the cod stock has already occurred in the sea around Newfoundland, which previously sustained a very important cod fishery, and there are still no signs that the stock is about to recover (Fu *et al*., 2001).

Cod spawns in huge aggregations of individuals, and it is expected that effective population sizes are also high. This again is expected to lead to very little random genetic drift and, consequently, genetic divergence among populations occurs at a slow rate. Further, cod may undertake long migrations and since there are few distinct boundaries in the ocean, there is a potential for considerable gene flow among populations. Consequently, the expectation is that limited drift and/or strong gene flow will lead to very low levels of genetic differentiation among populations, except over large geographical scales. The results of empirical studies are generally in accordance with this prediction, as exemplified by studies by Mork *et al.* (1985) and Pogson *et al.* (2001) on transatlantic scales. Nevertheless, allozymes, and in particular microsatellite DNA analyses, have also documented examples of weak but statistically significant genetic differentiation at more regional scales (e.g., Mork *et al.*, 1985; Ruzzante *et al.*, 1997; 1998; Hutchinson *et al.*, 2001) and a study employing microsatellite analysis of historical and contemporary samples has demonstrated that the genetic structure of populations is temporally stable at least at the scale of several decades (Ruzzante *et al.*, 2001). Cod in the Baltic Sea appears to be a special and very interesting case, as Baltic cod is substantially genetically differentiated from North Sea cod (Mork *et al.*, 1985; /17/).

The research on cod presented in this thesis focuses on two different issues. First, the use of individual assignment tests for identifying cod from the Baltic Sea, the North Sea and the Barents Sea has been explored (/17/). Second, since cod is a prolific mass spawner, it has been of interest to investigate the mating system of the species (/21/). Does a pelagically spawning fish like cod spawn more or less at random or does reproductive competition occur, leading to biased reproductive output at the level of individuals?

2.5 European otter (*Lutra lutra* L.)

The European otter is a mammal that belongs to the family *Mustelidae*. It is closely associated with aquatic habitats where it forages primarily on fish, but also amphibians, small mammals and birds. Unfortunately, the species has experienced drastic population declines over large parts of Europe (Macdonald & Mason, 1994). Otters have traditionally been subject to hunting, because they have been assumed to "compete" with humans for fish resources, but the more recent declines are a result of habitat destruction and pollutants.

The thesis contains two studies focusing on otters, but from very different angles. First, otters are nocturnal animals and are therefore only rarely observed directly. Information about the distribution of otters therefore is based on indirect signs, in particular scats. We developed a mitochondrial DNA based assay that allowed for distinguishing between scats from otter, American mink (*Mustela vison*) and polecat (*Mustela pretorius*) (/9/). Second, we were interested in looking further into the decline of Danish otters. Did the recent decline really appear very suddenly or was it the culmination of a decline taking place over a longer time span? What are the genetic relationships between the extant otter population and extinct otter populations from other parts of Denmark? In order to resolve these questions we analysed microsatellite markers from the extant population and from up to 100 year old historical samples from other parts of Denmark (/19/).

3. Molecular methods

The classical molecular (or rather biochemical) technique for studying genetic variation at co-dominant Mendelian inherited loci is allozyme electrophoresis. The technique was developed in the 1960s (Lewontin & Hubby, 1966) and was dominating until the early 1990s. In the early 1980s the first population genetic studies based on analysis of mitochondrial DNA emerged (e.g., Avise *et al*., 1979). Later, with the advent of the polymerase chain reaction (PCR) which allows for amplifying huge number of copies of specifically targeted DNA, a number of different techniques emerged, ranging from sequencing of the DNA of interest to methods analysing length polymorphisms, such as microsatellites. Just browsing through a randomly chosen issue of the journal *Molecular Ecology* will reveal a large number of terms and abbreviations denoting different techniques, such as RAPD, AFLP, SSCP, DGGE, etc. Here, I will restrict myself to explain the basics of the methods and markers that we have used, i.e. allozymes, mitochondrial DNA and microsatellites. I also provide an introduction to analysis of historical samples, which has been an important element in several of the papers of this thesis.

3.1 Allozyme electrophoresis

Allozyme electrophoresis denotes the technique for identifying genetic variation at the level of enzymes, which are directly encoded by DNA. The principle of the methodology is that mutations may lead to substitutions of amino acids in the enzyme, which may again result in a shift of conformation and net charge of the whole enzyme. Since allelic variation reflected in an enzyme may thus result in different properties, it is possible to identify different alleles by electrophoresis; tissue extracts are applied to a gel (usually starch) and an electrical current is applied. Different allelic variants of an enzyme may then migrate through the gel at a rate determined by the net charge and conformation of the enzyme. Finally, enzyme-specific histochemical staining is used to visualise specific enzymes, and different alleles are identified from different banding patterns.

The major advantage of allozyme electrophoresis is that it is technically reasonably uncomplicated and may be applied to any organism with only relatively minor adjustments to experimental

protocols. It allows for screening a large number of loci, often more than 30-40, however, usually many of the loci studied turn out to be monomorphic. In a species like Atlantic salmon this is a particularly important problem with only a handful of loci exhibiting polymorphism (e.g. Ståhl, 1987). Also, the level of variation at polymorphic loci is in most cases low, with individual loci usually exhibiting no more than two-three alleles. There are important demands concerning the freshness of tissue samples and many loci exhibit tissue-specific expression (e.g. some loci are only expressed in heart tissue) which renders it difficult to conduct non-destructive sampling.

Allozyme electrophoresis was previously the dominating technique for studies of genetic structure of populations, but today it has to a large extent been replaced by DNA techniques, in particular microsatellite DNA analysis. In this thesis the method has been used in one paper (/1/).

3.2 Mitochondrial DNA

Even though most DNA in eukaryote organisms is found in the nucleus, organelles (mitochondria and, in plants, the chloroplasts) contain their own DNA. This most likely reflects that the organelles were originally independent endosymbionts (Margulis, 1981). Mitochondrial DNA (in the following abbreviated mtDNA) has the special feature that it is haploid, maternally inherited, (mainly) selectively neutral and (mainly) non-recombining. Also, the mutation rate is higher (at least in some regions of the mtDNA molecule) than in most nuclear DNA regions. Some of the features of mtDNA that were previously taken for granted have, however, been questioned during recent years, such as selective neutrality (Rand, 1994) and its strictly non-recombining nature, and deviations from the latter assumption may lead to severe biases in phylogeny reconstruction (Schierup & Hein, 2000).

The fact that mtDNA is haploid and maternally inherited has some very important implications for its use as a genetic marker for population studies. The effective population size of mtDNA is only approx. ¼ that of nuclear loci, assuming an equal sex ratio (Birky *et al.*, 1983). As a consequence, mtDNA is subject to more drift than nuclear loci. In fact, there are some examples available demonstrating that the genetic composition of populations may be temporally stable at the level of nuclear loci, but temporally unstable at the level of mtDNA (e.g. DeSalle *et al.*, 1987; Hansen & Loeschcke, 1996a). The stronger drift operating on mtDNA also results in stronger genetic differentiation as compared to nuclear DNA (Birky *et al*,. 1989). Since mtDNA is maternally inherited this effect may be reinforced if gene flow is male-biased, whereas female-biased gene flow will tend to decrease the difference between differentiation at nuclear loci and mtDNA (Birky *et al.*, 1989). It follows that one must be careful about drawing conclusions, based on mtDNA analysis, about the overall genetic structure of populations in cases where gene flow may be expected to be sex-biased.

MtDNA is the classical genetic marker for phylogeographical studies (Avise, 1994). This is primarily due to a relatively high mutation rate and the lack of recombination, which makes it reasonably easy to reconstruct the phylogeny of different haplotypes. The assumption is then made that the phylogeny of haplotypes also reflects population history. Of course, this assumption should be subject to critical evaluation, as exemplified above, and it must also be considered that mtDNA essentially represents just one locus. Thus, it is not possible to average information from several independent loci. Keeping these reservations in mind mtDNA still remains the most useful genetic marker for phylogeography and recent statistical developments, in particular nested clade analysis (Templeton *et al.*, 1995; Templeton, 1998) have provided sophisticated tools for discriminating

between patterns of historical and ongoing gene flow and migration, range expansions and secondary contact.

Variation at mtDNA may be analysed in several different ways. The approach yielding maximum resolution is to sequence the mtDNA region of interest, the major disadvantage being that this is costly both in terms of time and money invested, in particular if large sample sizes are required. An alternative is to conduct a more coarse-grained screening, which does not necessarily detect all variation present, and all studies included in this thesis employing mtDNA analysis (/1/; /2/; /3/; /4/; /6/; /7/; /8/; /9/; /12/) have been based on such an approach. Briefly, we have PCR (polymerase chain reaction) amplified specific regions of the mtDNA molecule and then screened for variation using restriction enzymes, i.e. enzymes that recognise a specific sequence and then cuts the DNA at a specific site. This results in a number of DNA fragments, which can be visualised using gel electrophoresis and stains for DNA such as ethidium bromide. Mutations may lead to gain or loss of the sites recognised by the different restriction enzymes and, consequently, different banding patterns for different haplotypes.

Since mtDNA is so intensively studied and as sequences in some parts of the molecule are highly conserved across species, several sets of "universal primers" have been developed that allow for analysing the same mtDNA segments in a variety of species. We initially used such universal primers by Cronin *et al.* (1993) for analysing the mtDNA ND-1 and ND-5/6 segments in brown trout and the ND-1 segment in Atlantic salmon. However, amplification was sometimes suboptimal in brown trout, and in Atlantic salmon we were unable to amplify and analyse the ND-5/6 segment. Atlantic salmon generally shows low polymorphism. In order to identify more haplotypes (if present) and obtain a higher resolution of the phylogeny of haplotypes, we aligned the primer sequences for the ND-1, ND-3/4 and ND-5/6 segments originally published by Cronin *et al.* (1993) to the total sequence of the mtDNA molecule in rainbow trout (*Oncorhynchus mykiss*) (Zardoya *et al.*, 1995). There were a total of two mismatches for the ND-1 primers, five mismatches and one deletion for the ND-3/4 primers and five mismatches in the case of the ND-5/6 primers. Primers redesigned from the rainbow trout sequences resulted in much improved amplification of the three segments, both in Atlantic salmon, brown trout and European whitefish. The addition of the ND-3/4 and ND-5/6 segments to mtDNA analysis of Atlantic salmon resulted in a total of eleven haplotypes detected in a subsample of Atlantic salmon as opposed to five detected by analysing only the ND-1 segment (/7/). This illustrates that even though universal primers are useful it may nevertheless be advantageous to redesign primers for the particular group of organisms of interest, and that in organisms exhibiting low levels of variation, like Atlantic salmon, it may pay off to simply increase the range of sequence screened for variation.

3.3 Microsatellite DNA

Microsatellite DNA is non-coding nuclear DNA found throughout the genomes of eukaryotes (Jarne & Lagoda, 1996). Microsatellites consist of a short sequence motif, such as "TG", repeated a number of times, such as "TGTGTGTGTGTG.....". The sequence motif may consist of a single base, leading to a mononucleotide microsatellite, two bases leading to a dinucleotide microsatellite, three bases leading to a trinucleotide microsatellite, etc. In practise, most microsatellites employed in population genetic studies consist of di-, tri- and tetranucleotide repeats. In addition to these categories microsatellites may be subdivided into three types, "perfect" loci consisting of non-interrupted sequences of repeat units, such as $(TG)_{25}$, "compound" microsatellites consisting of sequences of different repeat units, such as $(TG)_{10}(TC)_7(TG)_{12}$ and "interrupted" microsatellites, consisting of repeat units interrupted by non-repetitive DNA, such as $(TG)_{10}ACATGATAC(TG)_{12}$.

The mutational processes at microsatellites are quite distinct from those occurring at other types of DNA. First, mutation rate is very high, with values from 10^{-5} to 10^{-3} reported in different studies (Jarne & Lagoda, 1996; Goldstein & Pollock, 1997). This leads to high levels of variability, with numbers of alleles at individual loci often ranging between 5 – 20 or more. Second, mutation does not occur according to an infinite allele mutation model (IAM), where each mutation leads to a new, unique allele. Instead, mutation involves primarily insertion or deletion of one or a few repeat units, presumably resulting from slippage of the DNA polymerase during replication. In other words, it appears that the state of a new allele is not independent of the state of the previous allele, i.e. prior to mutation. This also means that two alleles that are identical in state are not necessarily identical by descent, as they could be the result of different mutational events (homoplasy). It was initially suggested that mutations at microsatellite loci follow a strict stepwise mutation model (SMM), involving insertion/deletion of a single repeat unit. Later studies have, however, demonstrated that mutations involving several repeat units also do occur. The presently most favoured mutation model is the two-phase model (TPM) by DiRienzo *et al.* (1994), where most mutations involve insertion/deletion of a single repeat unit, but a fraction of mutations involve several repeats. However, even this model is unlikely to tell the whole story of microsatellite mutational processes (see, e.g., discussion in Jarne & Lagoda, 1996; Goldstein & Pollock, 1997; Estoup & Cornuet, 1999). First, mutation rate and processes may deviate among different loci, e.g. between "perfect" and "interrupted" loci. Second, mutation rate may not even be the same for different alleles at the same locus. Third, microsatellites do not mutate into infinite lengths; there are clearly some size constraints on microsatellite loci, but the actual mechanisms are not known. The heterogeneity and unknown factors concerning microsatellite mutational processes have important consequences for the use of statistics that make specific assumptions about mutation models (e.g., Goldstein *et al.*, 1995; Slatkin, 1996; Beaumont, 1999). In most cases, these statistics assume a strict stepwise mutation model, and it is important to consider how deviations from this model could affect the results. It is also a bit ironical that some of these statistics, e.g. the $(\delta\mu)^2$ distance (Goldstein *et al.*, 1995) have a very high sampling variance, and that some of the "older" distance measures, such as that by Cavalli-Sforza & Edwards (1967) actually perform better in retrieving the correct topology of a phylogenetic tree, unless a large number (>20) of microsatellite loci are studied (Takezaki & Nei, 1996; Tomiuk *et al.*, 1998).

Since the early 1990s microsatellites have gradually replaced allozymes as the preferred marker for population studies. There are several reasons for this, with the following presumably being the most important. First, microsatellite loci are typically short, in the range of approx. 80 to 400 base pairs. This makes it easy to amplify the loci using PCR, and the amplified products can subsequently be analysed on either "manual" sequencing gels or systems for automated sequencing. Second, the much higher variability at microsatellites as compared to allozymes results in increased power for a number of applications, ranging from analysis of kinship and parentage assignment to assignment of individuals to populations and detection of population bottlenecks (reviewed by Luikart & England, 1999). In this context, I find it important to stress that the real advantage of microsatellites as compared to allozymes does not so much lie in "traditional" analyses of genetic population structure, involving estimation of F_{ST} and construction of a dendrogram. It is the many new research opportunities that are now possible using microsatellite analysis that makes it really attractive to use this type of genetic marker.

Of course, despite the usefulness of microsatellite markers they are not without problems, and one of these, the presence of so-called "null alleles" (Pemberton *et al.*, 1995) deserves special

mentioning. Null alleles occur when mutations take place in the primer binding regions of the microsatelllite locus, i.e. not in the microsatellite DNA itself. This may result in one of the two primers being unable to anneal satisfactorily to the target DNA, and the allele with the mutation is consequently not PCR amplified. The presence of an amplified allele and a non-amplifying allele is therefore erroneously interpreted as a homozygote, and a null allele homozygote is interpreted as an individual which for technical reasons would not amplify, for instance due to low quality DNA. Even though methods are available that allow for estimating the frequency of null alleles, the presence of null alleles at a locus nevertheless causes severe problems, in particular in individual based analyses such as relatedness estimation and assignment tests, and most researchers prefer to discard loci exhibiting null alleles.

Microsatellites have been the preferred genetic marker in the majority of the studies included in this thesis, with applications involving general studies of genetic population structure (/10/; /20/; /23/) phylogeography (/8/), spatiotemporal genetic relationships (/5/; /10/; /18/; /22/; /23/), population assignment and admixture analysis (/5/; /12/; /14/; /15/; /17/; /18/; /20/; /22/), estimation of demographic parameters (/13/; /19/; /23/) and studies of kinship and behavioural patterns (/3/; /21/).

3.4 Analysis of historical samples

Most studies of genetic population structure operate on a spatial scale, i.e. populations from different geographical localities are sampled and their genetic relationships are subsequently analysed. However, it becomes meaningless to analyse the current genetic structure of populations without considering that the structure we observe is in fact a result of past events, i.e. gene flow, genetic drift, selection and mutation, both in the short and long term. Phylogeography is an almost extreme example of this. Inferences are made about events taking place, say, 10,000 or even more years ago, based on the current genetic composition of populations. One of the big challenges of phylogeography is therefore to be able to separate population structure from population history.

Obviously, it would be nice if samples were available dating far back in time, and fortunately development of techniques for analysing so-called “ancient DNA” has now made this a realistic possibility (e.g., Pääbo, 1989; Ellegren, 1991; Janczewski *et al.*, 1992; Zierdt *et al.*, 1996). Samples dating thousands of years back in time are in most cases extremely rare and can primarily be used for addressing problems in phylogeny reconstruction and, to some extent, phylogeography (see Consuegra *et al.*, 2002 for a recent example of analysis of several thousand year old Atlantic salmon samples, leading to results that challenge current phylogeographical hypotheses). On a shorter time scale, archived samples are sometimes available in sufficient numbers to allow for population level studies. This is particularly the case for commercially important fishes, where scales and otoliths have been collected over several decades with the purpose of determining age and growth patterns. Many of these samples have been stored even after the parameters of interest have been estimated, and these samples can now provide valuable sources of DNA (/11/).

The main problem with working with historical samples is the degradation of DNA that inevitably occurs over time. This reduces the total quantity of DNA, and the DNA that remains consists of short fragments, a few hundred base pairs or even less. This makes it a challenge even to be able to extract a sufficient quantity of DNA. We have tested several protocols in our laboratory and found that a combination of phenol-chloroform extraction and the use of microconcentrators (for both washing and concentrating the DNA) yields the most consistently successful PCR amplification (/11/). It should be noted, though, that in some other studies more crude extraction protocols have also yielded satisfactory results (e.g., Miller & Kapuscinski, 1997). Once DNA has been

successfully extracted the next step is to ensure that PCR amplification is reliable and reproducible. In particular, the low quantities of DNA is a concern, because this means that contamination by DNA from samples already amplified may easily out-compete the target DNA from the historical sample. It is therefore necessary to keep DNA extraction and PCR amplification of historical samples in as clean and isolated conditions as possible, to have special reagents reserved only for the use with historical samples and, in general, to do many replicates in order to ensure that the results are reproducible. Finally, when the data are subject to statistical analysis it is recommended to look for indications of artefacts, such as large allele drop-outs at microsatellite loci, where one allele (the shortest) is preferentially amplified at the expense of the other allele (the longest). Even though this may occur also in fresh samples, the highly degraded DNA of historical samples amplifies the problem because there are expected to be proportionally fewer intact copies of the larger alleles. Allelic drop-outs may be detected by testing for heterozygote deficits (Rousset & Raymond, 1995), but again replicating the molecular analysis may help to generally rule out the possibility of technical artefacts significantly affecting the results. However, the best way to avoid problems with large allele drop-outs and, in general, to avoid "gappy" data sets with many non-amplifying individuals is to analyse relatively short microsatellite loci. In our own studies, based on 50-90 year old scale samples, the limit is approximately 250 bp, and loci exhibiting alleles larger than this often do not amplify reliably (/11/).

In birds and mammals, bones, teeth, feathers and skins have been used as sources of historical DNA samples (e.g., Taylor *et al.*, 1994; Bouzat *et al.*, 1998; Beaumont *et al.*, 2001). In most cases, historical samples have not been stored in numbers comparable to that of archived scale and otolith samples in fishes. However, in the case of Danish otters a relatively high number of sculls have been stored at museums. The tooth root of otter canine teeth gets completely encapsulated. It turned out that canine teeth from archived sculls provided a reliable source of DNA. This allowed us to analyse temporal samples of otters dating more than one hundred years back in time, and comparing the past and contemporary genetic composition of Danish otters (/19/).

Analysis of historical samples provides a valuable tool for many aspects of population and conservation genetics research. It can shed light on the fundamental question whether or not the genetic structure of populations is indeed stable over time (e.g., Tessier & Bernatchez, 1999; Ruzzante *et al.*, 2001; /5/; /10/; /23/). Also, the availability of samples separated by long time spans facilitates estimation of effective population size using the so-called temporal method, where effective population size is estimated from the genetic changes that have occurred over time (e.g., Waples, 1989). This approach, based on historical samples, has been used for estimating effective population size in Northern pike (*Esox lucius*; Miller & Kapuscinski, 1997), rainbow trout (*Oncorhynchus mykiss*; Heath *et al.*, 2002) and brown trout (/23/). In a related context, historical samples have been used to assess if population bottlenecks and loss of genetic variation has occurred over time (e.g., Taylor *et al.*, 1994; Bouzat *et al.*, 1998; /19/). Finally, in the case of salmonid fishes where extensive stocking with exogenous fish has often taken place, historical samples have provided a very useful tool to assess if indigenous populations have been introgressed by stocked fish (Koskinen *et al.*, 2002a; /22/). This has even provided a practical tool for conservation and management of heavily stocked populations, where assignment tests based on contemporary and historical samples have made it feasible to identify non-admixed indigenous fish and use only these for supportive breeding (/18/).

4. Genetic structure of salmonid fish populations

Fishes exhibit a variety of ecological specialisations and life histories. Hence, it is not surprising that genetic differentiation among populations may range from F_{ST} values as small as < 0.01 in several marine and catadromous species (e.g., Ruzzante *et al.*, 1998; Wirth & Bernatchez, 2001) to more than 0.25 in some freshwater and anadromous species (e.g., Ryman, 1983; Allendorf & Leary, 1988; Ferguson, 1989; Koskinen *et al.*, 2001). It seems to be a general feature that marine fishes exhibit low genetic differentiation but high intrapopulation genetic diversity, and freshwater fishes exhibit high genetic differentiation but low intrapopulation genetic diversity, with anadromous fishes showing intermediate values (Ward *et al.*, 1994). This is the result of much higher effective population sizes in marine fishes relative to freshwater fishes and a higher potential for gene flow among marine populations.

However, populations may not only exhibit differentiation as a result of ongoing evolutionary processes, but also as a result of past migration and recolonisation events, such as postglacial recolonisation from different glacial refuges. This has in some cases resulted in very strong genetic differentiation among populations, even at relatively small geographical scales where different populations may represent descendants of different postglacial lineages (see, e.g., Bernatchez & Wilson, 1998).

Our studies of salmonid fish populations have primarily focused on analysing the genetic structure of populations at medium to small geographical scales, with a particular view to understanding the biological significance of this differentiation. We have, however, also been interested in assessing whether genetic differentiation among salmonid populations in Denmark could reflect larger scale phylogeographical patterns. Below, I describe this research in more detail.

4.1 Genetic differentiation at local geographical scales – brown trout and Atlantic salmon

Over the years, there have been numerous studies of the genetic structure of brown trout and Atlantic salmon. In brown trout, some of the first studies reported very strong genetic differentiation among populations (e.g., F_{ST} of 0.37 among Swedish trout populations; Ryman, 1983). Quite surprisingly, it was also found that there was no correlation between genetic and geographical distance between populations, i.e. no isolation by distance effect (e.g., Ryman, 1983; Crozier & Ferguson, 1986; Ferguson, 1989). It would be expected that gene flow among populations would primarily occur between neighboring populations, i.e. according to a stepping-stone model (Kimura & Weiss, 1964). Thus, one interpretation of the lack of isolation by distance is that (a) gene flow occurs according to an island model (Wright, 1931), i.e. gene flow is equally likely to occur between distant and neighboring population. Another possibility (b) is that gene flow is simply unlikely to occur among the studied populations due to impassable barriers. Closer inspection of some of the early studies does indeed show that several of the studied populations are unlikely to exchange migrants due to, e.g., impassable waterfalls. Finally, there is the possibility (c) that sampling of populations is simply not representative. An often used approach for sampling is to collect juveniles in the natal rivers, rather than sampling adult fish, and this could potentially lead to sampling of just a few families, a problem that is treated in more detail in 4.2.

Most brown trout populations in Denmark consist of a mixture of resident and anadromous trout. Furthermore, Denmark is a lowland region and all populations have originally been accessible to gene flow, though human-made constructions like weirs and dams have later significantly fragmented many river systems. Nevertheless, there are ample opportunities for studying the genetic structure of populations accessible to gene flow, in particular mediated by anadromous trout, and

this has been the topic of several studies. Using microsatellite markers we have found that genetic differentiation is significantly lower than reported in previous studies encompassing both landlocked and accessible populations (F_{ST} = 0.049 among populations in the Limfjord region, northern Jutland; /20/). This also corresponds well with a study based on mitochondrial DNA markers, encompassing fewer river systems but on a geographical scale extending from the Limfjord to the island of Bornholm in the Baltic Sea (/6/). In this study, a Φ_{ST} value of 0.30 was observed, and given that differentiation at the mitochondrial DNA level is expected to be approximately four-fold as compared to nuclear genes, this result corresponds well with a microsatellite F_{ST} on the order of 0.049 (/20/). It could then be argued that recent stocking activity has inflated differentiation, however, analysis of a limited number of historical samples from the 1910s-1950s, along with contemporary samples, also shows relatively low genetic differentiation, with a hierarchical analysis showing that genetic differentiation among populations (F_{CT}) was 0.032, whereas genetic differentiation among temporal samples within populations (F_{SC}) was even lower, 0.006 (/23/). The lower differentiation among anadromous populations relative to resident and landlocked populations has also been confirmed in other studies on comparative geographical scales employing allozymes or microsatellites, e.g. in Norway (F_{ST} = 0.077; Knutsen *et al.*, 2001) and Spain (F_{ST} = 0.054; Moran *et al.*, 1995).

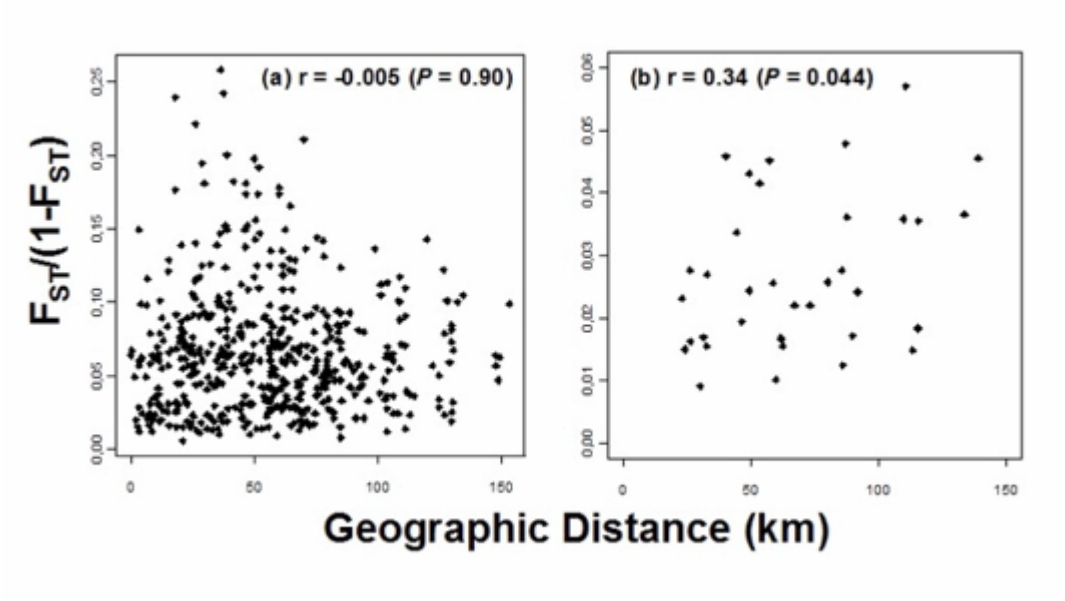

Fig. 3. Scatterplot of pairwise genetic [F_{ST}/ (1-F_{ST})] versus geographic distances (in km) for brown trout (*Salmo trutta*) from the Danish Limfjord region. (a) Scatterplot involving 32 individual tributary populations. (b) Scatterplot involving population samples pooled into 9 geographic regions. The significances of the correlations were tested using Mantel tests. (Figure from paper /20/).

The supposition of no isolation by distance does not seem to be valid in Danish anadromous brown trout. Thus, both using microsatellites (/20/) and mitochondrial DNA (/6/) isolation by distance was observed, even though dammed populations inaccessible to gene flow diverged strongly from neighbouring populations (/6/). It is interesting to note, however, that in both cases a significantly stronger pattern of isolation by distance was observed when samples from tributaries within rivers or even neighbouring rivers were pooled, than when they were analysed as distinct samples (see fig. 3). A similar pattern was observed by Moran *et al.* (1995) who observed isolation by distance when samples from different river systems were considered, but not when samples from within the same river systems were analysed. This could suggest that gene flow occurs according to a hierarchical

model, with a stepping-stone model predominating at a larger geographical scale, whereas gene flow at a microgeographical scale is more in accordance with an island model. Alternatively, it could suggest some added variance, e.g. due to sampling of a limited number of families, when individual samples are considered, but this microscale variance is levelled out when several samples from neighbouring populations are pooled.

Gene flow among anadromous populations also plays an important role for the level of intrapopulation genetic diversity. Ferguson *et al.* (1995) reported considerably higher levels of variation in Scottish anadromous trout compared to resident trout. In Danish trout populations considerably higher mitochondrial DNA variation was detected in populations accessible to gene flow relative to artificially dammed populations (/6/).

What can we then conclude about the genetic structure of anadromous trout compared to landlocked and resident trout? First, it does seem that the higher potential for gene flow in anadromous trout also results in actual gene flow causing relatively weaker genetic differentiation. Second, the supposition of no isolation by distance as observed in some studies of brown trout does not hold when anadromous populations are studied; there is indeed a positive relationship between genetic and geographical distance between populations. Third, the gene flow occurring among anadromous brown trout populations has important consequences for genetic variation within populations, with higher levels of variation in anadromous compared to resident populations. In this way, brown trout fits nicely into the general pattern of the distribution of genetic diversity in marine, anadromous and resident populations described by Ward *et al.* (1994).

Bouza *et al.* (1999) have provided a nice demonstration of these conclusions. They studied brown trout populations in Spain at the southern limit of the occurrence of anadromous trout. They observed a significant isolation by distance pattern and found that intrapopulation genetic variability decreased and genetic differentiation among populations increased when the degree of anadromy in populations diminished. It should be mentioned, though, that there are also examples of studies of anadromous trout populations with no apparent relationships between genetic and geographical distance, e.g. a study of Norwegian trout by Skaala (1992). In this particular case the explanation may be that there are so long geographical distances between trout populations in river flowing into different fjords that the populations are effectively isolated even though there are no other physical obstructions to gene flow.

Except for landlocked populations Atlantic salmon are anadromous. It is therefore surprising that many previous studies have failed to detect isolation by distance patterns in this species (e.g., Wilson *et al*., 1995). However, by analyzing microsatellite markers in historical (1910s to 1950s) samples of Danish Atlantic salmon a significant pattern of isolation by distance was observed (/10/). One explanation could be that overharvesting and habitat destruction leading to extensive genetic drift, along with stocking activity and escapes of farmed fish has blurred the original patterns of differentiation. A study by Koljonen *et al.* (1999) of Baltic Sea salmon supports this explanation. They found significant isolation by distance patterns among populations sustained by natural reproduction, but not among populations sustained by hatchery-rearing.

4.2 Family sampling – an important bias?

In many organisms the spatial distribution of closely related individuals is patchy instead of random. For instance, many fish species attach their eggs to specific substrates or hide them in nests or redds. Most species of trout and salmon spawn in distinct spawning redds dug in the gravel of the

river bottom. Upon hatching and emergence from the gravel the fry establish territories in the vicinity of the redd. Over time, strong density dependent mortality results in fewer surviving individuals from each family and, furthermore, the fish tend to move to other and perhaps deeper parts of the river. This is expected to lead to a more uniform distribution of fish representing different families. However, if a population is sampled by only catching fish from a short stretch of a river, and if the sample contains a large proportion of fry there is a considerable risk of sampling individuals representing only one or a few families. This could result in the sampled alleles not being representative of the true allelic distributions within the population, which may again blur the true genetic relationships among populations.

The problem of "family sampling" was first pointed out by Allendorf & Phelps (1981), and we decided to make an empirical assessment of the problem (/3/). We studied two brown trout populations in small rivers using analysis of mtDNA and microsatellites. In one of the samples seventeen out of eighteen individuals less than one year of age shared one particular mtDNA haplotype. Estimates of relatedness, based on microsatellite analysis, showed that these individuals most likely represented only three full-sib families. In the other sample there were no indications of many individuals belonging to just a few families. Also, in both samples trout older than one year of age exhibiting the same haplotypes generally were not closely related. Thus, the results show on the one hand that sampling of fry from a limited stretch of river may indeed lead to the sampling of just a few families. On the other hand, this is not necessarily a universal problem. If the physical conditions for spawning are good and if there is a high density of spawning fish, then a large number of families are expected to be represented, even within a short stretch of the river. A later study of Atlantic salmon (Mjølnerød *et al.*, 1999) has shown the same kind of pattern, whereas a study of 1+ salmon did not show a close spatial association of related individuals (Fontaine & Dodson, 1999), not unexpected given the higher age of the studied fish relative to our study. The recommendations for avoiding sampling of just a few families would be in the first place to sample adult fish. If this is not possible, then it is recommended to sample several age classes of juveniles (e.g. 0+, 1+ and 2+). If this is not possible and only 0+ individuals are available, then at least the sampling should encompass a long stretch of the river. This would make it unlikely that all sampled individuals are derived from just a few spawning redds.

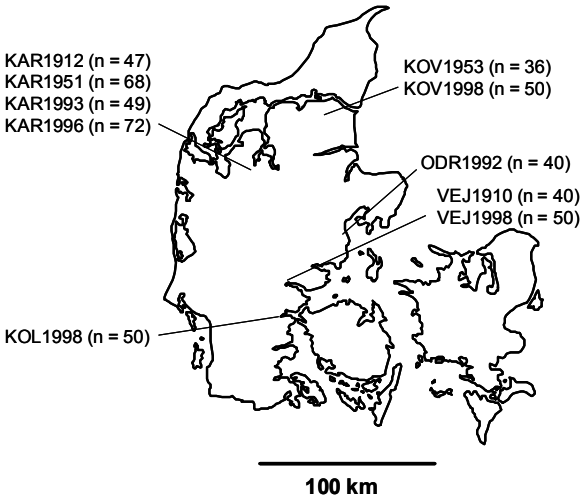

Fig. 4. Approximate location of brown trout populations in Denmark from which we analysed contemporary and historical samples. KOL = Kolding River, KAR = Karup River, KOV = Kovads River, ODR = Odder River, VEJ = Vejle River.

4.3 Spatiotemporal genetic structure

As described in the section on analysis of historical samples, most studies of genetic population structure operate only on a geographical scale, whereas the temporal scale of genetic differentiation is in most cases unexplored. This is also the case with salmonid fishes, although some studies have included sampling from several years and age classes in order to assess if the observed genetic composition of populations is stable over a time span from a couple of years to a decade (e.g., Jordan *et al.*, 1992; Laikre *et al.*, 2002). Recently, technical developments allowing for analysing historical samples, such as old scale samples, have greatly improved the possibilities for exploring the temporal dimension in population studies, as described in 3.4. We have used this approach for

studying the genetic composition of salmonid populations over long time spans, covering almost a century (from the 1910s to the 1990s). Both in Danish Atlantic salmon (/5/; /10/) and anadromous brown trout (/23/) it turned out that the genetic structure is remarkably stable over time, even despite strong human disturbance (see figs. 4 and 5 for results from anadromous brown trout; /23/). The smaller temporal as opposed to geographical differentiation among populations was also reflected in a hierarchical analysis of genetic diversity in anadromous brown trout populations, where 3.2% of genetic diversity was distributed among different populations, whereas a minor proportion (0.6%) was distributed among temporal samples from the same populations (/23/).

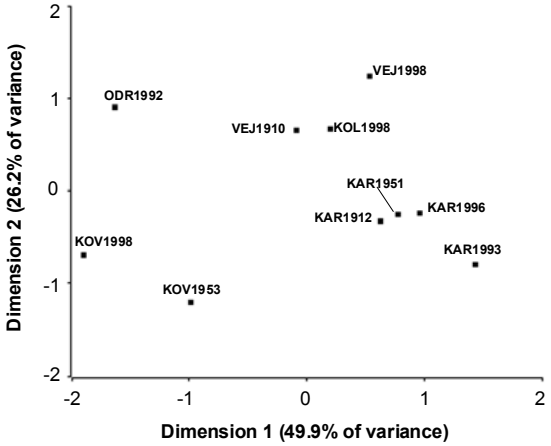

Fig. 5. Multidimensional scaling analysis of Nei's (1978) genetic distance, based on eight microsatellite loci, between historical and contemporary samples from Danish brown trout populations. Dimension 1 explains 49.9% of the variance and dimension 2 26.2% of the variance. See fig. 5 for approximate geographical location of the populations and sample codes. Note the close genetic relationships between the historical and contemporary KAR samples, historical and contemporary KOV samples and contemporary samples from the two neighboring rivers VEJ and KOL along with the historical VEJ sample.

Long-term temporal stability of genetic population structure was also observed in a study of landlocked salmon by Tessier & Bernatchez (1999). In particular, they found that one population diverged significantly from the others. This was not the result of a recent bottleneck, as evidenced by the analysis of historical samples, and a later study suggested that the divergent population was actually the result of a dual recolonisation event (Tessier & Bernatchez, 2000). In contrast, a study by Heath *et al.* (2002) of steelhead suggested that the genetic composition of populations was less temporally stable than observed in our studies and that of Tessier & Bernatchez (1999). The authors ascribed this outcome to geological unstability, in particular the presence of landslides possibly diverting and blocking water flows.

The temporal stability of genetic composition of trout and salmon populations obviously has important biological implications. It suggests that the effective population size is in fact high, and this combined with the fact that gene flow among populations is not sufficiently high to result in strong genetic differentiation over time, suggests that there is indeed an important potential for local adaptations. These issues are treated in more detail in later chapters.

4.4 Metapopulation structure

The concept of metapopulations has been increasingly applied in population genetics and ecology. Whereas metapopulations originally were defined as systems of populations linked by limited gene flow and experiencing extinction-recolonisation events there has been a tendency to user a broader definition, sometimes indistinguishable from that of a "conventional" subdivided population (see, e.g., Hanski & Gilpin 1991 for an overview). For this reason, I will here restrict the use of the term metapopulation to systems where there is a non-trivial probability of population turn-over, i.e. extinction and recolonisation.

Several different types of metapopulation models have been suggested, such as traditional island and stepping-stone models combined with the properties of extinction-recolonisation dynamics, and more specialised models such as source-sink systems, where populations with positive growth rates

(sources) supply migrants for populations exhibiting negative growth rates (sinks) (Gilpin & Hanski, 1991). The presence of extinction-recolonisation dynamics has important consequences for the level of genetic variation within populations and for the degree of genetic differentiation among populations. Genetic variation within populations is expected to be lower as compared to population systems with no turn-over (McCauley, 1993). This is a result of the repeated recolonisation events which essentially act as repated bottlenecks. Whether or not metapopulations exhibit stronger genetic differentiation compared to systems without turn-over is a more complicated issue and depends on the mode of recolonisation. Wade and McCauley (1988) analysed the consequences of extinction-recolonisation events for genetic differentiation in the island model of gene flow. They distinguished between propagule pool and migrant pool models of recolonisation. In the propagule pool model a number of individuals from the same source population found a new population. In this case founding will normally result in increased genetic differentiation, if the number of founders is less than the equilibrium population size. In the migrant pool model founders may be derived from all possible source populations, and extinction-recolonisation events may either reduce or increase genetic differentiation, depending on the relationship between number of founders and the number of migrants which are exchanged among existing populations.

Some studies targeted at salmonid fishes have taken a metapopulation perspective (e.g., Garant *et al.* 1900), but in general this is a relatively unexplored issue. One simple, but pertinent question is whether or not population turn-over really occurs that often under natural circumstances (see also Smedbol *et al.*, 2002). There is probably not a single universal answer to this question. For instance, it is possible that some geographical regions are geologically and climatically unstable, e.g. due to landslides (Heath *et al.*, 2002) or frequent droughts (Hansen & Loeschcke, 1996b). In such regions it is reasonable to assume a non-trivial probability of population extinction. However, in other more stable regions it is difficult to envisage that natural extinctions should occur frequently. Indeed, the relatively few studies undertaken that analyse the genetic composition of populations over long time spans using historical samples (Tessier & Bernatchez, 1999; /10/; /23/) do not suggest that population turn-over has taken place. Of course, this concerns a relatively small number of studied populations, but it should also be considered that it is difficult to find published examples of salmonid populations that have gone extinct for natural reasons. Even in the case of a species suggested to exhibit a metapopulation structure, the bull trout (*Salvelinus confluentus*), genetic data do not support this supposition (Kanda & Allendorf, 2001). In fact, these data suggest that the genetic population structure is stable and with clear signatures from postglacial recolonisation events.

Whereas natural turn-over of salmonid populations probably does not occur frequently, human-mediated extinctions due to pollution, habitat destruction and overharvesting unfortunately occur at a non-trivial rate. Thus, some salmonid population systems may now exhibit a metapopulation structure which would not be the case under pristine conditions. From a conservation perspective this is of course a regrettable situation, but from a population genetics point of view this provides a "natural laboratory" for studying modes of recolonisation. In the Danish Odder River system we studied two brown trout tributary populations which had been founded recently following extinction due to organic pollution (/1/). The study was based on allozyme and mitochondrial DNA analysis, and the genetic relationships among populations suggested that the tributaries had been founded by trout from neighboring tributary populations, i.e. in accordance with a propagule pool model. In one of the recolonised populations allelic and mitochondrial DNA haplotype frequencies were divergent from the neighboring populations. We performed computer simulations to assess if a contribution by straying stocked hatchery trout could explain this outcome, but found that the most likely

explanation was founding by a small number of local trout, i.e. founder effects. Knutsen *et al.* (2001) conducted a similar study of recolonisation patterns in Norwegian rivers, where acidification had extirpated the indigenous anadromous trout populations. In contrast to our study, they found that patterns of recolonisation were more likely to follow a migrant pool model, i.e. with recolonisers representing individuals from many different populations. The different results of the two studies are probably best explained by the fact that recolonisers in the Odder River system could be derived from other parts of the same system, i.e. just a few kilometres away. In the case of the Norwegian rivers, recolonisers would have to be individuals from other river systems and could therefore easily consist of mixtures of individuals from several populations. This shows that it may be difficult to generalise about recolonisation patterns; the patterns could depend on how far the potential source populations are situated from the sites to be recolonised.

4.5 Phylogeography

Phylogeography is concerned with testing biogeographical hypotheses, primarily at the intraspecific level, using molecular markers (e.g. Avise, 1994). This is done by analysing the geographical distribution of different alleles or mitochondrial DNA haplotypes, preferably along with analysis of the phylogenetic relationships among alleles/haplotypes. The first phylogeographical studies undertaken in the late 1970s and early 1980s used mitochondrial DNA as a genetic marker (e.g., Avise *et al.*, 1979) and in fact this has remained the preferred molecular marker for this purpose for reasons described in 3.2. There is, however, a growing interest in analysing nuclear genes, in particular in order to circumvent the problems associated with the special properties of mitochondrial DNA (see 3.2), and in the case of brown trout three recent studies have analysed the transferrin gene (Antunes *et al.*, 2002; Duguid, 2002) and internal transcribed spacer of ribosomal RNA genes (Presa *et al.*, 2002). Also, microsatellites have found some use in phylogeographical studies (e.g., Koskinen *et al.*, 2002b), but the specific mutational properties of microsatellites (see 3.3) tend to result in homoplasy (i.e. alleles that are identical by state are not identical by descent) and statistics designed to take this problem into account, such as the $(\delta\mu)^2$ distance by Goldstein *et al.* (1995), have been shown not to perform optimally unless large numbers of loci (20 or more) are analysed (Takezaki & Nei, 1998; Tomiuk *et al.*, 1998).

In the northern hemisphere repeated glaciations have had a very strong influence on the distribution of species and major lineages within species. The recurrent glaciations can be regarded as massive extinction-recolonisation events, where the flora and fauna during interglacial periods have recolonised from southern refuges. The last glaciation ended approx. 10-13,000 years ago and thus the present flora and fauna in previously glaciated regions are the result of recolonisation during the time elapsed since then. Several phylogeographical studies have detected very clear imprints from postglacial recolonisation events (see, e.g., reviews by Taberlet *et al.*, 1998; Bernatchez & Wilson, 1998; Hewitt, 1999). Thus, there is typically less genetic variation and a more shallow phylogeny of alleles and haplotypes in populations in recently glaciated regions compared to conspecific populations in regions that have not been glaciated, presumably a result of rapid northward expansion from the glacial refuges. Also, postglacial recolonisation from different distinct glacial refuges have in many cases brought divergent intraspecific lineages into secondary contact, in some cases resulting in hybrid zones, where hybridisation occurs but the differences between lineages are nevertheless maintained due to lowered fitness of hybrids (e.g., Hewitt, 1999).

Salmonid fishes are among the species that have received most attention for phylogeographical studies in Europe and North America (e.g., Bernatchez *et al.*, 1992; Bernatchez & Dodson, 1994; Bernatchez *et al.* , 1996; Bagley & Gall, 1998; Nielsen *et al.*, 1999; Garcia-Marin *et al.*, 1999;

Koskinen *et al.*, 2000; Bernatchez, 2001; Nilsson *et al.*, 2001; Antunes *et al.*, 2002; Presa *et al.*, 2002, just to mention a few). Our own contributions to phylogeography have focused on Atlantic salmon, brown trout and whitefish, with particular emphasis on assessing whether distinct lineages and contact zones are present in Denmark. The reason why Denmark is of interest is the geographical location of the country, right at the border between the Baltic Sea and the North Sea (and ultimately the Atlantic Ocean). Several studies of terrestrial organisms have shown that Scandinavia is a secondary contact hot spot zone, with seemingly one major expansion of organisms from an eastern refuge via Finland into Norway and Sweden, and another northward expansion from a south-western refuge via Denmark and further north into Norway and Sweden, where the two expansion waves have met and in some cases established contact zones (e.g., Taberlet *et al.*, 1998). Similar patterns could also be present in anadromous and freshwater fishes.

A study of variation in the mitochondrial ND-1 gene in Atlantic salmon from the northwestern Atlantic region; Danish Skjern River and from populations in western Sweden, Norway, Scotland, Iceland and Ireland, using restriction enzyme analysis, showed limited genetic variation (/2/). Further, there was no obvious relationship between the phylogeny of haplotypes and their geographical distribution. The interpretation of these results was that even if different recolonisations had taken place there were by now no distinct patterns left; the major factors affecting patterns of genetic differentiation were assumed to be genetic drift and gene flow. Since then, other mitochondrial DNA studies of Atlantic salmon from the same region have been conducted (Verspoor *et al.*, 1999; Nilsson *et al.*, 2001) and have basically reached similar conclusions for populations in the eastern Atlantic. The study by Nilsson *et al.* (2001), however, showed a distribution of mitochondrial DNA haplotypes in the Baltic Sea that was interpreted as reflecting dual recolonisation. The different haplotypes were very closely related, so the phylogeny of haplotypes was not informative, however, another study of Baltic salmon by Koljonen *et al.* (1999), based on allozymes, reached the same conclusion lending support to the dual recolonisation hypothesis.

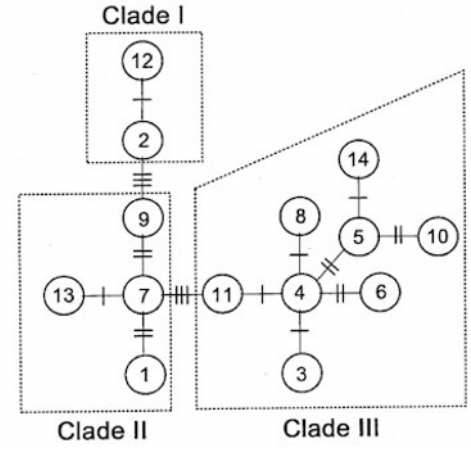

Fig. 6. Parsimony network showing the genetic relationships among mtDNA haplotypes observed in Danish brown trout. Bars across lines denote the number of mutations separating pairs of haplotypes. Clade I, II and III denote clades of haplotypes separated from each other by three mutational steps

In the case of Danish brown trout, we have analysed several populations using restriction enzyme analysis of the mitochondrial ND-1 and ND-5/6 genes (Hansen & Loeschcke, 1996b; /1/; /4/; /6/). In total, we have observed fourteen different haplotypes, the phylogeny of which does seem to be phylogeographically informative (/6/). Thus, the haplotypes represent three clades separated from each other by three mutational steps (fig. 6). In a survey of trout populations from river systems extending from the Limfjord in northern Jutland over two rivers with outlets into the Kattegat Sea to the island of Bornholm in the Baltic Sea we observed all clades in all rivers, but at highly different frequencies. Moreover, the clades exhibited a clinal distribution along the transect from northern Jutland to the Baltic Sea. This could indicate different directions of postglacial expansion of the different clades, although the number of populations studied made it difficult to reach any firm conclusions. Since paper /6/ was published there have been two other studies of mitochondrial DNA variation in brown trout from the Baltic region

(Wlodarczyk & Wenne, 2001; Laikre *et al.*, 2002), which in part analysed the same segments and used the same restriction enzymes. The distribution of haplotypes in these studies that would belong to the three clades described in /6/ provides little further support of a clinal pattern. This is not to say, however, that the phylogeny of the haplotypes is not phylogeographically informative. Bernatchez (2001) published the most comprehensive phylogeographical study of brown trout so far, based on mitochondrial DNA. Using nested clade analysis and mismatch distribution analysis he found strong signals of multiple recolonisation events in northern Europe from different refuges. Unfortunately, the segments of mitochondrial DNA analysed in Bernatchez' study could not be directly compared to our results, however, recent work by Duguid (2002) used analysis of the ND-1 and ND-5/6 segments in trout that had also been analysed by Bernatchez (2001). This has provided a "translation key" enabling me to compare our results to those of Bernatchez (2001), of course along with the results by Duguid (2002). Very interestingly, it turns out that clades 1-3 in (/6/) correspond to clades that Bernatchez (2001) have identified and that were hypothesized to have expanded from different glacial refuges. Duguid (2002) observed a surprisingly clear association of different clades with the "ferox" trout morph, accessible populations and landlocked populations, respectively, supporting the notion that there are still relatively "pure" remnants of the original recolonising glacial lineages in the British Isles. In contrast, our results (/6/) suggest that extensive mixing of the lineages has occurred in Denmark. This is hardly surprising, given that Denmark is all lowland and all populations have (at least historically) been accessible to gene flow. However, the new results by Bernatchez (2001) and Duguid (2002) do suggest that a much more detailed study of mitochondrial DNA variation among trout from Northern Europe could yield very interesting results concerning postglacial recolonisation routes and subsequent mixing of different lineages.

Coregonid fishes have been subject to several phylogeographical studies, in particular because of their high morphological diversity which has raised the question whether or not morphologically divergent forms represent distinct lineages. An example of this is found in Denmark, where two phenotypically divergent forms of whitefish are present, the "normal" whitefish (*Coregonus lavaretus*) and the North Sea houting (*C. oxyrhynchus*). As described in 2.3 North Sea houting is both morphologically and physiologically divergent compared to "normal" whitefish. It was previously distributed throughout the Wadden Sea area of the North Sea from the Netherlands to southern Denmark where it spawned in several large and medium-sized river systems. However, due to pollution, river regulation and establishment of impassable weirs most populations went extinct. In the 1980's the only remaining population was that of the Danish Vidaa River in western Jutland. The population has now recovered and reintroductions have taken place to a few other Danish river systems in the Wadden Sea area. We wanted to assess if the two forms or species represented two distinct phylogeographical lineages or if they originated from the same lineage but had diverged recently. Also, the geographical distribution of "normal" whitefish in Denmark is striking. They are distributed only in western Jutland, the Limfjord area, and in the Gudenaa River system in eastern Jutland.

In order to explain the geographical distribution of whitefish in Denmark it is necessary to focus on the possible recolonisation routes following the last glaciation. In postglacial times (11,000 - 13,000 years ago) melting water from the retracting ice in Jutland formed rivers that flowed from east to west. At that time the water level of the North Sea was low and the western Jutland rivers were tributaries to the Elbe River system which at that time advanced in a northwestern direction (fig. 7). The water level in the North Sea gradually increased and from c. 7,500 years ago the rivers of western Jutland became disconnected from the Elbe River. Some freshwater fishes, like grayling (*Thymallus thymallus*) and dace (*Leuciscus leuciscus*), are only distributed in Denmark in rivers that

were previously tributaries to the Elbe River, and postglacial recolonisation by these species probably took place via this waterway. The distribution of whitefish suggests that recolonisation took place in a similar way. However, unlike grayling and dace, whitefish appear to have been able to colonise the Limfjord area. At present the Gudenaa River flows into the Randers Fjord and ultimately into the Kattegat Sea, but for a long time (1000 years or more) following the retreat of the ice the outlet was in the Limfjord. This makes it possible that whitefish colonised the Gudenaa River at this time and remained in the river when the outlet changed direction to the Randers Fjord (see fig. 8). An alternative hypothesis is that two recolonisation events took place. Western Jutland and the Limfjord were recolonised as described above, but colonisation of the Gudenaa River took place from the Baltic Sea area.

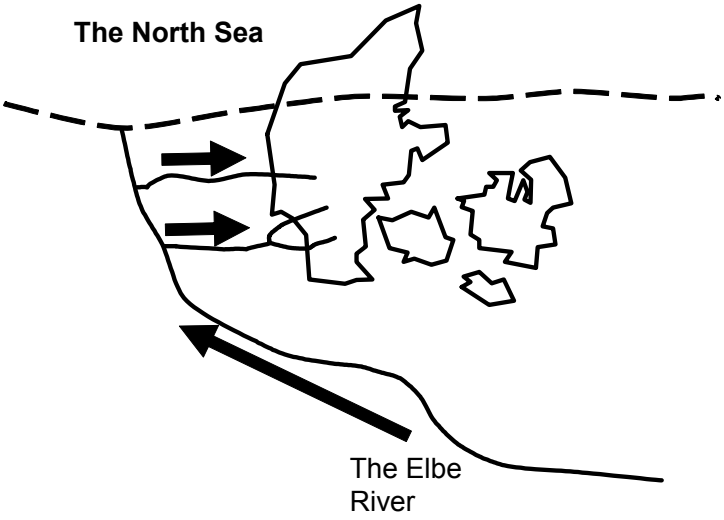

Fig. 7. Possible postglacial recolonisation history of whitefish in Denmark. The hypothetical colonisation routes by whitefish via the postglacial Elbe River and into rivers in western Jutland is indicated by arrows.

We analysed mitochondrial DNA (RFLP analysis of the mitochondrial ND-1 and ND-5/6 genes) and microsatellite markers in samples of Danish coregonids (whitefish and North Sea houting) along with two samples of anadromous whitefish from the Baltic Sea in order to assess the genetic relationships between North Sea houting and "normal" whitefish. The phylogeny of mitochondrial DNA haplotypes was shallow, with only one or two restriction sites separating haplotypes. All haplotypes observed in North Sea houting were also found in Danish whitefish and vice versa. In contrast, although the haplotypes observed in Baltic whitefish were closely related to those observed in Danish whitefish, all haplotypes were private (i.e. observed only in those samples) (fig. 9). The microsatellite data also showed close genetic relationships among all Danish populations, including North Sea houting, and considerably more distant relationships to the Baltic populations (fig. 10). These results show that Danish whitefish populations, including that from the Gudenaa River are most likely the result of one postglacial recolonization event. Thus, the hypothesis that all recolonization took place via the Elbe River system is supported, and other studies of freshwater fishes like grayling and perch (*Perca fluviatilis*) have since then lent further support to the importance of the Elbe River system as a major postglacial recolonization route (Nesbo *et al.*, 1999;

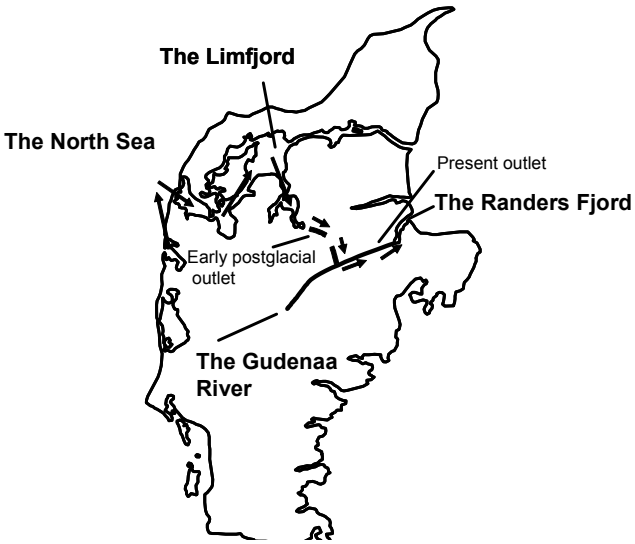

Fig. 8. Hypothetical colonisation routes by whitefish from western Jutland fjords into the Limfjord and via the Limfjord into the Gudenaa River, indicated by arrows. After approx. 1000 years the flow of the Gudenaa River changed direction to the present outlet in the Randers Fjord.

Koskinen *et al.*, 2000). However, the Baltic and Danish whitefish are also closely related, as evidenced by the mitochondrial DNA data, and may ultimately have originated from the same glacial refuge. Finally, North Sea houting and whitefish have diverged recently, within the past 10-13,000 years. This exemplifies the rapid divergence that apparently has taken place in several cases within this group of species and has sometimes even lead to the formation of species flocks (e.g., Douglas *et al.*, 1999).

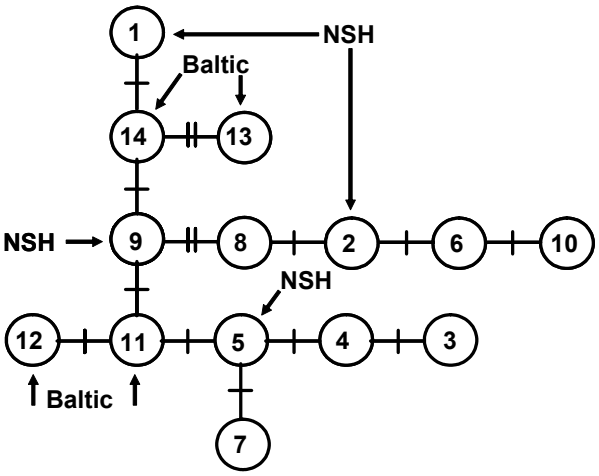

Fig. 9. Parsimony network showing the genetic relationships among the observed mtDNA haplotypes in the sampled *Coregonus* populations. Arrows indicate haplotypes observed in North Sea houting (NSH) and in populations from the Baltic Sea. Bars across lines denote numbers of mutation steps separating haplotypes.

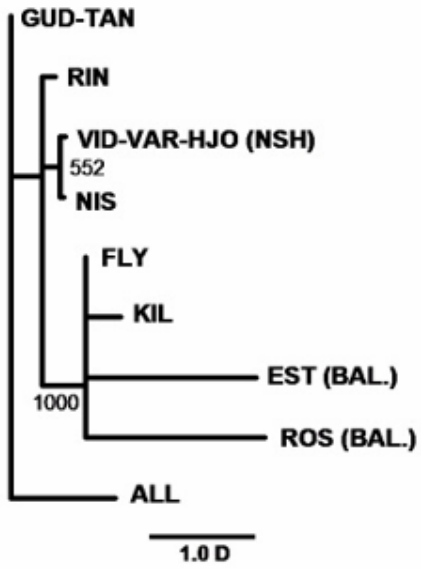

Fig. 10. “Hybrid" dendrogram (Angers & Bernatchez, 1998) showing the genetic relationships among the sampled *Coregonus* populations, inferred from microsatellite data. The hybrid dendrogram was constructed based on a topology inferred using Cavalli-Sforza & Edwards (1967) genetic distance, and with branch lengths based on Goldstein *et al.*'s (1995) $(\delta\mu)^2$ distance. Numbers denote bootstrap values (only bootstrap values exceeding 50% are indicated). "BAL" denotes Baltic populations and "NSH" denotes populations of North Sea houting. GUD = Gudenaa River, TAN = Lake Tange, RIN = Ringkøbing Fjord, VID = Vidaa River, VAR = Varde River, HJO = Hjortvad River, NIS = Nissum Fjord, FLY = Lake Flynder, KIL = Kilen, EST = Estonia (Baltic Sea), ROS = Rostock (Baltic Sea) and ALL = Alling River.

5. Demographic genetics: Estimating effective population size and detecting bottlenecks using molecular markers

The previous chapter was concerned with the use of molecular markers for analysing the genetic structure and differentiation among populations. However, molecular markers are also being applied increasingly for estimating demographic parameters within populations, i.e. by either directly estimating effective population size or by detecting changes in effective population size.

Effective population size, N_e, is a key parameter in conservation and population genetics, which determines the extent of genetic drift and inbreeding. It can be defined as the size of an “ideal”

population, with equal sex ratio and little variance of reproductive success among individuals, that would give rise to the same amount of inbreeding or genetic drift as occurs in the actual population of interest. There are several definitions of N_e, depending on which process is considered, but the two most important types of N_e are the inbreeding effective size (which measures the amount of inbreeding occurring) and the variance effective size (which measures the amount of drift occurring) (originally described by Wright, 1931). In many, but not all cases (e.g. when population size is changing over time or if a subdivided population is considered) the two measures give essentially the same result. Keeping a sufficiently high N_e is a main priority in conservation biology, in the long-term in order to maintain levels of genetic variation that ensures the evolutionary potential of populations and species, and in the short term to avoid a fitness decrease due to inbreeding depression (Franklin, 1980). In particular, it is important to avoid sudden drastic decreases of N_e, so-called bottlenecks, where deleterious recessive alleles become fixed at a rate at which their fixation can not be counteracted by selection (Frankham *et al*., 2002). Whereas the importance of inbreeding and inbreeding depression has for a long time been acknowledged in captive populations, its role in wild populations has been disputed, the argument being that inbreeding depression becomes an important factor at such low population sizes that extinction due solely to demographic factors is a much more overwhelming threat (Lande, 1988). However, recent studies have shown that inbreeding is certainly a factor of importance for the survival of populations in the wild. First, it has been demonstrated empirically that inbreeding can add significantly to the extinction risk of a population (Saccheri *et al.*, 1998). Second, it has been shown that inbreeding depression may particularly manifest itself when individuals are subject to stress (e.g., Bijlsma *et al.*, 2000). Thus, the present consensus is that populations probably do not go extinct due to inbreeding alone, but that inbreeding depression is a factor that in interplay with other negative factors can severely decrease the prospects for survival of populations. This makes it highly relevant to try to estimate N_e and detect population bottlenecks in both wild and captive populations.

N_e is nearly always considerably lower than the census population size due to differences in the number of males and females contributing to reproduction, variance in reproductive success and family size, and variance of N_e over several generations (Frankham, 1995). Consequently, even if census population size estimates are available it may be difficult to assess N_e due to the number and complexity of factors affecting it. However, N_e can be estimated in several ways using molecular markers. The so-called temporal method has proven particularly useful (e.g., Waples, 1989; Jorde & Ryman, 1995; Laikre *et al*., 1998). The basic principle is to sample a population at two or more points in time separated by a specified number of generations. Based on the changes in allele frequencies that have occurred during the interval it is then possible to estimate the variance effective population size. Of course, one should be careful with interpreting results based on the temporal method, because *all* changes in genetic composition that have occurred over time are ascribed to drift, which may in reality not be the case. For instance, strong gene flow from other populations could lead to temporal genetic differentiation that is not due to drift, thereby resulting in an erroneously low N_e estimate. This is a non-negligible problem in studies of salmonid fishes, where stocking with non-native fish could have affected indigenous gene pools.

Another approach is to estimate N_e from linkage or rather gametic phase disequilibrium (Hill, 1981; Waples, 1991b; Bartley *et al.*, 1992). Here, the principle is that genetic drift causes gametic phase disequilibrium and, conversely, estimating gametic phase disequilibrium yields an estimate of genetic drift which again yields an estimate of N_e. Again, some caution is necessary when

interpreting the results, as other factors than genetic drift, in particular population admixture, could lead to gametic phase disequilibrium and thereby result in a flawed N_e estimate.

Instead of directly estimating N_e another approach is to test for indications of a significantly lowered N_e (e.g., Luikart *et al.*, 1998a). A simple way of doing this is to compare samples taken from the same population at different times and test if there has been a loss of allelic diversity (e.g., Luikart *et al.*, 1998a; /11/). Other newer methods use various approaches for detecting shifts of allelic distributions within single samples that could indicate presence of a recent bottleneck (e.g. Cornuet & Luikart, 1996; Luikart *et al*., 1998b; Garza & Williamson, 2001). The most often applied method is probably the "Bottleneck test" developed by Cornuet & Luikart (1996). The test is based on the fact that a population bottleneck reduces both the number of alleles and *expected* heterozygosity (H_e). However, the number of alleles is reduced at a faster rate than H_e. Consequently, for some time after the bottleneck has taken place H_e is higher than expected given the alleles present at the loci, i.e. the population is not at mutation-drift equilibrium. Based on the alleles actually observed in the sample the program estimates the *equilibrium* heterozygosity (H_{eq}), assuming either an infinite allele mutation model (IAM), a step-wise mutation model (SMM), or a two-phase model of mutation (TPM), the latter two being the most relevant for microsatellite loci. The estimated equilibrium heterozygosity (H_{eq}) for each locus is then compared to the observed H_e and it is tested if a significantly high number of loci in a sample exhibit higher H_e than H_{eq}. Even though the method has proved to be useful it also has some drawbacks, including low statistical power unless a large number of loci are analysed, inability to detect bottlenecks that have occurred more than a few generations ago, and dependence on specific models of mutations, an issue that is still controversial in the context of microsatellite markers.

Finally, a different type of approach for detecting changes in N_e is to use the information contained in the gene genealogy. Such methods are not yet widely applied, but are potentially very useful. We have used one procedure by Beaumont (1999) for detecting population declines and expansions (/19/). It assumes a SMM and estimates the posterior probability distribution of several genealogical and demographic parameters, using Markov Chain Monte Carlo simulations, based on the observed distribution of microsatellite alleles and their repeat numbers. The procedure provides an estimate of the decline or expansion that has occurred in the population and it estimates the point in time (i.e. how many generations back in time) at which the population started to decline or expand. Even though it is intriguing to be able to quantify population declines and expansions and provide an approximate time scale for the process, based on just one sample, the method has some clear limitations. It is based on a model involving just one panmictic population, whereas in reality populations are often subdivided and gene flow may occur between genetically differentiated populations. Consequently, alleles may not coalesce in the population in which they were actually sampled. Also, the assumption of a strict SMM does not accurately reflect the mutation processes occurring at microsatellite loci, which should be taken into consideration in the interpretation of results.

To sum up, there are by now several methods available for estimating effective population size and detecting population bottlenecks using molecular markers, but they all seem to have their limitations. The best strategy is probably to apply more than one method and then compare the results to see if they point in the same direction (as was done in /13/ and /19/).

5.1 Genetic monitoring of brown trout supportive breeding

Captive rearing of fish and subsequent stocking into wild fish populations in order to increase the total population size has a long tradition worldwide. Salmonid fishes, in particular, have been subject to intensive stocking activity. There are basically two types of stocking, as determined by the type and origin of the fish stocked. First, the stocked fish may be derived from strains that are often non-native and have been reared in captivity for several generations (usually denoted domesticated strains). Second, the stocked fish may be offspring of local wild fish, so-called supportive breeding. For reasons treated in more detail in Chapter 9 most stocking activity of salmonid fishes in Denmark is now based on supportive breeding. However, even though this means that indigenous gene pools are not compromised by stocking of exogenous domesticated fish, it is necessary to ensure that stocking procedures do not lead to a lowered total effective population size. Ryman & Laikre (1991), Waples & Do (1994) and others have demonstrated that if the stocked fish are the result of a small effective number of breeders, and if the stocked fish make up a large proportion of the total number of fish (i.e. stocked + naturally reproduced), then the total N_e may be significantly decreased, even if N_e of the wild fish is in fact large. It should be noted, though, that recent studies have also shown that in the long term the consequences are not necessarily so severe provided that supportive breeding is successful in increasing census population sizes (Wang & Ryman, 2001; Duchesne & Bernatchez, 2002). Nevertheless, it is important to ensure that supportive breeding does not lead to reductions of N_e and genetic monitoring using molecular markers may provide a useful tool for this purpose.

We used microsatellite analysis for investigating supportive breeding practises in three Danish brown trout populations, the Karup, Skjern and Esrum Rivers (/13/). We used three different statistical procedures for detecting population bottlenecks and loss of variability: i) a randomization test for comparing allelic diversity between samples (/11/); ii) estimates of effective number of breeders (Nb) from gametic phase disequilibrium (Hill, 1981; Waples, 1991b; Bartley *et al.*, 1992); and iii) a test for assessing population bottlenecks based on detecting deviations from mutation-drift equilibrium (Cornuet & Luikart, 1996). In one of the populations, the Karup River, Nb appeared to be high in captively reared offspring of local wild trout, there were no signs of reduced allelic diversity in reared relative to wild-caught trout, and the tests for population bottlenecks did not yield a significant result, except when an infinite allele mutation (IAM) model was assumed. In fact, assuming this model resulted in significant outcomes for all populations, demonstrating that this mutation model is not valid for the studied microsatellite markers. In the two other populations there were, however, clear signs of low Nb among the reared fish. In one of the Skjern River samples Nb was estimated to 16 (95% CI 6-48) and in one of the Esrum River samples the Nb estimate was 13 (95% CI 6-29), the bottleneck test yielded a significant result for all mutation models considered, and there were significantly fewer alleles in the reared sample than in a sample of wild-caught fish from the river. In the Esrum River the low Nb in reared fish may be a particularly serious problem, as in some years blooms of toxic algae from the Esrum Lake eradicate large proportions of naturally reproduced trout. Consequently, stocked trout can make up a large proportion of the total trout population, leading to a severe reduction of the total N_e.

This study focused on just three trout populations subject to supportive breeding. Nevertheless, in two of three cases there were strong indications of reduced effective numbers of breeders, which obviously raises some concerns and stresses the need for conducting routine genetic monitoring of supportive breeding activities and for identifying the precise factors leading to reduced effective population sizes in reared trout. In the case of the Esrum River the observed low Nb and strong signals of a bottleneck were surprising, given that the anglers responsible for supportive breeding

assured that they had used a large number of parent fish. However, as part of the fertilization procedures they added sperm from several males simultaneously to the same batch of eggs, and Whithler & Beacham (1994) have demonstrated that this may lead to just one or a few males fertilising all eggs due to sperm competition.

There are only few other studies available, where the actual consequences of supportive breeding in terms of reduced N_e has been investigated. The most extensively studied case is that of winter-run chinook salmon (*Oncorhynchys tshawytsca*) in the Sacramento River USA. Here, both demographical and molecular approaches to estimating N_e have shown that the enhancement program has been successful in both increasing census population size and not decreasing effective population size (Hedrick *et al*., 1995; 2000).

5.2 Population decline of Danish otters

The European otter was previously distributed all over Denmark. However, population declines culminating from the 1960s led to the extirpation of the species from most parts of Denmark. Its present range of distribution encompasses almost exclusively the Limfjord area in northern Jutland, though this remaining otter population appears to have stabilized and the species is now again starting to expand its distributional range in Denmark. We wanted to look further into the dynamics of this population decline, and for this purpose we analysed microsatellite variation in both contemporary samples and historical samples, the latter represented by DNA extracted from canine teeth from archived skulls (/19/). The historical samples covered two time periods, i.e. 1883-1949 and 1960-63.

Levels of polymorphism were low with total numbers of alleles per locus ranging from two to five, both in the contemporary and, more surprisingly, in the historical samples. There were significantly fewer alleles in the contemporary relative to the historical samples at three of a total of nine polymorphic loci. Overall, however, there were few signs of the recent population declines having caused the low levels of variability observed in contemporary otters. One explanation could be that otters simply are not very polymorphic, a suggestion supported by other studies showing low variability in this species both at microsatellite loci (Dallas *et al.*, 1999) and at the level of mitochondrial DNA (Effenberger & Suchentrunk, 1999; Mucci *et al.*, 1999; Cassens *et al.*, 2000). This low variability could be due to founder effects associated with postglacial recolonization, or the effective population size of otters could simply be low due to its territorial behavior and the fact that it is a top predator and therefore occurs in relatively low densities. Another possibility is, however, that the low variability does in fact reflect population declines, but encompassing a longer time scale than covered by our historical samples. We used the method by Beaumont (1999) for detecting population declines and expansions, based on the inferred gene genealogy within a sample, and found that the data were most compatible with a severe population decline: The population was suggested to have declined to between 1 and 3% of its original size, based on information from both historical and contemporary samples. Also, this decline was suggested to have started approx. 2-3,000 years ago. Of course, such results should be treated with caution, as they depend heavily on the model used and the prior assumptions, such as that of a strict step-wise mutation model, which is unlikely to be fully valid for microsatellite loci. Nevertheless, the results from the comparisons of genetic variability in historical and contemporary samples and the results of the analyses using Beaumont's method are congruent in showing that population declines resulting in severe loss of genetic variation must have predated the time at which historical samples were taken. Also, a population decline that may date back even into prehistoric times does not seem unreasonable when it is considered that Denmark has been relatively densely populated by man for

approximately the past 2,000 years. Another riverine mammal, the European beaver (*Castor fiber*), went extinct in Denmark approximately 2,500 years ago, presumably due to man-induced habitat destruction or hunting (Aaris-Sørensen, 1988). Thus, the recent population decline of otters may only represent an acceleration of a negative development that has been going on for several centuries.

5.3 Effective population size in anadromous brown trout populations

In many fish populations N_e has previously been a "black box". Even though in some countries there is a long tradition for estimating census population sizes using electrofishing surveys or trap facilities, it is difficult to use these estimates for calculating N_e due to the unknown and potentially very important factors of variance in reproductive success among individuals and fluctuations in population size over time (Frankham, 1995). Furthermore, in some cases it may not even be feasible to obtain census population size estimates if rivers are deep and with unclear water, which can make electrofishing surveys highly unreliable. Estimating N_e using molecular markers and the temporal method appears to be the most sensible way to proceed. In particular, if temporal samples covering several generations can be analysed, this has a strong positive effect on the accuracy of the estimates. Analysis of historical samples is particularly useful for this purpose, and this approach has been used by Miller & Kapuscinski (1997) and Heath *et al*. (2002) for estimating N_e in northern pike and steelhead trout, respectively.

We wanted to estimate N_e of "typical" Danish anadromous brown trout populations (/23/). For this purpose we used microsatellite analysis of temporal samples from three Danish rivers (the Karup [samples from 1912, 1951, 1993 and 1996], Vejle [1910, 1998] and Kovads [1953, 1998] Rivers) and a new implementation of the temporal method (by Berthier *et al.*, 2002), which is a Bayesian and Markov Chain Monte Carlo based approach. Further, the method is based on coalescence theory; roughly speaking, it calculates the N_e that is most consistent with the inferred gene genealogy in a sample taken from a population at time t and going back to a sample from the same population but sampled at time t - T, where T denotes the number of generations separating the samples. The method allows for setting an upper limit, N_{emax} of N_e, and we assumed in most cases a N_{emax} of 1000, but also tried other values in order to assess if this would influence the results.

Table 1. Estimates of effective population size, N_e, using the temporal method by Berthier *et al.* (2002). T denotes the number of generations between temporal samples, N_{emax} denotes the prior information about the upper limit of N_e, and finally the median and 90% posterior probability intervals of N_e are listed. VEJ = Vejle River, KOV = Kovads River, KAR = Karup River.

Samples	T	N_{emax}	Median	90% posterior probability interval
VEJ1910 – VEJ1998	25	2000	1087	607 – 1837
VEJ1910 – VEJ1998	25	1000	795	537 – 980
KOV1953 – KOV1998	13	1000	547	296 – 924
KAR1912 – KAR1951	11	1000	799	489 – 980
KAR1912 – KAR1993	23	1000	614	378 – 930
KAR1912 – KAR1996	24	1000	671	417 – 949
KAR1951 – KAR1993	12	1000	456	253 – 856
KAR1951 – KAR1996	13	1000	530	301 – 897
KAR1993 – KAR1996	0.85	1000	84	35 – 761
KAR1993 – KAR1996	0.85	100	49	24 – 91

Estimates of N_e were high in the Vejle River and the Karup River, in the latter case at least until the 1950s, with 90% posterior probability intervals of N_e in both populations ranging from approx. 500 to 980, assuming $N_{emax} = 1000$ (Table 1). N_e estimates involving either the 1912 and 1951 samples from the Karup River and one of the contemporary samples, from 1993 and 1996, tended to be lower. A recent decline of N_e in the Karup River was clearly suggested by the low estimates involving the 1993 and 1996 samples (90% posterior probability intervals ranging from 35 – 761 and 24 – 91, assuming $N_{emax} = 1000$ and 100, respectively). Finally, the population in the Kovads River (KOV) exhibited a substantial N_e with a 90% posterior probability interval ranging from 296 - 924. This result is particularly striking, given that this is a small river, only approx. 3 km long, whereas the Karup and Vejle Rivers are relatively large river systems (by Danish standards), with lengths of the main rivers of approx. 50-60 km. We can thus conclude that N_e appears to be high in "typical" Danish anadromous trout populations, with N_e values clearly exceeding 50, the minimum short-term N_e suggested for avoiding inbreeding problems, and at least in the Vejle River and historical Karup River N_e is also above approx. 500, the minimum long-term N_e suggested for maintaining the evolutionary potential of populations (Franklin, 1980). We do not know the exact reason for the low N_e estimate in the contemporary KAR population. It could be a combination of factors, such as a general population decline due to habitat degradation combined with the use of too few parent fish for supportive breeding, as it is known that in some years in the late 1980s and early 1990s as few as 10-15 males and 20-30 females were used as broodstock.

There are still relatively few estimates available of N_e in wild salmonid populations, based on the temporal method, and most studies have been aimed at small, endangered populations (e.g., Waples, 1990). Heath *et al.* (2002) obtained N_e estimates ranging from 92 to 560 in steelhead populations in British Columbia. Jorde & Ryman (1996) estimated N_e in four Swedish resident brown trout populations. Compared to the present study N_e was relatively small (point estimates from 52 – 480). Laikre *et al*. (2002) used mitochondrial DNA analysis for estimating female effective population size of anadromous brown trout populations in small rivers on the Baltic island of Gotland. The estimates were generally low, ranging from 7 to 177, and presumably also correspond to low total effective population sizes. The most obvious explanation for the higher N_e estimates obtained for the Danish populations is that these populations inhabit larger rivers than those studied by Laikre *et al*. (2002) and that they are anadromous, compared to the resident populations studied by Jorde & Ryman (1996). The fact that a large proportion of the population migrates between the river and the sea means that the census population size of adult trout becomes less dependent on the availability of resources such as food and space in the river (e.g., Elliott, 1994).

6. The biological significance of genetic differentiation

As described previously brown trout is renowned for its strong genetic differentiation among populations. Even though the strong genetic differentiation in some cases can be ascribed to restricted gene flow among landlocked populations, it is also evident that there is a non-negligible degree of genetic differentiation among anadromous brown trout populations, despite the fact that some gene flow may occur among populations. The obvious question is then, what is the biological significance of this differentiation? Is it just a reflection of genetic differentiation due to random genetic drift and limited dispersal of individuals, but not reflecting any divergence at biologically important traits, or are populations likely to be adapted to local conditions? This question has become even more relevant with the advent of highly variable molecular markers, such as

microsatellites, which make it possible to detect so small genetic differences among populations that the biological importance may be questionable (e.g., Waples, 1998; Hedrick, 1999).

6.1 Direct demonstration of local adaptation

The question of local adaptation in salmonid populations has attracted much interest over the years. The most obvious way of addressing this issue is to try to demonstrate local adaptations in the wild, either by generally evaluating if individuals from a population exhibit higher fitness in their "home" environment than in a "foreign" environment, or by more specifically identifying traits that result in higher fitness in the native environment. Spectacular examples of the first approach are found in the former Soviet Union, where huge transplantation experiments with Pacific salmon species have been conducted, in many cases demonstrating lower fitness of the transplanted populations in the "foreign" environment (Altukhov *et al.*, 2000). The other approach, aimed at identifying traits reflecting local adaptations has been the most common. There are by now many examples available of differences among populations in traits that are assumed to represent local adaptations (e.g., Palm & Ryman, 1999; review by Taylor, 1991). In many cases, however, a definitive proof of local adaptation is lacking, as there are few studies demonstrating that differences in traits among populations are maintained by different selection pressures in different environments. A notable exception is a recent, very elegant study by Koskinen *et al.* (2002c). They used a combination of presumably neutral microsatellite markers and analysis of quantitative traits and showed that differences in life-history traits among introduced grayling (*Thymallus thymallus*) populations, derived from a common source population, must be the result of strong local selection rather than drift. This study points to a type of approach that is finding increased use in studies of local adaptation and demonstration of selection, i.e. comparisons of F_{ST} at presumably neutral markers and Q_{ST}, a measure of differentiation among populations at quantitative traits, analogous to F_{ST} (reviewed by McKay & Latta, 2002). However, as pointed out by Hendry (2002) it cannot be simply assumed that $F_{ST} = Q_{ST}$ in the case of neutrality of the trait in question; among other things this depends on the relationship between mutation rate and migration rate, and differences in mutation rate at the molecular markers used for estimating F_{ST} and the loci affecting the quantitative traits. Yet another approach for demonstrating local selection that is expected to be of much future importance consists in directly analysing quantitative trait loci, QTLs. In practical terms this means analysis of polymorphic molecular markers, such as microsatellites, that are tightly linked to a polymorphic locus that has an effect on a quantitative trait. QTLs are currently being identified in a number of species of commercial and/or scientific interest, including salmonid species like Atlantic salmon and rainbow trout (e.g. Perry *et al.*, 2001).

6.2 Assessing the potential for local adaptation

Whereas the approaches mentioned above are all aimed at directly demonstrating local adaptation, another more indirect way of assessing the *potential* for local adaptation consists in evaluating population parameters estimated from molecular data. Since local adaptation depends on the relative importance of local selection, gene flow and random genetic drift (which depends on effective population size, N_e), we can predict the potential for local adaption if we are able to estimate these parameters.

We used this type of approach for assessing the potential for local adaptation in "typical" Danish anadromous brown trout populations in relatively large rivers (/23/). First, we used N_e values estimated using the temporal method based on historical and contemporary samples (see 5.3). Based on this we assumed that N_e was somewhere between 500 and 1000, but perhaps with much lower values, e.g. 30, in populations that have been strongly affected by human impact. We then estimated

gene flow (among contemporary samples from five anadromous brown trout populations; the Karup, Kovads, Odder, Vejle and Kolding Rivers) from the relationship $F_{ST} = 1/(1 + 4N_em)$ (Wright, 1931), where N_e denotes effective population size and m denotes migration rate. We also estimated N_em using the private allele method by Slatkin (1985). The observed F_{ST} value corresponded to a gene flow estimate of $N_em = 6.5$ (4.6 - 10.6). A congruent result was obtained using Slatkin's (1985) private allele method, which yielded a N_em estimate of 5.1. By substituting N_e values of 500 and 1000 we then obtained estimates of m between approx. 0.005 – 0.02. However, since the methods used for estimating gene flow depend on a number of assumptions that are unlikely to be fulfilled in real populations (Whitlock & McCauley, 1999) we also assumed a higher migration rate, m, of 0.05, roughly corresponding to estimates of straying rates among brown trout populations obtained by tagging (e.g., Altukhov *et al.* 2000). Finally, we assumed three different selection coefficients, 0.1 (strong selection), 0.01 (modest selection) and 0.001 (weak selection.

We made the assumption that gene flow occurs according to a stepping-stone model (see 4.1), but we considered two types of stepping-stone models, i.e. a strict stepping-stone model, where gene flow occurs only between neighbouring populations, and a relaxed model, where dispersal also occurs over longer distances. To represent the latter we assumed that 60% of gene flow occurred between neighbouring populations and the remaining part occurred between populations separated by two (30%) and three (10%) population units or "stepping-stones". We then evaluated the potential for local adaptation using the approach by Adkison (1995), based on formulas in Nagylaki & Lucier (1980). I will not describe the formulas in detail here, but instead refer to these two papers along with /23/. However, there is one particular parameter that is particularly important to note, i.e. j, the spatial scale of selection. This parameter denotes the number of neighboring populations over which selection is similar. For instance, if j = 1 this means that selection is different in all populations/rivers, whereas if j = 4 this means that selection is the same over a range of four neighboring populations/rivers. We used these values, i.e. j = 1 and j = 4 in our calculations.

Table 2. Assessment of the potential for local adaptation, based on the approach and formulas from Nagylaki & Lucier (1980) and Adkison (1995). N_e denotes effective population size and m is migration rate. "Mode of gene flow" denotes the spatial pattern of gene flow: a, where all gene flow occurs exclusively between neighboring populations; and b, where 60% of gene flow occurs between neighboring populations, 30% between populations two "stepping-stones" apart and 10% between populations three "stepping-stones" apart. j denotes the number of populations over which selection regimes are the same, and s denotes the strength of selection. H denotes scenarios where genetic homogeneity is predicted, R denotes scenarios where random differentiation is predicted and, finally, A denotes situations where the prediction is local adaptation. H-A denotes situations intermediate between homogeneity and adaptation.

Scenario	N_e	m	Mode of gene flow	j	$s = 0.001$	$s = 0.01$	$s = 0.1$
A	30	0.01	a	1	R	R	A
B	30	0.01	a	4	R	R	A
C	500	0.01	a	1	H	H-A	A
D	500	0.01	a	4	A	A	A
E	500	0.01	b	1	H	H	A
F	500	0.01	b	4	H	A	A
G	500	0.05	a	1	H	H	A
H	500	0.05	a	4	H	A	A
I	1000	0.005	a	1	H	A	A
J	1000	0.005	a	4	A	A	A
K	1000	0.005	b	1	H	H	A
L	1000	0.005	b	4	H-A	A	A

The results of the analyses are shown in table 2. Assuming N_e of 30 and m of 0.01, local adaptation was predicted only to occur in the case of strong selection (s = 0.1; scenario A and B). Assuming N_e of 500 and m of 0.01, local adaptation was predicted in individual populations if selection was strong (s > 0.01; scenario C and E), but if the spatial scale of selection encompassed several populations (j = 4), then local adaptation was possible for smaller values of s (D and F). Assuming a higher m of 0.05 resulted in qualitatively similar predictions (G and H). As expected, assuming even higher N_e (1000) and lower m (0.005) resulted in increased possibilities for local adaptation (I – L). The mode of gene flow, i.e. whether gene flow occurred exclusively between neighbouring populations or could occur also over longer distances, also had a significant effect on the potential for local adaptation (C-D vs. E-F and I-J vs. K-L).

What are then the expected extent and geographical scale of local adaptation in the anadromous trout populations studied? There is presumably no simple answer, as it depends on the strength of selection, which is expected to vary considerably among traits (Endler, 1986). One important factor concerns whether the trait in question is polygenic or encoded by a single or few loci. Thus, in the case of quantitative traits where several loci have an effect on the trait, the selection acting on individual loci may be quite weak, even if the trait itself is subject to strong selection (Lynch, 1984). There are some examples of single locus "traits" possibly subject to differential local selection in salmonids, such as the major histocompatibility (MHC) class II B locus (Landry & Bernatchez, 2001) and the malic enzyme-2 (*MEP-2**) locus (Verspoor & Jordan, 1989) in Atlantic salmon. However, most morphological, behavioral and life history traits potentially involved in local adaptation are expected to be quantitative traits affected by several loci. It follows that unless selection is very strong, local adaptation involving such traits are primarily expected to occur at the scale of more populations experiencing similar selection regimes.

Is it realistic to assume that neighboring populations share the same selection regimes? In Denmark, all rivers flow through lowland landscapes and there are no dramatic shifts in geochemical properties. Consequently, some similarity of selection regimes, e.g. determined by water chemistry and temperature, at the scale of several neighboring rivers is not an unreasonable assumption. This needs not necessarily be the case, however, in regions exhibiting more geological variation than Denmark. For instance, among rivers with neighboring outlets into the sea some could be derived from mountainous areas with low water temperatures, whereas others could represent lowland drainages with higher water temperatures, thus potentially resulting in highly differing selection regimes.

A spatial scale of selection of four rivers/populations, as assumed in some of the scenarios, would in practical terms in Denmark correspond to rivers with outlets into the sea separated by waterway distances of approx. 40-75 km. For instance, at a distance of ≤ 40 km from the outlet of the Karup River there are five other rivers of comparable size, along with some other considerably smaller rivers. The fact that neighboring rivers flow into similar marine environments, such as the same or neighboring fjords, raises the issue of whether adaptation to the local marine environment could not also be important in addition to adaptation to riverine environments. Both in Utter (2000) and in /12/ it has been suggested that stocked non-native salmonids perform better adopting a resident life-history than if they become anadromous. This could imply that adaptation to the marine environment, or perhaps the transition from freshwater to the marine phase during smoltification, is important. Tagging data by Svärdson & Fagerström (1982) also suggest adaptation to the local marine environment, as brown trout populations from different parts of the Baltic Sea undertook

very different kinds of feeding migrations and in most cases retained their migratory behavior when transplantation experiments were conducted.

To conclude, we have assessed the *potential* for local adaptation, not the *actual* scale and extent of local adaptation. If this should be done it would require empirical studies, such as reciprocal transplantations to assess the performance of different populations in each other's local environment, or common garden experiments, where the performance and differences in possible adaptive traits could be compared under the same environmental conditions. However, keeping these reservations in mind our results are most consistent with a hierarchical distribution of local adaptations: Local adaptations maintained by weak selection at individual loci are most likely to be found on a larger geographical scale, whereas adaptations maintained by strong selection at individual loci could occur at the scale of individual populations. Such considerations could be used for more efficiently targeting future empirical studies of local adaptation at the appropriate geographical scale. Local adaptation at the level of individual rivers/populations is the current paradigm in salmonid evolutionary biology. Consequently, most studies of local adaptation are designed to find adaptations at the scale of individual populations, and of course the adaptations observed are of this type, whereas potentially important adaptations occurring at a larger geographical scale remain undetected because the studies have not been designed to detect them. Also, even if most local adaptations would in fact turn out to be distributed at a larger geographical scale rather than at the level of individual rivers, it is still important to conserve as many individual populations as possible. Extirpation of populations is equivalent to removal of migrational stepping-stones and may reduce the number of populations sharing similar selection regimes, thereby causing loss of local adaptations occurring at the scale of several neighboring populations. Consequently, even though limited resources may necessitate prioritization of some populations for conservation over others (e.g., Allendorf *et al*., 1997), it should be taken into consideration that maintaining the genetic structure of populations is also important. Otherwise, giving lower priority to some populations may in fact have negative consequences also for the populations that are highly prioritized for conservation.

7. Mating structure in fishes and its impact on genetic population structure

One of the important assumptions in many theoretical population genetics models, for instance the principle of Hardy-Weinberg equilibrium, is that of "random mating". However, it is questionable how many examples of true random mating can actually be found in real populations. The eternal struggle among individuals for passing their genes on to the next generations in order to maximize their fitness has led to sophisticated reproductive tactics in order to ensure reproductive success and make sure that gametes are not wasted on suboptimal partners. This is also the case in many fish species (reviewed by Taborsky, 1998). In several salmonid fishes, for instance, two different male reproductive tactics are observed. Some males are large (often anadromous), whereas others are small (down to 10-15 cm), so-called mature male parr (reviewed by Fleming, 1996). Large males compete and fight for access to females, whereas mature mature male parr fertilise eggs by adopting a sneaking behaviour and have been shown to have considerable reproductive success (e.g., Martinez *et al.*, 2000; Taggart *et al.*, 2001). There is even a recent study demonstrating higher sperm quality in Atlantic salmon mature male parr relative to adult males (Vladic & Järvi, 2001), though on the other hand there are also indications that adopting a mature male parr life history involves trade-offs resulting in susceptibility to aggression by adult males (Broberg *et al.*, 2000).

Another example of non-random mating in populations consists in mate choice based on variation at Major Histocompatibility Complex (MHC) genes. MHC genes encode glycoproteins on the surface of T-cells. The glycoproteins enable T-cells to recognize and eliminate foreign antigenes and MHC loci are thereby directly involved in the immune response. Thus, in Atlantic salmon it has been found that specific alleles at the MHC class IIB gene are involved in resistance against the disease furunculosis (Langefors *et al.*, 2001). Also, MHC has been shown to be involved in mate choice in a range of species, including mice and even humans (Wedekind *et al*., 1995), apparently through olfactory recognition. Assortative mating and mate choice reflecting MHC variation in individuals has also been observed in Atlantic salmon (Landry *et al.*, 2001).

Reproductive competition must in general be assumed to lead to increased variance of reproductive success among individuals, thereby leading to a low N_e/N ratio (i.e. the ratio between effective and census population size). However, the presence of mature male parr may increase total effective population size, because they have not been subject to the same mortality as older males and are therefore often found in high numbers at the spawning grounds. In Spanish Atlantic salmon this is thought to have been an important factor maintaining genetic diversity despite significantly decreased runs of adult salmon (Martinez *et al*., 2000).

7.1 Mating structure in cod, a pelagic marine spawner

Marine pelagic spawners, like cod, herring (*Clupea harrhengus*) and others could perhaps be examples of species exhibing true random mating. These fishes form large spawning aggregations that may consist of hundred thousands or millions of individuals, and it may be difficult to envisage that any distinct spawning behaviour other than synchronised release of gametes can occur. However, observations of cod in captivity have suggested that they do in fact exhibit specific mating related behavior, such as males courtshipping females and close ventral contact during mating (Brawn, 1961). Also, Hutchings *et al.* (1999) demonstrated aggressive interactions among males leading to a size correlated dominance hierarchy for access to females.

In order to estimate reproductive competition in cod males, an experiment was set up, where cod males and females of different sizes were allowed to spawn in enclosures (a total of six) rigged at sea (/21/). Patterns of individual reproductive success were then estimated by microsatellite based parentage analyses of offspring. The offspring were sampled from a large number of spawning events taking place over the whole spawning season.

The results showed that multiple males contributed sperm to most spawnings. However, siring frequencies were highly skewed among males, and this corresponded well to the male size rank; on average males sired higher proportions of offspring. This confirms that the size correlated male dominance hierarchy previously described for cod is also reflected in the actual reproductive success of individuals. Furthermore, the results indicated size assortative mating, as male reproductive success was dependent on the magnitude of the size difference between a female and a male (/21/).

All together, these results show that even a prolific mass spawner like cod does not exhibit “random mating”. Instead, mate choice and mating success are the result of specific behavioural interactions among individuals. The skewed reproductive output among males also has important consequences for the genetic population structure of the species. Cod has been shown to be subdivided into genetically distinct and temporally stable populations (Mork *et al*., 1985; Ruzzante *et al.*, 1998; 2001; Pogson *et al*., 2001; /17/). Given the huge census population sizes in cod and many other

marine fish species genetic drift is expected to be negligible, and it may in fact be considered surprising that detectable genetic differentiation is at all present at presumably neutral loci. It has been suggested that this could be the result of low ratios of effective to census population sizes (N_e/N) (Hedgecock, 1994). Low N_e/N ratios could result from high variance in reproductive success, and one mechanism could be that eggs and larvae from temporally and spatially separated spawnings experience large variations in physical and biological conditions. Thus, entire cohorts of recruits could be the product of relatively few spawnings ("sweepstakes selection"). Our results point to an additional factor, i.e. skewed reproductive success of individual males in a spawning aggregation, which could lead to high variance in reproductive success and thereby contribute to a low N_e/N ratio. Given that cod census population sizes (N) should be counted in millions it is unlikely that N_e is sufficiently small to lead to detectable genetic drift in cod populations on a short (ecological) time-scale. However, over longer time scales male reproductive skew could contribute to a detectable low N_e/N ratio and thus contribute to increasing genetic differentiation among populations.

8. Individual identification using molecular markers

Some of the most intriguing research possibilities provided by molecular markers such as microsatellites consist in the possibilities for individual identification. Given the high number of alleles present at microsatellite loci, the multilocus genotype of individuals can be regarded as a unique “genetic tag”, allowing for unambiguous identification of the individual just by sampling a small tissue sample. This genetic tagging approach was used by Palsbøll *et al.* (1997) for tracking the migration routes of humpback whales. Another type of individual identification consists of parentage assignment, i.e. assigning offspring to parents (e.g. Marshall *et al.*, 1998; reviewed by Wilson & Ferguson, 2002). Parentage assignment using molecular markers has become an invaluable tool in behavioural ecology, and among others was used in paper /21/ of this thesis. In some cases it is also of interest to assign individuals to species, for instance if only a small tissue sample or, as in paper /9/, a faecal sample is available from the individual in question. Finally, it is possible to assign individuals to populations based on the multilocus genotypes of individuals and the allelic composition of populations, i.e. so-called assignment tests (Paetkau *et al.*, 1995). In this chapter I will focus on the two latter issues, i.e. species identification and, in particular, assignment tests.

8.1 Species identification: Identifying scats from mustelid species

The most obvious ways of monitoring populations and species are by either observing them directly or by using some sort of capture-recapture method. However, in some cases the animals in question are so elusive that traditional approaches for monitoring are not feasible. The European otter, which is both shy and nocturnal, is an example of this. Consequently, monitoring of the species, such as determining its abundance and geographical distribution, has traditionally been based on signs of its presence, in particular spraints. Spraints have also been used for studying the feeding biology of the species by analysing skeletal residues (e.g., Jacobsen & Hansen, 1996).

Another mustelid species, the American mink (*Mustela vison*), has also recently attracted much interest in Europe, though for negative reasons. Mink is a North American species, but has been introduced in Europe where it is propagated in large numbers in fur-farms. Unfortunately, escapes occur regularly, and there have been concerns about the impact of escaped mink on local fish and wildlife populations (e.g., Lever, 1978). As is the case with otter the feeding biology of mink has

been studied by analysis of spraints. The spraints of the two species can in most, but not all cases be distinguished based on visual appearance and odour, but the situation is made considerably more complicated by the presence of a third mustelid, the polecat (*Mustela putorius*), the spraints of which are almost indistinguishable from those of mink.

Excrements have been used as a source of DNA from both the defaecator as well as residues of food items remaining in the faeces (e.g., Höss *et al.*, 1992; Paxinos *et al.*, 1997). Consequently, we focused on the possibility of extracting DNA and analysing species-specific DNA markers from excrements of the three mustelid species (/9/). The protocol would need to be based on the polymerase chain reaction (PCR) in order to be able to analyse minute amounts of DNA. Furthermore, otter, mink and polecat are known to feed on a variety of prey. Therefore, it was necessary to design PCR primers specific to these mustelid species that at the same time contained too many mismatches to allow for amplification of DNA from residues of prey (primarily fish, mice, amphibia and birds) in the spraints.

We developed a system for species identification based on species-specific variation in the mitochondrial cytochrome b gene. This was done by designing primers which matched the sequence of the three mustelid species but at the same time contained mismatches at important sites in sequences representative of important prey items (frogs, birds, rodents and fish; see fig. 11). The amplified product was 189 bp and contained sequence variation between the three mustelids that could be detected using the restriction enzymes *Taq* I and *Nla* IV. Because of the short size of the DNA segment and the expected low yields and poor amplification of DNA from spraints we labelled one primer with a flourescent dye which allowed for analysis using an automated sequencer. The system was first tested using DNA extracted from fresh tissue samples and was found to work well. Analysis of spraint DNA was also satisfactory, and revealed one case where an otter spraint had been misclassified as mink.

It was originally the intention to do individual identification of otters as well, based on microsatellite analysis of DNA extracted from spraints. However, the low microsatellite variability in Danish otters (/19/) would necessitate a large number of analysed loci. Furthermore, the number of mtDNA copies is always much higher than copies of nuclear DNA, so even successful amplification of a mtDNA segment does not guarantee amplification of microsatellite loci in highly degraded spraint DNA. Consequently, after some trials we decided not to proceed with this. Dallas *et al.* (2003), however, have recently demonstrated that analysis of microsatellite DNA from otter spraints is feasible, but the proportion of spraints yielding successful amplification is low, approx. 20%.

```
                                1           11          21
Mustela putorius                TTAGCCATAC  ACTATACATC  AGACACAGCC
M. vison                        ..........  ....C.....  .........T
Lutra lutra                     ..........  ....C.....  .......A..
Mus musculus                    ..........  ....C.....  ...T...ATA
Cairina sp.                     C.G..T..G.  ....C..CG.  .......T..
Rana japonicus                  C.G.......  ....C...G.  T..T..TT..
Salmo trutta                    C.A.......  ....C..C..  C..T.TCT.A
Anguilla anguilla               C.A.......  .T..T.....  .....TCT.A

31          41          51       ↓ 61          71          81    ↓
ACAGCCTTTT  CATCAGTCAC  CCACATCTGT  CGAGACGTCA  ACTATGGCTG  AATTATCCGA
..........  ..........  T..T..T..C  ........T.  .T.....T..  ...C..T...
........C.  ........G.  A........C  ..........  ....C.....  G.........
..........  .......A..  A.....T...  ........A.  .T..C..G..  .C.A......
CTT..T..C.  .C.....AG.  .A...CA..C  ...A.....C  .A........  .C.C.....C
CT...A....  ....TA..G.  ...T.....C  ..C..T....  ..A.C.....  .C.CC.T..T
..........  .C..T..TTG  ......T..C  ....T..T.  G...C.....  .C.C......
..T.....C.  .C.....AG.  T........C  ..........  .......A..  .C.A..T..C

91          101  ↑      111         121         131         141
TACATACACG  CAAACGGAGC  TTCCATATTC  TTTATCTGCC  TGTTCCTGCA  CGTAGGGCGG
..T.......  ....T.....  ..........  ...G.T...T  .......A..  T.....A..A
..........  ..........  C.........  ..C.......  .......A..  T.....A..C
..T.......  ..........  C..A.....T  .....T...T  .A.....T..  T..C..A..A
A..C.C....  .C..T..C..  C..AT.C...  ..C......A  .C.A......  .A.C..A..A
A.TC.C....  .C.....C..  C..AT.T...  ..C......A  .C.ATT.C..  .A.T.....A
A....T....  .T........  A..TT.C...  ........TA  .T.ATA.A..  TA.C.CC..A
A..C....T.  ....T.....  C..AT.C...  ..........  .A.A...C..  CA.T.CC..A

151         161         171         181
GGTTTATATT  ATGGATCTTA  TATATTCACC  GAAACATGA
..C.......  ..........  ......TC.T  .........
..CC.G..C.  .C........  .......C.T  .........
..C.......  .......A..  ..C...T.TA  .....C...
..C..C..C.  .C..C..C..  CC.G-----  ---------
..CC.T....  .C..C..A..  CC.C.A..AA  ..G......
..AC.C..C.  ....T..C..  CC...AT.AA  .....C...
..GC.T..C.  .C..C..A..  CC.T.AC.TA  .........
```

Fig. 11. Aligned sequences of a part of the mitochondrial cytochrome b gene from polecat (*Mustela putorius*), mink (*M. vison*), otter (*Lutra lutra*), house mouse (*Mus musculus*), duck (*Cairina sp*.), frog (*Rana japonicus*), brown trout (*Salmo trutta*) and European eel (*Anguilla anguilla*). Priming sites for the mustelid-specific primers are underlined. Restriction sites for the enzyme *Taq* I (recognition sequence TCGA) in polecat and mink are indicated by downward arrows, while the *Nla* IV restriction site (recognition sequence GGNNCC) in otter is indicated by an upward arrow.

8.2 Assignment tests

One of the most interesting and useful recent statistical developments in molecular population genetics is that of assignment tests, which allow for determining the population of origin of single individuals (reviewed by Waser & Strobeck, 1998; /16/). The original assignment test was devised by Paetkau *et al.* (1995), but since then numerous improvements and alternative, highly sophisticated approaches have been developed (e.g. Rannala & Mountain, 1997; Cornuet *et al.*, 1999; Banks & Eichert, 2000; Pritchard *et al.*, 2000; Dawson & Belkhir, 2001; Pella & Masuda, 2001). A common feature of all methods is that they make use of the huge amount of information

that is available in multilocus genotypes of individuals, i.e. the combined information from several loci within an individual. For each individual analysed some sort of probability (in its widest sense) of belonging to each of the possible populations is calculated. In the "classical" assignment test by Paetkau *et al.* (1995) this is done simply by calculating the likelihood of the individual in a population sample based on the observed allele frequencies and Hardy-Weinberg expectations (see /16/ for more details). The individual is then assigned to the population where its multilocus genotype is most likely to be derived from. Many of the assignment methods can be applied to two different kinds of sampling design (see fig. 12). First, they can be applied exclusively to individuals from samples taken from known populations (fig. 12a). This means that an individual may be assigned either to its "true" population sample or to a population sample other than the one that it was actually derived from. Second, individuals of unknown origin can be assigned to a set of baseline samples from known populations (Fig. 12b). An obvious pitfall in both types of design consists in the possibility that one or more individuals are derived from populations that have not been sampled. Cornuet *et al.* (1999) have partly solved this problem by developing a "simulation test", by which it can be estimated if an individual's multilocus genotype is likely to occur, given the observed allelic frequencies in a population sample. This allows for "accepting" or "rejecting" individuals in populations, and individuals that are "rejected" in all populations could be derived from populations that have not been represented by baseline samples.

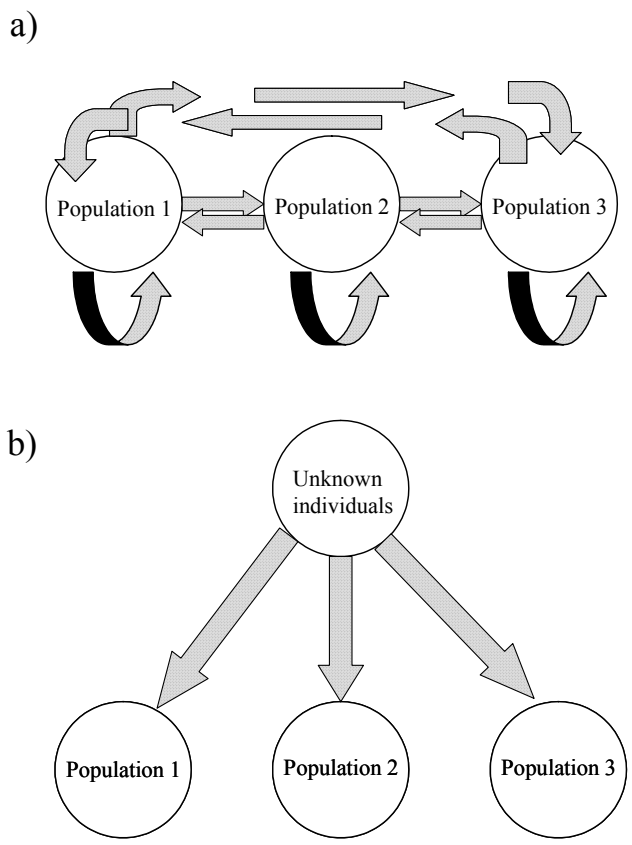

Fig. 12. Schematic representation of the two fundamental types of design of assignment methods. a) "Self-assignment". All individuals from all population samples are assigned. Thus, an individual may be assigned to the sample it was derived from or to another sample. b) "Assignment of unknown individuals". Individuals of unknown origin are assigned to a set of baseline samples.

Assignment of individuals to populations has been used in a number of the studies included in this thesis (/5/; /12/; /14/; /15/; /17/; /18/; /19/; /20/; /22/). The methods used have either been a modification of the "classical" assignment test by Cornuet *et al.* (1999) or the STRUCTURE approach by Pritchard *et al.* (2000). STRUCTURE is a highly useful and very versatile approach which is based on a different principle than the "classical" assignment test. It is a Bayesian, Markov Chain Monte Carlo (MCMC) based approach that uses model-based clustering for partitioning all individuals into groups, and the criteria for grouping individuals are to minimise Hardy-Weinberg disequilibrium and gametic phase disequilibrium between loci within groups. This ensures that the groups are as representative as possible of samples from single, random mating populations as Hardy-Weinberg disequilibrium and gametic phase disequilibrium would be expected if groups consisted of individuals from different populations. STRUCTURE allows for several kinds of analyses, including estimating the number of populations represented by a sample of individuals, assigning individuals to populations and estimating individual admixture coefficients, i.e.

estimating the proportion of an individual's genome that has been derived from one or the other population. In particular, the latter option has been useful for studies of the genetic impact of stocking domesticated salmonids into wild salmonid populations, where it is a crucial issue to assess whether or not interbreeding and introgression has occurred (/15/; /22/).

STRUCTURE also has the convenient feature that it can assign individuals both with and without using prior information on the population from which the individual was sampled. Thus, it is possible to assign individuals and estimate individual admixture coefficients with incomplete baseline data. For instance, if a sample is available of domesticated trout used for stocking a river along with a putative mixed sample of indigenous trout from the river and domesticated trout and "hybrids" between the two, then it is possible to assign individuals and estimate individual admixture proportions even without baseline data from the indigenous population prior to stocking (/15/; see also Beaumont *et al.*, 2001). Finally, STRUCTURE allows for incorporating different kinds of prior information, in particular prior information on migration rates among the populations under study. This, however, also illustrates one of the potential problems with the method. As in other types of Bayesian analyses it is important to critically assess the prior assumptions that the analyses are based on, as they can have significant influence on the results. Another more technical factor that one should be aware of is the fact that the method is based on detecting Hardy-Weinberg and gametic phase disequilibrium. Technical artefacts, such as null alleles and allelic drop-outs, may lead to such disequilibria which would then affect assignment precision and other results, such as estimates of the number of populations represented by the samples.

It is evident from the preceding lines that STRUCTURE is presently my preferred choice for doing individual assignment, but it is worth noting that there are a lot of developments going on in this area. PARTITION (Dawson & Belkhir, 2001) builds on rather similar principles as STRUCTURE and the software appears to be very user-friendly. The BAYES approach by Pella & Masuda (2001) is an innovative combination of mixed-stock analysis and assignment tests, where assignment of individuals to populations is conditioned on the estimated *genic* proportions of different source populations in the mixed assemblage.

8.3 Identification of indigenous Atlantic salmon in Denmark

Analysis of historical and contemporary samples combined with the use of assignment tests have played a key role in identifying remains of indigenous Atlantic salmon in Denmark (/5/; /18/). Until the 1920s-50s there were still indigenous salmon populations in seven rivers in western Jutland and a single river in eastern Jutland (the Gudenaa River). However, by the 1990s it was assumed that there was only one population left, in the Skjern River, whereas all other populations had succumbed to habitat destruction and overfishing. Even in the case of the Skjern River there were some suspicions that the original population had been extirpated. The population had declined severely during the past approx. 40 years, so it was suggested that the individuals present were merely the result of straying from other populations. In order to resolve this issue we analysed microsatellite variation in a sample from the present population and from historical samples collected in the 1930's along with samples from two "outgroup" rivers, one of which was the Swedish Ätran, the geographically closest remaining indigenous salmon population. Twenty eight of a total of 36 individuals from the contemporary sample were assigned to the original indigenous population. Only a single individual had a significantly higher likelihood of belonging to one of the two "outgroup" samples (as determined by a log likelihood ratio test using the program G-STAT (Siegismund, 1995)). Therefore, it was concluded that the present Skjern River population consisted of descendants of the indigenous population (/5/).

However, was it really true that the Skjern River population was the only remaining indigenous population? Catch statistics from other rivers in western Jutland suggested that there might still be remnants of indigenous salmon, as the numbers of salmon caught were too high to be ascribed only to strayers from other populations. Unfortunately, however, by then a stocking program had been initiated in western Jutland rivers, which involved exogenous salmon from populations from western Sweden (Ätran, Lagan), Ireland (Corrib, Burrishool) and Scotland (Conon). Even though historical samples were available from nearly all of the rivers which previously held indigenous salmon populations, the presence of large numbers of stocked exogenous salmon from different populations complicated the situation considerably. It was necessary to simulate a large number of individuals based on the observed allelic composition of the possible source populations (both indigenous salmon represented by historical samples and exogenous salmon used for stocking) and conduct assignment tests using these individuals (/18/). This was done first to assess the rate of misclassification that would inevitably occur; for instance, if 5% of individuals actually derived from an exogenous population would be misassigned as indigenous salmon, then it would be erroneous to claim that there were remains of indigenous salmon left, based on just a few individuals assigned to the indigenous population. Second, it was necessary to assess how "hybrids" between salmon from the different populations would be classified. If, for instance, Corrib-Ätran "hybrids" would have a genetic composition resembling indigenous salmon from the rivers in question, then this could lead to erroneous results. Assignment of simulated hybrids demonstrated, however, that this was not a problem in the present study, as "hybrids" showed intermediate probabilities of being assigned to the two parental populations and were not misclassified in other populations.

The assignments of simulated individuals were then compared to the assignment of the individuals actually sampled in the rivers suspected to hold indigenous salmon. It turned out that in two of the rivers (the Ribe and Varde Rivers) the number of individuals assigned to indigenous salmon (i.e. the historical samples) were significantly higher than expected by chance alone, and it was concluded that there were still remains of indigenous salmon in the two rivers, although the populations were small and highly endangered (/18/). Another interesting finding was that the proportion of fry assigned to the indigenous Ribe population was considerably higher than the proportion of adults classified as indigenous. This suggests that even though adult indigenous salmon only constituted a small fraction of the total salmon run, the indigenous salmon nevertheless exhibited higher fitness in their native environment compared to the stocked exogenous salmon, thus corroborating the view of the importance of local adaptation in salmonid fishes (e.g., Taylor, 1991).

Assignment tests are now applied on a routine basis for salmon electrofished in the Varde and Ribe Rivers during the spawning season in order to identify indigenous salmon and use only these for supportive breeding. Stocking with exogenous salmon has now ceased, but adult spawners resulting from the stockings that have already taken place will still continue to return for some years.

8.4 Assigning individual cod to populations

Marine fishes, like cod, are of very high economic importance, and at the same time they are in some cases so heavily exploited that strict fisheries regulations have been necessary to avoid collapses of the populations. If methods were available for assigning individuals to populations this could become an important tool for regulating fisheries and reveal illegal fishing on protected populations. However, as described in Chapter 4 genetic differentiation among populations of marine fishes is in most cases weak, though in some cases, like cod from the Baltic Sea and the

North Sea, levels of genetic differentiation have been observed that are comparable to differentiation among anadromous fishes (Mork *et al.*, 1985).

We analysed microsatellite variation at nine loci in samples of cod from the Baltic Sea, the North Sea and the northeast Arctic Ocean (/17/), and found unusually strong genetic differentiation among populations as compared to most other studies of marine fishes. Thus, pairwise F_{ST} values were 0.045 between cod from the Baltic Sea and the North Sea, 0.035 between the North Sea and the northeast Arctic Ocean, and 0.073 between the Baltic Sea and the northeast Arctic Ocean. When we self-assigned cod from the three populations, using STRUCTURE (Pritchard *et al*., 2000), assignment precision turned out to be very high, close to 100% even assuming prior information on migration rates as high as 10% (table 3). When we further assigned different samples from the Baltic Sea and the North Sea as "unknown samples" the proportion of correctly assigned individuals was 100% and 93%, respectively. Consequently, assignment is so reliable that testing just two or three individuals can provide an unambiguous conclusion about the origin of a sample that is claimed to originate from one of the three sampled populations, representing the majority of cod landings in the northeastern Atlantic. However, beyond the perspectives for management and conservation the results also point to many applications in ecological studies, for instance to study drift of cod larvae and juveniles from the spawning and nursery grounds and, along with mixed-stock analysis, to assess the extent of mixing of differentiated populations outside the spawning season (Ruzzante *et al.*, 2000).

Table 3. Number (percentage) of cod assigned to the population of origin. Shown are the number (percentage) of individuals with the highest probability of belonging to the population from which they were sampled, assuming three different migration rates. N is the number of individuals sampled from each population. "Unknown samples" are two samples each consisting of 30 individuals from the North Sea and the Baltic Sea, which were not included in the baseline samples. Individual assignment was performed using the STRUCTURE approach and software by Pritchard *et al.* (2000).

Population	North Sea	Baltic	Northeast Arctic
Baseline samples			
Migration rates			
1 %	156 (99)	152 (99)	69 (100)
5 %	156 (99)	152 (99)	69 (100)
10 %	153 (97)	151 (98)	68 (99)
N	158	154	69
Unknown samples	28 (93)	30 (100)	-

9. Genetic interactions between wild and domesticated fish

There is generally increasing awareness of the problems connected to elevating gene flow among populations and causing population admixture involving wild and domesticated populations, both in plants (e.g., Ellstrand *et al*., 1999) and animals (e.g., Gotelli *et al.*, 1994; Beaumont *et al.*, 2001; see also review by Allendorf *et al.*, 2001). However, even though the generality of these kinds of problems is increasing, the group of organisms that has been most subject to human-mediated changes of patterns of gene flow is fishes, and in particular salmonid fishes (Hindar *et al.*, 1991b; Hansen & Loeschcke, 1994; Ryman *et al.*, 1995). The reasons for this are twofold. First, large-scale aquaculture production has recently been boosted by the development of marine aquaculture facilities, where in particular Atlantic salmon are produced. The Atlantic salmon strains have been

subject to intensive selection breeding programs, but unfortunately there are frequent escapes of farmed fish which then enter rivers and have the potential to interbreed with local, indigenous populations (Heggberget *et al.*, 1993). Second, in order to compensate for population declines of salmonid fishes and enhance fisheries it has been common practise to stock hatchery-reared fish into wild populations. In some cases the fish stocked are derived from the local population, often denoted by the term "supportive breeding", but in many cases fish have been stocked that are of non-native origin and have been kept in hatcheries for many generations, often denoted as "domesticated" fish.

What are then the problems with stocking domesticated fish into wild populations? As described in Chapters 4 and 6 salmonid fishes exhibit complex patterns of genetic differentiation both at macro- and microgeographical levels and may possess specific adaptations to their native environment. Conversely, domesticated strains have been reared in captivity for several generations and have in many instances been found to exhibit reduced genetic diversity, both within and among populations (e.g. Garcia-Marin *et al.*, 1991; Stone *et al.*, 1997; /4/; /14/). Furthermore, it is a general concern with captive breeding that the selection regimes are different in captivity compared to a natural environment. This may lead to domestication selection that results in lowered fitness when individuals are introduced to the wild (Reisenbichler & Rubin, 1999; Lynch & O'Hely, 2001; Woodworth *et al.*, 2002). If domesticated fish are released or escape repeatedly in large numbers and interbreed with wild populations, this could lead to swamping of indigenous gene pools, which are then partly or fully replaced by the presumably non-adapted and less diverse gene pools of hatchery fish (Hindar *et al.*, 1991b; Hansen & Loeschcke, 1994). However, even though this may sound relatively simple from a theoretical point of view, there are in practise still very important gaps in the knowledge of the actual consequences of gene flow from domesticated strains to wild populations. Thus, controlled stocking experiments involving Atlantic salmon have demonstrated poor performance of domesticated fish in the wild (e.g. McGinnity *et al.*, 1997; Fleming *et al.*, 2000). Nevertheless, in some parts of their life cycle domesticated fish appear to have a competitive advantage over wild fish, presumably due to higher growth rates (McGinnity *et al.*,1997; Fleming *et al.*, 2000). Thus, domesticated fish have an overall lower fitness than wild fish, but are at the same time able to displace wild fish. This serves as an example to demonstrate that detailed studies are required to fully assess the impact of domesticated fish on wild fish populations.

In the two studies by McGinnity *et al.* (1997) and Fleming *et al.* (2000) the performance of F1 "hybrids" was mainly intermediate relative to the parental wild and domesticated populations, but the performance of F2 and further generations was not assessed. One way to assess the genetic impact of domesticated fish and increase the time scale beyond one or a few generations involves the use of genetic markers for opportunistic screening of wild populations subject to stocking or escapes of domesticated fish. This is the approach taken in the studies of this thesis. We have first aimed to genetically characterise the domesticated brown trout strains used for stocking (/4/; /14/), and next we have analysed stocked brown trout populations and compared the genetic composition to that of the stocked trout which has enabled us to estimate the genetic contribution of the stocked domesticated trout (/12/; /14/; /15/; /20/; /22/).

9.1 Genetic characterisation of brown trout hatchery strains used for stocking

Stocking practises involving releases of brown trout from hatchery strains into wild trout populations have occurred throughout Europe. In some cases in central and southern Europe the hatchery strains used for stocking have been imported from northern Europe and belong to the so-called Atlantic phylogeographical race, but the fish are stocked into wild populations that belong to

other phylogeographical races such as the Mediterranean and *marmoratus* races (e.g., Largiader & Scholl, 1996; Berrebi *et al.*, 2000). On the one hand, the strong genetic divergence between stocked fish and indigenous populations adds to the complexity of the conservation problem, because the two groups of fish may have been reproductively isolated from each other for hundreds of thousands of years. On the other hand, these stocking scenarios provide convenient genetic markers for identifying the genetic contribution by stocked fish, because wild populations and hatchery strains often are fixed for alternate alleles at allozyme loci, in particular *LDH-C1** (e.g. Moran *et al.*, 1991).

In Denmark there is some anecdotal information that one of the hatchery strains used for stocking was at one time mixed with trout imported from Switzerland. However, the vast majority of strains have been founded by trout from Danish rivers, though the precise time and source population is not always known. For investigating the genetic relationships among the most important hatchery strains used for stocking, we sampled the strains and at the same time collected information about the origin of the strains from the hatchery managers (/4/; /14/). It was in some cases difficult to provide exact information about the time at which the strains had been founded; several strains presently in use had been founded from other strains. However, most strains had originally been founded from wild populations in the 1960s or before, with a notable case of one strain that had been founded in the 1880s. If we tentatively assume a generation length of three-four years for hatchery trout this corresponds to at least ten generations of hatchery-rearing and in some cases considerably more.

We analysed the samples using PCR-RFLP analysis of mtDNA segments (/4/) and microsatellites (/14/). The inferred genetic relationships among strains in most cases corresponded well with the history of the strains, as informed by the hatchery managers. In particular, four of the strains were known to have a partly common origin and to have been mixed with each other on some occasions, and indeed they exhibited close genetic relationships. These strains are quantitatively the most important, supplying approximately 80% of stocked domesticated trout in Denmark and exports to other countries. From the point of view of biodiversity, the close genetic relationships among these strains may be problematic, as this means that a large number of genetically different populations are influenced by trout from one single gene pool as a result of stocking.

The analysis of mtDNA variation showed loss of variation in nearly all hatchery strains compared to wild populations (/4/). However, computer simulations showed that even with a fairly large number of female spawners per year (e.g., 50) some loss of variability at the mtDNA level would be expected to take place over time, simply because of the much smaller effective number of mtDNA as compared to nuclear DNA (1/4, given an equal sex ratio; Birky *et al.*, 1983). Thus, it was difficult to make firm conclusions about loss of variability in the strains based only on mtDNA markers. The microsatellite results (/14/) showed low levels of variation in three strains that also showed very low variability at the mtDNA level. However, in the other strains levels of variation was comparable to that observed in wild populations. Presumably, effective population sizes have been fairly large in most strains and exchanges of fish among strains have furthermore provided a buffer against loss of variation. We did, however, observe some temporal genetic differentiation between different cohorts from the same strains that could be interpreted as the result of low effective population sizes, but we were hesitant to make a firm interpretation of this result. Ryman (1997) has shown that a pulse of migrants during one spawning season could result in subsequent genetic divergence among cohorts that would require several generations before levelling out, and exchange of fish among strains could thus be one explanation of the result. Also, Waples and Teel

(1990) demonstrated that in species exhibiting overlapping generations allelic frequencies may fluctuate considerably among cohorts depending on the specific population dynamics, and this could also explain the small but statistically significant differentiation among cohorts.

What can we then conclude about Danish brown trout hatchery strains? First, they have been founded from local Danish populations, but the four most important strains share a common origin and show little differentiation. Second, there are some cases of loss of variation but also other cases where levels of variation are comparable to that of wild populations. Finally, they have been kept in captivity for several generations, and it is possible that domestication selection has resulted in changes at traits that may reduce the fitness of hatchery trout in the wild.

9.2 Stocking impact assessment using microsatellite DNA markers

In order to assess the impact of stocking domesticated trout into wild populations, and to identify if stocked populations have indeed been strongly introgressed by hatchery trout, we have conducted a series of studies using microsatellite DNA analysis (/12/; /14/; /15/; /20/; /22/). In most cases, the analyses have been based on contemporary samples from stocked populations and samples from the hatchery strains used for stocking, i.e. we have not had access to samples from the wild populations prior to stocking. Furthermore, as the hatchery strains have been founded from local Danish populations, although several generations ago, diagnostic markers separating domesticated and wild trout have not been available.

Initially, we tried out different approaches for assessing the genetic contribution of stocked domesticated trout in wild populations, including measures based on the frequencies of alleles observed in the hatchery strains that were not observed in the stocked populations ("maximum potential introgression rate"; /14/). However, it soon appeared that assignment tests were the optimal procedures to assess stocking effects when no data were available from the wild population prior to stocking. Assignment tests involving a sample of domesticated trout and a sample from the stocked population (including possible domesticated fish and their descendants) can provide an assessment of whether or not the stocked population has been significantly affected by domesticated fish. If all or nearly all individuals are assigned correctly to their sample of origin this indicates that the wild population has not been strongly affected by domesticated fish. However, the opposite result, i.e. a large proportion of "misassigned" individuals, does not necessarily prove that the wild population contains a large proportion of domesticated fish, as the domesticated strain and the wild population may for natural reasons exhibit close genetic relationships, thereby resulting in low assignment power. Despite this limitation we have found the approach useful (/12/; /14/; /20/; see also Fritzner *et al.*, 2001). Later, however, when STRUCTURE (Pritchard *et al.*, 2000) became available this has become the preferred method as it allows for assigning individuals to populations and estimating individual admixture proportions with incomplete baseline data (/15/; see also 8.2).

Even though we do not have a full geographical coverage of trout populations from all over Denmark we do have sufficient results to make some general conclusions about the impact of stocking domesticated trout into wild trout populations. In the majority, but not in all populations the genetic contribution by stocked domesticated trout is surprisingly low. This is particularly evident in the Limfjord region, where a number of populations have been stocked with domesticated trout, but still the vast majority of trout were assigned to the wild populations (/20/). This does not mean, however, that stocking has had no impact at all. There was a clear tendency towards a larger proportion of trout being assigned to the hatchery strains in populations where stocking was ongoing compared to populations where stocking with domesticated trout had ceased

at the time of sampling. This suggests that the genetic contribution by domesticated trout may disappear over time, presumably in response to selection. This conclusion is supported by results by Chilcote *et al*. (1986), Skaala *et al*. (1996) and Poteaux *et al*. (1998), who showed that natural selection was indeed acting against domesticated fish and their descendants.

Though little genetic contribution by stocked domesticated trout is the most commonly observed outcome we have also found examples of heavily introgressed populations. The Skjern River is a spectacular example of this (/22/; see next section), but also the Esrum River population in Sealand is an example of a population where domesticated trout have made a major contribution to the gene pool (/14/).

In conclusion, we have examples both of populations where stocking with domesticated trout has made a very limited permanent genetic contribution to the indigenous gene pools and of other populations where stocking has led to very strong introgression. This apparent lack of predictability of the outcomes is also reflected in other studies, where for instance Moran *et al.* (1991) observed almost no genetic contribution by domesticated trout, whereas Taggart & Ferguson (1986) and Berrebi *et al.* (2000) observed extensive interbreeding and introgression. Even within a single river system Garcia-Marin *et al.* (1998) have observed highly divergent degrees of introgression which they ascribed to differences in intensity of angling and thereby selection pressure against domesticated trout. In the next section I will look more into the factors that determine the outcome of stocking activity, exemplified by a study of two rivers subject to intensive stocking for more than a decade.

9.3 Long-term effects of stocking domesticated trout into wild trout populations

As discussed in the previous section there are examples of stocking activities leading to strong admixture of wild and domesticated fish and other examples where there are few if any genetic traces of stocked fish once stocking activity has ceased. All together, available evidence shows that spawning intrusion by domesticated salmonids into wild populations does occur, but also that there is selection acting against domesticated fish. Consequently, the final outcome, i.e. whether or not irreversible introgression or even gene pool displacement occurs, depends on a balance between the "immigration rate" of domesticated fish and the intensity of selection that acts against them. However, even though many studies of genetic interactions between wild and domesticated fish have been undertaken there are few empirical studies available that directly quantify the genetic impact on indigenous salmonid gene pools resulting from many years of repeated escapes or stocking with domesticated fish. What are the observed final outcomes? Has irreversible introgression occurred or are there examples where the genetic composition of populations has been relatively unaffected once immigration by domesticated fish has ceased?

In order to quantify how much domesticated fish have contributed to indigenous gene pools, information is required on the genetic composition of wild populations before and after influence by domesticated fish. The problem is usually to obtain data from the unaffected populations, as samples are in most cases not available that date so far back in time. However, we were fortunate to have access to large collections of trout scale samples from the 1940s-50s from two populations, the Karup and Skjern Rivers. The two populations experienced declines in the 1960's - 1980's due to habitat destruction and overfishing. From the beginning of the 1980's both rivers were subject to intense stocking with domesticated trout (of age classes 0+, 1+ and 2+) from the same strain. Later in the 1980's supportive breeding was undertaken, based on stocking offspring of locally caught wild fish. In the Karup River supportive breeding was based exclusively on stocking offspring of

wild trout caught each year in the river. In the Skjern River wild-caught fish and some of their offspring were maintained in a hatchery and this broodstock was occasionally supplemented with wild-caught spawners. Since 1991 (Karup River) and 1996 (Skjern River) there has been very little or no stocking with domesticated trout. I analysed variation at eight microsatellite loci in contemporary and historical samples of trout from the two rivers along with a sample from the hatchery strain used for stocking (/22/).

I estimated population level admixture proportions, i.e. the proportion of the contemporary gene pools derived from indigenous and domesticated trout, using a newly developed method (LEA; Chikhi *et al.*, 2001) that is able to take drift occurring in the populations into account. The results were surprising: In the Karup River the genetic contribution to the contemporary gene pool by domesticated trout was small (point estimate of 7 and 9% in two pooled samples taken in 1993 and 1996, respectively), whereas in the Skjern River domesticated trout made up between 57% and 88% in different samples (see table 4).

Table 4. Analysis of admixture proportions of domesticated trout (HAT) in contemporary samples of stocked populations, using historical samples and domesticated trout as baseline samples. The median of the posterior distribution of admixture proportions and 95% posterior probability intervals (2.5% and 97.5% quantiles, respectively) are listed. The analyses were performed using the program LEA by Chikhi *et al.* (2001). KA-W93 and KA-W96 denote samples of wild-caught anadromous spawners from the Karup River, taken in 1993 and 1996, respectively. SK-W00 denotes a sample of wild-caught anadromous spawners from the Skjern River, taken in 2000. SK-S00, SK-S95 and SK-S96 denote samples of trout from a broodstock, based on anadromous spawners sampled from the Skjern River. The samples represent the years 1995, 1996 and 2000, respectively.

	KA-W93	KA-W96	SK-W00	SK-S00	SK-S95	SK-S96
Admixture proportion of domesticated trout	0.07	0.09	0.57	0.82	0.88	0.62
95% posterior probability intervals	0.00 - 0.23	0.00 - 0.24	0.27 - 0.86	0.50 – 0.99	0.61 – 0.99	0.30 - 0.90

When I further analysed individual admixture proportions using STRUCTURE (Pritchard *et al.*, 2000), I found a distribution of individual admixture proportions in the Karup River samples that was very similar to the expected distribution for non-admixed trout (I simulated a number of non-admixed Karup River trout based on the allelic composition of the historical sample, estimated individual admixture proportions for these simulated "pure" indigenous trout and then used the results as a reference point for the expected distribution of individual admixture proportions if no admixture with domesticated trout had occurred). In contrast, in the Skjern River samples nearly all trout appeared to have been admixed with domesticated trout. Interestingly, though, a few individuals caught as ascending spawners had a high individual admixture proportion of the indigenous population and might represent non-admixed trout. This suggested that there may still be some remains of non-admixed indigenous Skjern River trout somewhere in the river system, and in an ongoing study we have indeed identified some tributaries with indigenous trout (M.M. Hansen *et al.*, unpublished results).

In the case of the Karup River detailed information was available on the number and age stages of stocked trout, both domesticated trout and offspring of local trout (supportive breeding). This information, along with an estimate of the natural production of trout in the river, was used for calculating the *expected* genetic contribution by domesticated trout to the present population

assuming equal survival and reproductive success of wild and domesticated trout. This was done by considering each year's stocking of trout as immigration into the wild population and then calculating the accumulated genetic contribution by domesticated trout over the years, taking demographic parameters into account (i.e. age-specific survival and smoltification rates and the proportion of different age classes among spawning trout). It turned out that in a sample of anadromous trout taken in 1993 a genetic contribution of 64% from domesticated trout was expected, far exceeding the estimated admixture proportion in the sample taken in 1993 (7%, with a 95% posterior probability interval ranging from 0 to 23%; table 4). Consequently, it can be safely concluded that the domesticated trout had performed much worse than indigenous trout, though it should of course be stressed that in addition to genetic factors a number of non-genetic factors related to circumstances of hatchery-rearing and handling in connection with stocking are likely to have contributed to the outcome.

Given the outcome of stocking domesticated trout into the Karup River, why do we then observe the completely opposite outcome in the Skjern River, i.e. strong introgression? There is less detailed information on the number of stocked domesticated trout in the Skjern River than in the Karup River, but the information that is available suggests that the number of stocked trout in both rivers is approximately the same. As stated previously, whether or not introgression occurs is expected to depend on the strengths of the opposing forces of migration and selection, i.e. the migration–selection balance (Haldane, 1930). This would imply that $m < s$ in the Karup River population, leading to removal of the genetic contribution by domesticated trout due to selection, whereas in the Skjern River population $m > s$, leading to introgression. Of course, selection against domesticated trout could differ in the two rivers due to differences in environmental conditions, though there are no obvious suggestions of factors that should favour domesticated trout in one river but not the other. However, a second factor suggests that selection has been weaker in the Skjern River compared to the Karup River. In both rivers supportive breeding (based on wild-caught spawners) has taken place at the same time as stocking with domesticated trout. Thus, interbreeding has likely occurred as a result of "forced matings" between indigenous and stocked domesticated trout accidentally sampled for supportive breeding. In the Karup River supportive breeding is based exclusively on wild-caught fish, and there has been no opportunity for domesticated trout and their descendants to have a complete life cycle in a hatchery environment and thereby escape natural selection. However, this has been possible in the case of the Skjern River due to the maintenance of a permanent broodstock.

There is also the possibility that the immigration rate by domesticated trout may have been higher in the Skjern River than in the Karup River. Even if approximately the same number of domesticated trout were stocked in the two rivers, there are some indications that the population size in the Skjern River was very small during the decline in the 1960's – 80's, and considerably smaller than the population size in the Karup River. Thus, the proportion of domesticated trout relative to indigenous trout has likely been larger in the Skjern River than in the Karup River, leading to a higher immigration rate in the Skjern River. In conclusion, both higher immigration rate and weaker selection in the Skjern River relative to the Karup River population can explain the differences in genetic contribution by domesticated trout.

The results of this study underpin previous conclusions that stocking activity may lead to a variety of outcomes ranging from no introgression to very strong introgression (e.g., Hindar *et al.*, 1991b). However, even though it was not possible to identify the exact reason for the different outcomes it nevertheless does appear that there are good biological explanations available of factors that could

have resulted in the observed patterns of introgression. After all, the outcome of gene flow from domesticated strains into wild fish populations may not be as unpredictable as previously assumed.

9.4 The role of life-history variation in genetic interactions between wild and domesticated salmonids

As described in 2.1 and 2.2 many salmonid fishes exhibit highly divergent life-history types even within the same populations. In particular, the presence of both anadromous and strictly resident forms within brown trout populations is striking. These life-history forms are subject to very different environmental conditions through important parts of their life cycle, and it is obvious to ask the question if this type of life-history variation also plays a role in the performance and fitness of stocked domesticated trout.

In order to resolve this issue we focused on the Karup River, where an upstream section of the river had been stocked with domesticated trout several years after stocking had ceased in the other parts of the river system. We used a combination of microsatellite (seven loci) and mtDNA analysis to estimate the contribution of hatchery trout among resident and anadromous trout from the stocked section (/12/). We analysed baseline samples taken from the strain of domesticated trout used for stocking and from wild trout from the downstream section of the river, which was assumed not to have been severely affected by domesticated trout (Hansen *et al.*, 1995; see also 9.3 and /22/). Furthermore, we analysed putative admixed samples of anadromous and resident trout caught in the stocked part of the river.

Using assignment tests (the Bayesian method by Cornuet *et al.*, 1999) more than 90% of individuals from the two baseline samples (domesticated and wild trout, respectively) were assigned correctly to their sample of origin. 93% of the anadromous trout from the stocked section were assigned to the baseline sample of wild trout, whereas 46% of the resident trout were assigned to the baseline sample of domesticated trout, and 54% were assigned to the baseline sample of wild trout. This demonstrated the near absence of domesticated trout among anadromous trout, but a significant proportion of domesticated trout among resident trout. However, it did not show whether or not the proportion of domesticated trout among resident trout was due solely to stocked trout, or if interbreeding between wild and domesticated trout had occurred.

To resolve this issue, hybrid indices (Campton & Utter, 1985) were calculated, based on the three loci that provided the best discrimination. This hybrid index can be regarded as a way of visualising the relative assignment probabilities in an assignment test involving two parental populations. The index may range between 0 and 1 and, ideally, depending on the presence of sufficiently diagnostic loci, the score of individuals from the parental populations is close to either 0 or 1 whereas the scores of hybrids are intermediate. Even though the power of discriminating between wild Karup River trout and domesticated trout was low, with hybrid index values far from 0 and 1, respectively, a bimodal distribution of hybrid index values was nevertheless clearly evident in the bar diagram involving the wild and domesticated baseline samples (fig. 13a). The distribution of hybrid index values for the sample of anadromous trout from the stocked section resembled what would be expected in a "pure" sample of wild trout (fig. 13b), whereas an excess of intermediate hybrid index values was observed among resident trout (fig. 13c) where in fact a bimodal distribution (as in fig. 13a) would be expected if no interbreeding took place. This showed that a large proportion of the resident trout were the result of interbreeding between wild and domesticated trout. Finally, when the results of assignment tests were combined with data from maternally inherited mitochondrial DNA it turned out that most "hybrid" resident trout were the result of interbreeding between

hatchery males and wild females, in accordance with the male-biased sex ratio usually observed in resident trout (Rasmussen, 1986).

How do we then explain the observed outcome of very few admixed individuals among anadromous trout, but extensive admixture among resident trout? A simple explanation of the results could be that all stocked domesticated trout became resident. Even though this is possible, there is no evidence of reduced smoltification among trout from the domesticated strain. It has in fact been one of the most important suppliers of smolts for stocking elsewhere in Denmark. It is also possible that stocked trout that become anadromous exhibit poorer homing compared to indigenous fish and stray to other rivers. However, we find it unlikely that this alone should result in a return rate close to zero, as suggested by the present results. It appears more likely that stocked domesticated trout perform poorer as anadromous trout than as resident trout, likely as a result of the differences in complexity of the two kinds of life cycles. The life-history of anadromous trout is highly complex and various factors, like timing of smoltification and migratory behavior in the sea, may contain genetic components and reflect specific adaptations (e.g. Svärdson & Fagerström 1982). In contrast, stocked domesticated trout that become resident experience a comparatively more stable environment and may exhibit higher survival rates.

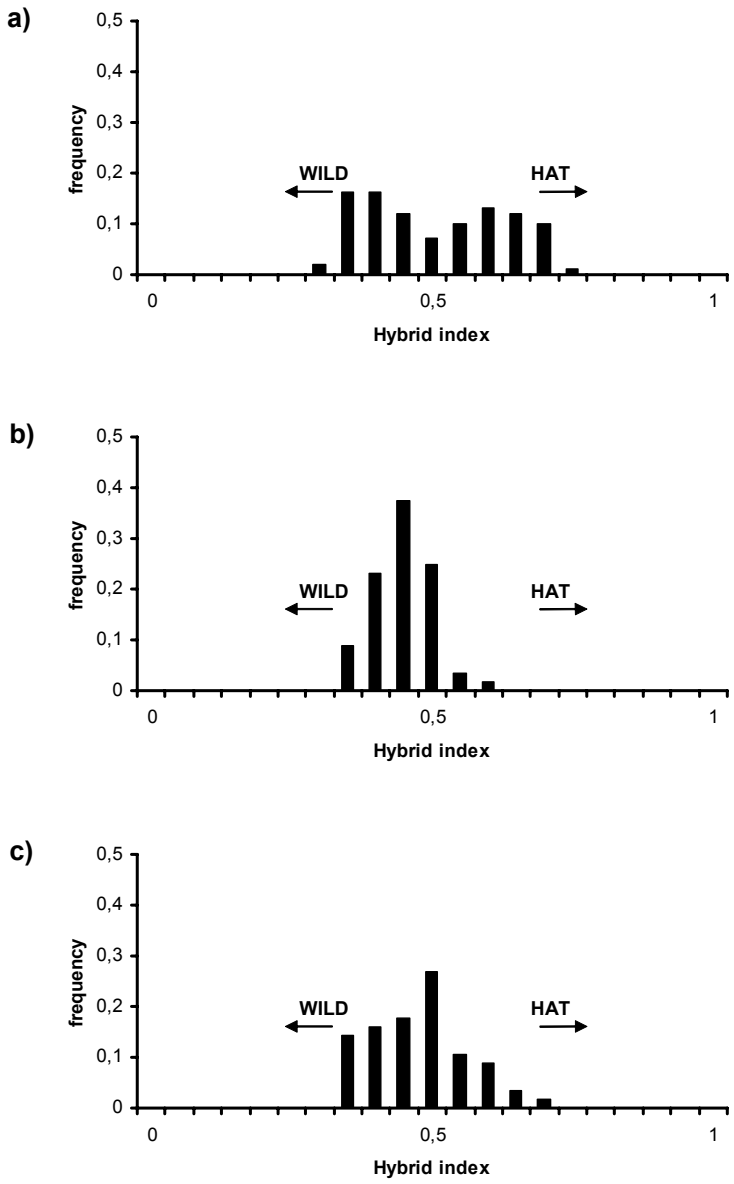

Fig. 13. Frequency distributions of hybrid indices of brown trout from the Karup River, Denmark, based on three microsatellite loci and calculated according to Campton and Utter (1985). The purpose of the analysis was to detect interbreeding between wild and hatchery trout. a) Hybrid indices of individuals from baseline samples of hatchery (HAT) and wild (WILD) trout pooled). b) Hybrid indices of anadromous trout from a stocked section of the Karup River. c) Hybrid indices of resident trout from a stocked section of the Karup River.

We have put forward the following hypothesis explaining the observed outcome: A proportion of stocked domesticated trout would smoltify and become anadromous trout, but only few would return to spawn, probably due to low survival. The majority of female domesticated trout would be included in this group. The other proportion of stocked domesticated trout, the majority of which are males, would become resident and exhibit higher survival than hatchery sea trout. Mature hatchery males, if able to reproduce, would in

most cases mate with wild female trout, most of which are anadromous trout. Differences in survival between anadromous and resident hatchery trout would thereby result in male-biased gene flow from domesticated trout to wild trout, as indicated by our results. It is tempting to speculate if the domesticated resident males with reproductive success are individuals that become mature male parr and adopt a "sneaky" strategy which allows them to successfully fertilise eggs from wild females. However, our results do not allow for investigating this issue, and this would probably require a controlled experimental set-up where the reproductive output of single individuals could be estimated.

After this study was published (/12/), Utter (2000) evaluated the patterns of introgression by stocked salmonid fishes into wild populations, based on results representing many studies and species. He reached basically the same conclusion as we did, i.e. resident populations appear to be more susceptible to introgression than anadromous populations. So, there are all together strong indications that life-history variation in salmonids plays an important role in determining whether or not introduction of domesticated fish into wild populations leads to strong introgression.

The overall conclusion that can be drawn both from a large number of studies, including those presented in this thesis, is that stocking with domesticated fish does not lead to a sustainable enhancement of populations and fisheries, and that it may in fact have negative consequences for the wild, indigenous populations. In Denmark, this has now led to the decision to abandon all stocking with domesticated salmonids. Future stocking activities will be based on offspring of local wild fish. In cases where the indigenous populations have been extirpated, the stocked fish will consist of fish from as geographically (and ecologically) proximate rivers as possible that sustain wild populations. In this way it will be possible to maintain a large recreative fishery and at the same time do as little harm as possible to the indigenous salmonid populations that we fortunately still have in Denmark.

10. Main findings and conclusions

In the introduction of the thesis I stated that the general aim of the research was to use molecular markers for analysing the genetic structure of populations and resolve issues of relevance for the management and conservation of populations and species. In this final chapter I sum up the main conclusions of the work, whereas the more detailed conclusions and findings can be found in the individual chapters and papers included in this thesis.

In the context of the genetic structure of salmonid fishes our results showed complicated patterns of postglacial recolonisation of Denmark. In whitefish, the results were most consistent with recolonisation from one glacial refuge, probably via the postglacial Elbe River (/8/). Even the phenotypically divergent North Sea houting appeared to be the result of the same recolonisation event as "ordinary" whitefish populations. In Atlantic salmon genetic variability at the mtDNA level was low in northern European Atlantic populations (/2/). It was concluded that the genetic differentiation observed was probably mostly a result of drift and gene flow rather than postglacial recolonisation events. The phylogeny of mtDNA haplotypes in Danish brown trout populations suggested the presence of three major clades, which could be interpreted as recolonisation from three different refuges (/6/), a result supported by the comprehensive study by Bernatchez (2001). Even though there was a weak signal of a cline in the distribution of clades among populations, going from the Limfjord and into the Baltic Sea, all three clades were represented in the populations studied, suggesting extensive mixing of the original lineages.

Our studies of local genetic population structure showed significant genetic differentiation among Danish anadromous brown trout populations (/1/; /6/; /20/; /23/), but weaker differentiation than observed among resident and landlocked populations with restricted gene flow. There was evidence of isolation by distance patterns, suggesting that gene flow occurs according to a stepping-stone model (/6/; /20/). There was also a significant pattern of isolation by distance among historical Atlantic salmon populations (/10/), and it was suggested that lack of isolation by distance among contemporary Atlantic salmon populations could be the result of human disturbance, due to e.g. population bottlenecks and transplantation of populations.

Analysis of historical samples proved to be a very useful tool for obtaining a deeper understanding of the genetic structure of Atlantic salmon and brown trout (/11/). In both species we found that the genetic composition of populations was remarkably stable, even over time spans of up to approx. 80 years (/5/; /10/; /23/). In anadromous brown trout populations we showed that the limited genetic drift over time corresponded to high effective population sizes (>300-500), and by also taking gene flow and selection coefficients into account this suggested that local adaptation was indeed likely to be present. However, the geographical scale at which local adaptation was expected to occur depended heavily on the strength of selection maintaining the adaptations. We argued that many local adaptations consist of quantitative traits, where the selection acting on individual loci may be weak. If so, this would imply that these adaptations are more likely to occur at the scale of several neighboring rivers rather than at the scale of individual rivers (/23/).

Apart from estimating effective population size in anadromous brown trout we also used microsatellite markers for estimating demographic parameters in a direct conservation context. In brown trout and other salmonids it is a matter of concern if supportive breeding of local populations results in a lowered total effective population size. In two out of three studied brown trout populations subject to supportive breeding there were strong indications of low effective population sizes and bottlenecks, showing that this is a problem that should be taken seriously (/13/). Historical samples were used to investigate the dynamics of population decline of European otter in Denmark (/19/). Even though the most recent decline has led to the extirpation of otters from nearly all of Denmark, except the Limfjord region in northern Jutland, we made the surprising observation that levels of genetic variation were low both in contemporary samples and in historical samples predating the 1960s. An analysis of demographical parameters, based on the genealogy of the observed microsatellite alleles, suggested that the population decline had started several centuries ago, and that the most recent decline was only an acceleration of a negative development that had been ongoing for a very long time.

Cod and many other marine fish species spawn pelagically in huge aggregations. There has previously been little knowledge about patterns of mate choice and reproductive competition. However, using microsatellite based parentage assignment it was possible to estimate mating patterns and reproductive success among cod in semi-natural experimental set-ups (/21/). Siring frequencies were highly skewed among males, and on average larger males sired higher proportions of offspring. Furthermore, there were indications of size assortative mating. Consequently, even a prolific pelagic spawner like cod does not exhibit random mating, and complicated behaviour and tactics are involved in reproduction.

Assignment of individuals to species or populations is among some of the most interesting new applications of molecular markers. We developed a molecular assay for distinguishing scats from

European otter, American mink and polecat, which could ultimately lead to more reliable species identification and monitoring of the species (/9/). Using assignment tests we were able to reliably assign cod from the north eastern Arctic Sea, the North Sea and the Baltic Sea to the correct population of origin, a result with many future perspectives, both in fisheries management and in ecological studies (/17/). Assignment tests, based on historical and contemporary samples, also led to the discovery of remains of indigenous Atlantic salmon populations in two heavily stocked rivers, where the populations were previously assumed to have been extirpated (/18/).

Genetic interactions between wild and domesticated trout has been a central theme for many of the studies in the thesis. Microsatellite and mtDNA analysis of the hatchery strains used for stocking revealed that even though several strains have been used for stocking, the four quantitatively most important strains are very closely related (/4/; /14/). Hence, stocking activity involving these strains means that a number of wild populations are influenced by large numbers of trout essentially derived from one source. There were also examples of strains where levels of genetic variation were reduced compared to wild populations, but this did not apply to all strains.

Based on microsatellite data from the domesticated strains and stocked populations we evaluated the genetic effects of stocking activity (/12/; /14/; /15/; /20/; 22/). The surprising, but very positive outcome was that there are still many wild trout populations left that have been little introgressed by domesticated trout, despite large numbers of stocked fish. There are, however, also examples of populations that have been strongly introgressed. By analysing microsatellite variation in historical and contemporary samples from two trout populations that had been stocked with approximately the same number of trout from the same domesticated strain, we observed two completely different outcomes (/22/). In one population there was very limited introgression despite intensive stocking activity, and we were able to show that the genetic contribution by domesticated trout was significantly smaller than expected, given the number of stocked fish relative to the size of the wild population. In the other population very strong introgression had occurred, and we pointed to several factors that could have tipped the immigration-selection balance in favour of the domesticated trout. The results underpin previous conclusions that stocking activity may lead to a variety of outcomes, depending on local conditions.

Finally, we assessed the role of life history variation in genetic interactions between wild and domesticated trout (/12/). We found that domesticated trout performed much better as resident than as anadromous trout and that domesticated trout adopting a resident life history may act as a vehicle leading to introgression, despite strong selection acting against domesticated trout that become anadromous.

In conclusion, analysis of molecular markers did indeed lead to new insights into issues related to genetic population structure and management and conservation of populations. Moreover, there are no signs that the progress in molecular biology and statistical genetics will cease. On the contrary, the many new developments commonly referred to as genomics will undoubtedly lead to even more research opportunities. I am sure there is a very exciting future awaiting all of us who are interested in molecular population genetics.

11. References

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Appendix – Papers included in the thesis

Paper 1. Hansen M.M. & Mensberg, K.-L. D. (1996). Founder effects and genetic population structure of brown trout (*Salmo trutta*) in a Danish river system. *Canadian Journal of Fisheries and Aquatic Sciences*, **53**, 2229-2237.

Paper 2. Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1996). Genetic structure of European populations of Atlantic salmon (*Salmo salar* L.) inferred from RFLP analysis of PCR amplified mitochondrial DNA. *Heredity*, **77**, 351-358.

Paper 3. Hansen, M.M., Nielsen, E.E. & Mensberg, K.-L.D (1997). The problem of sampling families rather than populations: Relatedness among individuals in samples of juvenile brown trout (*Salmo trutta* L.). *Molecular Ecology*, **6**, 469-474.

Paper 4. Hansen, M.M., Mensberg, K.-L. D., Rasmussen, G. & Simonsen, V. (1997). Genetic variation within and among Danish brown trout (*Salmo trutta* L.) hatchery strains, assessed by PCR-RFLP analysis of mitochondrial DNA segments. *Aquaculture*, **153**, 15-29.

Paper 5. Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1997). Analysis of microsatellite DNA from old scale samples of Atlantic salmon: A comparison of genetic composition over sixty years. *Molecular Ecology*, **6**, 487-492.

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Paper 7. Nielsen, E.E., Hansen, M.M. & Mensberg, K.-L. D. (1998). Improved primer sequences for the mitochondrial ND1, ND3/4 and ND5/6 segments in salmonid fishes. Application to RFLP analysis of Atlantic salmon. *Journal of Fish Biology*, **53**, 216-220.

Paper 8. Hansen, M.M., Mensberg, K.-L. D. & Berg, S. (1999). Postglacial recolonisation patterns and genetic relationships among whitefish (*Coregonus sp.*) populations in Denmark, inferred from mitochondrial DNA and microsatellite markers. *Molecular Ecology*, **8**, 239-252.

Paper 9. Hansen, M.M. & Jacobsen, L. (1999). Identification of mustelid species: Otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples. *Journal of Zoology*, **247**, 177-181.

Paper 10. Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1999). Genetic variation in time and space: Microsatellite analysis of extinct and extant populations of Atlantic salmon. *Evolution*, **53**, 261-268.

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Paper 12. Hansen, M.M., Ruzzante, D.E., Nielsen, E.E. & Mensberg, K.-L.D. (2000). Microsatellite and mitochondrial DNA polymorphism reveals life-history dependent interbreeding between hatchery trout and wild brown trout (*Salmo trutta* L.). *Molecular Ecology*, **9**, 583-594.

Paper 13. Hansen, M.M., Nielsen, E.E., Ruzzante, D.E., Bouza, C. & Mensberg, K.-L.D. (2000). Genetic monitoring of supportive breeding in brown trout (*Salmo trutta* L.), using microsatellite DNA markers. *Canadian Journal of Fisheries and Aquatic Sciences*, **57**, 2130-2139.

Paper 14. Hansen, M.M., Ruzzante, D.E., Nielsen, E.E. & Mensberg, K.-L.D. (2001). Brown trout (*Salmo trutta*) stocking impact assessment using microsatellite DNA markers. *Ecological Applications*, **11**, 148-160.

Paper 15. Hansen, M.M., Nielsen, E.E., Bekkevold, D. & Mensberg, K.-L.D. (2001). Admixture analysis and stocking impact assessment in brown trout (*Salmo trutta*), estimated with incomplete baseline data. *Canadian Journal of Fisheries and Aquatic Sciences*, **58**, 1853-1860

Paper 16. Hansen, M.M., Kenchington, E. & Nielsen, E.E. (2001). Assigning individual fish to populations using microsatellite DNA markers: Methods and applications. *Fish and Fisheries*, **2**, 93-112.

Paper 17. Nielsen, E.E., Hansen, M.M., Schmidt, C., Meldrup, D. & Grønkjær, P. (2001). Population of origin of Atlantic cod. *Nature*, **413**, 272.

Paper 18. Nielsen, E.E., Hansen, M.M. & Bach, L. (2001). Looking for a needle in a haystack: Discovery of indigenous salmon in heavily stocked populations. *Conservation Genetics*, **2**, 219-232.

Paper 19. Pertoldi, C., Hansen, M.M., Loeschcke, V., Madsen, A.B., Jacobsen, L. & Baagoe, H. (2001). Genetic consequences of population decline in European otter (*Lutra lutra*): An assessment of microsatellite DNA variation in Danish otters from 1883 to 1993. *Proceedings of the Royal Society of London, Series B Biological Sciences*, **268**, 1775-1781.

Paper 20. Ruzzante, D.E., Hansen, M.M. & Meldrup, D. (2001). Distribution of individual inbreeding coefficients, relatedness and influence of stocking on native anadromous brown trout (*Salmo trutta*) population structure. *Molecular Ecology*, **10**, 2107-2128.

Paper 21. Bekkevold, D., Hansen, M.M. & Loeschcke, V. (2002). Male reproductive competition in spawning aggregations of cod (*Gadus morhua*, L.). *Molecular Ecology*, **11**, 91-102.

Paper 22. Hansen, M.M. (2002). Estimating the long-term effects of stocking domesticated trout into wild brown trout (*Salmo trutta*) populations: An approach using microsatellite DNA analysis of historical and contemporary samples. *Molecular Ecology*, **11**, 1003-1015.

Paper 23. Hansen, M.M., Ruzzante, D.E., Nielsen, E.E., Bekkevold, D. & Mensberg, K.-L.D. (2002). Long-term effective population sizes, temporal stability of genetic composition and potential for local adaptation in anadromous brown trout (*Salmo trutta*) populations. *Molecular Ecology*, **11**, 2523–2535.