Population structure and local adaptations in marine fishes

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Population structure and local adaptations in marine fishes

PhD Thesis

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Preface

This thesis presents the main results from my PhD conducted at the Departments of Marine Ecology and Ecology & Genetics, University of Aarhus (AU) and at the Department of Inland Fisheries at the Danish Institute for Fisheries Research (DIFRES) in Silkeborg. The work has been focused on population structuring forces in marine fishes in general and on the genetic basis of local adaptations in particular. It’s been a lot of fun and hard work, and there are many people, who have contributed significantly to a positive outcome of these last years. I may forget some; please forgive me.

First and foremost I thank my family. This may not be the official order of doing things, but they do belong up here. Thanks Sidsel for always being there for me, to support and inspire me and for being the World’s best mom. Jonathan and Lærke, just because you are here!

Anyway, I would also like to thank all the people at the three departments where I have spent quite some time during the last years. Especially my supervisors Katherine Richardson, Volker Loeschcke, Peter Grønkjær and Einar Eg Nielsen who have all been very helpful; needless to say that I would most probably still be drifting around somewhere, had they not guided me with great academic and personal insight. I have especially been bothering Volker, Peter and Einar who have devoted a lot of time and energy to me - Einar in particular during the last hectic months. Thank you all very much!

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Jakob Hemmer-Hansen
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It is now well established that the World is facing its 6th mass extinction, believed to be driven mainly by human disturbance (Pimm et al. 1995; Thomas et al. 2004; Pimm et al. 2006). While terrestrial systems have been subject to attention from conservation biology and conservation genetics for many decades, the marine environment has only recently received increased attention in conservation contexts (Avise 1998), despite the fact that it is covering more than 70% of the World’s surface. It is now clear that marine ecosystems are as vulnerable to human disturbance as are terrestrial (e.g. Malakoff 1997; Solan et al. 2004) and that several marine species are now already extinct or at the brink of extinction (Powles et al. 2000, Dulvy et al. 2003, Reynolds et al. 2005). The increased awareness of the need for marine conservation has also led to the acknowledgement of a need to conserve intra-specific biodiversity, especially in relation to harvestable resources (Ryman et al. 1995; Nielsen and Kenchington 2001; Smedbol and Stephenson 2001; Ruzzante et al. 2006). Thus, there is an urgent need for knowledge of how selective forces operate in the sea to structure marine species into discrete population units. Because local populations may harbor a unique portion of the species’ genetic material allowing it to adapt to specific environmental components in present as well as in future times (e.g. Hilborn et al. 2003), the study of the genetic basis of local adaptations has been highlighted as worth special attention (Moritz 2002; van Tienderen et al. 2002). Studies of the genetic structuring of populations of marine fishes have benefited tremendously from the advent of highly variable neutral genetic markers in recent years (e.g. Carvalho and Hauser 1998), which has facilitated an exploration of structuring forces in this high gene flow environment (Waples 1998). However, while our knowledge of the interplay of demographical processes (gene flow, drift and historical processes) is improving rapidly these years, we still know very little about how selection may act to structure marine fish population, because the commonly applied markers are believed to be selectively neutral and hence not applicable for studies of adaptive population divergence.

Although technological developments have advanced our ability to study evolutionary processes at still finer scales, it should be remembered that processes at the species and population levels are highly interconnected. For instance, species specific physiological constraints may limit the ability of populations to adapt to environmental changes such as increasing temperatures (e.g. Somero 2005). Similarly, large scale climatic cycles will affect the levels of genetic diversity and sub-structuring within species observed today (Grant and Bowen 1998; Hewitt 2000). Furthermore, the degree of species integrity is likely to affect such processes, since continuous gene flow between species could result in genetic homogenization within species and might eventually lead to loss of local adaptations (e.g. Taylor et al. 2006). On the other hand, it is becoming increasingly clear that species are not homogenous genetic units and that it is only through an understanding of processes at the population level that we are able to predict the ability of species to adapt to future environmental changes.

Until recently it was relatively complicated, expensive and time consuming to demonstrate the genetic footprints of selection in non-model organisms such as marine fishes. While marine fishes still present researchers with many challenges in these types of studies,
recent increases in information about the organization and structure of marine fish genomes as well as technological developments in molecular biology have all provided new opportunities. Hence, it no longer appears an impossible task to examine adaptive population divergence in the marine environment.

**This thesis**

The overall theme of this PhD thesis is population structure and local adaptations in marine fishes. The purpose has been to elucidate the interplay of evolutionary forces in the sea at different hierarchical levels ranging from inter-specific hybridization to macro- and micro-geographical neutral structuring as well as adaptive population divergence within species. The aim has been to elucidate the scale and magnitude of population structure and local adaptations in marine fishes. It is only through an understanding of these interacting evolutionary processes on different hierarchical levels that we may gain a better understanding of how selection operates in this high gene flow environment.

We have used the European flounder (*Platichthys flesus* L.) as a model species for these studies, since several aspects of the species’ biology make it a suitable model to study the action of evolutionary forces in the marine environment. First of all, it shares many characteristics with other marine species, such as pelagic egg and larval stages and, presumably, large effective population sizes believed to result in limited structuring of marine fish populations. On the other hand, it is relatively stationary and coastal, which could lead to the prediction that structuring should be more pronounced in this species compared to other more oceanic species. Hence, this species may actually represent a case where true signals are clearly larger than random noise (cf. Waples 1998). Secondly, its wide distribution exposes it to diverse environmental conditions. As a coastal species spending much of its life in shallow waters, it is expected to be affected by spatial and temporal differences in environmental parameters making it a suitable model for studies of adaptive population divergence. Thirdly, as a euryhaline species, it represents an important contrast to other marine fishes studied so far. This facilitates comparative and contrasting analytical set-ups and should prove useful for validating earlier results from other species. And finally, local adaptations have actually been suggested in populations in the Baltic Sea, which are believed to have adapted their spawning strategy to life at low salinities (Aro 1989; Nissling et al. 2002), but the genetic signatures of these presumed adaptations have never been investigated.

The purpose of this general introduction is to review current knowledge of population structure and local adaptations in marine fishes to set the stage for a discussion of recent developments which could improve our understanding of the genetic basis of local adaptations in marine fishes. By evaluating available techniques in a marine fish perspective, I will try to highlight the approaches most likely to be useful for disclosing the genetic basis as well as the scale and magnitude of local adaptations in marine fishes in the coming years. This field has got the potential to contribute significantly to our understanding of evolution in general and of the outcome of the interplay of evolutionary forces in the sea in particular. Throughout this introduction I will integrate the results from my own PhD and highlight the areas where I believe that the results have contributed significantly to increase our understanding of these processes.
**Population structure in marine fishes**

The genetic structuring of populations of marine fishes has only received intense attention within the last 10-20 years. Historically, studies of the genetic structure of marine fish populations have followed technological developments, but have tended to lag somewhat behind studies in terrestrial environments (Avise 1998).

**Molecular markers**

Studies on haemoglobin polymorphism in Atlantic cod in the 1960s (Sick 1965a, 1965b) were among the first applications of biochemical markers in fishes. The advent of enzyme electrophoresis (Hubby and Lewontin 1966; Lewontin and Hubby 1966) brought with it an explosion of studies characterising genetic variation in natural populations of fishes, demonstrating genetic population structure for many species (e.g. Christiansen et al. 1976, Winans 1980, Grant and Utter 1980, Kornfield et al. 1982). Although allozymes are often complimented with DNA based markers today (e.g. González-Wangüemert et al. 2004; Maes et al. 2006; Pampoulie et al. 2004) they are occasionally applied separately to infer population structure in marine fishes (e.g. Stefani et al. 2003; Cimmaruta et al. 2005). However, several studies have found allozymes to be under selection (Hilbish and Koehn 1985; DiMichele et al. 1991; Schmidt and Rand 1999) and they should hence be used as neutral markers with caution. Modern DNA based techniques, particularly highly variable markers such as microsatellites, have revolutionized studies of marine fish population structure. These markers have been very useful for disclosing the often low levels of structure found in marine fishes (Waples 1998; DeWoody and Avise 2000) because of their associated high statistical power (Ryman et al. 2006; Waples and Gaggiotti 2006). Although still in its infancy, single nucleotide polymorphisms (SNPs) have been highlighted as a potential marker of choice in future studies of neutral population divergence (Morin et al. 2004). However, since these markers are often bi-allelic, more loci will be required in order to achieve the same power as in microsatellite studies (Kalinowski 2002), and the choice of marker should always be based on both theoretical and technical considerations.

**Statistical developments**

The development of highly variable genetic markers has brought with it a suite of useful statistical techniques for handling the wealth of information from these markers (e.g. Luikart and England 1999; Excoffier and Heckel 2006). This has allowed population genetic studies to move beyond the purely descriptive stage and has also resulted in interesting applications in marine fishes.

Assignment and individual admixture analyses can provide important information on interactions between populations by disclosing migration and hybridization (e.g. Hansen et al. 2001; Manel et al. 2005). Bayesian methods have been developed to assess the most likely number of populations represented by the data by minimizing levels of Hardy Weinberg and linkage disequilibrium within groups (e.g. Pritchard et al. 2000; Corander et al. 2003). These methods have also been applied in marine fishes (e.g. Nielsen et al. 2003; Nielsen et al. 2004; Manuscript IV), but it should be kept in mind that the power of these approaches for correctly identifying both true genetic structure and hybrid individuals decreases dramatically at the low levels of structuring (Latch et al. 2006; Vähä and Primmer 2006) often observed in marine fishes. Hence, in marine fishes individual admixture analyses have mostly been applied at the species level to identify species and hybrid individuals (e.g.
eels, Albert et al. 2006 and Maes et al. 2006; flatfish, Manuscript III).

Even though hybrid individuals can be difficult to identify with statistical certainty within species, these methods can provide important information about the genetic interaction among marine fish populations. For instance, they have been used to examine the distribution of individual admixture proportions in cod (Nielsen et al. 2003; Nielsen et al. 2005), turbot (Nielsen et al. 2004) and flounder (Manuscript IV) populations with the aim of examining evolutionary and ecological interactions among genetically differentiated populations. Hence, although the specific hybrid class of individuals could not be identified with high certainty, the distribution of individual admixture proportions gave important insights into the interactions of differentiated populations of these species.

Recently, the increased statistical power provided by genetic markers has been combined with other information in integrative approaches which hold great promise for providing increased understanding of population structure and ecological processes in nature. For instance, demographic and genetic information has been combined in a method aiming at identifying colonization events and processes (Gaggiotti et al. 2004), and the emerging field of landscape genetics combines geographical and genetic information to identify the largest genetic breaks among interconnected populations (Manel et al. 2003), which has also found its applications in marine fishes (e.g. Bekkevold et al. 2005; Jørgensen et al. 2005; Manuscript I). Future developments of these approaches could include information of oceanographic currents as both potential barriers to gene flow and as dispersal mechanisms (Galindo et al. 2006) and could be very useful in marine fishes exposed to such physical forces. A recently published method aims at combining geographic information with the power of Bayesian clustering algorithms to identify populations and migrants/hybrids in a geographical context (Guillot et al. 2005; Coulon et al. 2006). This could prove valuable for disclosing cryptic population structure in marine fishes, but, although this particular method has not been evaluated at low levels of structuring, it most likely suffers from the same problems as other clustering methods in these scenarios. Environmental parameters have been incorporated with genetic information in partial Mantel tests to examine if patterns of genetic structuring are best explained by geographic or environmental differences between populations (Bekkevold et al. 2005; Jørgensen et al. 2005; Manuscript I). This approach thus points to potential environmental factors, such as salinity and temperature, which could be important for shaping genetic structure in marine fishes, but do not show if populations are locally adapted since the markers applied are not subject to selection. Future developments of integrative approaches should be useful for studies of population structure in marine fishes, thereby moving this research area further by gaining important new knowledge of the interplay of evolutionary forces in the marine environment.

**Population structure**

The emerging picture from the studies conducted so far has been one of limited genetic structuring of populations. This is thought to be caused by the particular characteristics of the marine environment and its inhabitants such as few physical barriers to gene flow, large effective population sizes and high dispersal capabilities, both as adults and as pelagic eggs and larvae. Such features are generally seen as important for promoting high levels of gene flow (Ward et al. 1994; Waples 1998), which in turn decreases the signal:noise ratio (Waples 1998) and complicates studies of population subdivision in marine fishes (see Box 1).
However, despite the general picture of high levels of gene flow, recent studies have identified significant genetic structuring of marine fish populations. Several evolutionary mechanisms have been proposed to be important for generating the small but significant levels of genetic structuring in an environment without any obvious physical barriers to gene flow.

First of all, genetic structuring may be affected by processes not related to contemporary physical or environmental factors directly. In some cases, patterns of structuring have been found to be influenced by levels of inter-specific hybridization (e.g. Roques et al. 2001; Roques et al. 2002; Albert et al. 2006). We used individual admixture analyses of real and simulated data to identify hybrids of first and later generations between plaice and European flounder (Manuscript III). We found that introgression from plaice to flounder did occur beyond the first generation in flounder populations in nature, but at a relatively modest rate, implying efficient

Box 1. Statistical and biological significance in studies of marine fish population structure

The high basal levels of gene flow and the high statistical power of highly variable markers have resulted in concerns about the coupling between biological and statistical significance in high gene flow scenarios, such as in marine fishes (Waples 1998, Hedrick 1999). First of all, the high levels of gene flow often result in minute signals which could be difficult to detect with genetic markers, resulting in Type2 errors (not rejecting H0=”no structure” when it is in fact false). This problem is particularly important in relation to less variable markers, such as allozymes, but could also present a problem in microsatellite studies, e.g. if too few loci are used. For instance, we have found that populations of European flounder in the Baltic Sea are highly structured between different life history forms (Manuscript I). However, detailed analyses of samples from a particular locality revealed important biological signals most likely reflecting seasonally migrating individuals. These signals did not, however, translate into significant tests for population subdivision and could have gone undetected in a genetic study if less information about the biology of the species had been available (Manuscript IV).

Type1 errors (rejecting H0=”no structure” when it is actually true) is a more common problem with microsatellite markers; i.e. that tests are statistically significant but not reflecting true biological signals (Waples 1998, Hedrick 1999). Because the true signal is often very small, noise could bias conclusions seriously (Waples 1998). Noise in microsatellite studies is most often generated or inflated by non-random sampling (Waples 1998) or genotyping errors (e.g. Pompanon et al. 2005). However, unrecognized biological sources of noise could also bias results even if studies are perfectly designed. For instance, inter specific hybridization could result in biased results, particularly if introgression is unevenly distributed throughout the sampled range. Although hybridization between marine fish species is relatively sparsely studied, evidence is emerging that it could be relatively common between some species (Gardner 1997). While there was little evidence that introgression from plaice to flounder could have biased the results from our study of population structure in European flounder (Manuscript III), other studies have found high levels of introgression in some parts of a species’ distributional area (Roques et al. 2001; Buonaccorsi et al. 2005; Albert et al. 2006). If not recognized, such introgression could seriously bias surveys of population structure.

Thus, from the above it should be clear that an arbitrary significance cut-off (such as an alpha-level of 0.05) makes little sense without a thorough evaluation of the signals and patterns of structuring in the data set. Furthermore, results will often be easier to interpret if sampling is temporally replicated (Waples 1998). Our own studies on European flounder serve to illustrate these points.

However, despite the general picture of high levels of gene flow, recent studies have identified significant genetic structuring of marine fish populations. Several evolutionary mechanisms have been proposed to be important for generating the small but significant levels of genetic structuring in an environment without any obvious physical barriers to gene flow.
selection against hybridization between the two species in nature, so the effects of introgressive hybridization obviously depends on the specific setting.

The importance of including a species’ history when studying patterns of genetic variation in contemporary samples has repeatedly been stressed (e.g. Grant and Bowen 1998; Hickerson and Cunningham 2005) because any pattern observed today is the result of historical as well as contemporary processes. It has been suggested that many classical marine fishes (i.e. species with large effective population sizes, high migration potential and pelagic eggs and larvae, see Nielsen and Kenchington 2001) have shallow population histories in the Northern Atlantic because of recent colonization after the last glaciation (Grant and Bowen 1998; Pogson et al. 2001). In most species examined in detail, shallow histories have indeed been confirmed (e.g. Árnason 2004; Hoarau et al. 2004) implying young population ages and potential non-equilibrium between migration and drift. The effect of these factors could be to depress levels of structuring, thereby leading to an underestimate of the true level of population structure (e.g. Pogson et al. 2001). However, colonization processes also leave behind signatures which can be detected in contemporary populations, such as decreased levels of variability in populations in the extreme parts of the distributional area associated with founder events when new habitat was colonized (Hewitt 2000; Widmer and Lexer 2001). Such patterns have also been observed in marine fishes (Wilson 2006; Manuscript I).

Differences in life-history strategies have long been known to result in significant genetic structuring of salmonid populations (e.g. Taylor 1991), but few examples of populations with different life histories are found in marine fishes. One such example is Atlantic herring, which has been shown to be structured among population components with different spawning strategies (Bekkevold et al. 2005). The European flounder is another example with populations in the Baltic Sea exhibiting different life history characteristics. We found a sharp genetic break between these life histories, indicating that this is an effective barrier to gene flow in marine fishes (Manuscript I). We also found indications of mixing of different populations at some times of the year (Manuscript IV), suggesting significant ecological interactions among these differentiated populations.

A number of studies have also found contemporary physical drivers to be important for shaping the distribution of genetic variation between populations. Thus, geographic distance per se has been suggested to be important for creating patterns of isolation by distance in some instances (Pogson et al. 2001) and current systems have been found to be important for shaping both temporally stable (e.g. Ruzzante et al. 1999) and variable (e.g. Knutsen et al. 2004) genetic structure. Finally, environmental transitions have often been found to be associated with significant genetic structuring (Riginos and Nachman 2001; Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Johanneson and André 2006) implicating barriers to gene flow associated with adaptations to local environments. Thus, there appears to be numerous factors capable of structuring marine fish populations, but it has rarely been possible to evaluate the relative importance of these factors because often only a single factor has been investigated, or sampling schemes have not been extensive enough to allow the investigation of several factors. In a few cases, however, several mechanisms have been assessed simultaneously. These studies have found that several mechanisms are indeed interacting to structure populations within the same species (Riginos and Nachmann 2001; Roques et al. 2002). In the study of population structuring n
European flounder (Manuscript I), we also found evidence of the simultaneous operation of several factors, such as population history, life-history and current systems, demonstrating that the outcome of this interplay depends strongly on the specific setting. The effect of environmental differences has been evaluated specifically in relation to geographical distance in some cases (Bekkevold et al. 2005; Jørgensen et al. 2005; Manuscript I). These studies have found that environmental factors rather than geographical distance per se seem to be significantly associated with genetic structure. While, these results suggest that environmental parameters are responsible for generating genetic structure they do not prove that the associations are caused by restrictions in gene flow between locally adapted populations because the markers applied are believed to be selectively neutral.

**Local adaptations in marine fishes**

It is evident from the section above that we are now fairly sure that many species of marine fish are structured into discrete units. Often the genetic difference between these units are not large, but they are consistent (e.g. when comparing different species in the same area) and, importantly, appear to be temporally stable in many cases (e.g. Poulsen et al. 2006; Ruzzante et al. 2006). Conversely, we know relatively little about what this structure means in terms of adaptive population divergence, because very few studies have addressed this question specifically. The main reason for this lack of knowledge is probably the general time-lag between studies on marine and terrestrial conservation genetics (Avise 1998), but the general picture of high levels of gene flow in marine fishes has most likely also resulted in limited interest in this field, since gene flow is expected to hamper local adaptations. However, it is important to recognize that local adaptations arise and are maintained as a result of interplaying evolutionary forces. The large effective population sizes of many marine fishes tend to increase the importance of selection over the random process of genetic drift. The level of adaptive divergence is furthermore affected by environmental differences (i.e. the selection differential) between populations, so basically any outcome is possible, even in situations of high levels of gene flow. Since marine species often have very wide distributions it is likely that fish in different parts of the range may experience very different selection regimes (Palumbi 1994), thereby increasing the potential importance of natural selection.

**Evidence for local adaptations**

In fishes, local adaptations have mainly been investigated in freshwater and anadromous species where research has been devoted at identifying local populations, mainly for conservation purposes. In salmonids, for example, the marked genetic structuring found between different river or tributary populations is thought to reflect that many of these populations are also locally adapted (Taylor 1991; Adkison 1995; Church and Taylor 2002). Even though it is now widely accepted that genetically distinct salmonid populations are also locally adapted at the genetic level, it has only been thoroughly evaluated and demonstrated in a few cases that the genetic structure at neutral markers also translates into differences in the genes subject to selection and thereby directly involved in local adaptations (e.g. Koskinen et al. 2002; McGinnity et al. 2003; McGinnity et al. 2004). As is the case in marine fishes, this is mainly because such a demonstration is not straightforward. But unlike the case in many marine species, salmonids have traditionally been kept in aquaculture and have thus been easier to manipulate in large scale ecological and genetic studies.
Furthermore, the focus of the aquaculture industry on this group of fishes has led to an increased effort to map and understand the genetic architecture behind adaptive traits.

Only few studies have examined genetically based differences in adaptive traits between populations of marine fishes. One such example is found in the common killifish, *Fundulus heteroclitus*. Here, the molecular basis for changes in both enzyme transcription and activity has been identified (e.g. Schulte et al. 2000; Schulte 2001). For example, a single base mutation in the promoter region of the *Ldh-B* gene results in adaptive differences between Northern and Southern populations in the regulation in this gene. *F. heteroclitus* probably represents the most well studied marine species with respect to local adaptations. As a model species in comparative physiology and evolutionary genetics, most *Fundulus* research has compared Northern and Southern subpopulations (sometimes referred to as subspecies). These are believed to have diverged between 0.5 and 1 Million years ago (Bernardi et al. 1993). Combined with stationary behaviour, this translates into very high levels of population structuring at neutral markers (Pairwise $F_{ST} \approx 24\%$; Whitehead and Crawford 2006). Hence, while the research on *Fundulus* has provided exceptional insights into the action of natural selection in the marine environment, results may not necessarily be easily transferred to classical marine fishes, which often have younger population ages and lower levels of population structure/higher levels of gene flow, as evidenced in the sections above.

Studies of counter-gradient variation in life history traits have found that population differences are maintained by selection in several species (e.g. Conover 1998; Marcil et al. 2006). These results suggest that local adaptations may in fact be common in the marine environment despite phenotypic similarity between individuals from different populations (Conover 1998). However, while demonstrating local adaptations, these studies have not revealed anything about its genetic architecture.

In order to elucidate the genetic footprints of local adaptations, we applied a candidate gene approach to study local adaptations in European flounder (Manuscript II). We found strong indications of local adaptations to the Baltic Sea environment by comparing genetic differentiation at neutral microsatellite loci with that at a candidate gene locus for adaptive divergence. However, besides these few examples, there is a marked shortage on studies investigating the genetic basis of adaptive population divergence in marine fishes.

Studies on *Fundulus heteroclitus* and countergradient variation in general have been concentrated on latitudinal environmental gradients, which illustrates that temperature has been the most extensively studied environmental component in relation to local adaptations in marine fishes. However, many marine species inhabit other environmental gradients as well. Estuaries and lagoons represent interesting study systems with respect to temperature, salinity and also often pollutants. Salinity has been found to be an important selective force in many studies of allozyme polymorphisms in mussels (Koehn et al. 1980; Hilbish and Koehn 1985), and hence these systems could be good places to start searching for local adaptations in marine fishes. Other gradients, such as photoperiod, depth, degree of disturbance etc all represent interesting environmental parameters worth future investigations, but have so far not been investigated.

**Methods to detect local adaptations**

The complicated interplay between genetic and environmental components of phenotypic variation makes studies of the genetic basis of
local adaptations in non-model organisms very challenging (e.g. Falconer and Mackay 1996). Various approaches have been and can be applied, ranging from demonstrating the presence of local adaptations at the phenotypic level to indirect quantification of its genetic component and investigations of the causing polymorphisms at the level of DNA itself.

Neutral genetic markers
It has been debated if neutral markers can be used to predict the level of population divergence at loci under adaptive evolution (e.g. Merilä and Crnokrak 2001; Reed and Frankham 2001; McKay and Latta 2002). However, even if such a correlation should exist, it is most likely not perfect because different selective forces are affecting the two types of loci. Therefore, most researchers who are interested in local adaptation of fishes and employ neutral genetic markers are restricted to make inferences about the potential for local adaptations. Valuable insights into the conditions permitting local adaptations have been obtained by incorporating genetic and ecological or environmental information in models to predict the scenarios in which local adaptations are most likely to occur. For example, if populations are small, genetic drift will be more important than local selection and hence local adaptations are not predicted at the population level. However, if several populations are connected through some gene flow and furthermore experience the same selective regimes, then adaptations to the common environmental conditions are much more likely (e.g. Hansen et al. 2002). However, this particular approach could be difficult to apply in many marine fishes, which are often exposed to clinal rather than discrete environmental variation, complicating a separation into distinct environmental groupings.

Important information have been gained with the ability to retrieve DNA from archived samples, since it is now possible to assess the temporal stability of population structure without the need for a long-term sampling effort (e.g. Hutchinson et al. 2003, Poulsen et al. 2006). Even though the period assayed may be quite short on evolutionary time scales, this approach does offer some unique possibilities, since local adaptations are clearly more likely to arise if population structure is temporally stable. Furthermore, and as noted earlier, some studies have demonstrated a significant correlation between genetic structure and environmental parameters, indicating that the specific variables may be involved in driving adaptive population divergence (Bekkevold et al. 2005; Jørgensen et al. 2005; Manuscript I). Clearly temporally stable population structure associated with drastic environmental transition indicates local adaptation, but does not prove it, since the markers applied are not directly affected by natural selection. It is therefore expected that research on local adaptations in marine fishes will apply other methods targeted at answering these questions more specifically in the future.

Transplantations and common gardens
A quite simple, but often logistically complicated, method to assess the degree of adaptation to different habitats is to conduct (reciprocal) transplants and compare fitness of introduced and native populations in the respective environments (Endler 1977). This is most easily done in model or laboratory held species even though some large scale experiments have been conducted on salmon (Altukhov and Salmenkova 1990). In an extensive study, Hendry et al. (2002) also used transplantations to assess the importance of selection and gene flow for creating genetic differences between lake and stream ecomorphs of sticklebacks. In marine fishes, this approach has rarely been applied, and although transplants have been conducted in some
species (e.g. Schmidt 1917) its applicability seems fairly limited in most species.

An alternative to transplants is to bring individuals from different populations under common environmental conditions. This strategy has been applied in Atlantic silversides and other species to study countergradient variation in life history traits. One of the best known examples is growth rate in Atlantic silverside, where populations show adaptive and clinal variation in growth rates (reviewed in Conover 1998). The findings from silversides have been confirmed in other traits and species (e.g. Conover et al. 1997; DiMichele and Westerman 1997; Salvanes et al. 2004; Marcil et al. 2006), indicating that this may in fact be a general phenomenon.

**Outbreeding depression**

An assessment of the severity of outbreeding depression is another approach, which does not necessarily employ genetic markers. Hybrids from crosses between presumed adaptively diverged populations are expected to show reduced fitness when compared to the original populations. This reduction is mainly caused by the break-up of coevolved gene complexes, and hence may first become assessable in F2 hybrids (Gharrett and Smoker 1991). Many studies have concentrated on salmonids, where some have assessed outbreeding depression in crosses between different natural populations (e.g. Gharrett et al. 1999; Gilk et al. 2004), while other studies concern the presumed detrimental effects that non-native fish may have on wild conspecific populations. A recent study showed that both hybrids from farm x wild crosses, backcrosses to both kinds of parents and the F2s had a marked reduction in lifetime fitness in nature, underlining the potential devastating effects that farmed fish could have on wild fish populations (McGinnity et al. 2003).

The long generation times of many marine fishes makes the methods based on common gardens reviewed here logistically difficult to employ in this group of species. Furthermore, while these methods demonstrate that populations may be locally adapted, they reveal little about the genetic architecture underlying these adaptations. Other approaches must be taken to achieve this goal.

**QST vs. FST**

The magnitude of adaptive divergence can be assessed by comparing the differentiation of presumed neutral loci (FST) with the differentiation at the genetic component of the quantitative genetic variance (QST). This approach was first proposed by Spitze (1993), who used it to demonstrate adaptive divergence of *Daphnia* populations, but has since been applied in a large range of species.

The approach requires the separation of the various components of the quantitative phenotypic variance (Falconer and Mackay 1996). Only the additive genetic variance (VA) responds to selection, so a QST vs. FST comparison will need a fairly good estimate of VA, which is often assayed in common garden experiments. Once an estimate of VA is obtained, the comparison with the variance of the neutral markers is straightforward, the idea being that a QST larger than the FST would implicate adaptive differentiation, while QST=FST signals differentiation due to neutral evolutionary forces (genetic drift) only. A QST smaller than FST implicates the operation of stabilizing selection on the traits studied (Spitze 1993).

This approach is obviously most useful in species which are easily kept in common gardens. Hence, it has also primarily been applied in plants or, for instance, *Drosophila* or *Daphnia* among animals (reviewed in Merilä and Crnokrak 2001; McKay and Latta 2002). Few studies have applied the approach in fishes. Koskinen et al. (2002) showed that QST for many life history traits in small grayling populations only isolated a few (10-20)
generations ago greatly exceeded the \(F_{ST}\) estimates from microsatellites, implying that natural selection had been responsible for the rapid divergence observed between these populations. Likewise, Rogers et al. (2002) found that \(Q_{ST}\) for behaviour related traits was significantly larger than \(F_{ST}\) between whitefish ecomorphs.

There are, however, several potential problems with this approach. One is that \(Q_{ST}\) may in fact not equal \(F_{ST}\) under neutrality, for instance in situations with high mutation rates and low migration rates. Furthermore, different conclusions could be reached depending on the types of neutral markers applied, because different markers have very different mutation rates and mechanisms (Hendry 2002). Another problem is that \(Q_{ST}\) estimates have been found to be quite imprecise under most study designs realistically carried out in marine fishes (O’Hara and Merilä 2005). Finally, \(V_A\) estimates are environment and population specific (Falconer and MacKay 1996) making generalisations from such studies difficult.

To our knowledge, this approach has never been applied in marine fishes, and although it could relatively easily be done in aquaculture species, the approach seems to have lost some of its popularity in recent years, probably because of some of the problematic issues mentioned above. Furthermore, logistical constraints will seriously limit the number of marine fish species, where this approach could be applied.

**Gene expression**

A change in gene expression is regarded as an important component of an organism’s ability to acclimate and adapt to novel or changed environmental conditions (Ferea et al. 1999; Schulte 2004; Whitehead and Crawford 2006b).

Hence, the analyses of expression of specific target genes have been studied in individuals from different populations in common environments to investigate adaptive differences. A very illustrative example among marine fishes is the common killifish (Fundulus heteroclitus), which has been found to show consistent differences between Northern and Southern populations in gene expression for a number of genes of adaptive importance (e.g. Schulte et al. 2000, Fangue et al. 2006). Furthermore, a large number of genes have been investigated in other marine fishes, primarily those of interest to aquaculture (e.g. Deane and Woo 2005; Olsvik et al. 2006; Hall et al. 2003).

Techniques to study global gene expression are now also available in species of little relevance for drug discovery or as models for development (Gibson 2002). Studies in non-model species offer unique possibilities for dissecting the evolution of gene transcription between and within species (Ranz and Machado 2006), thereby making it possible to explore the genetic basis of adaptive divergence in gene expression (e.g. Whitehead and Crawford 2006b).

Microarrays have been developed for a few fishes (e.g. Atlantic salmon, Rise et al. 2004; killifish, Oleksiak et al. 2002; carp, Gracey et al. 2004; zebrafish, Ton et al. 2002; catfish, Li and Waldbieser 2006; medaka, Kimura et al. 2004), but unfortunately only for very few marine species (European flounder, Williams et al. 2003; Japanese flounder, Kurobe et al. 2005).

Until now, microarrays have only been used on natural populations in Fundulus heteroclitus among marine fishes. These studies have contributed significantly to an understanding of how genetic variation in gene expression is distributed within and between natural populations (Whitehead and Crawford 2006b). Oleksiak and colleagues (2002) thus found a correlation between variation in gene expression within and between populations for most genes, when comparing Northern and Southern populations of Fundulus heteroclitus; a pattern also seen in primates and believed to
reflect neutral evolution of gene expression (Khaitovich et al. 2006). However, Oleksiak et al. (2002) also identified interesting outlier loci, which were not influenced by genetic drift alone. Some of these genes furthermore showed similar expression patterns in Southern populations of a sister species of F. heteroclitus, while being different from Northern F. heteroclitus. These expression patterns further support the conclusion they are under adaptive evolution. Similarly, Whitehead and Crawford (2006a) have recently identified differentially expressed genes, which could not be explained by drift alone, along a latitudinal temperature gradient in F. heteroclitus, supporting the conclusions from earlier studies of local adaptations via gene expression in the species.

However, analysis of global gene expression is not without its problems either. First of all, studies rely on the assumption that gene expression is heritable, but this is rarely tested in the specific setting (Gibson and Weir 2005), because studies in non-model organisms are almost always carried out in one generation only. Hence, it is difficult to rule out non-additive genetic effects such as maternal effects, epistatic interactions and gene by environment interactions (Gibson and Weir 2005).

Furthermore, it has been argued that there may be little coherence between mRNA expression levels and actual activity of the protein in the cells, which is the resulting phenotype (Feder and Walser 2005). A correlation between mRNA and protein activity is often an assumption not tested in microarray studies, but if it proves be the case that the two levels are not correlated, gene expression data should be interpreted with great caution. Finally, it should be noted that measures of gene expression produces a phenotype of an individual. Hence, to be useful for demonstrating local adaptations in marine fishes, a controlled environment is required, much like any of the other phenotype based approaches discussed above.

It is doubtful, if microarrays will be available for many marine species in the near future. This shortage on resources could limit the method’s applicability as a general tool for studying local adaptations in marine fishes. However, cross species hybridization to cDNA arrays has been reported to be successful (Renn et al. 2004), so this technical obstacle may be overcome in species where arrays are available in close relatives. Alternatively, new methods to study quantitative gene expression at a global level have recently been developed, and these could prove to be very valuable alternatives to microarrays in non model organisms. Although differential display (Liang and Pardee 1992) and cDNA-AFLP (Bachem et al. 1996) have been available for some years, they have primarily been used to identify specific candidate genes which are differentially expressed. However, it was recently suggested that these techniques could also be used to study quantitative gene expression (Breyne et al. 2003; Venkatesh et al. 2005), and they have in fact been shown to produce data of equal quality to those obtained from microarrays (Breyne et al. 2003). Since differential display and cDNA-AFLP do not require specialized equipment and could be applied to all species relatively easily, these latest developments appear very promising for marine fish. Results from Fundulus are promising for future applications of this approach in marine fishes, provided that technical resources, such as non-array based techniques to study gene expression, become available for studies in non-model organisms.

Genome scans
Recently, much attention has been centred on ways to identify markers potentially under selection in genome scans (reviews in Luikart et al. 2003; Storz 2005) applying microsatellites (Kauer et al. 2003, Vasemägi et
al. 2005), AFLPs (Campbell and Bernatchez 2004) or single nucleotide polymorphisms (SNPs) (Akey et al. 2002). The principle behind these approaches is genetic hitch-hiking, where a marker linked to a mutation under selection shows non-neutral patterns of variation (Maynard Smith and Haigh 1974). The classical Lewontin-Krakauer test is based on the assumption that loci under positive selection will show increased levels of population divergence (Lewontin and Krakauer 1973). Thus, based on the observed distribution of $F_{ST}$ values across the panel of loci studied, outliers can be detected based on comparisons to expectations under neutrality. This particular test has now been almost totally abandoned because of heavy criticism over its potential sensitivity to population structure (reviewed in Guinand et al. 2004; Vasemägi and Primmer 2005). However, new developments (e.g. Beaumont and Nichols 1996; Vitalis et al. 2001) have revived this analytical framework by introducing simulations to assess departures from neutral expectations taking population structure into account. An alternative strategy also based on principles of genetic hitch-hiking is to search for loci showing reduced variability in certain populations. Thus, the LnRH and LnRV tests (Schlötterer 2002; Kauer et al. 2003) were developed specifically for microsatellites to identify loci showing abnormal variance in heterozygosity or repeat lengths when conducting pairwise population comparisons.

The usefulness of genome scans has recently been questioned, particularly because of potential problems with ascertainment bias, population sub-structure and large numbers of false positives (Kelley et al. 2006; Teshima et al. 2006) and hence they should be applied with caution, and results should be verified by alternative analyses. For example, similar signatures from independent population comparisons strongly suggest a true signal rather than any of the alternative explanations (e.g Storz and Dubach 2004; Vasemägi et al. 2005; Bonin et al. 2006).

To our knowledge, genome scans have never been conducted on marine fishes, probably because fairly dense genome coverage is required for these approaches to be useful. Even though fish genomes are unlikely to reach the marker resolution currently applied in human genome scans (e.g. ~ 1.5 Million SNPs, Kelley et al. 2006), these approaches could be useful for studying local adaptations in marine fish populations if other types of markers are applied. Thus, Campbell and Bernatchez (2004) applied AFLPs in genome scans in whitefishes and identified several loci potentially undergoing adaptive evolution. Alternatively, microsatellite markers linked to EST sequences were applied in a genome scan of salmon populations to show that some of the loci had apparently diverged adaptively (Vasemägi et al. 2005). These resources should provide short cuts to cover a large part of marine fish genomes at relatively modest time and economic costs.

**QTL mapping**

If marker locations within the genome are known, the genome scan approach can be further refined to identify specific genomic regions involved in adaptive divergence. QTL mapping identifies genomic regions that are influencing a given quantitative trait and is hence a first step in understanding the genetic architecture of complicated phenotypic traits. Classic QTL mapping strategies requires an elaborate breeding programme in combination with adequate genetic markers such as microsatellites, AFLPs or SNPs (Mackay 2001). The successful identification of QTL allows inferences about the genetic architecture of local adaptations. QTL mapping has been combined with mRNA expression data, so-called “genetical genomics” (Jansen and Nap 2001), to construct eQTL (expression QTL) maps. These have recently been highlighted as
a useful tool for studying adaptive divergence (Vasemägi and Primmer 2005) by revealing the genetic architecture of transcriptional regulation. However, eQTL mapping has until now only been applied in model organisms (e.g. Yang et al. 2006).

In fishes, linkage maps have been constructed for well studied model species, such as sticklebacks (Peichel et al. 2001), cichlids (e.g. Albertson et al. 2003; Streelmann et al. 2003) and whitefish (Rogers et al. 2001). In non-model fishes, single QTL have mostly been identified in species of relevance to aquaculture, and the traits involved have primarily been closely related to production, e.g. heat or cold resistance (Perry et al. 2001; Cnaani et al. 2003), spawning time (Sakamoto et al. 1999; O’Malley et al. 2003), growth (Cnaani et al. 2003; Reid et al. 2005) or disease resistance (Fuji et al. 2006). Once identified these QTL may be very useful for studies of the action of selection in natural populations of these or closely related species (Reid et al. 2005; Somorjai et al. 2003).

However, it is unknown if QTL identified under laboratory conditions are also important in natural populations experiencing very different environments (Malmberg et al. 2005; Slate 2005). In principle, it is possible to estimate relatedness based on molecular markers in wild populations (Ritland 2000) so the requirement for a known pedigree can be met under these conditions and hence QTL mapping can be done in wild populations (Slate 2005). This approach has successfully been applied in e.g. red deer (Slate et al. 2002). However, the very large effective population sizes and high levels of within population genetic variance make this an almost impossible task in most marine fishes.

Furthermore, even if QTL studies are successful, there is still a very long way to identifying the genes and nucleotides involved in local adaptations in marine fishes, since markers are often anonymous and single QTL may cover several thousand bases as well as several genes or regulatory regions (e.g. Flint and Mott 2001; Erickson et al. 2004). Thus, the progress from QTL to QTN (Quantitative Trait Nucleotide, Mackay 2001) is an overwhelming task in species other than e.g. human and model organisms, for which genetic markers are abundant and/or which are easy to manipulate genetically (e.g. Glazier et al. 2002; Flint et al. 2005; Salvi and Tuberosa 2005). Even in these species, certain QTL, such as regulatory regions and other non-coding regions have practically been impossible to isolate at the nucleotide level (Salvi and Tuberosa 2005). Since these regions are in fact targeted by the eQTL strategy, it could by especially problematic to get all the way from eQTL to eQTN in these cases. Furthermore, strategies aiming at dissecting QTL into genes and gene interactions have only recently been applied in model species, where they have shown that the genetic architecture of quantitative traits may be even more complex than previously thought (e.g. Kroymann and Mitchell-Olds 2005; Malmberg et al. 2005). These findings are not encouraging for the applicability of the methods in non-model species.

As is the case with some of the approaches mentioned earlier, QTL-based strategies require common garden set-ups. Furthermore, it is required that the species can be kept and bred in the laboratory and that a dense set of markers is available. These are the main obstacles preventing the strategies reviewed here from becoming a general tool for dissecting the genetic basis of local adaptations in marine fishes.

There does not seem to be any marine fishes used as model species for systematic QTL mapping at the moment, but valuable resources may be generated in e.g. Japanese flounder (Fuji et al. 2006) or some gadoids in the years to come. AFLP markers, in particular, should be useful for mapping of both QTL and
eQTL (Vuylsteke et al. 2006) in marine fishes if these approaches are applied.

Admixture mapping
An alternative to QTL mapping is admixture mapping in wild admixed populations (Rieseberg and Buerkle 2002; Darvasi and Shifman 2005; Vasemägi and Primmer 2005). Admixture mapping removes the dependency on a known pedigree and takes advantage of linkage disequilibrium built up during many generations (Rieseberg and Buerkle 2002), but it still requires a dense marker map in the species under investigation and therefore suffers from some of the same potential problems as the QTL mapping approach in relation to marine fishes.

Until now, admixture mapping has primarily been applied in humans (Darvasi and Shifman 2005) and plants. One of the best known examples from plants involves admixture mapping in hybrid zones between two sunflower species (Rieseberg et al. 1999), but hybrid zones in forest trees have also been proposed to be suitable for admixture mapping (Lexer et al. 2004; Lexer et al. 2006). Hybrid zones constitute very suitable settings for admixture mapping because a large variety of hybrid categories can be found in a narrow zone of contact between genetically distinct populations (Rieseberg and Buerkle 2002).

Intra-specific hybrid zones have recently been described in marine fishes (Nielsen et al. 2003; Nielsen et al. 2004), and this setting should provide a very valuable resource for admixture mapping in several marine fish species, since many species are found to be significantly structured across the same environmental gradient (Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Johanneson and André 2006), providing good opportunities to conduct comparative studies.

Genetic variation in candidate genes

The study of variation in candidate genes is the most direct way of studying complex quantitative traits (Mackay 2001). Genetic variation in candidate genes is subject to a number of different analytical strategies, depending on the kind of variation or marker assessed.

Variation can be assessed on the allele frequency or nucleotide level, examples of these types of tests are the Ewens-Watterson test (Watterson 1977) and Tajima’s D-test (Tajima 1989) respectively. However, these tests are very sensitive to population demography and population subdivision and consequently have limited power for detecting the footprints of selection (Nielsen 2001). Alternative and more powerful tests are based on comparisons of synonymous to non-synonymous substitutions (reviewed in e.g. Skibinski 2000; Yang and Bielawski 2000). These tests are not confounded by population demographics and structure because they are focusing on a specific and short segment of the genome (Nielsen 2001). Refinements of these methods have increased power significantly by allowing specific sites to be tested rather than the entire gene (e.g. Swanson et al. 2001; Ross and Rodrigo 2002), thereby allowing functional interpretations of observed patterns of genetic variation (e.g. Swanson et al. 2001). Although originally developed and typically used for interspecies comparisons, also in fishes (see e.g. Bargelloni et al. 1998; Ford 2001), these tests could potentially be used to detect adaptive sequence divergence between populations within a species. However, this has only very rarely been attempted in fishes (e.g. Ford 2000), but with the continued efforts to refine and increase statistical power of these methods, they should be useful in the future.

Another classical way of inferring natural selection is the association of certain gene products with environmental parameters (Endler 1977). Studies on enzyme polymorphisms have illustrated the power of
this approach for inferring natural selection (Berry and Kreitman 1993; Gardner and Kathiravetpillai 1997; Gardner and Palmer 1998; Storz and Dubach 2004), but despite its potential it has not received much attention in marine fishes. A noteworthy exception is the haemoglobin 1 locus, which has been found to be non-randomly distributed between populations of cod (Sick 1965a,b), and especially temperature selection has been proposed to act on the haemoglobin genotypes (Karpov and Novikov 1981; Brix et al. 1998; Petersen and Steffensen 2003). Many of the above studies have identified allele frequency clines along latitudinal clines, illustrating that environmental parameters associated with latitude may have been important for shaping the observed distribution of genetic variation in the candidate genes.

We used a candidate gene approach to study adaptive divergence of European flounder populations (Manuscript II). We compared genetic variation in nine microsatellite loci with that at a markers linked to Hsc70, a heat shock cognate gene, and found that variation at the latter marker was non-neutrally distributed between populations. This strongly indicated adaptive divergence at this locus and therefore suggested local adaptations in the flounder populations.

The usefulness of the candidate gene approach for finding genes involved in specific diseases has been questioned (reviewed in Tabor et al. 2002). Since phenotypes often have a very complex genetic basis, single studies have had difficulties in identifying specific genes significantly associated with a given phenotype (e.g. Aguirre-Hernández and Sargan 2005). However, it should be emphasized that there is a fundamental difference between the classical candidate gene approach in disease research and the approach suggested here. The main difference is that in the latter case we need not be focused on elucidating the genetic basis of specific phenotypes, but can focus on identifying abnormal behavior of the genetic markers themselves. Hence, by choosing genes expected to be important for local adaptations without focusing on specific phenotypes (which are probably not even known), we may increase our ability to identify patterns of genetic variation which is non-neutral.

A major problem with the candidate gene approach is ascertainment bias, which has been a major concern in genome wide surveys of genetic diversity in humans (Clark et al. 2005). Because SNPs are often identified in a sub-sample of individuals, rare alleles could be missed with consequences for estimation of parameters such as diversity and population differentiation (Nielsen 2004). Ascertainment bias can potentially be a problem when choosing candidate markers for local adaptations if markers are not chosen from a random and representative sub-sample of the total sample (van Tienderen et al. 2002; Morin et al. 2004). However, ascertainment bias cannot be totally eliminated unless all individuals are sequenced for all candidate genes. Since this is not a realistic strategy for the coming years, ascertainment bias should be taken into account when designing candidate gene studies. The good thing is, however, that experiences from the human genome projects have illustrated the severity of the problem as well as its impact on various downstream analyses under different ascertainment schemes (Nielsen 2004). Therefore, it is now possible to design sampling and screening for genetic variation based on acceptable levels of ascertainment bias. In general, it is important that markers are chosen based on a random sample of individuals from many populations in the survey (Morin et al. 2004). Furthermore, critical evaluation of results in relation to environmental components suspected to be involved in shaping the observed genetic variation should aid in assessing if observed patterns are indeed real or artefacts.
Another problem similar to QTL approaches is that even if a particular gene can be identified as a very likely candidate for being responsible for the genetic basis of local adaptations, there may still be a long way to understanding the true functional and physiological meaning of the observed genetic variation (e.g. Schulte 2001). Since not all amino acid substitutions are equally important to the function of a protein, knowledge of sequence differences may still be quite far away from the functional importance of these differences (Golding and Dean 1998). Moreover, the signal of selection may not stem from variations in the genes themselves but from variation in regulatory elements, which would obviously require very detailed knowledge about the genes’ regulatory machinery. However, even when complete knowledge about the functional relationships between gene variants is not available, suitable candidate genes can still provide valuable information as a first step towards an understanding of adaptive divergence of populations (Ford 2002).

Even though this approach has only been applied in a few marine fishes, it does appear promising for future studies for several reasons. First of all, it can be applied to all species, provided that they can be accessed for sampling. Secondly, there appear to be plenty of suitable candidate genes to choose from (see e.g. Ford 2002, Moran 2002 and Box 2). Thirdly, tools are now also available for discovering genetic polymorphism in natural populations (e.g. Till et al. 2006) and these strategies should provide researchers with access to genetic resources also in non-model fishes. And finally, the high level of gene flow often observed between populations of marine fishes (Ward et al. 1994; Waples 1998) should actually provide good opportunities for detecting outlier loci based on the distribution of genetic variation within or between populations.

### The importance of a neutral background

Regardless of the methodology adopted for the study of local adaptation it is of major importance to be able to rule out the possibility that an observed pattern is simply caused by random evolutionary forces (Landry and Bernatchez 2001; Moran 2002). This problem will obviously not affect all strategies to the same degree, but is particularly important to have in mind in candidate gene studies and studies of gene expression. Genome scans and QTL/admixture mapping approaches should take random forces into account by searching from deviations from the neutral signals in the data set.

The simplest way to ascertain that observed patterns are truly caused by natural selection is to generate a neutral background from markers expected to behave neutrally. The choice of marker for the neutral background is however not without its difficulties either. Even though microsatellites may be the neutral genetic marker of choice these years, they may not be particularly well suited as a neutral background for candidate gene studies applying other marker types such as SNPs. This is because different mutation rates and processes of the two types of markers could make interpretations of results difficult, unless results are very clear and supported by evidence other than different signals from the two marker types.

Strategies for separating global gene expression data into adaptive and neutrally evolving components have also been developed. Whitehead and Crawford (2006a) used a neutral baseline generated from microsatellites to estimate the variation between populations accounted for by phylogenetic relationships (genetic drift) before moving on to consider outliers associated with specific environmental parameters.
Box 2. How to find the needle in the haystack

A very critical step in any candidate gene strategy is to choose suitable candidates. Different approaches can be taken to find candidate genes for adaptive population divergence in marine fishes. First of all, since several studies have shown that allozymes may in fact not be selectively neutral, the known or suspected function of some allozymes makes them a very useful tool to infer the action of natural selection. Furthermore, the function of many allozymes is relatively well described, which makes these markers a good starting point for candidate gene studies. The studies on adaptive divergence in Ldh-B genes in *Fundulus heteroclitus* is probably the best example of this approach. In this species, several enzymes, including Ldh-B, show clear allele frequency clines with latitude (Powers and Place 1978), suggesting that the locus is under selection. Through a series of studies, the genetic basis of differences in enzyme activity has been dissected to identify specific responsible mutations (Schulte *et al.* 2000; Schulte 2001). While this example illustrates the power of the candidate gene approach, it also highlights the complexities involved in demonstrating the genetic basis of observed physiological differences, since the studies have been conducted over a period of more than 20 years.

An alternative strategy is to isolate novel candidate genes in the species under study. Two different strategies could be applied for this purpose. One strategy is to focus on genes with a central position in signalling pathways. These so-called “hub-genes” integrate signals from many different pathways/environmental components and have therefore been suggested to be good candidate genes for human disease (Lehner *et al.* 2006). Lehner *et al.* (2006) identified six genes with chromatin modelling function to be important in *C. elegans* and suggested that the same genes may well be important in other animals as well. Alternative hub-genes could be Heat shock protein genes or other stress hormones, since these genes are also integrating signals from many pathways (Rutherford and Lindquist 1998; Feder and Hofmann 1999; Queitsch *et al.* 2002; Sørensen *et al.* 2003). The newly discovered microRNAs (reviewed in e.g. He and Hannon 2004) have also been found to be both conserved between species (Pasquinelli *et al.* 2000; Berezikov *et al.* 2005) and to have central roles as regulators of the expression of many genes and pathways (e.g. Lim *et al.* 2005). Hence, these genes, or their target sites (Plasterk 2006), could also be interesting candidates in this category. Since these genes seem to be so essential for cellular functions they would also be expected to be under heavy selective constraints, allowing little variation within species. While this may be a discouraging fact, it also means that any genetic variation detected has a higher likelihood of having a phenotypic consequence.

Another strategy could be to apply candidate genes with a well defined and understood function in relation to specific environmental parameters. MHC in salmonids (e.g. Miller *et al.* 2001) is a classical example of this category. Moran (2002) and Ford (2002) suggest numerous other candidate genes which could be studied in natural populations of fishes (primarily salmonids). Alternatively, genetic polymorphisms with known phenotypic effects isolated in aquaculture species could be applied in natural populations, since many genes of interest to aquaculture, such as growth (e.g. Tao and Boulding 2003) and maturity and spawning (e.g. Leder *et al.* 2006), would also be useful for studies of local adaptations in marine fishes. Although relatively few marine fishes are subject to intense aquaculture efforts, candidate gene sequences should be applicable for primer design in closely related species (Ford 2002), especially if the chosen candidate genes are well conserved through evolution. However, if a specific markers is not already available, it could be difficult to identify causing polymorphisms, even if good candidate genes for a specific phenotype are available (e.g. Aguirre-Hernández and Sargan 2005), maybe because phenotypic effects of genetic variation in these genes are buffered by hub-genes, such as heat shock proteins (Rutherford and Lindquist 1998), thereby reducing selective constraints on the genes.
Alternatively, variation within and between populations can be compared for the expression of each gene separately. Generally, it is expected that neutral evolution will result in a correlation between the two measures, and outliers can then be identified as either showing more or less variation between populations than expected under neutrality (Olesiak et al. 2002; Whitehead and Crawford 2006a). These strategies have been proposed as standard tools for separating gene expression data into neutral versus selected components (Whitehead and Crawford 2006a).

**Complicating factors**

As evidenced by the sections above, studies of the genetic basis of adaptive population divergence are very complicated and time consuming in most animals. However, marine fishes present researchers with several specific challenges, which deserve elaboration before moving on to discuss future developments within the field.

First of all, it will be difficult if not impossible to keep many marine species under controlled environmental conditions for several generations, as required by the phenotype based approaches discussed above. The reasons for this are that we know relatively little about the biology of many species and that many marine fishes have long generation times and special space or habitat requirements, which complicates studies in common garden environments. However, the few marine fishes already kept in aquaculture could be good candidates for these types of approaches (see next section).

Secondly, there is a general lack of genomic resources for marine fish species. Only few species are subject to systematic gene sequencing (Cossins and Crawford 2005), and classical marine species are not among the targeted species. Moreover, no marine fish genomes have yet been mapped to any reasonable resolution (Clark 2003; Cossins and Crawford 2005). We therefore know relatively little about the organization and structure of fish genomes in general and marine fish genomes in particular.

Thirdly, all fish species are believed to have gone through at least one full round of genome duplications besides several independent and lineage specific duplications (Volff 2005). While many paralogous gene copies have since been lost, several hundreds are still functional, some having acquired new functions (Volff 2005). Several gene copies make both gene expression and sequence polymorphism based strategies more complicated because it may be difficult to separate different copies from different alleles within a copy. Na⁺K⁺-ATPase genes may be an interesting case of acquisition of new functions by gene duplications in fishes (Cutler and Cramb 2001). For instance, recent studies have shown that different isoforms may be important under different environmental conditions (Richards et al. 2003; Bystriansky et al. 2006; Morrison et al. 2006), illustrating one of the many complex aspects of gene regulation, which may be important for adaptations to local environments (Schulte 2004). Obviously, such complexity could pose serious technical and interpretational problems if undetected.

**Future perspectives**

Undoubtedly, future studies of population structure in marine fishes will apply more neutral markers, since these are getting still cheaper and faster to develop and genotype (e.g. Brumfield et al. 2003; Morin et al. 2004). However, while this will increase statistical power, it will not change the basic signal of little structure in many marine fish species. Since research the last few years has confirmed that there is genetic structuring among marine fish populations, it now seems to be the time to
move on to try and understand what this structure means. To achieve this goal, integrative approaches, such as combinations of neutral genetic and demographic, geographic or environmental information seem very promising. Furthermore, research specifically targeting the genetic basis of local adaptations is warranted, since we know very little about the scale and magnitude of adaptive population divergence in this high gene flow environment. For studies of the genetic basis of adaptive divergence in marine fishes to be successful, it would obviously be worthwhile focusing on resources already available as well as the special attributes of marine fishes, which provide good opportunities to study local adaptations, instead of trying to adopt strategies not easily applicable to marine fishes.

As emphasized above, several aspects of the biology of marine fishes and our understanding of this, makes it difficult to keep many of the species in captivity for several generations. This limits the number of species for which common garden set-up can be applied. However, a few marine species are subject to increasing aquaculture efforts (e.g. gadoids, Rosenlund and Skretting 2006), providing valuable settings to conduct these studies. As advocated by Vasemägi and Primmer (2005) there should be great advantages in combining some of the techniques discussed in the sections above in integrative approaches. For example, the application of EST linked markers in mapping studies could aid in identifying specific candidate genes within the QTL (Erickson et al. 2004). Alternatively, specific candidate gene markers could be used in combination with QTL mapping (Leder et al. 2006), thereby increasing the candidate status of specific QTL and genes. Rogers and Bernatchez (2005) have integrated traditional QTL analyses with a genome scan to identify QTL most likely to be associated with the ecological differentiation of dwarf and normal ecomorphs of whitefishes. In another integrative strategy, Wayne and McIntyre (2002) combined mapping with microarray analyses to narrow the range of potential candidate genes for avariole numbers in Drosophila. In cases where microarrays are not available, alternative techniques, such as cDNA-AFLP, hold great promise for future applications in marine fishes. These techniques could also be used for discovering genes important for local adaptations (e.g. Cui et al. 2006). While these integrative strategies are promising for narrowing the range of candidates responsible for complex phenotypes and local adaptations in general it is doubtful if they will be applicable to most marine fishes in the nearest future because of time, space and economical constraints. However, less ambitious aims, such as studying the genetic architecture of local adaptations on a more coarse resolution (e.g. Rogers and Bernatchez 2005), should be feasible in some marine fishes in the near future.

In contrast to common garden based strategies, natural populations can be studied in all species, provided that they can be accessed for sampling. Furthermore, since several attributes of wild fish populations makes them ideal for applying some of the strategies outlined in the sections above, these settings hold great promise for future studies.

Since many marine fishes are distributed over very large geographical areas they often also experience diverse environmental conditions, which should provide excellent opportunities to study the effect of different environments on genetic structuring. To exploit this opportunity first and foremost requires that sampling is truly targeting a significant part of the environmental distribution of the species. If properly designed genome scan and candidate gene approaches should be very useful for disclosing adaptive population divergence in samples from natural populations.
Normally a dense distribution of markers over the entire genome is required for genome scans to be efficient. In most marine fishes such a map is not readily available, making a complete genome scan problematic. However, the newly developed AFLP markers (Vos et al. 1995) appear to be a particularly promising in non model organisms, since they are fast, reliable and provide reasonable genome coverage (Bensch and Åkeson 2005). While, until now, only applied in a few cases in marine fishes (e.g. eels, Albert et al. 2006), the advantages of AFLPs should make these markers preferable in marine fishes in the future (see e.g. Campbell and Bernatchez 2004). Other methods for identifying markers of potential interest have been proposed. For example, Vasemägi et al. (2005) used EST-linked microsatellites in a genome scan of Atlantic salmon populations and identified several potential candidate genes for adaptive population divergence. Since EST resources are building up quite rapidly in a number of fish species or can be generated relatively easily (e.g. Chen et al. 2005; Cossins and Crawford 2005) this strategy could be very useful for identifying candidates under selection in genome scans.

An alternative approach is to adopt a hybrid candidate gene/multi-locus approach by selecting specific candidate genes suspected to be important for adaptive population divergence and then survey these specific genes as well as putatively neutral markers for genetic variation in natural populations. This method would provide hints on genes and processes directly involved in adaptation and aid in finding specific nucleotides responsible. Such a strategy was recently used by Anderson et al. (2005) to study adaptive divergence of malaria parasites in response to drug treatments. The authors compared putatively neutral SNPs with SNPs in known genes for drug resistance and found that the latter clearly exhibited non-neutral behavior, strongly indicating adaptive divergence. We used a similar approach for studying adaptive divergence in European flounder by comparing genetic structuring at neutral microsatellite markers to structuring at a Hsc70 gene, which was chosen as a candidate gene for adaptive divergence (Manuscript II), and found strong indications of local adaptations in flounder populations. The results from the study suggest that this approach has a great potential in non-model species such as many marine fishes, since relatively few markers are required. In future studies, it is expected that marker development will proceed at increased speeds, making it relatively easy to isolate and characterize genetic markers such as SNPs in non-model species (e.g. Till et al. 2006). These techniques should be very useful, since specific genes of interest can be targeted in combination with putative neutral regions.

Furthermore, it would be logical to take advantage of the neutral baselines which have already been generated for a large number of species. These data sets represent valuable information which could be applied in combination with candidate gene variation. In this case, neutral markers and markers in candidate genes should preferably be comparable with respect to mutational properties and mutation rates. Since many studies of population structure have applied microsatellites, it would be preferable to use this marker type linked to candidate genes, for instance applying the EST based strategy proposed by Vasemägi et al. (2005). It should, however, be kept in mind that the original studies were not necessarily designed to capture the total environmental heterogeneity experienced by the species. Original sample material may therefore need to be supplemented with new samples from poorly covered areas in order for this strategy to be successfully applied.

Another excellent setting available in marine fishes is found in some of the hybrid zones already described (e.g. Schulte 2001;
Nielsen et al. 2003; Nielsen et al. 2004). These areas and the species living in them should provide a good opportunity for applying an admixture mapping strategy in wild populations. Furthermore, the fact that hybrid zones may be shared between several species (Nielsen et al. 2004; Johanneson and André 2006) will be a very good setting for conducting comparative studies which could further strengthen the evidence that genetic divergence is truly adaptive (see e.g. Schulte 2001) and which could shed important light on the generality versus species specificity of the genetic architecture underlying local adaptations. The previously mentioned advantages of AFLPs make these markers well suited for admixture mapping strategies in marine fishes.

Finally, it would be very interesting to add a temporal dimension to studies of local adaptation in marine fishes, for instance in relation to human induced evolutionary forces such as climate change and fishery induced selection. In this case, only DNA based techniques would be useful, since archived material (such as scales, otoliths and bones) should be used for comparisons to contemporary samples. This opportunity has not previously been explored in marine fishes, but it could relatively easily be applied, if markers are designed to amplify in samples of partly degraded DNA. Earlier studies using microsatellites (Ruzzante et al. 2001; Hutchinson et al. 2003; Poulsen et al. 2006) indicate that this should be a feasible approach applied to candidate genes as well.

Thus, there appears to be plenty of opportunities for designing high-quality studies of the genetic basis of local adaptations in marine fishes. Particularly studies in natural populations appear promising, since these strategies are in principle feasible in any species. Such studies would gain considerable power if the same signals are detected in independent population comparisons (e.g. Storz and Dubach 2004; Bonin et al. 2006), and marine fishes do provide several opportunities for designing comparative studies within and across species. But regardless of the strategy adopted, there is a need for a deeper physiological understanding of local adaptations. In order to achieve this goal, it will be necessary to initialize cross disciplinary projects which are integrating population genetics with evolutionary and physiological genetics. These research fields have largely been asking and answering similar questions, but have tended to communicate very little across disciplines, for instance by publishing in different scientific journals. An integration of these areas would improve our understanding of the genetic and physiological basis of local adaptations from individual cells to systems in the marine environment (Pörtner 2002). By providing new insights into how selection operates in marine fishes, these studies will further our understanding of evolution in this high gene flow environment and should provide indispensable tools for management of marine biodiversity in the future.
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Manuscript I

Evolutionary factors shaping the genetic population structure of marine fishes; lessons from the European flounder (Platichthys flesus L.)

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Evolutionary factors shaping the genetic population structure of marine fishes; lessons from the European flounder (*Platichthys flesus* L.)

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Running title: Population structure in European flounder
Abstract

A number of evolutionary mechanisms have been suggested for generating low but significant genetic structuring among marine fish populations. We used microsatellites and recent developments in landscape genetics and coalescence based estimation of historic gene flow and effective population sizes to assess temporal and spatial dynamics of the population structure in European flounder (*Platichthys flesus* L). We found highly significant genetic differentiation among samples covering a large part of the species’ range (global $F_{ST} = 0.024$, $P < 0.0001$). In addition to historical processes, a number of contemporary evolutionary drivers were found to be associated with genetic structuring. Physical drivers, such as oceanographic and bathymetric barriers, were most likely related with the extreme isolation of the island population at the Faroe Islands. A sharp genetic break was associated with the change in life-history from pelagic to benthic spawners in the Baltic Sea. Furthermore, partial Mantel tests showed that environmental transitions rather than geographic distances *per se* were associated with genetic structuring among Atlantic populations (latitude controlling for geography: $r=0.764$, $P=0.002$, geography controlling for latitude: NS). These results support the hypothesis of environmental differences as a major structuring force in marine fishes and highlight that the magnitude and scale of structuring generated by a specific driver depend critically on its interplay with other structuring forces. We suggest evaluating all potential evolutionary drivers simultaneously, emphasizing the importance of investigating species with wide geographical and ecological distributions, such as the European flounder, to increase our understanding of evolution in the marine environment.
Introduction

Understanding evolution in marine organisms requires knowledge of the genetic population structure as well as the biotic and a-biotic drivers responsible for creating patterns of neutral and adaptive genetic divergence. During the last decades the availability of highly variable genetic markers has facilitated the gathering of large quantities of data on the scale and magnitude of population structuring in marine fishes. These studies have generally confirmed that population structure in marine fish species is best explained by a low structure-high gene flow scenario (Ward et al. 1994; Waples, 1998; DeWoody & Avise 1999; Hutchingson et al. 2001; Hoarau et al. 2002a; Mariani et al. 2005). Still, small but significant, and usually temporally stable, genetic structuring has been demonstrated for many species of marine fishes (Ruzzante et al. 1999; Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Jørgensen et al. 2005).

A number of potential mechanisms have been suggested to explain how population structure can evolve in an environment without any obvious physical boundaries to gene flow. Historical processes associated with colonization of new habitat after the last glaciation has been recognized as important for shaping present day genetic structure (Grant & Bowen 1998; Gysels et al. 2004; Hickerson & Cunningham 2005; Wilson 2006) and certain life-history characteristics, particularly restricted dispersal of egg and larval stages, have been found to be associated with increased structuring of populations (Waples 1987; Bernardi 2000). Physical structuring factors, such as geographical distance, oceanic current systems and bathymetric barriers to gene flow have also been proposed to be important (Ruzzante et al., 1999; Lundy et al., 2000; Pogson et al. 2001; Knutsen et al. 2004). Furthermore, environmental transitions have been found to be associated with genetic structuring of populations (Lundy et al. 1999; Riginos & Nachman 2001; Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Johanneson & André 2006), implicating that populations are locally adapted to their native environment. Although many mechanisms have been proposed, it has been difficult to assess the relative importance of these evolutionary forces, since mechanisms have often been investigated on a case by case basis. Important exceptions to this general picture can be found. Thus, Riginos & Nachman (2001) investigated the effects of several potential evolutionary drivers among highly structured populations of Axoclinus nigricaudus in the Gulf of California and found interacting effects from biogeography, geographic distance and habitat quality. Similarly, Roques et al. (2002) found evidence for the effects of both historical and contemporary forces in structuring redfish (Sebastes mantilla) populations in the Northern Atlantic. Hence, until now only few studies have identified multiple mechanisms - and their relative importance - for shaping population structure in a “classical” marine fish species, i.e. a species with a wide distribution, large population sizes, high fecundity and high dispersal potential for juveniles and adults (Nielsen & Kenchington 2001).

The European flounder has a very wide geographical and ecological distribution. It inhabits coastal areas throughout the North Eastern Atlantic from the White Sea to the Mediterranean Sea and as one of only a few marine fish species, it is also found in the inner parts of the brackish Baltic Sea. The flounder is very tolerant to low salinities and is often found migrating to rivers and lakes for extended periods before returning to the sea to spawn. Accordingly, it is uniquely suited for studying the simultaneous action of several evolutionary drivers for shaping population structure in a potential high gene flow species.

The flounder offers a very good opportunity for evaluating the effect that different life-history strategies may have on the genetic structuring of populations because different strategies are found within the same species in different parts of the distributional area. While the “normal” form spawn...
pelagic eggs at off-shore spawning grounds, the so-called “Bank-flounders” (sometimes referred to as a sub-species of *P. flesus*) in the innermost parts of the Baltic Sea appear to have adapted to the brackish environment by spawning benthic eggs (Solemdal 1973; Aro 1989; Drevs *et al.* 1999). This adaptation assures that eggs are not exposed to anoxic conditions in the deeper parts of the Baltic Sea. The distributions of the two types of flounders overlap around the island of Gotland in the central Baltic Sea, but the level of interbreeding between the two types is not known. This setting permits studying genetic differentiation between and within two life-history forms, which has only rarely been possible in marine fish species.

The life-history of the flounder also allows studying the effect of geographic distance *per se* on genetic structuring. Adults are relatively stationary and are primarily migrating locally between feeding and spawning grounds (Aro 1989). Juveniles are sedentary in brackish or in fresh water. Eggs are only pelagic for a few weeks and larvae have been shown to be able to utilize estuarine currents for selective tidal stream transport (Campos *et al.* 1994; Bos 1999) enabling them to reach their preferred nursery grounds even before metamorphosis. It therefore seems reasonable to assume that the potential lifetime dispersal of a single individual does not include the full distributional area of the species, which provides a good opportunity for evaluating the effect of geographical distance on genetic structuring.

Oceanic current systems and bathymetric features could also be expected to affect the structuring among populations of flounder. European flounder is a coastal species and rarely migrates large distances over deep seas (Aro 1989), and hence it is expected that island populations should be particularly isolated or even missing. Therefore, the inclusion of the Faroe Island population, isolated by deep Atlantic waters and a circular current system (Jákupsstovu & Reinert 1994; Joensen *et al.* 2000) allows an assessment of the importance of these potential barriers to gene flow in the marine environment. In particular, they facilitate an assessment of the strength of these barriers compared to sharp environmental clines and geographic distance *per se*.

The flounder is exposed to a number of highly pronounced but very different environmental gradients following south-north and east-west transects, thereby allowing an evaluation of the relative importance of environmental differences for shaping its genetic structure. Along a latitudinal transect in the Atlantic part of the distributional area, several environmental parameters vary, the most obvious being related to temperature, light and seasonal cycles. Another important gradient is the Atlantic-Baltic Sea transition which has been studied intensively in other species. Flounders in the central and western Baltic Sea show several special physiological features thought to reflect adaptations to the brackish environment. For instance egg shell thickness and sperm mobility appear to be adapted to allow efficient reproduction at the lower salinities in these regions (Solemdal 1967; Solemdal 1973; Nissling *et al.* 2002). Similar adaptations have also been described in other species in this region (Vallin *et al.* 1999; Ojaveer & Kalejs 2005), however, with its unique tolerance to low salinities the flounder represents an important contrast to the marine species studied in the North Eastern Atlantic so far.

The aims of the present study of population structure in the European flounder were to assess the relative importance of several evolutionary drivers acting to shape the genetic structure of marine fishes. We combine a method for indirect estimation of gene flow and effective population size with landscape genetic analysis and an assessment of the temporal and spatial dynamics of the population structure. In particular, we first of all evaluate the relative importance of historical versus contemporary processes. We then assess the levels of structuring within as well as between life-history strategies before moving on to study the importance of physical forces, such as geographical distance and oceanographic currents. Finally, we concentrate on selective forces associated with environmental
differences in two different scenarios from the species’ distribution. Understanding the outcome of the interplay of these structuring forces in a high gene flow setting is central to gain a better understanding of the magnitude and scale of population structuring in the marine environment and will hence improve our ability to manage marine biodiversity efficiently.

**Materials and Methods**

**Sampling**

Samples of approximately 50 European flounders were collected by research vessels or local fishermen (see Figure 1 and Table 1 for sampling locations) both in 2003 and 2004. Among the sampled locations were two secluded Danish fjords and Lake Pulmanki in Northern Norway and Finland, which is connected to the Barents Sea via the river Teno. Most samples were collected during the spawning season and primarily comprised of mature or spawning individuals (Table 1). In some cases, however, samples consisted of immature adults or juveniles. Gill filaments or fin clips (app. 0.5*0.5 cm) were collected from each individual and stored in 96% ethanol for subsequent DNA extraction.

European flounder is known to hybridise with plaice (*Pleuronectes platessa* L.) in some parts of the distributional area. The frequency of hybrids varies between localities but is generally believed to be highest in the western part of the Baltic Sea (Sick *et al.* 1963). A large quantity of these hybrids could potentially bias the results of the population genetic analyses conducted here. Hence, a sample of 18 North Sea plaice collected in 2003 was included to aid in the identification of hybrid individuals.

**DNA analyses**

DNA was extracted with standard HotSHOT (Truett *et al.* 2000), Chelex (Estoup *et al.* 1996) or DNeasy (Qiagen) techniques. PCR was performed on DNA extracted by all three methods from fifteen randomly chosen individuals in order to test for discrepancies between genotypes obtained with the different extraction methods.

Nine microsatellite loci were employed for the genetic analyses. LIST1001 (Watts *et al.* 1999, GenBank accession number: AF149831), PL142 and PL167 (Hoarau *et al.* 2002b, accession numbers: AF406750 and AF406751), originally developed for plaice but worked well on flounder following modifications of PCR conditions. StPf1001, StPf1002, StPf1004, StPf1005, StPf1015 and StPf1022 (Dixon *et al.* unpublished, accession numbers: AJ315970, AJ315975, AJ315973, AJ315974, AJ538313, AJ538320) were all developed for European flounder. PCR was applied with standard reagents and thermal cyclers and PCR products were analyzed on a Pharmacia ALFexpress automated sequencer following the manufacturer’s recommendations. Standard size ladders and individuals of known genotypes were run on each gel to minimize scoring error.

**Statistics**

The programme MICRO-CHECKER (van Oosterhout *et al.* 2004) was used to check for potential technical problems such as null alleles, stuttering and large allele drop-out and null-allele frequencies were estimated according to Chakraborty *et al.* (1992). Exact tests for departure from HW equilibrium (Guo & Thompson 1992) were performed per locus and sample in GENEPOP (Raymond & Rousset 1995). $F_{ST}$ (Weir & Cockerham’s (1984) θ), their confidence intervals and significances were estimated using the program FSTAT (Goudet 1995), which was also used to estimate allelic richness (ElMousadik & Petit 1996).
A hybrid neighbour joining tree was constructed with the PHYLIP package (Felsenstein 2004). The tree topology was based on Cavalli-Sforza’s chord distance (Cavalli-Sforza & Edwards 1967) with statistical support from 1000 bootstrapped data sets. Branch lengths were based on (δμ)² (Goldstein et al. 1995) calculated with the program POPULATIONS (Distributed by Olivier Langella, available from http://bioinformatics.org/project/?group_id=84). These genetic distance measures have been proposed to be particularly suitable to represent tree topology and evolutionary distances between populations based on microsatellite markers (Takezaki & Nei 1996).

The program BARRIER (Manni et al. 2004) was used to assess the largest breaks in the genetic structure across the species’ range. BARRIER identifies barriers to gene flow based on geographic and genetic relationships among populations. In order to assess the robustness of barriers, 100 matrices of Weir & Cockerham’s θ (1984) were generated by bootstrapping over loci. The robustness of barriers is proportional to the number of times each barrier is supported by the 100 data sets. Each data set supports a number of barriers of decreasing order, reflecting the relative strength of the detected barriers.

We used simple linear regression to examine a potential association between genetic diversity and latitude. Partial Mantel tests were performed in order to test if the genetic structure observed in a subset of the samples is best explained by the geographic or environmental distance between samples. Geographic distance was calculated as the shortest coastal distance between samples. It has been shown that it can be very difficult to separate the effects of different environmental parameters if they are highly correlated and potentially interacting (Sarup et al. 2006). Hence, we used differences in latitude and longitude from the sample positions as proxies for environmental differences between samples, since these measures are expected to capture much of the environmental variation among the samples included in the partial Mantel tests. Partial Mantel tests were done controlling for latitude and longitude separately and significance was assessed with 1000 permutations. The partial Mantel tests were conducted with the software IBDWS (Jensen et al. 2005).

The program MIGRATE (Beerli & Felsenstein 2001) was used to estimate population specific Θ (=4Neμ, where μ is the mutation rate across loci) and pairwise scaled migration rates, M (=m/μ, where m is the migration rate). This program uses a coalescence based likelihood and MCMC method to estimate Θ and M values and allows for the assessment of asymmetric rates of migration. We stress that MIGRATE estimates a scaled historic effective population size and migration rate. Hence, this approach will pick up any signal affecting these parameters in the history of the populations and we do therefore not attempt to take the estimates at face value. Neither do we find it reasonable to multiply the estimates with some arbitrarily chosen mutation rate to reach estimates of actual effective population size and migration rate, but are rather comparing patterns and relative values throughout this paper.

We applied the “heating scheme”, which runs parallel Markov chains at different temperatures. A cold chain is sampling locally, while hotter chains move more freely and can therefore explore more of the genealogy space. This scheme should allow for a more efficient exploration of the genealogy space by swapping states between chains. This approach should be useful in situations with flat likelihood surfaces (Beerli 2004) such as would be expected in marine fishes. Initial trial runs indicated that a plateau in likelihood values of chains was reached after approximately 10 short chains. To be conservative, 12 short and 3 long chains were run in the subsequent analyses. Short and long chains were run for 500 and 5000 sampled genealogies or until 50 and 500 new genealogies had been found in cases with low acceptance ratios. The only exception to this general setting was for locus StPf1001, which was run until 25 and 250 new trees were accepted for short and long chains respectively, because
the algorithm found very few acceptable genealogies for this locus. The two years were kept separate in order to assess the reliability of the method in the case of marine fish species showing relatively little evidence of genetic divergence across the range of samples. Three runs, each consisting of a number of sub-runs, were conducted for each year. Parameter estimates in the first sub-run were based on $F_{ST}$ estimates, while subsequent sub-runs had estimates from the preceding sub-run as starting values. The runs were stopped when the majority of estimates were found to be within 95% confidence intervals in successive sub-runs, and final parameter estimates were then obtained by combining the long chains in the final sub-run.

Results

Genetic variation

DNA extractions with the three different methods yielded consistent genotypes in 14 of the 15 chosen individuals; the 15th individual was discarded from further analyses because of DNA of poor quality not giving consistent PCR amplification. In total 5 out of 1086 individuals (0.46%) were discarded from analyses because of poor quality DNA. No locus or population had more than 4% missing genotypes, indicating that genotyping success was high in the remaining individuals. The MICRO-CHECKER analyses provided little evidence of technical problems with only 7 of 198 tests conducted (3.5%) showing evidence of minor null allele problems. Potential null alleles were found for locus StPf1004 in Bor03 (null-allele frequency: 0.08), Aer03 (frequency: 0.06) and Aer04 (frequency: 0.07), for locus List1001 in Bor04 (frequency: 0.17), for StPf1022 in Irs04 (frequency: 0.07), StPf1001 in Bor03 (frequency: 0.06) and Pl142 in Rin03 (frequency: 0.04). All loci amplified well under standard conditions in the sample of North Sea plaice. In this sample potential null alleles were identified in loci StPf1004, StPf1005 and StPf1022 (frequencies of 0.31, 0.15 and 0.15 respectively), which were all developed for European flounder. These potential null-alleles were not affecting our ability to detect hybrids between the two species. Hybridisation with plaice did not present a significant problem for the analyses conducted in this study, since rates of introgression from plaice to flounder were found to be low and relatively evenly distributed throughout most of the flounder’s distributional range. Only one out of 1063 flounders genotyped was identified as a potential F1 hybrid. This individual was discarded from the data set.

When conducting the exact test for deviations from Hardy-Weinberg equilibrium, we found 13 significant tests among the total 198 tests conducted (Appendix A). The apparent deficiency of heterozygotes in a few loci in the western Baltic Sea samples is most likely not indicative of a Wahlund effect, since a simulated sample consisting of a mechanical mixture of pure North Sea and inner Baltic Sea flounders did not deviate significantly from Hardy Weinberg expectations. This reflects a general lack of statistical power for detecting true Wahlund effects in marine fishes and suggests that the heterozygote deficiencies in these samples are caused by technical problems as indicated by the MICRO-CHECKER analysis rather than by mechanical mixing of individuals from divergent populations. However, since such potential problems are not substantial and furthermore not biasing the analyses conducted here, we retained all loci for further analyses.

The allelic richness was highest in the North Sea/Irish Sea samples and tended to decline in the extreme parts of the distributional area. The Faroe Island samples had exceptionally low levels of genetic diversity (Appendix A). Overall there was a weak but significant association between allelic richness and latitude ($r^2=0.19$, $P=0.04$). However, this association was no longer significant when the extreme Faroe Island population was excluded from analysis ($r^2=0.13$, $P=0.12$).
**Temporal stability**

No pairwise comparisons involving temporal samples from the same locality were significant. $F_{ST}$ ranged from -0.003 between the Bay of Biscay and Faroe Islands samples to 0.003 between the Thyborøn samples (Appendix B), thus indicating that the signals of genetic differentiation detected in this study were temporally stable over the short time period covered. In spite of this overall similarity, temporal samples were kept separate for two main reasons. First of all, it allowed for an assessment of the reliability of the results obtained from the different analyses. Since the 2004 data set covered a much larger part of the distributional area it was also expected to capture more of the total genetic variation in the species compared to the narrower sampling scheme in 2003. By keeping the years separate it was possible to evaluate the effect of this difference in sample coverage between the two years. This is expected to be particularly important in the MIGRATE analyses, since this approach has been shown to be relatively sensitive to “low signal” data sets (Abdo et al. 2004), such as expected in the present case. Secondly, small but non significant differences were detected between temporal replicates, for example between the Thyborøn and Gotland samples. By pooling these samples, important information about the short term temporal dynamics could be lost. Hence while the overall conclusion is that the population structure detected here is temporally stable, we find it more useful for analytical purposes to keep temporal samples separate.

**Population structure**

The hybrid tree (Figure 2) showed a strong partitioning of samples into distinct population groups. The Faroe Islands, Bay of Biscay and benthic spawning flounders in the inner Baltic Sea were clearly isolated from the remaining populations, which grouped together with lower bootstrap support. Additionally, the evolutionary distances (branch lengths of $(\delta \mu)^2 \approx 6$ and 14 respectively) to the inner Baltic and Lake Pulmanki populations were very large compared to the distances among the remaining populations.

The overall $F_{ST}$ was 0.024 (95% CI: 0.018-0.031) and highly significant ($P<0.0001$). Examining the pairwise $F_{ST}$ estimates (Appendix B) it is evident that some contributed much more to this overall pattern than others. The highest pairwise $F_{ST}$ values were from comparisons involving the Faroe Islands, the Bay of Biscay, Lake Pulmanki and the benthic spawners in the inner Baltic Sea. Although the differences between populations within the area from the North and Irish Seas to the western Baltic Sea were fairly small ($F_{ST}$ ranged from 0 to app. 0.01), most of these comparisons were still statistically significant. In comparison, the pairwise $F_{ST}$ between the plaice and flounder samples was around 0.2.

The landscape genetic analysis identified four barriers with high bootstrap support in 2004 (Figure 1 and Table 2). All data sets except one supported the barrier around the Faroe Islands as the most important one. This barrier was followed by barriers of decreasing importance around Lake Pulmanki, Bay of Biscay and the benthic spawning populations at Gotland and Turku (Table 2). In addition, a barrier separating Trondheim and the western Baltic Sea could be detected with lower bootstrap support. Computation of additional barriers resulted in a random scatter of barriers on the map. In 2003 the barrier around the Faeroe Islands was also identified as the strongest barrier supported as the first order barrier by 96 of 100 data sets. This barrier was followed by the barrier isolating the benthic spawning flounder populations in the Baltic Sea. A third barrier with less support was identified north of the Bay of Biscay and between the Thyborøn and Limfjord/Erø samples. The apparent isolation of the Limfjord population could potentially be an artefact resulting from an intense
supportive breeding program in this area using a limited number of parent fish to produce a large
number of juveniles (J. Støttrup, Danish Institute for Fisheries Research, personal communication).

Partial Mantel tests were conducted on a subset of samples excluding samples from the Faroe
Islands, Gotland and Turku. This was done since these samples showed clear evidence of structuring
caused by factors other than geographical distance per se (see discussion). Excluding these samples
allowed an in-depth analysis of potential structuring forces in the remaining set of samples. Among
these samples there was a clear and highly significant pattern of isolation by distance in 2004 (r=0.705,
P=0.002) but not in 2003 (r=0.008, P=0.495) (Table 3). There was also a significant association
between both environmental variables and genetic distance in 2004 (latitude: r=0.886, P=0.001,
longitude: r=0.703, P=0.009). When controlling for environmental variability in 2004, geographic
distance was no longer significant, while latitude was still highly significant when controlling for
gEOGRAPHY (r=0.764, P=0.002). Likewise, longitude was not significant when controlling for geography
(Table 3).

Estimates of scaled population sizes and migration rates
The consistency between different runs of the program MIGRATE was markedly better in 2004 than in
2003, probably because of a better coverage of the total distributional area and hence a larger signal in
the data set. Because of the uncertain estimates from the 2003 data, this data set was not used for
further analyses and the reported estimates are from the 2004 data set only. These results stress the
importance of a broad coverage of the distributional area of the species under investigation.
Furthermore, they illustrate that the application of this particular method may be problematic in studies
of genetic structure in marine fishes, which often exhibit even lower levels of structuring than the ones
reported here. At the very least, validation by several independent runs of MIGRATE should be
conducted in order to check the reliability of estimates.

Estimates of scaled long term effective population size (Θ) (Table 4 for a summary of all three
runs and supplementary tables S1-S3 for individual runs with estimates and 95% confidence intervals)
show that the Faroe Islands and Lake Pulmanki populations are clearly the smallest in the data set.
Trondheim is relatively small, while the populations in the North Sea (Thy) and western/central Baltic
Sea (Årø and Bor) represent larger populations. The three different runs gave inconsistent estimates of
the sizes of Ringkøbing Fjord, Turku, Gotland, Bay of Biscay and Irish Sea. Turku, Årø and Thyborøn
were always net donors of migrants, while the Faroe Islands and Lake Pulmanki populations were
consistently net receivers. There also appeared to be asymmetrical migration from the large North Sea
and western Baltic Sea populations into the southernmost population in the Bay of Biscay.

When examining specific population pairs, some additional signals were common to all three
runs. One was an asymmetrical migration from Thyborøn in the North Sea into the central Baltic Sea at
Bornholm, while the western Baltic Sea population (Årø) appeared to donate migrants both into and
out of the Baltic Sea. Gotland also consistently received more migrants from the western Baltic and
North Sea populations than it donated.

Discussion
Overall, there is clearly large and highly significant genetic structuring among the samples of European
flounder used in this study, which is supporting the occurrence of local populations for the species.
However, the level of structuring is unevenly distributed throughout the geographical range, and the
analyses conducted indicate that different factors may be responsible for generating these signals of
genetic structuring.

**History and founder events**

In general, populations from the extreme parts of the distributional area showed signs of reduced allelic
richness, which could be the result of founder events when new habitat was colonised after the last
 glaciation (Hewitt 2000; Widmer & Lexer 2001). Results from the MIGRATE analysis clearly
corroborate a signal of founder events in the Faroe Islands and Lake Pulmanki by estimating these
populations to be significantly smaller than the remaining populations in the data set. The larger \( \delta \mu \)^2
distances to Lake Pulmanki as well as to the benthic spawning populations in the inner Baltic Sea are
also consistent with episodes of elevated genetic drift, possibly reflecting founder events. While the
Baltic Sea populations appear to have reduced genetic diversity compared to their Atlantic neighbours,
these populations do not, however, seem to be particularly small as based on estimates of \( \Theta \) from
MIGRATE.

Although the large evolutionary distances to these populations are consistent with founder
events, they could possibly also reflect an alternative evolutionary history, such as a colonisation from
different glacial refuges. Some studies have proposed a possible glacial refuge in the southern North
Sea in addition to the well established refuge near the Iberian peninsular (e.g. brown trout, García-
Marín et al. 1999; Atlantic salmon, Verspoor et al. 1999; common goby, Gyse1s et al. 2004), and it is
not impossible that such ice free areas in the southern North Sea could have allowed both freshwater
and coastal marine species, including European flounder, to sustain local populations during the last ice
age.

The \( \delta \mu \)^2 distances of app. 8 and 13 between the bank-flounder populations and Lake Pulmanki
and their closest neighbours would imply an average mutation rate of app. 1 – 2*10^{-3}, assuming a linear
relationship between \( \delta \mu \) and time for microsatellites (Goldstein et al. 1995), a generation time of
three years and a divergence time of 10,000 years since the last glaciation. This estimate seems to be
high compared to other estimates for teleost microsatellites (2*10^{-4} in zebrafish, Shimoda et al. 1999;
~4*10^{-3} in pink salmon, Steinberg et al. 2002), implying that these populations could have diverged
before the last glaciation. However, the proposed founder events may well have inflated the genetic
distances to these populations, thereby inflating the above mutation rate estimates. Furthermore,
classical marine fishes, such as Atlantic cod (Árnason 2004) and plaice (Hoarau et al. 2004), show
shallow mtDNA genealogies, providing little evidence for different glacial lineages in the North
Eastern Atlantic in these species. Hence, while we cannot exclude different glacial refugia as
responsible for generating parts of the signals detected here, it seems highly unlikely that this should be
the only factor involved in generating the signals of three (and potentially four if the Faroe Islands are
included) glacial lineages in the flounder. It appears more plausible that the large genetic distances to,
and marked genetic isolation of, these populations were generated by relatively recent events rather
than by different glacial histories.

**Life-history strategy**

We did not find elevated levels of structuring among populations spawning benthic eggs, which is
expected to reduce gene flow relative to a “pelagic egg” life history strategy. Waples (Waples 1987)
found a close relationship between dispersal capacity of eggs and larvae and levels of genetic
structuring in a large range of coastal species, but so far it has not been possible to compare levels of
divergence between life-history strategies with different dispersal capacities within the same species. In
the present case, the largest levels of structuring are found among populations with the high gene flow
life-history characteristics. While this finding may be contradictory to expectations it does illustrate
that life-history strategy per se may not be a good predictor of the expected levels of population
structure when several evolutionary drivers are considered simultaneously. On the other hand, the
known differences in life-history strategies do seem to be a very potent structuring force in European
flounder. The sharp break in genetic constitution among Baltic Sea populations is found between the
two different forms and indeed it is no surprise that a change from pelagic to benthic spawning habitat
would result in considerable reductions in gene flow between populations. Different life-histories are
also found in Atlantic herring, where population-components spawn during different times of the year.
The genetic and environmental base of this complex life-history switching is largely unknown, but
recently Ruzzante et al. (2006) have shown that these spawning components remain genetically distinct
despite extensive seasonal mixing at feeding grounds, implicating strong homing behaviour and
temporally stable population structure. Bekkevold et al. (in press) demonstrated that sympatric winter
and spring spawning components may in fact have different origins. Some winter spawning groups
were found to be genetically identical to the sympatric spring spawners, implying a plastic response in
a local population, while others were genetically more similar to winter spawning herring from distant
localities. In the latter case, the sympatric spawning components apparently have different geographical
origins, further illustrating the complexity of the Atlantic herring population structure in the North
Eastern Atlantic. While the degree of geographical overlap between the two life-history strategies in
European flounder is not known in detail, the genetic break observed in the inner Baltic Sea is
substantial and implicates significant reductions in gene flow between the two forms.

The evidence of ecological differentiation in European flounder demonstrates that rapid
adaptive divergence might also be possible under a high gene flow scenario in the marine environment.
If the splitting of “pelagic” and “benthic” flounder in the Baltic Sea happened after the initial
colonisation of the Baltic Sea it must have happened within the last app. 8,000 years, after the brackish
state of the Baltic Sea was established by salt water intrusion through the Danish straits (Bjorck 1995).
Similar examples of rapid evolution based on ecological differentiation (Orr & Schmidt 1998) have
also been found in cichlids (e.g. Galis & Metz 1998; Albertson et al. 2003), sticklebacks (McKinnon &
Rundle 2002) and whitefish (e.g. Lu & Bernatchez 1999). If the “benthic spawning” life-history did
evolve in response to the low saline environment, the selective forces would have acted on reproductive
strategies and generated reproductive isolation directly. Alternatively, reproductive isolation could have
acted as a reinforcement mechanism after an initial divergence in response to selection for other traits
(Schluter 2001), such as different migration patterns and/or delayed spawning time in response to
longer and more severe winters in the innermost Baltic Sea. Sub-species of European flounder have
also been described in the Mediterranean Sea, where P. flesus luscus and P. flesus italicus are believed
to have diverged from P. flesus about 2 My ago (Galleguillos & Ward 1982; Borsa et al. 1997), but so
far no detailed study has included the Bank-flounders in the Baltic Sea, which have apparently diverged
much more recently.

Geographical distance
Geographical distance per se does not seem to be very important for structuring populations of
European flounder, since we find no indication of isolation by distance when controlling for
environmental differences between samples. This result is caused mainly by the fact that populations
all the way from the western Baltic Sea to the Irish Sea are very similar genetically but still some of
them are separated by large geographic distances. The effects of other evolutionary drivers as well as
random noise are overriding any structuring effect of geographic distance in the present case. Genetic
structuring has also been found to be significantly associated with environmental parameters rather
than geographical distance in Atlantic herring (Bekkevold et al. 2005; Jørgensen et al. 2005). In these
cases structuring is associated with the marked environmental differences between the Baltic Sea and
the North Sea rather than with transitions within one of the two areas. Several species do show a
pattern of genetic isolation by distance in the North Eastern Atlantic. Geographic distance has been
found to be significantly associated with genetic structure in cod (Hutchinson et al. 2001), plaice
(Haarau et al. 2002a) and Atlantic herring (Mariani et al. 2005). These signals were mainly driven by
single divergent populations in the Barents Sea (cod), Iceland (plaice) and English Channel and Norway
(herring). Structuring among the remaining populations was low and only weakly associated with
geographic distance. A more detailed analysis of potential structuring along a latitudinal cline has not
been possible in these species because of a lack of samples along the cline.

Oceanography and bathymetry
The marked genetic isolation of the Faroe Islands population is in agreement with the expectations
based on oceanographic and bathymetric conditions in this area. Deep trenches and anti-cyclonic
circulation systems surrounding the islands (Jákupsstovu & Reinert 1994; Joensen et al. 2000) suggest
retention of egg and larvae and limited adult migration and thus function as effective barriers to gene
flow. Hoarau et al. (2002a) found that the Icelandic plaice (P. platessa L.) population was isolated from
all other populations in the North Eastern Atlantic and found no evidence of a separate
phylogeographic lineage at Iceland (Hoarau et al. 2004). Both European flounder and plaice are near
the margin of their distributions in these cases. Combined with limited gene flow across the deep areas
in the Atlantic, this pronounced physical isolation of island populations of flatfishes is the most
parsimonious explanation for their genetic isolation.

Environmental differences
We found that environmental differences coincided with population structure in two different cases,
highlighting the importance of these drivers for generating genetic structure in marine fish species. One
case was the abrupt change in genetic constitution in the inner Baltic Sea, while the other was a more
general association between environmental change and genetic structuring along a latitudinal cline in
the Atlantic. In both cases the effect of geographical distance was found to be of minor importance.
The clear isolation of the benthic spawning populations in the inner Baltic Sea is of a similar
magnitude to what has been observed at the transition zone to the Baltic Sea in other marine species
(Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Johanneson & André 2006) and
suggests that the levels of divergence observed in these species represent significant levels of local
adaptation to the Baltic Sea environment. As an euryhaline species the flounder appears to tolerate the
steep salinity gradient at the transition zone and, accordingly, it does not present a major barrier to gene
flow. Instead, the major break is found at even lower salinities, where the gradient is less steep and is
clearly related to the marked change in life-history strategy believed to be an adaptation to the
environment in the inner Baltic Sea. Migration rate estimates from MIGRATE place the bank flounder
population sampled at Turku as a net donor of migrants, which further supports the status of this
population as a self-sustaining local population rather than a sink population.
The association between latitude and genetic structuring, when excluding populations at the
Faroe Islands, Gotland and Turku (benthic spawners) provide further evidence in support of
environmental differences as an important evolutionary force in marine fishes. This suggests that some
environmental differences associated with latitude are more important than geographical distance per se in shaping the genetic relationships among these populations. We can only hypothesize about potential factors of importance, since we do not know the specific environmental agents involved. The temperature gradient, both with respect to mean and variance in annual temperatures, is the most obvious latitudinal gradient in this area. This gradient has been proposed to be important for generating observed patterns of counter gradient variation in fitness related traits found in several marine fish species inhabiting latitudinal clines (Conover et al. 1997; Dimichele & Westerman 1997; Conover 1998; Salvanes et al. 2004). However, other environmental components, such as day length and seasonal cycles, could also be of importance for individual reproductive success.

It should be noted that the observed pattern of isolation by latitude is highly influenced by Lake Pulmanki. This population is divergent from the remaining populations both with respect to genetic and latitudinal differences. The apparent founder event in Lake Pulmanki could bias theses analyses. Exclusion of Lake Pulmanki from the partial Mantel tests does, however, not affect the conclusion of latitude being more important than geography (geography, controlling for latitude: $r=0.1025$, $P=0.339$. Latitude, controlling for geography: $r=0.7926$, $P=0.033$). Furthermore, there is also well supported evidence of a barrier to gene flow to the Bay of Biscay, and hence the pattern of isolation by latitudinal gradients rather than by geographical distance seems to be well supported.

Subtle structure of potential biological significance

The subtle structuring between the North Sea and the central Baltic Sea (Bor) populations appears to be temporally stable and could hence be of evolutionary significance. Migration seems to be asymmetric from the North Sea (Thy) to Bornholm, while Ærø is a net donor of migrants like the large population in the North Sea. This may indicate that Bornholm represents the extreme edge of the Atlantic/North Sea population-complex. Significant structuring indicate some reduction in gene flow between the North Sea and central Baltic Sea, but it is unknown if this reduction is sufficient to allow adaptations to local environments in these areas. However, unpublished results from ongoing work on gene expression and genetic variation in candidate gene loci indicate that the populations of European flounder in the western and central Baltic Sea are subject to different selective pressures and may indeed be genetically adapted to local environments (P. Foged Larsen, Danish Institute for Fisheries Research, personal communication; J. Hemmer-Hansen et al. unpublished results).

As is the case with most studies of genetic structure in marine fish species, it is unknown if the system has yet reached drift-migration equilibrium. Hence, a pattern of little genetic differentiation between populations could simply be the result of a non equilibrium situation rather than a signal of high levels of gene flow. In this case, we would overestimate the levels of gene flow to the central Baltic Sea. However, this study also clearly illustrates that sufficient time has passed to attain very high levels of structuring among other populations of European flounder, so it seems reasonable to conclude that gene flow from the Atlantic into the western and central Baltic Sea is relatively high in European flounder compared to other species in this region.

Conclusions

We found evidence within one single species of marine fish for the simultaneous action of many of the evolutionary drivers which have so far been reported in independent studies for different species. In general, we found the largest genetic distances and reductions in gene flow to populations in the extreme parts of the species’ distributional area. While historical processes associated with colonization following the last glaciation have most likely affected this signal, it is evident that several other
evolutionary drivers are also acting to structure populations of European flounder. The genetic
signature of each driver is not easily predicted but rather seems to be dependent on its interplay with
other drivers in each specific scenario. Interestingly, we do not find any effect of geographical distance
per se. We do, however, find that physical forces such as oceanographic currents and bathymetric
obstacles to migration are coinciding with marked levels of genetic structuring. Moreover, we find that
environmental factors associated with environmental transitions are potent structuring forces in the
marine environment. These drivers are capable of generating patterns of structuring of similar or larger
magnitude as observed between populations with different life-history strategies, supporting the
evidence for the importance of these factors for driving population sub-structuring in marine fish
species.
References


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Author Information Box

This work represents a part of Jakob Hemmer-Hansen’s PhD thesis. He is interested in population structure and evolution of marine fishes in general and the genetic basis of local adaptations in particular. The focus of Einar Eg Nielsen’s research is on population structure and evolution of marine and anadromous fishes. Peter Grønkjær is a fisheries and fish biologist working on population biology and recruitment in marine fishes and Volker Loeschcke has a broad interest in evolutionary genetics and has been working on a number of species form fruit flies to fishes.
Figure legends

Figure 1. Map of sampling locations and major barriers to gene flow. Numbers refer to sample names in Table 1 and barrier letters to barriers identified in Table 2.

Figure 2. Hybrid tree based on Cavalli-Sforza’s chord distance for topology and $(\delta \mu)^2$ for branch lengths. The tree is rooted with the North Sea plaice sample. The distance to the plaice sample is truncated to enhance graphical presentation of the remaining flounder populations. The $(\delta \mu)^2$ distance between the plaice and flounder samples is approximately 1700.
Table 1: Samples of European flounder. Location numbers refer to Figure 1. Mature/maturing individuals are ripe and/or running.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample name</th>
<th>Date</th>
<th>App. position</th>
<th>Sample size</th>
<th>Spawning strategy</th>
<th>Proportion maturing and mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Turku 2003</td>
<td>Tur03</td>
<td>May</td>
<td>22° E, 60° N</td>
<td>54</td>
<td>Benthic</td>
<td>100</td>
</tr>
<tr>
<td>2. Turku 2004</td>
<td>Tur04</td>
<td>June</td>
<td>22° E, 60° N</td>
<td>50</td>
<td>Benthic</td>
<td>24</td>
</tr>
<tr>
<td>3. Gotland 2003</td>
<td>Got03</td>
<td>April</td>
<td>18.5° E, 57° N</td>
<td>46</td>
<td>Benthic</td>
<td>App. 50</td>
</tr>
<tr>
<td>4. Gotland 2004</td>
<td>Got04</td>
<td>June</td>
<td>19.5° E, 58° N</td>
<td>48</td>
<td>Benthic</td>
<td>0</td>
</tr>
<tr>
<td>5. Bornholm 2003</td>
<td>Bor03</td>
<td>March</td>
<td>16° E, 55.1° N</td>
<td>55</td>
<td>Pelagic</td>
<td>100</td>
</tr>
<tr>
<td>6. Bornholm 2004</td>
<td>Bor04</td>
<td>March</td>
<td>16° E, 55.1° N</td>
<td>53</td>
<td>Pelagic</td>
<td>100</td>
</tr>
<tr>
<td>7. Ærø 2003</td>
<td>Aer03</td>
<td>Feb-Mar</td>
<td>10° E, 55° N</td>
<td>52</td>
<td>Pelagic</td>
<td>100</td>
</tr>
<tr>
<td>8. Ærø 2004</td>
<td>Aer04</td>
<td>Mar</td>
<td>12° E, 55° N</td>
<td>52</td>
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<td>100</td>
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<td>9. Thyborøn 2003</td>
<td>Thy03</td>
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<td>8° E, 57° N</td>
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<td>10. Thyborøn 2004</td>
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<td>8° E, 57° N</td>
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<td>19</td>
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<tr>
<td>11. Ringkøbing Fjord 2003</td>
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<td>90</td>
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<td>Rin04</td>
<td>Feb</td>
<td>8.3° E, 55.96° N</td>
<td>50</td>
<td>Pelagic</td>
<td>10</td>
</tr>
<tr>
<td>13. The Limfjord 2003</td>
<td>Lim03</td>
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<td>8.59° E, 56.5° N</td>
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<td>0</td>
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<td>14. Irish Sea 2003</td>
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<td>-4° E, 54° N</td>
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<td>Bis03</td>
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<td>-2.3° E, 47.20° N</td>
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<td>18. Bay of Biscay 2004</td>
<td>Bis04</td>
<td>Okt</td>
<td>-2.3° E, 47.20° N</td>
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<td>20. Lake Pulmanki 2004</td>
<td>Pul04</td>
<td>Jun-Jul</td>
<td>28.02° E, 70.01° N</td>
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<td>Pelagic</td>
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<td>21. Faeroe Islands 2003</td>
<td>Far03</td>
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<td>-6.45° E, 62° N</td>
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<td>Pelagic</td>
<td>84</td>
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<tr>
<td>22. Faeroe Islands 2004</td>
<td>Far04</td>
<td>Feb-Jul</td>
<td>-6.45° E, 62° N</td>
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<td>Pelagic</td>
<td>56</td>
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<td>23. Plaice 2003</td>
<td>Pla03</td>
<td>March</td>
<td>9° E, 57.3° N</td>
<td>18</td>
<td>Pelagic</td>
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Table 2: Location of major barriers to gene flow identified by the program BARRIER. The barrier order reflects the strength of the barrier. The numbers given are the number of bootstrapped $F_{ST}$ matrices supporting a barrier. Note that numbers do not always add up to 100 because some rare barriers are supported by a few matrices. Barrier letters refer to Figure 1. Barrier e) refers particularly to the separation of Trondheim and western Baltic Sea; fewer matrices support the separation of Trondheim and the North Sea. Barrier f) is a barrier between the Thyborøn and Limfjord/Ærø and samples.

<table>
<thead>
<tr>
<th></th>
<th>1st order</th>
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<td>Barrier e</td>
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Table 3 Mantel and partial Mantel tests on a subset of samples, excluding samples from Turku, Gotland and the Faroe Islands.  
Significance was assessed with 1000 permutations.

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<th>Year</th>
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</table>
Table 4. Final estimates of $\Theta$ and $M$ from the three MIGRATE runs. Results with 95% confidence intervals from individual runs are given in supplementary tables S1-S3.

<table>
<thead>
<tr>
<th>Donor population</th>
<th>$\Theta$</th>
<th>$M$</th>
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<tr>
<td></td>
<td>Tur</td>
<td>Bor</td>
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<tr>
<td><strong>First run</strong></td>
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<td></td>
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<tr>
<td>Turku</td>
<td>0.238</td>
<td>17.67</td>
</tr>
<tr>
<td>Second run</td>
<td>0.332</td>
<td>14.53</td>
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<td>Third run</td>
<td>0.321</td>
<td>16.04</td>
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<td><strong>First run</strong></td>
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Figure 1.
Figure 2.
## Appendix A

Estimates of genetic diversity and exact tests for deviation from Hardy Weinberg proportions

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**Locus PI167**

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#### Locus StPf1005

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| Na            | 15    | 17    | 17    | 17    | 14    | 14    | 15    | 14    |
| Size range    | 164-214 bp | 164-222 bp | 166-226 bp | 164-222 bp | 166-210 bp | 158-206 bp | 162-226 bp | 166-210 bp |
| H₀            | 0.833 | 0.864 | 0.897 | 0.86  | 0.945 | 0.857 | 0.771 | 0.867 |
| Hₑ            | 0.886 | 0.907 | 0.895 | 0.909 | 0.891 | 0.893 | 0.891 | 0.904 |
| HWE exact test| 0.1927 | 0.0604 | 0.9784 | 0.2243 | 0.0177 | 0.8516 | 0.0176 | 0.2253 |

| Population    | Bis03 | Bis04 | Tro04 | Pul04 | Far03 | Far04 | Pla03 |
| Na            | 16    | 16    | 17    | 17    | 9     | 10    | 12    |
| Size range    | 166-222 bp | 166-218 bp | 164-214 bp | 164-194 bp | 166-214 bp | 166-214 bp | 262-326 bp |
| H₀            | 0.938 | 0.872 | 0.918 | 0.853 | 0.886 | 0.912 | 0.667 |
| Hₑ            | 0.891 | 0.923 | 0.886 | 0.833 | 0.814 | 0.827 | 0.926 |
| HWE exact test| 0.624 | 0.2471 | 0.8271 | 0.4611 | 0.9708 | 0.1737 | 0.0381 |
## Appendix A continued.

### Locus StPf1015

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**Locus StPf1002**

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### Appendix B: Pariwise F_{ST}s with 95% confidence intervals (above diagonal) and P-values for their significance (below diagonal)

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**Supplementary table S2.** Final estimates and 95% confidence intervals of $\Theta$ and $M$ from the second MIGRATE run.

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Adaptive divergence in a high gene flow environment: *Hsc70* variation in the European flounder (*Platicthys flesus* L.)

Jakob Hemmer-Hansen, Einar Eg Nielsen, Jane Frydenber and Volker Loeschcke

In review for publication in Molecular Biology and Evolution
Adaptive divergence in a high gene flow environment: Hsc70 variation in the European flounder (*Platicthys flesus* L.)

Research Article

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Key words: Local adaptation, adaptive evolution, candidate genes, marine fish, European flounder, Heat shock cognate gene.

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Running head: Adaptive divergence in European flounder
Abstract

Understanding local adaptations and their genetic basis in marine organisms is central to improve our knowledge of how evolution operates in the sea. Marine fishes have recently been subject to a number of studies applying highly variable genetic markers. These studies have often found weak genetic differentiation between local populations. However, only rarely have marine fishes been subject to studies of adaptive population divergence by applying markers potentially under selection. Hence, the extent to which marine fishes are adapted to local environmental conditions is presently unknown. In this study, we applied a candidate gene approach to investigate adaptive population divergence in the European flounder (*Platichthys flesus* L.), a coastal flatfish distributed throughout the Northeastern Atlantic. We contrasted patterns of genetic variation in a presumably neutral microsatellite baseline to patterns from a heat shock cognate protein gene, *Hsc70*, which was chosen as a candidate gene for adaptive population divergence. Using two different neutrality tests, we found that the microsatellite data set most likely did represent a neutral baseline. In contrast, *Hsc70* strongly deviated from neutral expectations, indicating that this locus was under selection or linked to a locus under selection. When estimating standardized levels of population divergence, the discrepancies between the two data sets were particularly striking in pairwise comparisons involving populations in the Western and central Baltic Sea. These results strongly indicate the presence of flounder populations under local selective pressures and they suggest that the populations under study are locally adapted. To our knowledge, this is the first demonstration of the genetic basis of adaptive population divergence in a marine fish species, illustrating that adaptive evolution is possible in marine fishes despite high levels of gene flow. Our results also suggest that the candidate gene approach is potentially very useful for demonstrating local selection in non-model organisms such as most marine fish species.
Introduction

Marine fishes have been subject to an increasing number of population genetic studies applying neutral genetic markers in recent years. These studies have generally confirmed that gene flow between populations of many marine fishes is high (Ward et al. 1994; DeWoody and Avise 1999; Waples, 1998). Since gene flow is expected to hamper adaptive population divergence, these results indicate that local adaptations should be rare or absent in marine fishes. On the other hand, the wide distributions over diverse environments and large effective population sizes of many marine fish species would tend to favor the effects of natural selection over the random effect of drift and the homogenizing effect of gene flow (Palumbi 1994). Despite the high levels of gene flow, a number of studies have identified significant, albeit small, levels of genetic structuring among populations of marine fishes, primarily with the aid of highly variable genetic markers such as microsatellites (Ruzzante et al. 1999; Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Jørgensen et al. 2005; Ruzzante et al. 2006). However, because these markers are presumable neutral any inferences about the evolutionary significance of the results in terms of adaptive population divergence have been based on evaluating the likely conditions under which populations would be allowed to diverge adaptively. Thus, evolutionary scenarios with temporally stable genetic structuring among populations could permit relatively strong local selection to override the effects of drift and gene flow, resulting in temporally stable adaptive population divergence, i.e. local adaptations.

Only rarely have marine fishes been subject to studies of population differences at loci believed to be directly affected by selection. One classical example of a selected gene in marine fishes is the hemoglobin locus in Atlantic cod (*Gadus morhua*). This locus shows clinal allele frequency variation along a latitudinal cline in the Northeastern Atlantic as well as differences between the North Sea and the Baltic Sea (Sick 1965a; Sick 1965b), and physiological experiments strongly suggest that natural selection is maintaining this variation (Brix et al. 1998; Petersen and Steffensen 2003). Few other examples of genes suggested to be under selection in marine fishes can be found (reviewed in ICES 2006) such as the vesicular membrane protein gene Pantophysin, which also shows non-neutral patterns of variation in Atlantic cod (Pogson 2001; Pogson and Fevolden 2003). None of these studies have, however, attempted to compare patterns of population divergence for genes of presumed adaptive value to a background of neutral genetic markers, which is important since it allows for a separation of the effects of natural selection from demographic effects, i.e. migration and genetic drift (Landry and Bernatchez 2001; Moran 2002).

In this study we examine genetic variation in a candidate gene for adaptive divergence of European flounder (*Platichthys flesus* L.) populations in combination with a neutral baseline from genomic microsatellite loci. The European flounder is a wide ranging euryhaline flatfish species inhabiting a large part of the Northeastern Atlantic including the brackish Baltic Sea. As a coastal species inhabiting shallow areas the flounder is more exposed to spatial and temporal environmental differences in parameters such as temperature, salinity and light compared to other species found in deeper and more stable marine habitats. Furthermore, the flounder shows an unusual tolerance to low salinities; e.g. it is often found migrating to rivers for long periods before returning to the sea to spawn. In European waters flounders are exposed to two major environmental gradients. One gradient is a gradual environmental transition with latitude in the Atlantic parts of the distributional area. The other is a more abrupt change between the marine North Sea and brackish Baltic Sea. Flounders in the innermost Baltic Sea are believed to have adapted to the local environment by changing from the normal pelagic spawning strategy to benthic spawning near the coast (Aro 1989).
This adaptation assures that eggs are not exposed to oxygen poor water in the deeper parts of the Baltic Sea. Furthermore, and as seen in other marine fishes in the Baltic Sea, such as cod (Vallin et al. 1999), the flounders in the Western Baltic Sea show distinct physiological characteristics. Thus, increased egg volumes and higher sperm mobility at lower salinities compared to neighboring marine populations in the North Sea are believed to reflect local adaptations to the brackish environment in the Baltic Sea (Nissling et al. 2002; Ojaveer and Kalejs 2005). Accordingly, due to the flounder’s unique geographical and ecological distribution it is very well suited for studying the potential effects of environmental parameters on adaptive divergence among populations.

In this study we use a heat shock protein gene as a candidate gene for local adaptations in the European flounder. Heat shock cognate 70 (Hsc70, sometimes denoted Hsc71) is a member the heat shock protein 70 (Hsp70) gene family of molecular chaperones. These genes have been found in every organism from bacteria and plants to humans and play a central role in the cellular stress response system by assuring correct transport and folding of damaged proteins (Feder and Hofmann 1999). Hsp70s have been found to be expressed in response to a large variety of stressors in fishes, such as elevated and lowered temperature, osmotic stress, radiation and heavy metals (reviewed in Iwama 1998; Basu et al. 2002), illustrating their ubiquitous role in the cellular stress response. The function of Hsc70 is believed to be similar to that of Hsp70 except that these genes are primarily constitutively expressed (Freeman and Morimoto 1996). The few genomic sequences available from fishes show that the Hsp70 genes are highly conserved through fish evolution (Basu et al. 2002), which indicates that they are under heavy selective constraints, probably because of their central role in maintaining cellular homeostasis.

Our aims were to study local adaptations in marine fishes by investigating adaptive genetic divergence among European flounder populations covering the majority of the species distribution. We compared standardized levels of genetic differentiation for a Hsc70 linked indel and a set of neutral microsatellite markers. Despite the great potential for this candidate gene vs. neutral variation approach to disclose the genetic basis of adaptive population divergence among natural populations it has, to our knowledge, never been applied in marine fish species.

Materials and Methods

Sampling and DNA extraction

Samples of European flounder were collected in 2003 and 2004 covering most of the species’ distributional range (Fig. 1 and Table 1). We collected samples form the Baltic Sea (Turku, Gotland, Bornholm and Årø), Danish Fjords (Ringkøbing Fjord and the Limfjord) and the Atlantic/North Sea (Thyborøn, Irish Sea, Bay of Biscay, the Faroe Islands and Trondheim). Finally, we collected flounders from Lake Pulmanki which is connected to the Barents Sea via app. 100 km of the river Teno, through which flounders presumable need to travel to the sea to spawn. DNA was extracted from ethanol stored fin or gill tissue by Chelex (Estoup et al. 1996), DNeasy (Qiagen) and hotSHOT (Truett et al. 2000) methods.

Genome walking and screen for genetic variation

A fragment of the gene coding the European flounder Hsc70 gene was obtained by genome walking using the DNA walking SpeedUp™ kit (Seegene). Primers were designed to walk upstream from aligned sequences of the 5’ end of other fish Hsp70 genes. Obtained sequences were subsequently used to design primers for additional walks upstream and downstream from the known sequence.
Between two and four individuals from different populations were used for an initial screen for genetic variation. These individuals were chosen to cover the total distributional area of the species. All fragments were cloned in the TOPO TA cloning kit (Invitrogen) prior to sequencing.

PCR and genotyping

Nine highly polymorphic microsatellites developed for plaice (LIST1001, GenBank Acc. no. AF149831, Watts et al. 1999; PL142 and PL167, Acc. no’s AF406750 and AF406751, Hoarau et al. 2002a) and European flounder (StPf1001, StPf1002, StPf1004, StPf1005, StPf1015 and StPf1022, Acc. no’s AJ315970, AJ315975, AJ315973, AJ315974, AJ538313, AJ538320, Dixon et al. unpublished) were amplified under standard PCR conditions and genotyped on an ALFExpress automated sequencer (Amersham Biosciences). Other primers were designed to amplify a fragment containing a 21 bp. indel in intron1 of the flounder Hsc70 gene (Forward 5’ - GAG ACA TGT GAG GGA TCC CTC C – 3’; Reverse 5’ - CAT CAT TCT TGC TGG AAA CAA GC – 3’). These fragments were amplified under standard PCR conditions and genotyped on agarose gels.

Statistical analysis

Departures from Hardy-Weinberg equilibrium were tested with the exact test implemented in the software GENEPOP (Raymond and Rousset 1995) for microsatellites and Hsc70 genotypes separately. Temporal stability was assessed by estimating pairwise FST, their confidence intervals (by bootstrapping over loci) and significance between temporal samples within localities in the program FSTAT (Goudet 1995). We calculated G’ST (Hedrick 2005) to facilitate comparisons of population structuring between the microsatellite and Hsc70 data sets. G’ST is a standardized measure of population divergence independent of levels of heterozygosity (Hedrick 1999, 2005). This standardized measure was used because the markers applied in this study (microsatellites versus indel) are expected to differ markedly in levels of heterozygosity and hence in maximum levels of population divergence attainable (see Hedrick 2005). Multidimensional scaling (MDS) plots of G’ST were constructed with the program Vista 5.6.3. (Young 1996) to visualize the relationships among populations. MDS plots were made for microsatellites and Hsc70 separately. Pairwise comparisons of G’ST for microsatellites and Hsc70 were conducted to examine which population pairs showed the largest discrepancy between the two marker types, i.e. where selection could be inferred. For this purpose, we calculated 95% confidence intervals for microsatellite G’ST by bootstrapping over loci. Point estimates of G’ST from Hsc70 were then evaluated in relation to these confidence intervals.

We used two different tests to assess the assumption of microsatellite neutrality, since conclusions regarding the outlier status of particular microsatellite loci are more robust when supported by different analytical approaches (e.g. Vasemägi and Primmer 2005). The simulation based test by Beaumont and Nichols (1996) implemented in the program FDIST2 and the LnRH test by Schlötterer (Schlötterer 2002; Kauer et al. 2003) were both developed to identify outlier loci in genome scans, but differ in their approaches and assumptions in a number of ways. The FDIST2 test is based on the assumption that outlier loci will show increased levels of population structuring if they are under diversifying selection or closely linked to a locus which is, i.e. genetic hitch-hiking (Maynard Smith and Haigh 1974). The method compares actual levels of differentiation at individual loci in relation to heterozygosity to a simulated distribution of loci generated from observed levels of population differentiation. We carried out the simulation under both the Stepwise Mutation Model (SMM), which should be well suited for microsatellites, and the Infinite Alleles Model (IAM), which should describe the mutational process of an indel better. Beaumont and
Nichols (1996) have shown that the type of marker applied (i.e. mutational mechanisms involved) has little effect on the simulated distribution under neutrality. Consequently, this method was used both to evaluate the neutrality of microsatellites as well as the outlier status of Hsc70. Hsc70 was not, however, used to generate the expected neutral distribution, since we expected this locus a priori to be under selection.

The LnRH test assumes that microsatellite loci which are linked to a gene of adaptive importance subject to a selective sweep will show reduced levels of diversity within the populations subject to selection (Schlötterer 2002, Kauer et al. 2003). The LnRH test therefore compares relative levels of heterozygosity between loci in pairwise population comparisons. Since the LnRH test was specifically developed for microsatellite loci it has not been evaluated in situations where mutational mechanisms vary between loci and, accordingly, it was only used to assess the assumption of neutrality of the microsatellite baseline.

Results

Genome walking and screen for genetic variation

The genome walking produced a sequence of 1586 bp. This sequence showed the highest similarity to Japanese flounder (Paralichthys olivaceus) Hsc70 mRNA followed by other Hsc70/Hsc71 and Hsp70 genes in a Blast search (Altschul et al. 1997). Based on an alignment with the Japanese flounder Hsc70 mRNA, we assume that the European flounder sequence contains part of intron1, the entire exon1 and a part of intron2 of a Hsc70 gene. Other genomic sequences from fish contain an untranslated exon0 upstream of the translational start site. This exon is located app. 1640 and 1270 bp upstream in two Hsc70 genes from Rivulus marmoratus (Park et al. 2001; Lee 2004) and app. 1830 bp upstream in rainbow trout (Zafarullah et al. 1992). Based on comparisons with these few available fish genomic sequences and the fact that gene prediction softwares failed to identify any transcriptional start sites in the European flounder sequence (results not shown), we find it unlikely that we have reached this potential untranslated exon0 by the genome walking in European flounder. The partial sequence of European flounder Hsc70 has been deposited in GenBank under accession number XXXXXX. The initial screen for genetic variation identified a 21 bp deletion in intron1 in individuals from the Irish Sea, Turku and Lake Pulmanki. This indel was subsequently genotyped in all individuals.

Genetic variation

Exact tests showed that no locus or population exhibited consistent deviations from Hardy-Weinberg equilibrium (Appendix A). Only one pairwise comparison between temporal samples was statistically significant (Thy03 versus Thy04 for microsatellites, FST=0.006, P=0.04). However, this is not more than expected by chance in sixteen tests and it does not change the overall picture of temporal stability over the two years studied. Temporal samples from the same localities were therefore pooled for the remaining analyses.

Population structure

The overall FST for microsatellites was 0.024 (95% CI 0.018-0.031) and highly significant (P<0.0001), while the overall FST for Hsc70 was 0.139 and also highly significant (P<0.0001). As evidenced by Figure 1, the genetic variation at Hsc70 seemed to be highly unevenly distributed throughout the distributional range. Populations in the Baltic Sea and Lake Pulmanki had very low
frequencies of the shortest allele, while the remaining populations had higher and more variable frequencies of this allele. Examining the relationships between populations, it was evident that the two marker types showed highly discordant patterns of structuring.

The microsatellite MDS plot mirrored the geographical relationship between the populations and revealed a clear separation of populations in the inner Baltic Sea (benthic spawners), Faroe Islands, the Bay of Biscay and Lake Pulmanki (Fig. 2). The Western and central Baltic Sea samples were positioned between the Atlantic and inner Baltic Sea samples, while the remaining populations were grouping together. The MDS plot based on Hsc70 (Fig. 3) identified the sample from Bornholm in the central Baltic Sea as very distinct, reflecting the extremely low frequency of the short allele at this locality (see Fig. 1). Furthermore, there was a clear grouping of populations in the Baltic Sea with Lake Pulmanki. The Faroe Island population was similar to the North Sea populations, while Ringkøbing Fjord, Trondheim and the Bay of Biscay were all quite unique.

The pairwise comparisons in Figure 4 revealed the largest discrepancies between the two data sets in comparisons involving the Western and central Baltic Sea populations showing particular high levels of divergence from the Atlantic and North Sea populations for Hsc70, but high similarity employing microsatellites.

When populations were divided into two major groups based on environmental similarity (Baltic Sea samples and Lake Pulmanki versus all remaining Atlantic Sea samples), there was a highly significant difference in allele frequencies between the two groups (Mann-Whitney U-test, P=0.006). Moreover, allele frequencies were more variable among the populations in the Atlantic group as evidenced by larger standard deviations in this group (0.09 for the Atlantic group versus 0.05 for the Baltic Sea/Lake Pulmanki samples).

Neutrality tests
We found little difference between the results obtained by simulations under the two mutation models (SMM versus IAM) as also noted by Beaumont and Nichols (1996) when analyzing simulated data. Consequently, we report only results obtained under the SMM. Results based on the estimated mean FST of 0.024 (see above) showed that only one microsatellite locus (Pl167) fell slightly outside the upper 95% confidence level, while the Hsc70 locus showed much higher levels of population differentiation given its level of heterozygosity (Fig. 5). We also conducted the simulations for the lower bound on FST (lower confidence level of 0.018), resulting in one additional locus (StPf1002) falling slightly outside the upper 95% confidence interval of the simulated loci. When the simulations were done with the upper bound on FST (0.031), no microsatellite loci were outliers while Hsc70 was still identified as an extreme outlier (results not shown). The LnRH test on all 594 pairwise comparisons (66 population comparisons in 9 loci) revealed no loci as conspicuous outliers (Fig. 6). As expected, some loci were in the outer 5% of the distribution of Standardized LnRH values. These data points were primarily from loci StPf1004, StPf1001, Pl167 and StPf1005 in different pairwise population comparisons.

Discussion
We found that the Hsc70 linked marker was clearly identified as an outlier locus in terms of levels of population divergence when compared to a presumed neutral baseline. The clear discrepancy between signals from the microsatellite baseline and Hsc70 strongly suggests that natural selection is affecting the distribution of genetic variation at Hsc70. In contrast, we found no convincing
evidence for non-neutrality of any of the microsatellites used to represent the neutral baseline in this study. These results imply adaptive population divergence of the Hsc70 gene in European flounder or at a closely linked locus. Importantly, it is evident that such adaptations are possible even in a background of very high levels of gene flow as inferred by the population similarity at microsatellite loci.

Whitehead and Crawford (2006) recently identified adaptive differences in gene expression among highly structured populations of the intertidal Fundulus heteroclitus based on comparisons to a neutral baseline from microsatellites. F. heteroclitus is the most well studied marine fish species with respect to adaptive population divergence (e.g. Schulte et al. 2000; Schulte 2001) but it is not a typical marine species in many respects. There is a deep phylogeographic split between Northern and Southern populations of F. heteroclitus, which are believed to have diverged between 0.5 and 1 Million years ago (Bernardi et al. 1993) and consequently show very large levels of population structuring at neutral markers (Pairwise F_ST ≈ 0.24; Whitehead and Crawford 2006). In contrast, many classical marine fish species (i.e. species with very large effective population sizes, pelagic eggs and larvae and highly mobile adults; Nielsen and Kenchinton 2001) have younger population ages (Grant and Bowen 1998, Árnason 2004, Hoarau et al. 2004) and show much lower levels of structuring between populations (Pairwise F_ST typically not above 0.05, e.g. Ruzzante et al. 1999; Haorau et al. 2002b; Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005). To our knowledge this very large group of marine fishes has until now never been subject to studies aiming at disclosing the genetic basis of local adaptations.

The signal of local selection at Hsc70 is particularly strong in the Western and central Baltic Sea and Lake Pulmanki populations. This may suggest that these populations are locally adapted at Hsc70, or alternatively at a locus closely linked to Hsc70. The populations in the Western and central Baltic Sea (Ærø and Bornholm) are very similar to the North Sea and Irish Sea populations when assessed with neutral microsatellites, but markedly different at the Hsc70 linked marker. The grouping of Lake Pulmanki with the Baltic Sea populations with respect to Hsc70, but not microsatellites, further supports the conclusion that selection is involved in shaping the inter-population relationships at Hsc70 since Lake Pulmanki share many environmental characteristics with the Baltic Sea, such as low winter temperatures, fluctuating seasonal temperatures and low salinity. Finally, we note that variation in Hsc70 allele frequencies is much larger among the Atlantic Sea samples than among the Baltic Sea and Lake Pulmanki samples. This further supports the conclusion that directional selection is stronger at the latter localities, thereby reducing the overall variation in allele frequencies among populations. The apparent reduction in selective pressures in the Atlantic Sea would thus allow allele frequencies to vary more freely around 0.5.

Based on the results from this study we are not able definitively to point to a specific environmental variable driving selection at Hsc70. However, variation in a number of environmental parameters can be found within the studied area. In the narrow zone connecting the marine Atlantic and the brackish Baltic Sea there is a drastic environmental transition with respect to temperature and salinity in particular. Generally, both annual and intra-annual temperature variations are larger and annual minimum temperatures are lower and in the Baltic Sea than in the nearby North Sea (e.g. Becker and Pauly 1996; Siegel et al. 2006). Therefore, temperature fluctuations could be an important selective agent driving adaptive divergence at Hsc70, since both Hsp70 and Hsc70 have been found to be expressed in response to temperature changes in fishes (Basu et al. 2002; Deane and Woo 2005; Fangue et al. 2006). An alternative selective driver could be annual minimum temperatures. Although the majority of studies on heat shock gene expression conducted so far have concentrated on elevated temperatures, heat shock proteins have also been shown to be induced by
cold stress in fishes (Iwama et al. 1998; Ali et al. 2003, Place et al. 2004; Place and Hofmann 2005a). Studies done in some Antarctic notothenoids suggest that these species may have a very high need for cellular protection against denatured proteins, since it appears that their Hsp70s have shifted from an induced to a constitutive expression pattern (Place et al. 2004). Furthermore, the Hsc70s from the same group of species have been found to retain chaperoning activity at lower temperatures compared to temperate species in the same genera (Place and Hofmann 2005b; Place and Hofmann 2005c), suggesting adaptation to cold environmental conditions at the molecular level. While these species inhabit extremely cold environments of sub-zero temperatures, less extreme conditions have also been found to induce Hsc70 in other species. Ali et al. (2003) have, for instance, shown that expression of Hsc70 was increased 7.5 – 10 fold in carp muscle when fish were exposed to a temperature drop from 12°C to 5°C, indicating that Hsc70s may also have an important role in protecting cells against cold stress in species exposed to less extreme temperatures in nature.

Ambient salinity is a second and very important environmental parameter distinguishing the brackish Baltic Sea and freshwater Lake Pulmanki populations from the remaining marine populations in this study. Osmotic stress has been found to induce heat shock protein expression in fish (Smith et al. 1999; Deane and Wo 2004). Thus, Sea bream (Sparus sarba) has been found to induce Hsc70 expression in response to both hypo- and hyperosmotic conditions indicating a central role for Hsc70 in the response to osmotic stress in fishes (Deane and Wo 2004).

A third potentially important environmental component is aquatic pollution, since heat shock proteins have also been found to be induced in response to elevated levels of e.g. heavy metals (Basu et al. 2002; Ali et al. 2003). However, while the Baltic Sea is quite heavily polluted this is highly unlikely to apply to Lake Pulmanki, since this lake is located far from any major urban areas. We therefore regard it as more likely that temperature and/or salinity is the selective agent involved. Since these parameters are highly correlated among the samples in the present study, we are not able to differentiate between them. Indeed they may not be mutually exclusive but could both be involved in generating the observed distribution of genetic variation at Hsc70.

Interestingly, the distribution of genetic variation at Hsc70 is not perfectly correlated with temperature and/or salinity parameters, since the innermost Baltic Sea populations (Gotland and Turku), which experience the most extreme environmental conditions, i.e. lowest temperatures and salinities, do not have the most extreme Hsc70 allele frequencies. These populations are believed to have adapted to the extreme environment in the inner Baltic Sea by changes in spawning strategy from spawning pelagic to benthic eggs (Aro 1989; Drevs et al. 1999). Hence, the Bornholm sample represents the most extreme Baltic Sea sample of populations with the “normal” life-history strategy. These results suggest that selection at or near Hsc70 is very strong in the pelagic spawning populations in the Western and central Baltic Sea, while less intense in the benthic spawning populations. This could indicate that Hsc70 acts as a “first defense” against the stressful conditions at low/fluctuating temperatures and/or low salinities, while more complex biochemical adaptations in addition to the known behavioral adaptations could be found in populations at the more extreme environmental conditions.

Other studies conducted on fishes have found relatively high levels of structuring at neutral markers in the transition zone between the North and Baltic Seas (Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005; Johanneson and André 2006). These results have generally been believed to reflect restrictions in gene flow between populations. The results from the present study differ from previous findings in that levels of neutral structuring across this transition zone are relatively low, implying higher levels of gene flow between flounder populations in the two regions. Still, we have demonstrated that populations of European flounder in the Western and central Baltic
Sea are under heavy local selective pressures at a candidate locus for environmental adaptation and consequently probably locally adapted. These results indicate that the high levels of structuring at neutral markers observed in this region in other species are indeed indicative of strong barriers to gene flow caused by local adaptations.

While the Hsc70 allele frequencies in Lake Pulmanki are as extreme as in the Baltic Sea, it is more difficult to interpret this signal. Since these fish are most likely a component of the Barents Sea population, the signal could be reflecting allele frequencies in the Barents Sea or be specific to Lake Pulmanki, i.e. reflecting selective constraints on fish migrating to the lake. However, without a Barents Sea reference population it is not possible to know where the signal was generated.

The use of different marker types in the present study could introduce statistical and interpretational problems, particularly related to downward bias of microsatellite divergence between the North and Baltic Seas caused by e.g. size homoplasy (Estoup et al. 2002) and departure from migration-drift equilibrium (see e.g. Pogson et al. 2001). However, it appears highly unlikely that these mechanisms should have had a major impact on the patterns observed for the following reasons. First of all, it is clear that some populations of flounder are strongly differentiated (e.g. pairwise microsatellite G’ST values between 0.1 and 0.2), which shows that enough time has elapsed to allow populations to diverge significantly. Furthermore, the fact that many marine organisms are highly structured at neutral markers across the same transition zone imply that time has been sufficient to structure populations of other species in this specific environmental setting. The diverging pattern observed for European flounder could thus be explained by either unique mutational properties for flounder microsatellites (e.g. extreme size homoplasy and/or mutation rates) or simply higher levels of gene flow in flounder compared to other marine fishes. The first explanation would require very extreme mutational properties for flounder microsatellites, for which there is no evidence. On the contrary, observed levels of variability, numbers of alleles and allele size ranges are similar to what has been found in other marine fishes. It is, however, well known that European flounder displays a unique tolerance to low salinities compared to other marine species. This would explain why flounders are less structured across the steep salinity gradient in the transition zone compared to other species in the area. It therefore seems likely that the similarity of the North Sea and Western/central Baltic Sea flounder populations with respect to microsatellites truly reflects high levels of gene flow rather than non-equilibrium situations or microsatellite mutational mechanisms. Finally, it should be noted that ascertainment bias could have influenced the observed pattern of variation in Hsc70. However, the Hsc70-polymorphism was initially identified in several individuals from different populations. Furthermore, as evidenced by the microsatellite data set, the populations which are grouping with respect to Hsc70 apparently belong to very different population components within the species. Thus, the grouping with environmental similarity rather than with geographic or historical proximity strongly suggest that a biological or evolutionary explanation is more likely responsible for generating the observed pattern than is ascertainment bias.

In summary, we cannot rule out that the potentially confounding mechanisms mentioned above may have influenced the patterns of structuring observed in this study. However, their effects are most likely very modest and they cannot account for the very clear picture of a discordant distribution of genetic variation between the two marker types. Local selection remains the single most likely explanation for the observed patterns, which is suggesting local adaptations in the populations under study.

Our study thus strongly suggests adaptive evolution even in a background of high gene flow. Since many marine fishes demonstrate very low levels of genetic differentiation, local adaptations
may be much more widespread in the marine environment than previously believed from evaluating the distinctness of marine fish populations from neutral markers alone. Still, there is a need for further studies confirming the generality of the patterns observed here. Such knowledge of the scale and magnitude of local adaptations in marine fishes is central to improve our understanding of how evolution operates in the sea. Furthermore, knowledge of the genetic basis of local adaptation will add to our ability to manage biodiversity efficiently in relation to human impact such as exploitation and global warming by improving our ability to predict how distribution and abundance of species will change in response to human mediated evolutionary forces.

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1 Young FW. 1996. ViSta: the visual statistics system. Research Memorandum 94-1(b) 2d. ed. L.L.

2 Thursone Psychometric Laboratory, University of North Carolina, Chaper Hill, NC.

4 cloning and characterization of a constitutively expressed heat-shock-cognate Hsc71 gene from
Figure legends

Figure 1. Map of sampling locations and \(Hsc70\) allele frequencies. Sample numbers refer to sample names in Table 1. Black proportion in pie charts denotes frequencies of the long allele.

Figure 2. Multidimensional scaling plot of \(G'_{ST}\) (Hedrick 2005) calculated based on the microsatellite data set. Sample names refer to Table 1.

Figure 3. Multidimensional scaling plot of \(G'_{ST}\) (Hedrick 2005) calculated based on the \(Hsc70\) data set. Sample names refer to Table 1.

Figure 4. Pariwise point estimates of \(G'_{ST}\) for microsatellites (♦) with confidence intervals calculated by bootstrapping over loci and point estimates of \(G'_{ST}\) for \(Hsc70\) (□).

Figure 5. Results from the simulations with the FDIST2 program under the Stepwise Mutation Model. Shown is the distribution of \(F_{ST}\) values (mean and 95% confidence intervals) from 500000 simulated loci as well as the actual values of individual microsatellite loci (○) and \(Hsc70\) (□).

Figure 6. Results from the LnRH tests. Shown are all pairwise population comparisons by microsatellite loci. Locus Pl42 (■), StPf1004 (◇), List1001 (♦), Pl167 (▲), StPf1015 (▲), StPf1005 (□), StPf1022 (○), StPf1002 (x) and StPf1001 (●). Dashed lines represent +/- 1.96*σ.
Table 1. Location and sample size of sampled populations.

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<th>Sample year</th>
<th>Approximate position</th>
<th>Total sample size</th>
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Figure 2
Figure 3
Figure 4
## Appendix A.

### Locus PI142

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<th>Bornholm</th>
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<th>Thyborøn</th>
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### Locus List 1001

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### Population The Limfjord Irish Sea Bay of Biscay Trondheim Lake Pulmanki Faroe Islands

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### Population The Limfjord Irish Sea Bay of Biscay Trondheim Lake Pulmanki Faroe Islands

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## Locus StPf1005

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</table>

Population The Limfjord Irish Sea Bay of Biscay Trondheim Lake Pulmanki Faroe Islands

| Ho         | 0.735 | 0.542   | 0.618    | 0.714 | 0.667    | 0.180            |
| He         | 0.600 | 0.634   | 0.543    | 0.678 | 0.615    | 0.185            |
| HWE exact test | 0.2385 | 0.3307  | 0.3896   | 0.9617 | 0.4011   | 0.1977           |

## Locus StPf1022

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Population The Limfjord Irish Sea Bay of Biscay Trondheim Lake Pulmanki Faroe Islands

| Ho         | 0.941 | 0.819   | 0.909    | 0.918 | 0.853    | 0.920            |
| He         | 0.892 | 0.887   | 0.915    | 0.886 | 0.833    | 0.820            |
| HWE exact test | 0.1036 | 0.0344  | 0.2568   | 0.8531 | 0.4852   | 0.1488           |
### Appendix A continued

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Population | The Limfjord | Irish Sea | Bay of Biscay | Trondheim | Lake Pulmanki | Faroe Islands |
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Population | The Limfjord | Irish Sea | Bay of Biscay | Trondheim | Lake Pulmanki | Faroe Islands |
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<tbody>
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Manuscript III

Evidence of genetic introgression from plaice (*Pleuronectes platessa* L.) to European flounder (*Platichthys flesus* L.) populations

Jakob Hemmer-Hansen and Einar Eg Nielsen

In preparation
Evidence of genetic introgression from plaice (*Pleuronectes platessa* L.) to European flounder (*Platichthys flesus* L.) populations

Jakob Hemmer-Hansen¶*#ψ and Einar Eg Nielsen#

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* Department of Marine Ecology, University of Aarhus, Finlandsgade 14, 8200 Aarhus N, Denmark

# Danish Institute for Fisheries Research, Department of Inland Fisheries, Vejlsøvej 39, 8600 Silkeborg, Denmark

Key words: inter-specific hybridization, genetic introgression, F1 hybrids, flatfish, microsatellites, individual admixture analyses.

ψ Corresponding author. Tel. +45 89423315, Fax +45 89422722, email jhh@difres.dk
Introduction

Inter-specific hybridization and genetic introgression has been documented and analyzed in detail in a large number of terrestrial animals (e.g. Goodman et al. 1999; Beaumont et al. 2001; Payseur and Nachman 2005; Verardi et al. 2006) and plants (e.g. Rieseberg 1999; Rieseberg et al. 2003, review in Arnold et al. 1999). Classical hybrid zone theory typically regard hybridization as an evolutionary dead end because different models of stable hybrid zones assume that hybrids are either less fit than both parental species (e.g. tension zones, Barton and Hewitt 1985) or only more fit at intermediate environments in restricted geographic regions (e.g. bounded hybrid superiority, Moore 1977). Recently, a number of examples have illustrated that hybridization may be an important source of evolutionary novelty (e.g. Arnold et al. 1999; Salzburger et al. 2002; Rieseberg et al. 2003; Seehausen 2004,) and hence hybridization is no longer by default regarded as a detrimental process (Arnold et al. 1999). In contrast, the outcome of hybridization events appears to be highly dependent on ecological and evolutionary contexts (e.g. Arnold et al. 1999; Barton 2001). However, one potential outcome of intensive genetic introgression could be the break down of species barriers (e.g. Taylor et al. 2006) or the prevention or prolongation of complete species’ splits (because of continuous events of gene flow), since introgression by definition involves an integration of species’ genomes.

Although several marine fishes have characteristics, such as wide distributions, mass spawning and external fertilization, which should make this group of species good models to study hybridization in the marine environment, quite few marine fishes have been subject to detailed studies (Gardner 1997). Even fewer studies have examined genetic introgression among marine fish species (Gardner 1997), probably because backcrosses are often difficult to identify based on morphological characters alone. However, the advent of molecular markers suitable for identifying hybridization beyond the first generation has resulted in a number of recent studies confirming hybridization and introgression between marine fish species in nature. Interesting examples include redfish (Roques et al. 2001), rockfish (Seeb 1998; Buonaccorsi et al. 2005), eels (Albert et al. 2006) and flatfish (She et al. 1987). In most examples, hybridization was found to be geographically restricted, reflecting the contact zones between species with different geographical or ecological distributions. Apart from the interesting case of hybridization between European and American eels (Albert et al. 2006), introgressive hybridization in marine fishes has only rarely been investigated in species which are potentially able to hybridize throughout most of their reproductive ranges.

In this study, we examine genetic introgression between two closely related flatfish species, European flounder (Platichthys flesus L.) and plaice (Pleuronectes platessa L.), sharing a large part of their geographical distribution. Specifically, we are investigating introgression from plaice to European flounder populations throughout most of the flounder’s distributional range in order to assess the spatial dynamics of introgression from plaice to flounder.

European flounder and plaice are both coastal Pleuronectid flatfishes inhabiting the Northeastern Atlantic - overlapping distributions. The major difference between their distributions is in the Baltic Sea, where flounders are found in the innermost parts while plaice are only found in the westernmost parts of the brackish sea. Although closely related, there is a major biological difference in the ability of the species to tolerate reduced levels of salinity; while plaice is a typical marine species European flounder tolerates brackish or even fresh water for long periods of time and particularly juvenile stages are often found in fresh water. Spawning localities and times have been investigated rather extensively in North Sea plaice by egg and larval surveys (Harding et al. 1978) and tagging experiments (Hunter et al. 2003) revealing that major spawning ground are located in the Southern North Sea and that plaice seem to home to specific spawning areas. Early larval stages of European
flounder are found at the same locations in the North Sea and English Channel (Campos et al. 1994, Grioche et al. 1999) indicating that flounder and plaice may use the same spawning habitats. Both species spawn in early spring but peak spawning of flounder seems to be delayed a few months compared to plaice. The same patterns have been observed in the Danish Belt Sea connecting the North Sea to the Baltic Sea (Heegaard 1947). Thus, it seems reasonable to conclude that at least time and place of reproduction in the two species are overlapping to some extend, providing opportunities for hybridization.

Naturally occurring hybrids between the two species have been known for more than hundred years (Gottsche 1835 reviewed in Ubisch 1953), and they appear to be easily recognized based on their intermediate phenotypes (e.g. Pape 1935; Sick et al. 1963). Both F1 and backcrosses have been produced in the laboratory (Pape 1935; Ubish 1953; Sick 1973), which shows that the species are at least capable of hybridizing beyond the first generation. In an early study, Sick et al. (1963) investigated hemoglobin patterns in both parental species as well as in putative hybrids from the Danish Belt Sea. They found distinct differences between the three classes, confirming the presence of F1 hybrids in nature and also identified a single aberrant individual which could have been a later generation hybrid. Sick et al. (1963) suggested that hybridization is most frequent in the Western Baltic Sea and in the Danish Belt Sea, but that F1 individuals may reproduce inefficiently in nature resulting in limited genetic introgression between the two species. However, the geographical extend of hybridization has so far not been thoroughly investigated. Furthermore, the degree of introgression in natural populations has not been investigated, and hence it is generally unknown if the species hybridize beyond the first generation in nature.

In this study, our aim was to assess the magnitude and geographical distribution of genetic introgression from plaice to European flounder. Specifically, our purpose was to search for potential later-generation hybrids among putative flounders caught throughout the distributional range of the species. We applied highly variable microsatellite markers, which are well suited for detecting later generation hybrids, and a combination of individual admixture analysis and data simulations to detect signals of introgressive hybridization between the two species.

Materials and methods

Samples

A total of 1093 European flounder were collected in 2003 and 2004 from localities covering most of the distributional range of the species. Detailed population genetic analysis of these population samples have been submitted for publication. In order to assess the level of introgression from plaice to flounder, the flounder samples were supplemented with a sample of 49 plaice from the North Sea collected in 2003 and a sample of 9 putative hybrids collected in the Bay of Århus in 2006 (Figure 1 and Table 1). Fin clips or gill tissue was stored in 95% ethanol before DNA extraction with DNeasy (Qiagen), Chelex (Estoup et al. 1996) or HotSHOT (Truett et al. 2000) methods.

Microsatellite analyses

We used three microsatellite loci developed for plaice (List1001, Watts et al. 1999 and Pl142 and Pl167, Hoarau et al. 2002a) and five developed for European flounder (StPf1001, StPf1002, StPf1004, StPf1005, StPf1022, Dixon et al. unpublished) in the present study. These loci worked well in both species with minor modifications of PCR conditions. PCR products were analyzed on an AlfExpress automated sequencer (Amersham Biosciences).
**Statistical analyses**

Genetic diversity within samples was assessed by observed (Ho) and expected (He) levels of heterozygosity, number of alleles, allele size ranges and allelic richness (ElMousadik and Petit 1996) estimated with the program FSTAT (Goudet 1995). Conformances to Hardy-Weinberg expectations were tested for each locus in ARLEQUIN (Excoffier et al. 2005) and null allele frequencies according to Chakraborty et al. (1992) were estimated with the program MICROCHECKER (van Oosterhout et al. 2004). The distribution of genetic variance within and between species was assessed by the Analysis of MOlecular VAriance (AMOVA) framework implemented in ARLEQUIN. Pairwise estimates of Weir and Cockerham’s (1984) $\theta$ were estimated in FSTAT.

We used the model based Bayesian clustering algorithm implemented in the software STRUCTURE (Pritchard et al. 2000) to assess the population and individual levels of genetic introgression, specifically focusing on introgression from plaice to flounder. We first ran STRUCTURE on the total data set representing all sampled individuals in order to estimate the most likely number of populations. This was done by estimating the likelihood under assumptions of the number of populations (K) ranging from 1 to 8 in 10 independent runs for each K. Subsequently, the flounder samples were grouped into corresponding populations based on their population level admixture proportions. Throughout we will refer to the groups identified by STRUCTURE as “populations” and the sampled plaice and flounder individuals as “samples”.

Other studies have compared levels of introgression in populations in allopatry and in sympathy in order to separate signal from noise, i.e. assess if the signals detected reflect true introgression rather than random noise or population sub-structure (see Borge et al. 2005 for a recent example). However, this approach was not possible in the case of plaice-flounder introgression because the two species are found at the same locations throughout most of the geographical range covered. The inner Baltic Sea is the only place, where allopatric flounder populations could be expected, but these populations of flounder are highly differentiated from the populations in the remaining study area (see results) and hence they could not be used as an unbiased non-introgressed baseline. Therefore, we approached the question through comparisons of real and simulated data as outlined below. First, we simulated 1000 individuals from each of the four populations (three flounders and one plaice, see results) identified by STRUCTURE. This was done by randomly drawing alleles from the average allele frequency distributions estimated by the first ten program runs. Subsequently, these simulated populations were used to generate simulated F1 and backcrossed hybrids in all three flounder populations (100 individuals in each category) in the program HYBRIDLAB (Nielsen et al. 2006). These simulated individuals (4600 in total) were then included with the real sample data in a second round of ten STRUCTURE runs in order to compare admixture proportions between real and simulated data. The number of populations was fixed at four, since this was the number originally identified by STRUCTURE. If the original data set was truly structured, and if STRUCTURE was able to pick up the signal, we would expect the simulated data to show higher levels of population structure (i.e. less introgression) than the original data set, because the program would have identified immigrants and introgressed individuals as belonging to a different population than the one in which they were placed. Therefore, we assumed that a smaller level of introgression in a simulated population (for instance from simulated plaice to simulated Atlantic flounders) indicated that the signals detected among the real data (i.e. levels of plaice-introgression in the real Atlantic samples) were of biological significance rather than just random noise. Averages over the ten runs were also used to compare individual admixture proportions in all flounder samples in a boxplot, allowing an assessment of the distribution of outliers (i.e. high plaice admixture proportions) within and between populations. We chose to focus our analyses on F1 and backcross hybrid categories only, since these categories represent realistic targets.
given the levels of genetic divergence between the plaice and flounder samples and the fact that a relatively modest number of loci were used in this study (Vähä and Primmer 2006).

To assess the power of the approach for detecting individual hybrids, we evaluated the distribution of individual level admixture proportions in the simulated parental and backcrossed individuals as suggested by Vähä and Primmer (2006). This was done in order to set a q-value threshold above which individuals could reliably be considered hybrids rather than miss-classified parentals. The thresholds were chosen in order to balance the false positive rate (i.e. parentals wrongly identified as hybrids) with the expected number of missed backcrosses at the threshold.

Results

Genetic diversity

Overall genetic diversity was high in both flounder and plaice samples (He between 0.277 and 0.934 in flounder and between 0.562 and 0.960 in plaice). The plaice sample tended to be more variable than most flounder samples for the majority of the eight loci as also evidenced by higher allelic richness in the plaice sample (Appendix A). No flounder population deviated systematically from Hardy Weinberg expectations, while the plaice sample deviated significantly in three (StPf1001, StPf1004 and StPf1005) and was nearly significant in two (Pl142 and StPf1002) of the eight loci (Appendix A). These deviations from Hardy Weinberg expectations were caused by heterozygote shortage potentially because of null alleles. The deviation was particularly pronounced at locus StPf1004 with a null allele frequency of 0.2268. Locus StPf1005 also showed evidence of null alleles with frequencies of 0.0384. However, while null alleles may lower power to detect hybrids, they may not necessarily be a major problem for the purpose of hybrid identification, since their effects were distributed over all alleles. We therefore retained all microsatellite loci for further analyses.

Plaice and flounder had overlapping allele size ranges for all except one locus. Thus, alleles at locus StPf1022 were roughly 100 bp longer in plaice than in flounder. Although size ranges were overlapping among the remaining loci, the two groups did have different allele frequency distributions for the majority of loci (Appendix B).

Population structure

Results from the AMOVA showed that a much larger proportions of the genetic variation was distributed between species (16.06%, P=0.0000) than between samples of European flounder (2.12%, P=0.0000). These results thus also show a significant genetic structuring of flounder samples, albeit of minor magnitude than the structuring between species.

Results from the 10 independent STRUCTURE runs were highly consistent and all returned K=4 as the most likely number of populations represented by the data. These groups corresponded to one plaice and three flounder populations (Faroe Islands, Turku and Gotland in the inner Baltic Sea and all remaining Atlantic/Western Baltic Sea samples), confirming the structuring of flounder populations detected by the AMOVA. We refer to these three sample groups as “Faroe Islands”, “Atlantic” and “Baltic”.

Power analyses

Evaluating the different false positive rates with respect to power of detecting hybrid individuals (Table 2) it was evident that power to detect F1 individuals in all populations was very high. Among the backcrosses, the proportion of missed individuals was only reduced substantially when moving from a
false positive rate of 0.1% to 5% in the Atlantic and Baltic populations. However, at a false positive rate of 5%, the q-value cut-off for designating individuals as hybrids was very low in these populations (about 0.015). Since we were more interested in examining geographical differences in the level of introgression than in identifying every hybrid present in the data set, we chose a q-value cut-off for designating individuals as introgressed at the more restrictive false positive rate of 1%. This was done because this cut-off represented a reasonable compromise between power to detect hybrids and expected number of false positives (i.e. about 8 individuals among the samples in the Atlantic population, 2 in the Baltic population and below one in the Faroe Islands population).

Introgression

STRUCTURE identified all presumed F1 individuals as admixed between the flounder and plaice groups, having a plaice admixture proportion of approximately 0.5. The nine individuals were primarily identified as hybrids between plaice and Atlantic flounders, but some also had a contribution from both Baltic and Faroe flounders (Figure 2). The apparent split of the flounder proportion into three populations most likely reflects a lack of statistical power than true introgression among the flounder groups.

When including both simulated and real data in the same STRUCTURE runs (ten independent runs), there was a consistent difference in the levels of population admixture between the simulated and real data sets (Table 3). When examining the introgression between the three inferred populations of flounder it was evident that the real data set contains considerable more admixture than the simulated data set, reflecting genetic introgression among the real flounder samples. With respect to introgression from plaice to flounder, a signal of introgression was only present in the Atlantic population, since this population showed less introgression in the simulated compared to the real data set. In contrast, both Faroe and Baltic flounder appeared to show little signs of introgression from plaice. Furthermore, there was no signal of introgression from any of the flounder groups to plaice, despite the fact that absolute levels of population admixture from flounder to plaice were larger than from plaice to flounder (Table 3).

Apparent levels of population introgression from plaice to the Atlantic flounders was low, averaging 0.9% over the ten STRUCTURE runs (Table 2). Among the putative flounders, one individual from the Westerschelde estuary (Wes_2) had a plaice q-value of app. 0.5 indicating that this individual was a hybrid. The multi-locus genotype of this individual (Pl142: 147/165, StPf1004: 154/166, List1001: 84/86, Pl167: 170/190, StPf1005: 99/103, StPf1022: 194/262, StPf1002: 177/177, StPf1001: 251/251) was fully compatible with having one allele from each parental species, suggesting that it was most likely a first generation hybrid. In addition to this single individual, a number of flounders showed signs of having a portion of their genome of plaice ancestry. This signal was particularly evident among the Atlantic samples as evidenced by the number and magnitude of outliers in these samples (Figure 3). Among the Atlantic samples only the Thyborøn and the Limfjord samples did not show signs of significant outlier individuals, while very few apparent outliers were present in the Baltic Sea samples (Gotland and Turku) and in the Faroe Islands population. The differences in individual admixture proportions between populations was statistically significant (Atlantic versus Baltic, P=0.0001; Atlantic versus Faroe, P=1.1E-30, Baltic versus Faroe, P=5.6E-11, Mann-Whitney U-tests). These findings are in accordance with results from the population level admixture proportions (Table 3).

Table 4 lists all individuals with q-values above the q-value threshold corresponding to a false positive rate of 1%. There were no individuals above the threshold in either of the Faroe or Baltic samples, reflecting the absence or very limited extend of introgression in these populations. Besides the
putative F1 hybrid, Wes_2, twenty individuals from the Atlantic populations had q-values above the threshold. These were distributed among several of the populations in this group, mirroring results from the boxplot in Figure 3. Approximately eight of these outlier individuals were expected to be false positives at a false positive rate of 1%, which indicates that 1.5% (12/817) of the individuals in the Atlantic population had a portion of their genome of plaice ancestry.

Discussion

The results from this study have documented the presence of first generation hybrids between plaice and European flounder as well as of backcrosses to flounder in natural populations of flounder. Comparisons of simulated and real data and the geographical distribution of introgression suggest that the signal of introgression is most likely a true biological signal rather than random noise. Likewise did introgression not simply seem to reflect the genetic distance between flounder and plaice samples or the level of genetic variation within flounder samples, since samples closest to plaice (Trondheim, Thyborøn, Westerschelde) were also among the most variable and did not show signs of increased levels of introgression (Appendix A and Figure 3). Furthermore, increased variation in these samples would be expected to increase the overall level of population introgression (i.e. all individuals appearing more admixed) rather than resulting in an increased number of outliers which was actually observed. Hence, these results show that genetic introgression does occur from plaice to European flounder in nature. Importantly, evidence of introgression was found through most of the geographical range surveyed in this study. This suggests that hybridization is occurring wherever the two species co-occur and not specifically in e.g. the Western Baltic Sea which has previously been identified as a hybridization hot-spot because of the observation of relatively large numbers of first generation hybrids (Sick et al. 1963). It should be noted that our results can not be taken as an estimate of the frequency of first generation hybrids in nature, since sampling was specifically targeting presumed pure flounders and may hence have avoided collecting F1 hybrids. In contrast, they are expected to reflect true levels of introgression beyond the first generation, because backcrosses are generally impossible to separate from pure individuals based on phenotypes alone. Our results add to the increasing number of recent studies confirming that hybridization may be a common phenomenon in marine fishes (Gardner 1997; Seeb 1998; Roques et al. 2001; Bounaccorsi et al. 2005; Albert et al. 2006; Wilson 2006; Yaakub et al. 2006), but differ from most previous studies by investigating introgression between species which are potentially able to hybridize throughout most of their distributional ranges.

The rate of introgression from plaice to flounder seems to be relatively low as evidenced by low levels of population admixture (0.9%) as well as the relatively few individuals which were identified as significantly introgressed (1.5%) among the flounders. These results are in contrast to some of the studies of introgression between marine fish species conducted so far. Thus Roques et al. (2001) found levels of introgression of 15% between redfish species in the Northwestern Atlantic. Similarly, extensive hybridization and introgression beyond the first generation has recently been found between American and European eels at Icelandic localities (Albert et al. 2006).

The relatively low introgression from plaice to European flounder may be surprising given that time and place of spawning is presumably largely overlapping and that the species are fully capable of producing viable and fertile offspring as evidenced by both field and laboratory studies (Pape 1935; Sick et al. 1963; This study). This indicates that selection is preventing extensive introgression from plaice to flounder thereby maintaining species barriers. Selection against hybridization can occur at both pre and postzygotic stages. Although time and place of spawning may be overlapping, spawning
behavior could limit the extent of hybridization between the two species. Flatfishes are known to exhibit courtship behavior preceding spawning, often involving pairing of male and female followed by ventral mounts (Forster 1953; Stoner 1999). Some studies have also suggested assortative mating by female choice (Carvalho et al. 2003). Although such barriers to gene flow have not been reported specifically with respect to plaice-flounder hybridization, it is clear that spawning behavior in flatfish is complex and could hence serve as an important barrier to gene flow between species.

However, F1 hybrids have been reported to constitute a relatively large part of flounder catches in some areas (Pape 1935; Sick et al. 1963), indicating that these pre-zygotic barriers, if present, are far from complete in nature. Since frequent hybridization has been reported in these areas for more than hundred years, there is little reason to believe that these patterns should have changed to a lower frequency of F1 hybrids today. Nevertheless, we find little evidence of large levels of introgression in these same areas which indicates reduced reproductive fitness of first generation hybrids in nature (e.g. McGinnity et al. 2003). An alternative explanation, not excluding the first, could be outbreeding depression caused by the break-up of co-adapted gene complexes in later generation hybrids (Burton et al. 1999; Edmands 1999; Gharrett et al. 1999). The most pronounced difference between the life histories of the two species is the ability of flounders to tolerate fresh water for prolonged periods of time. Juvenile flounders prefer sites with fresh-water run off and are often migrating to rivers and lagoons where they spend the first few years of their lives (Kerstan 1991; Bos 1999), whereas juvenile plaice are found in coastal marine nursery areas (Zijlstra 1972). It is plausible that selection would act against plaice genotypes not adapted to fresh water at this life stage, thereby preventing their introgression in the flounder genome. However, since the fitness of hybrids have never been studied in detail it is difficult to point to specific mechanisms responsible. Nevertheless, it appears highly likely that selection must be involved in preventing extensive gene flow between the two species.

It should be noted that the estimates of the number of introgressed individuals are most likely underestimates, since we expect to miss app. 10% of the simulated backcrosses in the Atlantic populations at a q-value threshold of 0.036. It is clear from simulation studies (e.g. Vähä and Primmer 2006) that the eight loci applied here represents close to the minimum required for efficient hybrid identification at levels of divergence between the parent populations of app. 0.16-0.2 (see Figure 3). Hence more loci with discriminating power could be applied in future studies to increase power of hybrid detection. However, the results also show that power is sufficient to detect first generation hybrids with great certainty and backcrosses with reasonable success, and we find it unlikely that introgression should be considerably larger than observed here. This is supported by the fact that we find only one plaice-specific allele from locus StPf1022 among the putative flounders (Wes_2), which is unexpected if introgression is truly pervasive. On the other hand more than one allele could be expected if introgression occurs at the rate suggested here, and it cannot be excluded that a few additional plaice specific alleles were lost due to technical problems such as large allele drop-out in hybrids. Differential amplification success of plaice and flounder alleles in hybrids was observed at a locus which was omitted from the study. However, locus StPf1022 amplified well in all known hybrids indicating reasonable amplification success across species.

The apparent lack of introgression from flounder to plaice could suggest asymmetrical rates of introgression between the two species. However, it should be noted that the single plaice sample may not have been sufficient to thoroughly investigate introgression in this direction. There was a tendency for STRUCTURE to underestimate the discreteness of the plaice sample by assigning alleles not present in the real plaice sample (such as flounder alleles at locus StPf1022) non-zero allele frequencies. This may have resulted in the detected signal of more introgression in the simulated data. At the individual level, there was in fact a single individual (Pla_18) which may have been a backcross.
to plaice (plaice q-value of app. 0.63, results not shown). This may indicate that backcrossing occur both ways in nature.

In this study, we were able to take the overall population structuring among the flounder samples into account, but could not do so for plaice. It is unlikely, however, that missing plaice samples are biasing the results regarding introgression form plaice to Atlantic flounder, because plaice has been found to show very low levels of population structure throughout the range of the Atlantic samples in this study (Hoarau et al. 2002b). Faroe plaice, however, appears to be significantly different from the plaice in the North Sea (Hoarau et al. 2002b) and it cannot be excluded that a Faroe plaice sample could have changed conclusions regarding introgression in Faroe flounders slightly. However, the very low levels of genetic diversity in Faroe Island flounders is not compatible with substantial levels of introgression from plaice since this would be expected to increase levels of diversity by introducing new alleles to the population.

To summarize, we find evidence of hybridization and genetic introgression from plaice to European flounder in nature. Results suggest that introgression occurs wherever the two species co-occur supporting the conclusion from other studies that hybridization and introgression may be widespread in marine fishes. However, despite the long known ability for these species to hybridize introgression seems to be of limited magnitude. Notably, there was not an increased signal of introgression in areas where intensive hybridization has been reported to occur. This is strongly suggesting that selection against hybrids at some stages of the life histories of the species are involved in maintaining species barriers.
References


Payseur BA, Nachman MW. 2005. The genomics of speciation: investigating the molecular correlates of X chromosome introgression across the hybrid zone between *Mus domesticus* and *Mus musculus*. Biological Journal of The Linnean Society 84: 523-534.


Figure legends

Figure 1. Sampling locations for samples of flounders, plaice and hybrids. Sample abbreviations refer to positions in Table 1.

Figure 2. Individual level admixture proportions of nine putative hybrids collected in the Bay of Århus in 2006.

Figure 3. Boxplot of individual level admixture proportion (plaice proportion) in flounder samples. Samples are arranged from left to right with increasing Fst to the plaice sample. Population labels are 1: Trondheim (Fst=0.159), 2: Thyborøn (Fst=0.161), 3: Westerchelde (Fst=0.161), 4: Ringkøing Fjord (Fst=0.164), 5: Bay of Biscay (Fst=0.167), 6: Ærø (Fst=0.169), 7: Lake Pulmanki (Fst=0.169), 8: Irish Sea (Fst=0.170), 9: Bornholm (Fst=0.177), 10: The Limfjord (Fst=0.182), 11: Turku (Fst=0.186), 12: Gotland (Fst=0.186), 13: Faroe Islands (Fst=0.225). The F1 hybrid from Westerschelde has been removed to enhance graphical presentations of the remaining individuals.
Table 1. Samples of flounder, plaice and hybrids with approximate position and sample sizes.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Sample year</th>
<th>Approximate position</th>
<th>Total sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Turku (Tur)</td>
<td>2003 and 2004</td>
<td>22º E, 60º N</td>
<td>104</td>
</tr>
<tr>
<td>2. Gotland (Got)</td>
<td>2003 and 2004</td>
<td>19 º E, 57.5 º N</td>
<td>94</td>
</tr>
<tr>
<td>3. Bornholm (Bor)</td>
<td>2003 and 2004</td>
<td>16º E, 55º N</td>
<td>108</td>
</tr>
<tr>
<td>4. Ærø (Aer)</td>
<td>2003 and 2004</td>
<td>10º E, 55º N</td>
<td>104</td>
</tr>
<tr>
<td>5. Thyborøn (Thy)</td>
<td>2003 and 2004</td>
<td>8º E, 57º N</td>
<td>114</td>
</tr>
<tr>
<td>6. Ringkøbing Fjord (Rin)</td>
<td>2003 and 2004</td>
<td>8.3º E, 55.96º N</td>
<td>119</td>
</tr>
<tr>
<td>7. The Limfjord (Lim)</td>
<td>2003</td>
<td>8.59º E, 56.5º N</td>
<td>55</td>
</tr>
<tr>
<td>8. Westerschelde estuary</td>
<td>2003</td>
<td>3.7º E, 52.4º N</td>
<td>48</td>
</tr>
<tr>
<td>9. Irish Sea (Irs)</td>
<td>2003 and 2004</td>
<td>-4º E, 54º N</td>
<td>98</td>
</tr>
<tr>
<td>10. Bay of Biscay (Bis)</td>
<td>2003 and 2004</td>
<td>-2.3º E, 47.20º N</td>
<td>88</td>
</tr>
<tr>
<td>11. Trondheim (Tro)</td>
<td>2004</td>
<td>11º E, 65º N</td>
<td>49</td>
</tr>
<tr>
<td>12. Lake Pulmanki (Pul)</td>
<td>2004</td>
<td>28.02º E, 70.01º N</td>
<td>34</td>
</tr>
<tr>
<td>13. Faroe Islands (Far)</td>
<td>2003 and 2004</td>
<td>-6.45º E, 62º N</td>
<td>78</td>
</tr>
<tr>
<td>14. Plaice (Pla)</td>
<td>2003</td>
<td>9º E, 57.2º N</td>
<td>49</td>
</tr>
<tr>
<td>15. Hybrids (Hyb)</td>
<td>2006</td>
<td>10.30º E, 56.30º N</td>
<td>9</td>
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</table>
Table 2. Estimated q-value cut-offs and missed backcrosses in the simulated datasets under different false positive rates. Shown are the average values from ten STRUCTURE runs.

<table>
<thead>
<tr>
<th>False positives</th>
<th>0.5%</th>
<th>1%</th>
<th>5%</th>
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<tr>
<td>q-value cut-off</td>
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<tr>
<td>Atlantic</td>
<td>0.048</td>
<td>0.036</td>
<td>0.015</td>
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<tr>
<td>Faroe</td>
<td>0.082</td>
<td>0.059</td>
<td>0.028</td>
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<tr>
<td>Inner Baltic</td>
<td>0.082</td>
<td>0.052</td>
<td>0.014</td>
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Missed F1 individuals at threshold

<table>
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<tr>
<th></th>
<th>0%</th>
<th>0%</th>
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<tbody>
<tr>
<td>Atlantic</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Faroe</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Inner Baltic</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
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</table>

Missed backcrosses at threshold

<table>
<thead>
<tr>
<th></th>
<th>11%</th>
<th>9%</th>
<th>7%</th>
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<tbody>
<tr>
<td>Atlantic</td>
<td>9%</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td>Faroe</td>
<td>13%</td>
<td>11%</td>
<td>5%</td>
</tr>
<tr>
<td>Inner Baltic</td>
<td></td>
<td></td>
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Table 3. Population level admixture proportions for simulated and real data sets (mean and standard deviation from ten STRUCTURE runs).

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of individuals</th>
<th>Atlantic</th>
<th>Faroe</th>
<th>Baltic</th>
<th>Plaice</th>
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</thead>
<tbody>
<tr>
<td>Simulated Atlantic Atlantic</td>
<td>1000</td>
<td>0.875 (0.006)</td>
<td>0.051 (0.003)</td>
<td>0.067 (0.004)</td>
<td>0.007 (0.001)</td>
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<tr>
<td>Simulated Atlantic Atlantic</td>
<td>817</td>
<td>0.838 (0.006)</td>
<td>0.066 (0.003)</td>
<td>0.087 (0.004)</td>
<td>0.009 (0.001)</td>
</tr>
<tr>
<td>Simulated Faroe Faroe</td>
<td>1000</td>
<td>0.044 (0.001)</td>
<td>0.930 (0.002)</td>
<td>0.018 (0.001)</td>
<td>0.007 (0)</td>
</tr>
<tr>
<td>Simulated Faroe Faroe</td>
<td>78</td>
<td>0.049 (0.001)</td>
<td>0.923 (0.002)</td>
<td>0.024 (0.001)</td>
<td>0.004 (0.001)</td>
</tr>
<tr>
<td>Simulated Baltic Baltic</td>
<td>1000</td>
<td>0.088 (0.003)</td>
<td>0.026 (0.002)</td>
<td>0.880 (0.004)</td>
<td>0.006 (0)</td>
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<tr>
<td>Simulated Baltic Baltic</td>
<td>198</td>
<td>0.210 (0.006)</td>
<td>0.053 (0.002)</td>
<td>0.731 (0.006)</td>
<td>0.006 (0)</td>
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<tr>
<td>Simulated Plaice Plaice</td>
<td>1000</td>
<td>0.022 (0)</td>
<td>0.013 (0.001)</td>
<td>0.014 (0.001)</td>
<td>0.951 (0.001)</td>
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<tr>
<td>Simulated Plaice Plaice</td>
<td>49</td>
<td>0.016 (0.001)</td>
<td>0.010 (0.001)</td>
<td>0.009 (0.001)</td>
<td>0.966 (0.001)</td>
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Table 4. Mean individual flounder q-values from 10 STRUCTURE runs. Shown are all individuals from the samples belonging to the Atlantic population with q values above the population specific q value threshold corresponding to a false positive rate of 1%. Broken line indicates 0.5% threshold. The simulated individuals from the Atlantic population with q-values above the 1% threshold are also presented. No individuals from the Faroe, Gotland and Turku samples had individual q values above the 1% threshold.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Atlantic population</th>
<th>q values</th>
<th>Simulated Atlantic</th>
<th>q values</th>
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<tbody>
<tr>
<td>1</td>
<td>Wes_2</td>
<td>0.4826</td>
<td>0.1476</td>
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<td>2</td>
<td>Irs_20</td>
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Figure 1
Figure 2.
Figure 3.
## Appendix A. Summary statistics and tests for Hardy Weinberg Equilibrium

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### Population Westerschelde Irish Sea Bay of Biscay Trondheim Pulmankijärvi Faroe Islands Plaice
| Na         | 21    | 24      | 22       | 22   | 13       | 11         | 19           |
| Allelic richness | 18.55 | 19.46   | 17.40    | 18.43 | 12.97    | 9.12       | 15.99        |
| Size range | 147-203 | 147-229 | 139-193  | 139-199 | 147-189 | 155-185    | 129-193      |
| Ho         | 0.844 | 0.915   | 0.871    | 0.959 | 0.903    | 0.857      | 0.833        |
| He         | 0.927 | 0.933   | 0.906    | 0.933 | 0.901    | 0.808      | 0.887        |
| HWE exact test | 0.367 | 0.164   | 0.636    | 0.732 | 0.956    | 0.623      | 0.055        |

### Locus StPf1004

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### Population Westerschelde Irish Sea Bay of Biscay Trondheim Pulmankijärvi Faroe Islands Plaice
| Na         | 18    | 23      | 18       | 15   | 15       | 8          | 17           |
| Allelic richness | 14.40 | 15.54   | 13.33    | 12.83 | 14.36    | 6.87       | 15.95        |
| Size range | 138-192 | 136-188 | 134-188  | 132-192 | 144-192 | 138-192    | 144-194      |
| Ho         | 0.809 | 0.796   | 0.805    | 0.857 | 0.879    | 0.782      | 0.486        |
| He         | 0.770 | 0.799   | 0.790    | 0.809 | 0.882    | 0.752      | 0.914        |
| HWE exact test | 0.984 | 0.427   | 0.129    | 0.415 | 0.093    | 0.564      | 0.000        |
### Locus List1001

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Appendix B. Allele frequency distributions of plaice and pooled flounder samples.

### Allele Frequency Distributions

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Appendix B continued
Manuscript IV

Ecological and genetic interactions between flounder (*Platichthys flesus* L.) populations in the Baltic Sea

Jakob Hemmer-Hansen and Einar Eg Nielsen

In preparation
Ecological and genetic interactions between flounder (*Platichthys flesus* L.) populations in the Baltic Sea

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**Key words:** Life-history variation, local adaptations, seasonal migration, microsatellites, individual admixture proportions, *Platichthys flesus*.

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Introduction

The advent of highly variable genetic markers, such as microsatellites, has resulted in the demonstration of highly significant genetic structuring among marine fish populations (e.g. Ruzzante et al. 1999; Nielsen et al. 2001; Nielsen et al 2003; Knutsen et al. 2003; Bekkevold et al. 2005). These findings have resulted in increased efforts to understand the evolutionary mechanisms responsible for generating and maintaining significant genetic differences between populations in an environment without any obvious barriers to gene flow. Hence, several physical forces, such as current systems and habitat suitability have been found to be important for maintaining genetic structure in marine fishes (e.g. Ruzzante et al. 1999; Riginos and Nachmann 2001; Taylor and Hellberg 2003). Furthermore, environmental transitions have been highlighted as an important structuring force, potentially because gene flow is limited between locally adapted populations (Nielsen et al. 2003; Nielsen et al. 2004; Bekkevold et al. 2005).

Recently, the interaction between genetically differentiated populations has also been investigated in more detail. For instance it has been found that populations of cod and turbot apparently interact in hybrid zones in an area of sharp environmental transition between the marine North Sea and the brackish Baltic Sea (Nielsen et al. 2003; Nielsen et al. 2004). These findings and the fact that many other organisms show high levels of genetic structuring across the same environmental gradient (Johanneson and André 2006) indicate a role for natural selection in maintaining these population differences.

Another interesting case of interactions between genetically differentiated populations has been described in Atlantic herring (Bekkevold et al. 2006; Ruzzante et al. 2006), where sympatric feeding components utilize different spawning areas and spawn at different times of the year. The genetic component of these life-history differences is largely unknown, but it has been shown that, at least in some instances, the divergence between spawning populations are maintained despite intense mixing at feeding grounds (Ruzzante et al. 2006), suggesting very complex population structure, intra-annual migration patterns and interactions among genetically differentiated populations.

The European flounder is one of a few marine fishes also found in the innermost parts of the Baltic Sea. As is the case for many other fishes in the Baltic Sea (Nissling and Westin 1997; Nissling et al. 1999, Nissling et al. 2002; Ojaveer and Kalajs 2005), populations in the Western and central parts have decreased egg shell thickness resulting in eggs being able to stay buoyant at lower salinities compared to populations in the highly saline North Sea (Nissling et al. 2002). However, in the innermost parts of the Baltic Sea, eggs are smaller and heavier. This physiological change is associated with a change in spawning strategy to benthic spawning in coastal habitats, which are the only areas where ambient oxygen levels allow survival of the eggs (Solemdal 1967; Nissling et al. 2002), and hence this life-history change is regarded as an important adaptation to local environmental conditions in the inner Baltic Sea. The distributions of the “pelagic” and “benthic” types of flounder overlap around the island of Gotland in the central Baltic Sea. Tagging studies have shown that flounder populations are relatively stationary in the area, but that seasonal migrations between spawning and feeding areas do occur (Aro 1989).

We have previously identified highly significant genetic structuring of flounder populations with different life-history strategies in the Baltic Sea (Manuscript in preparation). However, that study also indicated temporal differences between samples from the island of Gotland. In this study, we examine the spatial and temporal population structure of flounder populations in the Baltic Sea with a view to investigate potential interactions among populations exhibiting very different life-history strategies. We apply highly variable microsatellite markers, which are well suited to disclose minute
levels of genetic structuring, in combination with both population and individual level admixture analyses to examine potential interaction among populations at a local scale.

**Materials and Methods**

**Sample information**
A total of 524 adult flounders from five locations along a transect from the North Sea to the inner Baltic Sea were caught for genetic analyses (Figure 1). Most fish were sampled in the spawning season. However, some samples were taken near the end of the spawning season (e.g. Thy04, Tur03 and Got03) and were therefore not solely comprised of mature individuals. A single sample from Gotland 2004 was collected after the spawning season and consisted entirely of individuals which had already spawned (Table 1). Samples of gill tissue were stored in ethanol prior to DNA extraction using Chelex (Estoup et al. 1996), HotShot (Truett et al. 2000) or DNeasy (Qiagen) techniques.

**Molecular markers**
Nine microsatellite loci were employed for the genetic analyses. LIST1001 (Watts et al. 1999, GenBank accession number: AF149831), PL142 and PL167 (Hoarau et al. 2002b, accession numbers: AF406750 and AF406751), originally developed for plaice but worked well on flounder following modifications of PCR conditions. St Pf1001, St Pf1002, St Pf1004, St Pf1005, St Pf1015 and St Pf1022 (Dixon et al. unpublished, accession numbers: AJ315970, AJ315975, AJ315973, AJ315974, AJ538313, AJ538320) were all developed for European flounder. PCR was applied with standard reagents and thermal cyclers and PCR products were analyzed on an ALFexpress (Amersham Biosciences) automated sequencer following the manufacturer’s recommendations. Standard size ladders and individuals of known genotypes were run on each gel to minimize scoring error.

**Statistical analyses**
Exact tests for departure from Hardy-Weinberg equilibrium (Guo & Thompson 1992) were performed per locus and sample in GENEPOP (Raymond & Rousset 1995). $F_{ST}$ (Weir and Cockerham’s (1984) $\theta$), their confidence intervals and significances were estimated using the program FSTAT (Goudet 1995), which was also used to estimate allelic richness (ElMousadik & Petit 1996). Temporal stability was assessed by comparing temporal samples within localities. Population relationships were visualized by a multidimensional scaling plot of Nei’s $D_A$ (1983) created in Vista 5.6.3. (Young 1996). Furthermore, differences between years in the pairwise $F_{ST}$ were examined by estimating pairwise $F_{ST}$ from Thyborøn to the Baltic Sea samples for the two years separately. We used the Bayesian model based clustering algorithm implemented in the program STRUCTURE (Pritchard et al. 2000) to estimate the most likely number of populations in the data set and in a sub-set consisting of samples from Bornholm, Gotland and Turku. The program Admix1.0 (Bertorelle & Excoffier 1998) was used to estimate population level admixture proportions according to Roberts and Hiorns (1965) in populations of intermediate geographical position between the two most extreme populations (Thyborøn and Turku), which were used as baselines. Subsequently, we used STRUCTURE to estimate individual level admixture proportions in all samples using simulated Thyborøn and Turku samples (each of 1000 individuals) as fixed baselines. Simulated samples were generated by randomly drawing alleles according to the estimated allele frequency distributions in the two samples in the program HYBRIDLAB (Nielsen et al. 2006). Individual admixture proportions were subsequently compared by Mann Whitney $U$-tests to examine spatial as well as temporal differences.
**Results**

*Genetic variation*
Genetic variation was high in all samples, expected levels of heterozygosity ranging from app. 0.3 at locus List1001 to between 0.8 and 0.9 in many of the other loci. The populations in the innermost parts of the Baltic Sea tended to have lower levels of genetic diversity as evidenced by lower levels of allelic richness at these localities (Appendix). No sample or locus deviated systematically from Hardy Weinberg expectations.

*Population structure*
The $F_{ST}$ among all samples was 0.016 (95% CI: 0.008 – 0.025) and highly significant ($P < 0.0001$). The structuring of samples was also evident in the MDS plot (Figure 2) where samples grouped in two major aggregations according to their spawning strategy. Most of the variation (72%) was explained by dimension one along which samples were distributed largely corresponding to their geographical relationships. The geographically extreme Thyboron and Turku samples were positioned near the ends of this axis with the remaining Baltic Sea populations in between. Along axis one, all temporal samples from the same locality were grouping together except for the Gotland samples which were relatively far apart. Dimension two explained 8% of the variance.

No temporal samples from the same locality were significantly differentiated. Among the spatial samples, the largest differences were found in comparisons of samples in the two groups comprised of the different spawning strategies, although significant differentiation was also found between samples in the Western and central Baltic Sea and samples in the North Sea (Table 2). When estimating pairwise $F_{ST}$ between Thyboron and samples in the Baltic Sea, there was a large shift between Bornholm and Gotland, mirroring the picture from the MDS plot. However, this shift was less extreme in 2004, where the transition to the inner Baltic Sea sample at Turku appeared more gradual (Figure 3).

The clustering algorithm in STRUCTURE returned the highest posterior probability of two populations in the total data set. These two populations corresponded to the same structure identified in the MDS plot and in spatial estimates of population structuring, but with some introgression between samples in the two populations. Individuals in the Thyboron and Turku samples were found to assign most strongly to the two populations identified by STRUCTURE. Hence temporal samples form these localities were pooled to represent baseline populations for calculations of admixture proportions in intermediate samples in the Baltic Sea. We also ran STRUCTURE using only samples from Bornholm, Gotland and Turku to examine potential population interactions at Gotland in more detail, but in this case, the most likely number of populations was one, reflecting decreased power of this approach at low levels of structure and a modest number of loci (Latch et al. 2006). Hence, we carried out all analyses with the original data set.

*Population and individual admixture*
Using pooled samples from Thyboron and Turku as baselines for population admixture analyses, the intermediate samples were all admixed to some degree, the proportion from Turku in the samples increased from the Western to the central Baltic Sea samples (Table 3). Result were highly consistent for temporal samples from Ærø and Bornholm, while the Gotland samples showed a somewhat different pattern; here the 2003 sample appeared almost as non-admixed (i.e. pure Turku), while the
2004 sample had a significant Thyborøn proportion. The Gotland samples were the only samples where standard deviations did not overlap between the temporal samples.

When estimating individual level admixture proportions, it was also evident that the samples from Ærø and Bornholm were very similar, both within and between localities while the distribution of individual admixture proportions at Gotland were significantly different between years (Table 4). At Gotland the distribution of individual level admixture proportions were more sigmoid in 2004 than in 2003, reflecting an increased number of fish with extremely low Turku admixture proportions in 2004 (Figure 4).

Discussion

We found strong indications that the two temporal samples from Gotland deviated significantly, while all other temporal samples were very similar. The analyses conducted indicate that the sample from Gotland from 2004 contained a significant input of flounder genotypes from the Southern and/or central Baltic Sea, while the sample from 2003 was very similar to Turku in the inner Baltic Sea. This result is unlikely to be an artifact by sampling at different locations in the two years, since the samples in 2004 were actually collected further to the North than the 2003 sample (see Figure 1). Hence, these results indicate relatively strong temporal shifts at the Gotland locality, as evidenced by both population level admixture and the distribution of individual level admixture proportions, despite the fact that the two Gotland samples were not significantly differentiated when estimating population divergence with Weir and Cockerham’s \( \theta \).

These temporal differences could be explained either by differences between years, i.e. a temporally unstable population structure, or by temporal changes within years, for instance caused by migrating individuals. Interestingly, a recent study on Baltic Sea turbot (Florin and Höglund 2006) also found temporal changes among Gotland samples, but otherwise found little evidence of population structure in the Baltic Sea. The authors suggested that population structure is highly unstable and largely dependent on the frequency of high saline inflows and the length of intervening stagnation periods. Furthermore, they noted that the minor changes at Gotland could be caused by subtle temporal differences in larval dispersal or different contribution of cohorts in the three years under study. However, this study only had temporal replicates of samples from Gotland and hence it was not possible to assess if the temporal shifts was a general phenomenon as suggested by the authors. In the case of European flounder, temporal changes were only found at Gotland. If large scale movements, e.g. following a major inflow in 2003, would have allowed “pelagic” populations to move further into the Baltic Sea, we would have expected to see the shifts in the other samples as well. This was clearly not the case, since all other temporal samples were very stable. Hence, while we cannot exclude the possibility of a local environmental shift near Gotland, we believe that the results point to an alternative explanation, i.e. variation within years for instance in caused by feeding migrations. This theory is supported by the fact that the sample from Gotland in 2003 was the only one without mature or spawning individuals. Since this sample was collected later in the year, it is plausible that it could have contained individuals on feeding migrations. Tagging studies have shown that adult flounders do perform annual migrations along the Swedish and Russian coasts (Aro 1989), a picture supporting the proposed intra-annual movements seen in this study.

Results indicate that a significant part of the sample at Gotland in 2004 is of “pelagic” origin (e.g. the individuals with very low Turku admixture proportions in Figure 4), which could suggest mixing of flounders with different life-history strategies at feeding grounds. This was indeed suggested by Solemdal (1973) who caught two flounders with physiological characteristics matching each of the
two flounder types near Stockholm in October. This locality is also near the proposed overlap of the
two flounder types and Solemdal suggested that the two types could mix during feeding seasons. We
attempted to model the distribution of simulated hybrid versus mechanically mixed Gotland
populations applying the method outlined in Nielsen et al. (2003). However, our power was not
sufficiently high (too few loci and relatively modest levels of differentiation) to statistically
differentiate between the expected distributions under the two scenarios, i.e. both mechanically mixed
and hybrid populations had similar distributions of individual admixture proportions. Hence, it was
difficult to determine with statistical certainty that the individuals with very low Turku admixture
proportion in our study truly were immigrants from a “pelagic” population further to the south, or
alternatively from the Gotland basin, which may also support some spawning of the “pelagic” type.
However, the mixing of different life-history forms is supported by the observations by Solemdal and
by tagging studies showing migrations in the directions proposed here (Aro 1989).

Different life-history strategies are well known among salmonids where differences in run-time
and migration patterns are believed to result in high levels of structuring among populations (e.g.
Taylor 1991). Among marine fishes intra specific life-history variation has been described in Atlantic
herring, where complex spawning components utilize different spawning areas and seasons (e.g.
Bekkevold et al. 2006; Ruzzante et al. 2006). Ruzzante et al. (2006) found a temporally stable pattern
of structuring of spawning components exhibiting different spawning behaviors, despite the seasonal
mixing of these components in feeding areas. On the other hand, comparable spawning groups have
been found to be both genetically homogenous and genetically structured across life-history breaks in
other areas (Bekkevold et al. 2006), suggesting very complex demographic and evolutionary scenarios
in Atlantic herring. While the degree of geographical overlap between the two life-history strategies in
European flounder is not known in detail, and the genetic component of the differences has never been
investigated, the genetic break observed in the inner Baltic Sea appears to be relatively sharp,
suggesting significant reductions in gene flow between the two forms.

If the results from the present study do indeed indicate seasonal mixing of genetically different
life-history forms, then there must be strong pre – or post-zygotic barriers to hybridization between the
two forms, since their distributions are apparently overlapping, at least at some times of the year. In the
present case, the most obvious barrier to gene flow is migration patterns and spawning habitat, since
the two types are apparently utilizing different spawning habitat. However, other mechanisms, such as
assortative mating (Carvalho et al. 2003), has also been described in flatfishes and could be operating
in order to prevent interbreeding. However, even if interbreeding should occur, a number of post-
zygotic mechanisms could reduce fitness of hybrids. For instance, interbreeding of locally adapted
populations has been shown to result in outbreeding depression and the subsequent loss of local
adaptations (Gharrett et al. 1999; Edmands 1999; Taylor et al. 2006). Such effects could easily be
imagined, for instance in relation to well described physiological adaptations, such as egg buoyancy

However, while we cannot conclude that there is a mechanical mix of different life-history
forms in 2004, the results from this study most likely show a considerable seasonal mixing of fish from
different areas in the Baltic Sea. If population structure is truly stable as suggested by the temporal
stability of all remaining samples, this would indicate significant homing of adults to spawning
grounds, a also suggested in Atlantic herring (Ruzzante et al. 2006) and as seen from tagging studies
applying electronic data in, for instance, plaice (Hunter et al. 2003). These results are largely in
agreement with earlier tagging studies, which have also demonstrated seasonal movements of flounders
in the Baltic Sea (Aro 1989, Bagge and Steffensen 1989). However, these studies have concluded that
flounders in the Baltic Sea are relatively stationary, implicating that these movements are not
substantial. This does not seem to be the case in the present study, since the genetic shift observed
between years is substantial and seemingly involves many individuals.

In order to elucidate the sharpness of the genetic break between the two life-history forms in the
Baltic Sea, future studies should aim at a more detailed sampling scheme around the island of Gotland.
Preferably, samples from both coastal and deep waters should be collected in the spawning season to
examine fine scale differences at the time of spawning. In addition, temporal sampling throughout the
season could help considerably in establishing if the pattern of intra-annual movements suggested here,
is a general phenomenon. Future studies could also gain substantial power by combining information
from genetic markers with physical and physiological measures of e.g. egg buoyancy, egg shell
thickness and sperm mobility (Solemdal 1967, 1973; Nissling et al. 2002) on the individual level.

In conclusion, we believe that this study has demonstrated significant intra-annual movements
of flounder individuals and populations in the Baltic Sea. These results suggest that ecological and
genetic interactions between these populations may be quite considerable despite substantial life-
history differences between them.
References


Young FW. 1996. ViSta: the visual statistics system. Research Memorandum 94-1(b) 2d. ed. L.L. Thursone Psychometric Laboratory, University of North Carolina, Chaper Hill, NC.
Figure legends

Figure 1. Map of sample sites

Figure 2. MDS plot of Nei’s $D_A$ (1983)

Figure 3. Pairwise $F_{ST}$ from Thyborøn in the North Sea to all Baltic Sea samples in 2003 (▲) and 2004 (■).

Figure 4. Individual Turku admixture proportions in the Gotland samples from 2003 (▲) and 2004 (■). Note that there are 46 individuals in Got03 while there are 48 in Got04. The two populations are presented together for comparative purposes.
Table 1. Sampling locations. Sample names correspond to geographical presentation in Figure 1.

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<th>Sample size</th>
<th>Spawning strategy</th>
<th>Proportion maturing and mature</th>
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<td>Benthic</td>
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<td>2. Turku 2004</td>
<td>Tur04</td>
<td>June</td>
<td>22º E, 60º N</td>
<td>50</td>
<td>Benthic</td>
<td>24</td>
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<td>3. Gotland 2003</td>
<td>Got03</td>
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<td>18.5º E, 57º N</td>
<td>46</td>
<td>Benthic</td>
<td>App. 50</td>
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<td>4. Gotland 2004</td>
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<td>55</td>
<td>Pelagic</td>
<td>100</td>
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Table 2. Pairwise F_{ST} with 95% CI above diagonal and their associated P values for the permutation test for significance below diagonal.

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Table 3. Population level admixture proportions (Roberts and Hiorns 1965) with standard deviations.

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Table 4. P values from pairwise Mann Whitney U-tests on individual admixture proportions. Simulated Thyborøn and Turku populations were used as fixed baselines.

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Figure 1
Figure 3
Figure 4
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Summary

This thesis consists of five main parts; a general introduction to knowledge and future perspectives for studies of population structure and local adaptations in marine fishes, and four manuscripts presenting the major findings from my PhD.

The first manuscript describes the analyses of population structure in European flounder (*Platichthys flesus*) on both large and local geographical scales. Using microsatellite genetic markers, we found high levels of genetic structuring between different flounder populations. Importantly, these differences apparently had very different causes, some being driven by changes in life-history characteristics, while others were more likely to be associated with physical oceanographic forces or gradual environmental changes. This study thus adds to our understanding of how evolutionary forces may interact in the sea to structure species of marine fishes into discrete and reproductively isolated units.

The second manuscript focuses specifically on adaptive population divergence. By applying a candidate gene approach we found strong indications of adaptive population divergence of flounder populations despite seemingly high levels of gene flow between populations. This approach has not previously been applied in marine fishes, and the results indicate that adaptive divergence and local adaptations are indeed possible in the high gene flow marine environment. A result which is encouraging for future studies of local adaptations in marine fishes, of which we know very little.

In the third manuscript, we are investigating potential genetic introgression from plaice (*Pleuronectes platessa*) to flounder populations in different parts of the distributional area of the flounder. We found strong indications of significant introgression throughout the areas where the two species have overlapping distributions. However, levels of introgression were low, indicating relatively strong selection against hybridization between the two species. Hence, in contrast to other studies which have demonstrated high levels of introgression in hybrid zones between other species, there seems to be considerable selective constraints on plaice-flounder hybridization. This could allow the two species to remain genetically separate despite apparently sharing distributional and spawning areas.

The forth manuscript examines the stability of population structure on a local scale in the Baltic Sea. Specifically, we are investigating interactions between populations with different life-history characteristics; some populations are spawning pelagic eggs, while others are spawning benthic eggs. We found relatively strong temporal shifts at the same locality between samples collected in two different years and at different times of the year (one close to time of spawning, the other in the feeding season). Hence, the results indicate substantial intra-annual movements and potential mechanical mixing at feeding grounds of individuals from populations exhibiting very different life-history characteristics. These findings thus add to our understanding of how genetically differentiated populations may interact genetically and ecologically.