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# Population genetics of plaice (Pleuronectes platessa L.) in Northern Europe <br> Hoarau, Galice Guillaume 

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## Population genetics of plaice (Pleuronectes platessa L.) in Northern Europe



Galice Hoarau

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Cover: Dyle Hervé-Staakman
The research reported in this thesis was performed at the Department of Marine Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

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## Rijksuniversiteit Groningen

# Population genetics of plaice (Pleuronectes platessa L.) in Northern Europe 

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Chapter 1
GENERAL INTRODUCTION


Fig. 1. ICES fishing areas (www.ices.dk).

Geographical areas used in fisheries management (i.e. ICES areas www.ices.dk, Fig. 1) do not typically coincide with the actual distribution areas of biological fish stocks simply because the stock may move seasonally between fixed management areas, or because separate fish stocks may occur in a single management area. This results in a mismatch between the spatial scale of management and biological reality (Daan 1991, Pawson and Jennings 1996). Uniform exploitation of a resource composed of numerous subpopulations/stocks, for example, can lead to the erosion of a section of that resource (Bailey 1997).

The North Sea area is under heavy fishing pressure. About 400 Dutch trawlers are operating in this area. The entire bottom of the North Sea is trawled several times per year, which dramatically affects the benthic community as well as demersal fish (Rijnsdorp et al. 1998). Since the 1980s the stock has been managed under the European Common Fishery Policy (CFP) which sets the Total Allowable Catch (TAC) for individual species. The subsequent collapse of stocks such as cod illustrates the failure of such a quota management policy, especially in the case of flatfish mixed fisheries (Daan 1997). For North Sea plaice, the European Commission recently proposed a $40 \%$ reduction of the TAC since plaice stocks are considered to be outside of the safe biological limit (ICES). Despite this advice, 2004 TACs were only reduced by 5 to $20 \%$ (europa.eu.int; www.pvis.nl).

Plaice (Pleuronectes platessa), together with sole (Solea spp.), are the main target species for the European beam-trawl-fishery. Heavy fishing pressure over the past fifteen years has reduced landings by $>50 \%$, from 170.000 t in the 1989 to 82.000 t in 2001 (Rijnsdorp and Millner 1996, ICES) (Fig 2). Concern for collapse of the fishery has raised questions about plaice stocks.


Fig. 2. Landings of plaice (in thousands of tonnes) for the North Sea between 1900 and 2001. Modified from Rijnsdorp and Millner (1996).

The concept of stock has many meanings in the fisheries context-as an abstract and undefined unit, as a physical location in which the fish are found and, as is the case here, as a genetic unit defined as a group of randomly mating individuals (Carvalho and Hauser 1994).

The population structure of a species is the result of a complex interaction between the life history characteristics (i.e. pelagic larval stages, homing behaviour), demographic parameters (i.e. recruitment, growth, reproduction, mortality) and genetic processes (i.e. drift, gene flow, selection, mutation). The population dynamics (life history and demography) of many commercial fish species have been well studied since the end of the $19^{\text {th }}$ century. However population genetic data for such species are only now becoming available to complete the full picture of population structure-the most comprehensive definition of a stock.

As part of the NWO-Prioriteit Programme "Sustainable Use of Marine Natural Resources" the three most general questions posed for this thesis were:

1. What is the spatial scale of population genetic structure in plaice throughout its range in Northern European waters?
2. To what extent are spawning grounds connected to specific nursery grounds?
3. To what extent is there a mismatch between the spatial scale of genetic stock and management of those stocks?

From the population genetics perspective the initial questions included:
4. What insights about the mating system (especially philopatry) of plaice can be gleaned from the genetic data?
5. What is effective population size ( Ne ) of plaice?
6. Is there evidence for fisheries effects on genetic diversity and genetic structure?

## Plaice, Pleuronectes platessa L

Plaice (Fig 3) is a flatfish inhabiting shallow waters ( $<100 \mathrm{~m}$ ) from the Western Mediterranean (although occurrence in the Mediterranean and the Black Sea is doubtful) to Iceland and the White Sea (Wimpenny 1953) (Fig 4).


Fig. 3. European plaice, Pleuronectes platessa.


Fig. 4. Distribution map of plaice (source FAO www.fao.org).

Box 1. About flatfish.

Flatfish belong to the Pleuronectiformes (Teleostei). The monophyly of the lineage is supported by morphological (Chapleau 1993) as well as molecular analyses (Berendzen and Dimmick 2002). Although fossil dating places flatfish origins in the lower Eocene ( 50 Ma BP), species diversification was apparently sudden (Chanet 1997). A rapid radiation within this order is supported by the lack of resolution of the phylogenetic relationships. Some 570 to 620 species comprise 123 genera and 14 families (Chapleau 1993, Nelson 1994). About three-quarters of these species are tropical. About ten species of flatfish are commercially exploited in temperate Northern European waters :

Scientific name<br>Scophthalmus rhombus<br>Scophthalmus maximus<br>Pleuronectes platessa<br>Platichthys flesus<br>Limanda limanda<br>Microstomus kit<br>Glyptocephalus cynoglossus<br>Hippoglossus hippoglosus<br>Solea solea

| English | Dutch |
| :--- | :--- |
| Brill | Griet |
| Turbot | Tarbot |
| Plaice | Schol |
| Flounder | Bot |
| Dab | Schar |
| Lemon sole | Tong schar |
| Witch | Witje |
| Halibut | Heilbot |
| Sole | Tong |

Plaice, like all flatfish, have a unique morphology, characterised by an asymmetrical skull with both eyes on the same side of the head. Initially, plaice larvae are similar to larvae of other fish with a bilateral symmetry. However, early in their development, they begin side-swimming, one eye starts moving to the opposite side of the head, and the two sides of the body develop differentially. Gradually, the larvae lose their swim bladder, which promotes the transition to the demersal existence (Fig 5). Adult plaice are benthic, often hiding under mud or sand with only the eyes protruding.


Fig. 5. Development of a flatfish from egg to juvenile (from Hovenkamp 1991).

Adults roam freely over feeding grounds from April to November. Tagging data have been used to model the spatial dynamics (De Veen 1978, Rijnsdorp and Pastooors 1995) in which migration distances of 300 km were relatively common. Migration rates of $>20 \mathrm{~km}$ in a single day have also been documented from electronic tags (Metcalfe and Arnold 1997). Apparently plaice do not travel in schools, as do many pelagic species. As the spawning season nears, adults move towards the spawning grounds, which are well known throughout the North Sea (Harding et al. 1978) (Fig 6).


Fig. 6. Schematic representation of life cycle in plaice. A: offshore spawning area (grey); B: inshore nursery area (stippled); C: offshore adult feeding and spawning areas. The arrows on the map indicate residual currents (from Rijnsdorp et al. 1995).

Spawning occurs in winter, from December to March. Fecundity is high with 20,000-600,000 eggs/female (Rijnsdorp 1991). Mating is assumed to occur at random in large aggregations. The pelagic egg and larval phase lasts from 3-4 months (Harding et al. 1978) during which time the larvae gradually drift towards
the nearest coastal nursery grounds. The larvae gradually metamorphose into demersal juveniles (0-class), with the familiar flatfish morphology, and settle at a size of 10 to 15 mm in shallow coastal areas (Edwards and Steele 1968, Zijlstra 1972) which they enter using selective tidal transport (Rijnsdorp et al. 1985). Young plaice remain in the nursery areas for 1-2 years before migrating to off-shore, deeper water, where they join adult feeding populations. The main nurseries are located in the Wadden Sea and Southern Bight of the North Sea. Recruitment dynamics, including the underlying oceanographic mechanisms for these areas, have been extensively studied by Van der Veer (1986) and Van der Veer et al. (1990, 2000).

## Fish population genetics

## Population genetic surveys in high dispersal fish

Although the concept of stock identification is straightforward, the life history and ecology of a species (Waples 1998) can greatly affect the ability to actually identify genetic stocks in nature. In high dispersal marine species, which includes most fish, high gene flow and thus large scale genetic homogeneity of populations has been considered axiomatic. In this view, high fecundity, passive dispersal of larvae and active migration of adults can lead to dynamic and seemingly unstructured populations. Conversely, physical barriers to dispersal such as depth, temperature or salinity boundaries, or hydrodynamic eddies favouring larval retention; or strong philopatry are factors that can promote population differentiation. Only recently has it become apparent that subtle genetic substructure exists between these ends of the spectrum (Wirth and Bernatchez 2001, Nielsen et al. 2004).

A large number of population genetic studies of commercially important fish have used allozymes or mitochondrial DNA (mtDNA) as molecular markers. In most cases low or no differentiation was detected (reviewed in Waples 1998). Whether failure to find differentiation was due to a lack of sufficient resolving power of the markers or to the biology of the fish has not always been clear. With the arrival of highly polymorphic microsatellite loci in the middle 90s, (see e.g. Jarne and Lagoda 1996) the potential for detecting weak differentiation in high gene flow species increased substantially. Over the past five years studies in high
dispersal fish such as cod (Nielsen et al. 2003), European seabass (Bahri-Sfar et al. 2000) and eel (Wirth and Bernatchez 2001, Maes and Volckaert 2002), have routinely detected population substructure.

## Genetic structure in North Atlantic-Mediterranean flatfish

A few studies of broad-scale geographic differentiation have been done on commercially important flatfish (Table 1). For flounder (Platichthys flesus/stellatus) strong differentiation has been found among Atlantic, Western Mediterranean and Adriatic populations. Strong differences were also found within the Mediterranean. In contrast, differences within the North Atlantic region were much lower but indicated some isolation by distance and differentiation within the North Sea, usually north and south of the Dogger Bank, has been (Borsa et al. 1997).

In sole (Solea vulgaris [=S. solea]), Kotoulas et al. (1995) detected significant East-West differentiation across the Mediterranean and North-South differentiation along the European Atlantic coast but were unable to demonstrate any differentiation within the North Atlantic "zone". The between-region differences found in flounder and sole are generally attributed to narrow, larval-temperature-tolerances of these two flatfish species as compared with other flatfish of the region.

In halibut (Hippoglossus hipoglossus) and in brill (Scophthalmus rhombus) lack of differentiation within most North Atlantic populations has been reported. In turbot (Scophthalmus maximus) subtle differentiation between the Baltic and the North Sea have been reported recently (Nielsen et al. 2004).

Prior to this thesis, the only attempts to characterise genetic stock structure of plaice in the North Sea region were by Purdom and Thompson (1976) and by Ward and Beardmore (1977). These authors used five allozyme loci to compare samples from the Irish Sea, Bristol Channel and southern North Sea. They were not able to establish any differences (Table 1).
Table 1. Genetic structure studies in flatfish along the North Atlantic continental shelf ( ${ }^{*} P<0.05$, ${ }^{* * *} P<0.001$, ns $=$ not
significant, n.t. = not tested).

|  | Author | Marker | Mean population differentiation $\theta / G_{\mathrm{ST}} / \Phi_{\mathrm{ST}}$ | Locations | Conclusion |
| :---: | :---: | :---: | :---: | :---: | :---: |
| $\overline{\text { Pleuronectes }}$ platessa | Ward and Beardmore 1977 | Allozymes 5 loci | not quantified | Bristol Channel, North <br> Wales, North Sea <br> (location not specified) | no subdivision. Mentions some differences in alleles between Irishand North Sea |
|  | Purdom and <br> Thompson <br> 1976 | Allozymes 5 loci | not quantified | Western English Channel, Irish Sea, North Sea | no subdivision |
|  | Hoarau et al. this study (chapter 3) | Microsatellites 6 loci | $0.0086^{* * *}$ for all populations $0.0014^{\text {ns }}$ for shelf populations | Norway to Iceland and to Bay of Biscay | Only on/off continental shelf differentiation |
|  | Hoarau et al. this study (chapter 4) | mtDNA <br> haplotypes | $0.0383^{* * *}$ for all populations $0.0132^{* * *}$ for shelf populations | Norway to Iceland and to Bay of Biscay | On/off continental shelf differentiation. Weak differentiation within the continental shelf |
|  | Watts et al. 2004 | Microsatellites 7 loci | $0.019^{\text {ns }}$ | Irish Sea | no subdivision |


| Platichthys | Borsa et al. |
| :--- | :--- | :--- | :--- | :--- | :--- |
| flesus |  |

Table 1 continued

| Scophthalmus maximus | Blanquer et al. 1992 <br> Bouza et al. <br> 1997 <br> Coughlan et al. <br> 1998 <br> Nielsen et al. 2004 | Allozymes 17 loci <br> Allozymes 14 loci <br> Microsatellites <br> 3 loci <br> Microsatellites <br> 8 loci | $0.000^{\text {ns }}$ $0.012^{\text {ns }}$ $0.009^{\text {ns }}$ $0.032^{* * *}$ North Sea and Baltic | Mediterranean to Kattegat Galician coast Ireland and Norway Inner Baltic to North Sea | Aegean Sea was the only one different from all others no subdivision <br> no subdivision <br> Significant between North Sea and Baltic |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Scophthalmus. rhombus | Blanquer et al. 1992 | Allozymes 17 loci | not given <br> Nei distance tree only | Mediterranean/Portugal/ Brittany-Kattegat | three regional groups identified |
| Solea vulgaris [=S. solea] | Kotoulas et al. 1995 | Allozymes 12 loci | $0.0147^{* * *}$ for all regions <br> Atlantic only $0.005^{\text {ns }}$ | Mediterranean to Normandy only (no North Sea samples) | IBD across regions but not within regions |
| Solea solea | Exadactylos et al. 1998 | Allozymes 27 loci | $0.0262^{\mathrm{ns}}$ <br> (no pairwise comparisons made) | Mediterranean to Normandy (3 North Sea) | no differentiation detectable |
| Solea solea | $\begin{aligned} & \text { Cabral et al. } \\ & 2003 \end{aligned}$ | Allozymes 7 loci | 0.013 | Portugal | IBD |
| Solea solea | Exadactylos et al. 2003 | RAPD | $0.193^{* * *}$ | Irish Sea, North Sea, Bay of Biscay | English Channel is a barrier to gene flow |
| Hippoglossus hippoglossus | $\begin{aligned} & \text { Foss et al. } \\ & 1998 \end{aligned}$ | Allozymes 4 loci | not given <br> Nei distance tree | Norway, Iceland, Faeroes, Greenland | east-west axis <br> Norway apart for the three others |
| Synaptura <br> lusitania | $\begin{aligned} & \hline \text { Cabral et al. } \\ & 2003 \end{aligned}$ | Allozymes 10 loci | 0.259 n.t. | Portugal |  |

## Census vs. effective population size

Over-exploitation and subsequent collapse of most major fisheries worldwide has made it clear that marine stocks are not inexhaustible. In $2002,75 \%$ of the world's marine stocks were fully exploited or overexploited (FAO, www.fao.org). Unfortunately, even after the collapse of such major fisheries as cod and herring (Hutchings 2000), the perception remains that marine fishes, particularly pelagic fishes, are resilient to large population reductions. The popular wisdom is that even a commercially "collapsed" stock, will still consist of tens of millions of individuals so that given time, it will recover. Coupled with this notion, is the idea that fisheries can, therefore, have little effect on the genetic diversity of stocks. This view is beginning to change.

Census population size $(N)$ refers to the total number of individuals in the population. Effective population size $(N e)$ refers to that subset of individuals in the population that successfully contribute to the next generation. Factors that affect Ne are discussed later. Populations with small $N e$ values can suffer a reduction in genetic diversity, mainly as a consequence of faster genetic drift, which leads to loss or fixation of alleles (Nei et al. 1975).

In marine species (especially invertebrates and fish), high fecundity together with very large variance in reproductive success can lead to an $N e$ several orders of magnitude smaller than $N$ (Hedgecock 1994). Indeed the most recent estimates of $N e$ in marine fish suggest sizes that are three to five orders of magnitude smaller than $N$ (Turner et al. 2002, Hauser et al. 2002, Hutchinson et al. 2003). With such a small $N \mathrm{e} / N$ ratio, even species with a very large $N$ may suffer loss of genetic diversity due to fishing pressure. In short, millions and millions of individuals do not guarantee maintenance of genetic integrity.

## Estimating Ne

Effective population size is notoriously difficult to estimate. The most reliable way is by temporal methods in which the same population is sampled through a time course in which it can/must be assumed that changes in allele frequencies are solely due to genetic drift. In other words, it must be assumed that there is no migration, no selection and no mutation. Even if these assumptions are accepted, the problem of sampling remains. Fortunately, historical DNA extracted from archived material such as scales (Nielsen et al. 1999, Hauser et al. 2002, Ardren and Kapuscinski
2003), fin clippings (Heath et al. 2002) and otoliths (Hutchinson et al. 2003, this study) solves this problem by providing the long term temporal sampling needed.

Classic methods for the calculation of Ne are based on changes in the standardised variance in allele frequency $(F)$ (e.g. Nei and Tajima 1984, Waples 1989). The problem with these $F$-statistic approaches is that as they are summary statistics and therefore do not use all of the information in the data (Wang 2001) thereby reducing accuracy. Moreover, when applied to microsatellite data, these $F$ based methods have difficulty dealing with rare alleles, which are common in microsatellites and are sensitive indicator of genetic drift (Frankel and Soulé 1981). Furthermore the upper limits of $95 \%$ confidence interval often reach infinity ( Luikart and Cornuet 1999), thus limiting the interest of these methods especially when $N$ e is large.

More recent methods for the estimation of Ne utilise maximum likelihood (ML) (e.g. Williamson and Slatkin 1999, Anderson et al. 2000, Wang 2001, Berthier et al. 2002). Although computationally intensive, these approaches which search for values that will maximise the likelihood function, in our case the $N e$ that best fits the change in allelic frequencies in a model of only genetic drift are more accurate and less biased than F- based methods (Williamson and Slatkin 1999, Wang 2001, Berthier et al. 2002) because they used all the information in the data.

## Factors affecting Ne

Variance in reproductive success due to sweepstakes recruitment is considered to be the most important parameter for species with high fecundity and high initial mortality (type III survival curves) (Hedgecock 1994, Turner et al. 2002), which are typical traits of marine fish. At equilibrium, the variance effective population size, as a function of $N$, the census size and of $V_{\mathrm{k}}$, the variance in the number of offspring per parent, is $N \mathrm{e}=(4 N-4) /\left(V_{\mathrm{k}}+2\right)$ (Crow and Denniston 1988). With high levels of female fecundity ( $20,000-600,000$ eggs•female ${ }^{-1}$ ) (Rijnsdorp 1991) offset by high levels of daily mortality for eggs (up to 20\%) (Rijnsdorp and Jaworski 1990) and juveniles (up to 4\%) (Van der Veer et al. 1990), plaice falls into the type III category. Therefore, sweepstakes recruitment is probably the main factor reducing Ne for this species. However, additional factors can affect Ne , variance in reproductive success due to mating behaviour, uneven sex ratios and fluctuation of population size.

The importance of mating behaviour is often overlooked in marine fish mainly due to a lack of data. For plaice the commonly accepted model of free spawning in large, seasonal aggregations suggests little or no courting behaviour. In fact, very little is known about plaice mating behaviour in the wild due to the difficulties of observation. However, recent observations of captive flatfish indicate complex mating behaviours including the possibility of female choice (Konstantinou and Shen 1995, Stoner et al. 1999). If correct, this could increase the variance in reproductive success and would have a large effect on effective population size.

As there is sexual dimorphism in maturation and growth, as well as a difference in the natural mortality and vulnerability for fishing, the heavy fishing pressure in the North Sea has removed the larger adult specimens from the population more strongly in males than females. Consequently the sex ratio in plaice is slightly skewed towards females (Rijsndorp 1994), potentially leading to a reduction of Ne .

Fluctuations in population sizes are probably not very important in plaice compare to species like sardines or anchovies (Gaggiotti and Vetter 1999) as the long reproductive live span of plaice with overlapping generations creates a buffer against such fluctuations.

## Fisheries effects on genetic diversity and life history traits

By reducing $N$ overall and/or by disrupting reproductive behaviour, fishing pressure can reduce Ne , and, therefore, the genetic diversity. Such a loss of diversity can lead to a loss of adaptability. Empirical evidence is accumulating: Smith et al. (1991) compared virgin and heavily exploited stocks of Orange Roughy (Hoplostethus atlanticus) in New Zealand and found an overall decrease in heterozygosity; a reduction in genetic diversity has also been reported in the New Zealand snapper (Pagrus auratus) (Hauser et al. 2002) and in the North Sea cod (Gadus morhua) (Hutchinson et al. 2003) both of which have a $N \mathrm{e} / N$ ratio of $10^{-5}$. These two latter cases illustrate the fact that $N e$ in fish can be small enough to suffer a loss of genetic diversity under heavy fishing pressure.

It is usually considered that a Ne of 500 individuals is necessary to maintain genetic diversity for long term adaptability. With a $N \mathrm{e} / N$ ratio of $10^{-5}$, this means that population with $N$ less then 50 millions could already suffer loss of adaptability. How much such loss of genetic diversity contributes to the lack of recovery observed in most marine fisheries (Hutchings 2000) is still unclear.

Box 2. Genetic diversity.

There are several ways to estimate genetic diversity. Allelic diversity or allelic richness is calculated by counting the average number of alleles per locus across several loci. However, this measure is dependent upon sample size. If this is not a problem, then the measure is particulary sensitive to the detection of bottlenecks.
A better measure, which is not sample size dependent, is the expected heterozygosity ( He ) also called gene diversity. He is the chance that two randomly chosen alleles from a population will be different. This can be expressed by the relationship

$$
H \mathrm{e}=1-\sum p^{2}{ }_{i}
$$

where $p_{\mathrm{i}}$ is the frequency of the $i$ th allele. Values range from 0 to 1 . However, this measure too has some drawbacks. As the frequencies of alleles at a locus approach equality, the measure become less sensitive. Moreover, when multi-allelic, He can vary as a function of the chosen loci and not necessarily as a result of actual biological diversity. Therefore, it is important to utilise loci with comparable levels of polymorphism and to take care in summary comparisons among independent studies that have used different loci.

Fisheries, acting as a selective pressure, also affect life history traits. Age and size of first reproduction, growth rate and reproductive output are heritable and can thus respond to selection by fishing pressure (Heino and Godo 2002). All organisms, which suffer an increase in mortality from a particular life history stage onward are likely to show a fisheries-induced adaptive change in either maturation or growth/reproductive investment or both. What the quantitative response will be is dependent on the particular life history characteristics of the species under consideration. Fisheries induced selection is expected to favour individuals that can reproduce at a younger age. A decrease in size at first maturation and an increase in fecundity as a response to fishing pressure has been demonstrated in several heavily exploited species (Law and Rowell 1993, Rochet 1998, Sinclair et al. 1999, Olsen et al. 2004) and in North-Sea plaice (Rijnsdorp 1993, Grift et al. 2003).

## Plaice population genetics

## Migration hypotheses and plaice stock structure

Five hypothetical migration models among feeding, spawning and nursery grounds (Fig 7) have been proposed indicating the range of stock structures that might be predicted. At one extreme (Model A), a population/stock/cohort would maintain its cohesion throughout the entire life cycle loop. Such an extreme model, with totally discrete units, is not realistic but is shown here for heuristic purposes. Such populations would be characterised by little or no gene flow, and strong differentiation as compared with other populations. At the opposite extreme (Model E), there is only one large panmictic population regardless of the stage in the life cycle loop. Gene flow would be complete with no measurable differentiation. Such a model is often proposed for pelagic oceanic species. Between these extremes, Model B corresponds to the original hypothesis for plaice as discussed above. Populations have designated "home" spawning and nursery grounds but mix freely on the feeding grounds. In this model, strong differentiation is expected among spawning and nursery grounds but not among feedings grounds. In Model C, populations have different feeding and spawning grounds with mixing occurring on nursery grounds. Strong differentiation is detectable among feeding and spawning grounds but not among nurseries. In model D , populations have different spawning grounds but mixing occurs on both the feeding and nursery grounds. Population differentiation is detectable only among spawning grounds.


Fig. 7. Hypotheses for migration in plaice. Open and closed circles indicate individuals from two different populations. Model B corresponds to our original model for plaice. See text.

## The question of philopatry to the natal spawning grounds

Tagging studies have shown high fidelity of individual plaice to a particular spawning ground (De Veen 1978, Hunter et al. 2003), which suggests that there is some form of social structure. The question is: how do plaice know how to find their spawning ground given that spawning occurs in the open sea with larvae quickly advected from the natal site? Arnold and Metcalfe (1995) suggested that immature plaice make a "dummy run" migration to the spawning grounds following that of mature fish to learn the location of the spawning ground, associating some feature of the mature spawning fish, perhaps a pheromone, with some permanent topographical feature of the sea bed. Strong philopatry involving larval attachment would promote strong population differentiation (Model A), but it seems more likely that homing behaviour is learned from older spawners, as in herring (Corten 2002). Such an explanation would consistent with no/weak population differentiation.

## Kinship and inbreeding

Due to their large census size and the lack of knowledge about the mating system, the possibility of kinship and inbreeding in marine fish has seldom been considered. However as we have discussed, effective population sizes can be small and the mating system complex. Therefore, kinship and inbreeding do require our attention. Inbreeding is the result of mating between genetically related individuals (related by common ancestry).

Inbreeding is unavoidable in very small isolated populations but it can also occur if mating does not occur at random; for example if there is a family structure (clan) and if mating is more frequent within the extended family. Inbreeding is a concern for conservation biology because of its potentially detrimental effect on fitness. Inbred individuals often suffer from inbreeding depression, a lower fitness compared to non-inbreds (Charlesworth and Charlesworth 1987).

Box 3. Kinship.
Kinship also refers to genetic relatedness among individuals. For example, full-siblings share both parents (relatedness coefficient of $1 / 2$ ), Half-sibs share one parent (relatedness coefficient of $1 / 4$ ). Totally unrelated individuals have a value of zero. The relatedness coefficient is the probability that two alleles sampled randomly from two different individuals in the same population are identical by descent (i.e., correlation of alleles between individuals).

Box 4. Inbreeding.
Inbreeding is the result of mating between genetically related individuals. There are two variations. In a very small random mating population it is called panmictic inbreeding. In a large non-random mating population, in which assortative mating occurs it is called partial inbreeding. Partial inbreeding can be detected by comparing the proportion of heterozygotes observed and expected under Hardy-Weinberg equilibrium. An inbreeding coefficient $F_{\text {IS }}$ can be calculated. It is the probability that two alleles at a locus within a single individual are identical by descent (i.e., correlation of allele within an individual).
Example :


Joke and Piet are sister and brother. They are kin. They share the common allele A, inherited from their mother Anna. Jan is the offspring of a brother-sister mating between Joke and Piet. Jan is inbred. His two alleles AA are identical by descent.

Sampling: issues and tissues
The ability to detect population subdivision depends upon the ability to accurately sample populations, thus avoiding Wahlund effects caused by overlaps among subpopulations or the effects of mixed populations on the feeding grounds. To test for population differentiation across its biogeographic range, we had to assume (at least a priori) that physical distance or topographic barriers would improve the chances of sampling different populations. In addition we also sampled by life stages, i.e. on the feeding grounds, spawning grounds and in nurseries.

DNA was extracted from blood or muscle tissue. Historical DNA was obtained from dried tissue still intact on otoliths. Temporal DNA samples were taken from the extensive otolith collections made available to us by the fisheries institutes HAFRO (Reykjavik, Iceland) and RIVO (IJmuiden, The Netherlands).

Otoliths (fish ear bones) are small structures of calcium carbonate found in the head of all Teleostei which provide a sense of balance to the fish. To the fisheries biologist, the otolith is a very important tool as growth rings (annuli) record the age and growth increments of a fish from the date of hatching to the time of death. Both HAFRO and RIVO have extensive collections dating from the 1920s. Otoliths are typically bloody and stored immediately in paper envelopes without cleaning. High quality DNA can be easily extracted from the dried otoliths (Hutchinson et al. 1999).

Box 5. Wahlund effect

A Wahlund effect is a type of sampling artefact that occurs when two or more subpopulations are mistakenly sampled as one. Homozygotes will be more frequent and heterozygotes will be less frequent than expected based on the allele frequency in the combined populations. The magnitude of the departure from expectations is directly related to the magnitude of the variance in allele frequencies across populations. The effect will apply to any mixing (spatial or temporal) of samples in which the subpopulations combined have different allele frequencies.

Example:


- Two sub population with $\mathrm{N}_{1}=\mathrm{N}_{2}=16$
- The two subpopulations are at Hardy-Weinberg equilibrium (HWE)
- Two alleles A and B, at frequencies p and q respectively

$$
\begin{array}{cc}
\text { Sub pop 1 } & \text { Sub pop 2 } \\
\mathrm{p}_{1}=0.25 & \mathrm{p}_{2}=0.75 \\
\mathrm{q}_{1}=0.75 & \mathrm{q}_{2}=0.25 \\
& \\
\mathrm{AA}=\mathrm{p}_{1}{ }^{2} \times \mathrm{N}_{1}=1 & \mathrm{AA}=\mathrm{p}_{2}{ }^{2} \times \mathrm{N}_{2}=9 \\
\mathrm{BB}=\mathrm{q}_{1}{ }^{2} \times \mathrm{N}_{1}=9 & \mathrm{BB}=\mathrm{q}_{2}{ }^{2} \times \mathrm{N}_{2}=1 \\
\mathrm{AB}=2 \mathrm{p}_{1} \mathrm{q}_{1} \times \mathrm{N}_{1}=6 & \mathrm{AB}=2 \mathrm{p}_{2} \mathrm{q}_{2} \times \mathrm{N}_{2}=6
\end{array}
$$

If the two subpopulations are mistakenly sampled as one:

$$
\begin{gathered}
\mathrm{N}=\mathrm{N}_{1}+\mathrm{N}_{2}=32 \\
\mathrm{p}=0.5 \\
\mathrm{q}=0.5
\end{gathered}
$$

Observed
AA=10
$\mathrm{BB}=10$
$\mathrm{AB}=12$

Expected under HWE
$A A=p^{2} \times N=8$
$\mathrm{BB}=\mathrm{q}^{2} \times \mathrm{N}=8$
$\mathrm{AB}=2 \mathrm{pq} \times \mathrm{N}=16$

There are fewer heterozygotes than expected. The more the allelic frequencies (here p and q ) differ among the subpopulations, the stronger this heterozygote deficiency will be.

## Microsatellites

Microsatellites are short DNA sequences (2-5 base pairs), tandemly repeated and scattered throughout the genome of most eukaryotes. They are nuclear, co-dominant, single-locus, and highly polymorphic. For example, 10-20 alleles/ locus is common although hypervariable loci with up to 100 alleles/locus are also possible. In the present study the number of alleles ranged from 5 to $40 /$ locus. Length variation in repeat number allows for easy genotyping, especially with automated methods that permit direct sizing of the DNA fragments (alleles). In short, microsatellite loci are the current marker of choice for population genetic studies requiring allele frequency data, such as studies of population differentiation and the mating system (Jarne and Lagoda 1996).

## Box 6. Microsatellites

1. Sequence of a cloned microsatellite allele. The microsatellite is shown in boldface, the flanking regions in italic.
CCTATGGATCGCTGGAGACACACACACACACACACACACACGTCAAAAGGCAATT
Forward PCR primer
Reverse PCR primer
2. The number of repeats varies among alleles and among individuals. Below is an example from a diploid individual that happens to be heterozygote.

Allele 1
Allele 2

3. Fragments are fluorescently labelled and detection occurs by electrophoresis on a high resolution gel that is scanned by a laser.


Microsatellite fluorogram. This individual is heterozygote.
Despite all of their advantages, microsatellites are not without problems. Loci must be developed on a species-specific basis (although cross-species amplification is sometimes possible) which require considerable time and expense. Development of 6-10 suitably polymorphic loci takes from 3-9 months in fish depending upon specific optimisation problems encountered. A more serious problem with microsatellite loci is their mutation model and their rapid mutation rate. Earlier generation codominant markers (allozymes) were assumed to evolve relatively
slowly and under an infinite alleles model (IAM). In contrast, microsatellite loci have mutation rates estimated between $10^{-3}$ and $10^{-5} /$ generation combined with a step-wise mutation model (SMM) (Ohta and Kimura 1973) in which the repeat length may expand or contract through replication slippage (Levinson and Gutman 1987). Fast mutation combined with forward and backward mutation may lead to homoplasy and saturation of a locus (Nauta and Weissing 1996). In order to account for this SMM mutation model, Slatkin (1995) developed $R_{\text {ST }}$ for estimating population differentiation. However, as more data became available, it appears that most microsatellites do not follow a strict SMM (Webster et al. 2002) and other models, such as a Two Phase Model (Dirienzo et al. 1994), have been proposed. It is now recognised that for population differentiation studies the traditional $F_{\mathrm{ST}}$ (Wright 1969) is still preferred over $R_{\text {ST }}$ (Gaggiotti et al. 1999).

Other potential problems with microsatellites are technical artefacts including null alleles, stuttering and large allele dropout.

Box 7. Scoring problems with microsatellites.
Microsatellite loci do not always amplify properly. This produces technical artefacts that must be identified and eliminated.

- Null alleles are the result of non-amplification of some alleles because of mutation in the flanking regions where the PCR primers must anneal. Use of lower stringency PCR condition can help to identify and (sometimes) correct the problem. Redesigning the PCR primers may also be required in some cases.
- Stuttering is due to Taq polymerase slippage during PCR amplification. All polymerases stutter on microsatellites at a rate characteristic of the enzyme, the locus and the PCR conditions. Stuttering can lead to difficulty in determining the correct allele sizes on the fluorograms. Altering the conditions of PCR and including T4 polymerase can help to reduce this problem.

- Large allele drop-out is the result of preferential amplification of the shorter allele in a heterozygote. In general, the larger alleles amplify less efficiently than the shorter one. Again relaxation of the stringency can help to minimise this problem.
The software package Microchecker (Van Oosterhout et al. 2003) can be used to test for all of these potential artefacts.


## Mitochondrial DNA

Animal mtDNA is a small ( $15-18 \mathrm{~kb}$ ), usually circular DNA molecule. MtDNA is widely used in evolutionary biology for both phylogenetic and population genetic studies (Avise 1994, Ballard and Whitlock 2004). The success of mtDNA is due to some of its characteristics such as maternal transmission, putative lack of recombination, and relatively high mutation rate. It also requires much less developmental effort to obtain PCR primers for a new species, as compared with microsatellite development. In the present study, we used the mitochondrial control region (D-loop).

In contrast with microsatellites, which are (mostly) nuclear and therefore (usually) diploid and biparentally inherited, animal mtDNA is haploid and, with a few exceptions (e.g. Gyllensten et al. 1991), maternally inherited. Because of these differences in ploidy and inheritance, the mitochondrial effective population size $\left(N \mathrm{e}_{(\mathrm{mt})}\right)$ is four times smaller than for nuclear loci (Birky et al. 1989), and thus more susceptible to the effects of genetic drift. Consequently, estimates of population differentiation can actually be higher for mitochondrial markers (see discussion in Crochet 2000, but also Birky et al. 1989 for possible exceptions). This feature was of special interest in the present study. Another advantage of mtDNA's smaller $N \mathrm{e}$ is that it will reach migration-drift equilibrium sooner than will nuclear DNA (Birky et al. 1989). This is relevant for species that are known to be part of the recent recolonisation of the North Atlantic following the last glacial maximum (last 1810,000 years). The use of mtDNA could, therefore, increase the chance of detecting population differentiation.

MtDNA, in combination with nuclear markers, provides a way to investigate sexbiased dispersal. Recent tagging data (L. Bolle personal communication) suggest that there is no sex-biased dispersal in plaice but population genetics data are needed to confirm this. This approach has been successfully used in several species, mainly mammal and birds (Pusey 1987). Very few studies have looked at sex-biased dispersal in fish. No sex-biased dispersal was found in blue marlin (Buonaccorsi et al. 2001) whereas brook trout (Hutchings and Gerber 2002) and white sharks (Pardini et al. 2001) showed male-biased dispersal.

## Outline of the thesis

The development of new microsatellite loci for plaice is described in chapter 2. Evidence for population differentiation throughout Northern Europe, including Iceland, was first explored using the microsatellite loci in chapter 3 and then with mtDNA haplotypes in chapter 4. In both analyses, adult and juvenile (=young-of-the-year) samples were taken $(\mathrm{N}=480)$ from 11 locations. In both analyses hypothesised connections among feeding, spawning and nursery grounds were determined and differences in the properties and resolving power of the two markers compared.

Unexpectedly, a strong heterozygote deficiency was found in both adults and juveniles that could not be explained on the basis of laboratory or sampling artefacts (i.e. null alleles or Wahlund effects). This result led us to investigate the possibility of relatedness and, ultimately, inbreeding-a condition not usually considered in species with large census populations. We first sampled and genotyped temporal cohorts of juveniles on a biweekly basis at Balgzand in the Dutch Wadden Sea and at Alftanes in Iceland (chapter 5). These results were consistent with small effective population sizes and inbreeding. In chapter 6 we continue the investigation of possible fisheries-induced inbreeding and low effective population size using archived historical DNA extracted from otoliths (fish ear bones) dating from the 1920s to 2002.

In chapter 7 we report on a spin-off project on flounder (Platichtys flesus) which reportedly hybridises with plaice. The analysis of the mitochondrial DNA control region revealed extensive heteroplasmy. Subsequent sequencing of the region revealed the first direct evidence for recombination in the mitochondrial DNA of a vertebrate.

In chapter 8 I summarise the main conclusions from the project, pose a number of new questions and offer a few prospects for the fate of plaice.

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## Chapter 2

# NEW MICROSATELLITE PRIMERS FOR PLAICE, Pleuronectes platessa L. (TELEOSTEI : PLEURONECTIDAE) 

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#### Abstract

Seven microsatellite loci were developed for Pleuronectes platessa, a commercially important flatfish found throughout northern European waters. The number of alleles per locus ranged from 11-33 and heterozygosities from $0.74-0.96$. Cross reactivity was tested against six other flatfish species. Significant amplification was found in all species from one to all seven of the loci.


Plaice (Pleuronectes platessa) is one of the most important flatfish for North Sea fisheries. Its life history (e.g. Van Der Veer 1986, Rijnsdorp and Pastoors 1995) is of interest because high dispersal of pelagic eggs and larvae, as well as adult migration are expected to reduce population differentiation; whereas adults faithful to several discrete spawning grounds could promote population isolation. Previous studies of plaice using allozymes did not reveal any evidence for population differentiation (Purdom et al. 1976, Ward and Beardmore 1977). Microsatellite markers may, therefore, be more appropriate genetic makers for exploring population structure and the mating system.

A size selected library ( $300-800 \mathrm{bp}$ ) was prepared and screened simultaneously with synthetic $(\mathrm{GT})_{15}$ and $(\mathrm{GA})_{15}$ oligonucleotides radiolabeled with $\left[\gamma^{32} \mathrm{P}\right]$ ATP, as described in Brooker et al. (1994). Approximately 250 positive clones were selected after two rounds of screening and the 50 showing the stronger signal were sequenced. Fifteen sets of primers were designed of which 7 were selected according to the clarity of the banding patterns. Polymorphism testing was done against a panel of 48 individuals collected from a broad geographical range (Table 1).

Total genomic DNA was extracted using a CTAB protocol modified from De Jong et al. (1998). PCR amplification was carried out in $10-\mu \mathrm{l}$ volumes containing 1 $\mu 1$ of $1 / 10$ diluted DNA, 1X reaction buffer (Promega), 0.2 mM of each dNTP, 2 $\mathrm{mM} \mathrm{MgCl} 2,0.25 \mathrm{U}$ Taq DNA polymerase (Promega) and each primer at the concentration indicated in Table 1. One primer of each pair was end-labelled with a fluorescent dye.

PCR amplification was performed in either a PTC100 (MJ Research) or a Mastercycler gradient (Eppendorf) thermal cycler and consisted of an initial denaturation at $94{ }^{\circ} \mathrm{C}$ for $1 \mathrm{~min} ; 3$ cycles of denaturation at $94^{\circ} \mathrm{C}$ for 1 min , annealing at $48-58^{\circ} \mathrm{C}$ for 1 min , and extension at $72^{\circ} \mathrm{C}$ for 30 s . This was followed by 30 cycles of denaturation at $94^{\circ} \mathrm{C}$ for 20 s , annealing at $48-58^{\circ} \mathrm{C}$ for 15 s , and extension at $72{ }^{\circ} \mathrm{C}$ for 12 s ; and a final extension at $72^{\circ} \mathrm{C}$ for 10 min . In order to reduce genotyping error, the PCR products of loci PL52, PL142 and PL167, were treated with T4 DNA polymerase to remove the extra bases added by the Taq polymerase (Ginot et al. 1996). After PCR amplification, 0.4 U T4 polymerase (Promega) was added to the PCR products and the mixture was incubated at $37^{\circ} \mathrm{C}$ for 30 min . PCR products were visualized using an ABI Prism-377 automatic
Table 1. Characteristics of 7 microsatellites loci developed for Pleuronectes platessa. $N=48$. HE and HO are expected and observed heterozygosity. T4 indicate the use of T4 polymerase treatment of PCR product (see text).
$\left.\begin{array}{lllllllllll}\hline \text { Locus } & \text { Repeat motif } & \text { Primer sequences (5'-3') } & \begin{array}{l}\text { Size range } \\ (\mathrm{bp})\end{array} & \begin{array}{l}\text { No. of } \\ \text { alleles }\end{array} & \text { HE } & \begin{array}{l}\text { H0 }\end{array} & \begin{array}{l}\text { Accession } \\ \text { number }\end{array} & \begin{array}{l}\text { Annealing } \\ \text { temp. }\end{array} \\ \hline \text { PL06 } & (\mathrm{GT})_{7} \mathrm{TT}(\mathrm{GT})_{25} & \text { F: AACTGATTTACTTTACTTTGTC } \\ \text { concentration }\end{array}\right)$
Table 2. Cross species amplification of the microsatellites developed for Pleuronectes platessa. + : amplification, - : no amplification or unreadable peaks, ? : not tested.

|  | PL06 | PL09 | PL52 | PL92 | PL115 | PL142 | PL167 |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Pseudopleuronectes americanus | + | + | + | + | + | + | + |
| Platichtys flesus | $?$ | + | $?$ | + | + | $?$ | $?$ |
| Microstomus kit | $?$ | - | $?$ | + | + | $?$ | $?$ |
| Limanda limanda | $?$ | + | $?$ | + | $?$ | $?$ | $?$ |
| Paralichthys lethostigna | - | - | - | + | + | - | + |
| Solea solea | $?$ | - | $?$ | + | - | $?$ | $?$ |

sequencer (Applied Biosystems) with an internal lane standard and GENESCAN ${ }^{\text {TM }}$ software (Applied Biosystems) for determination of allele sizes.

Observed and expected heterozygosity was computed using GENETIX software (Belkhir et al. 2000). The characteristics of the 7 loci are described in Table 1. No linkage disequilibrium was detected among the 21 pairwise comparisons. Cross species amplification was tested on 6 other flatfish species (Table 2).

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Chapter 2

## Chapter 3

# POPULATION STRUCTURE OF PLAICE (Pleuronectes platessa L.) IN NORTHERN EUROPE: MICROSATELLITES REVEALED LARGE SCALE SPATIAL AND TEMPORAL HOMOGENEITY 

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#### Abstract

Philopatry to spawning grounds combined with well-known migratory patterns in the flatfish Pleuronectes platessa (plaice) has led to the hypothesis that regional populations may reflect relatively discrete, genetic stocks. Using six microsatellite loci we genotyped 240 adult individuals collected from locations in Norway, the Faeroe plateau, the Irish Sea, the Femer Baelt, Denmark, and the southern North Sea; and 240 0-class juveniles collected from five nursery-ground locations in Iceland, Northwest Scotland, two sites in the Wadden Sea, and the Bay of Vilaine in Southern Brittany. The mean number of alleles/locus ranged from 5.3-20.4 with a mean of 13.9. Expected heterozygosity was uniformly high across all locations (multi-locus $H \mathrm{e}=0.744 \pm 0.02$ ). Pairwise comparisons of $\theta$ among all 11 locations revealed significant differentiation between Iceland and all others $\left(\theta=0.0290^{* * *}\right.$ to $0.0456^{* * *}$ ) which is consistent with the deep-water barrier to dispersal in plaice. In contrast, no significant differentiation was found among any of the remaining continental-shelf sampling locations. This suggests that regional stocks are themselves composed of several genetic stocks under a model of panmixia which persists even to the spawning grounds. The presence of significant heterozygote deficiencies at all locations (not due to null alleles) suggests a temporal Wahlund effect yet the absence of significant population differentiation among continental shelf localities makes this explanation alone, difficult to reconcile. Sampling of eggs at the spawning grounds will be required to resolve this issue. Causes of the mismatch between genetic and geographic stocks are discussed in the context of high gene flow.


## Introduction

The spatial- and temporal scales over which sub-populations of a species can be identified and the strength of gene flow among them are of fundamental importance to conservation and management of marine fisheries (Carvalho and Hauser 1994, Ward and Grewe 1994). Sub-populations or stocks, may possess novel characteristics that promote differences in growth rate, fecundity and disease resistance that, taken together, contribute to species-level, long-term adaptability and survival. Although the concept of stock identification is straightforward, the life history and ecology of a species (Waples 1998) can greatly affect the ability to actually identify genetic stocks in nature. Physical barriers to dispersal such as depth, temperature or salinity boundaries, or hydrodynamic eddies favouring larval retention; or strong philopatry are all factors that can promote population differentiation (Grant and Bowen 1998). Conversely, high fecundity, passive dispersal of larvae and active migration of adults can lead to dynamic and seemingly unstructured populations characteristic of many pelagic species (Waples 1998). Only recently has it become apparent that subtle genetic substructure exists between these ends of the spectrum (Wirth and Bernatchez 2001).

Plaice, Pleuronectes platessa L. (Teleostei : Pleuronectidae), is a commercially important flatfish species in European, continental-shelf-waters and especially in the North Sea. The species is found from the White Sea to the western Mediterranean (though now uncommon) and on off-shelf islands as far west as Iceland, down to a depth of 100 m (Nielsen 1986). Heavy fishing pressure over the past decade has reduced landings from 150.000 t in the 1980 s to 90.000 t in the late 1990s (Rijnsdorp and Millner 1996, ICES 2000). Concern for collapse of the fishery has raised questions about the number of populations as well as the overall genetic health of plaice.

Spawning of plaice occurs from December to March in offshore waters at a number of distinct spawning grounds. Plaice, like other flatfish, produce large numbers of passively dispersed, pelagic eggs and larvae. Fecundity is high with 20600 thousand eggs per female (Rijnsdorp 1991). The duration of the pelagic egg and larval phase typically lasts about three to four months (Harding et al. 1978). At the end of the pelagic phase, larvae metamorphose into demersal juveniles, with the familiar flatfish morphology, and settle at a size of 10 to 15 mm in shallow coastal
areas (Edwards and Steele 1968, Zijlstra 1972). They remain for about one to two years in these so-called nursery areas and thereafter migrate towards deeper waters to join the adult populations.

Plaice are more mobile than might be predicted from their demersal lifestyle. Tagging data have been used to model their spatial dynamics (De Veen 1978; Rijnsdorp and Pastoors 1995). Results showed a coarse North-South migration of adults within the North Sea with a stop at several well-known spawning grounds in early winter. Migration distances of 300 km were relatively common although migration rates of $>20 \mathrm{~km} /$ day have been recorded for some individuals using electronic data storage tags (Metcalfe and Arnold 1997). Despite the roaming lifestyle across the feeding grounds by adults, plaice also exhibit reproductive homing behaviour (De Veen 1978) which has been hypothesized to promote the maintenance of separate genetic stocks.

The influence of homing behaviour on population differentiation has been mainly studied in anadromous (e.g. salmon: Tallman and Healey 1994, shad: Waters et al. 2000, sturgeon: Stabile et al. 1996) and fresh-water species (e.g. walleye: Stepien and Faber 1998). However, this behaviour has also been suggested for pelagic marine species such as mackerel (Nesbo et al. 2000) and herring (McQuinn 1997). Several discrete spawning grounds have been identified for plaice in the North Sea and adjacent waters (Harding et al.1978). Although tagging studies indicate that adults return to spawning grounds year after year, important questions remain as to whether the chosen spawning ground is the natal spawning ground, or if individual fish return to the same spawning ground throughout their lives.

Most population genetic studies of commercially important fish have used allozymes or mitochondrial DNA (mtDNA) as molecular markers. In most cases low or no differentiation could be detected (reviewed in Waples 1998). Whether failure to find differentiation was due to a lack of sufficient resolving power of the markers or to the biology of the fish has not always been clear. With the advent of highly polymorphic microsatellite loci, (see e.g. Jarne and Lagoda 1996) the potential for detecting weak differentiation in high gene flow species has increased substantially (Waples 1998). Microsatellites have been able to detect a greater degree of population structure than allozymes or mtDNA in cod (Ruzzante et al. 1998), herring (Shaw et al. 1999b), squid (Shaw et al. 1999a) and hake (Lundy et al. 2000).

A few studies of broad-scale geographic differentiation have been done on commercially important flatfish (Table 1). For flounder (Platichthys flesus/stellatus) strong differentiation has been found among Atlantic, Western Mediterranean and Adriatic populations. Strong differences were also found within the Mediterranean. In contrast, differences within the North Atlantic region were much lower but indicated some isolation by distance. In sole (Solea vulgaris $[=S$. solea]), Kotoulas et al. (1995) detected significant East-West differentiation across the Mediterranean and North-South differentiation along the European Atlantic coast but were unable to demonstrate any differentiation within the North Atlantic "zone". A similar lack of significant differentiation within most North Atlantic populations has been reported for turbot (Scophthalmus maximus), halibut (Hippoglossus hipoglossus), brill (Scophthalmus rhombus) and plaice (Pleuronectes platessa) (Table 1).With the exception of turbot, all of the species had high heterozygosities indicative of large population sizes. Strong between-region differentiation in flounder and sole has been attributed to larval temperature tolerances which are narrower in these two flatfish species. Differentiation within the North Sea region, usually north and south of the Dogger Bank, has been reported in flounder (Platichthys flesus) (Borsa et al. 1997) and whiting (Merlanguius merlangus) (Rico et al. 1997). Older studies in plaice (Purdom and Thompson 1976, Ward and Beardmore 1977) compared only three locations (Irish Sea, Bristol Channel and southern North Sea) with five allozyme loci and were unable to establish differences.

The aim of the present study was to test the hypothesis that geographic stocks of plaice represent different genetic stocks linked by gene flow under a model of panmixis and isolation by distance. We first tested for population differentiation in plaice across its biogeographic range in the northern Atlantic, including on and off the continental shelf. We then examined a number of locations within the North Sea and Irish Sea. Finally, we compare differentiation between spawning, nursery and feeding ground locations.
Table 1. Comparisons of genetic structure studies based on allozymes, mtDNA haplotypes and microsatellites in flatfish along the North Atlantic continental shelf ( $* P<0.05, * * * P<0.001$, ns $=$ not significant, pop = population).

|  | Author | Marker | Number of locations sampled | $\begin{aligned} & \text { Genetic diversity } \\ & \text { H or He } \\ & \hline \end{aligned}$ | $\begin{aligned} & \text { Departure from } \\ & \text { HWE } \\ & \hline \end{aligned}$ | Mean population differentiation $\theta G_{\mathrm{ST}} / \Phi_{\mathrm{ST}}$ | Locations | Conclusion |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Plaice |  |  |  |  |  |  |  |  |
| Pleuronectes platessa | Hoarau et al. this study | msats 6 loci $($ mean Na /locus $=13.9)$ | $\begin{aligned} & 11 \\ & (\mathrm{~N}=48 / \mathrm{pop}) \end{aligned}$ | 0.743 (high) | All populations, all loci | $0.0086^{* * *}$ for all populations 0.0014 ns for shelf populations | see text | subdivision only between continental shelf and non-shelf populations no subdivision. Mentions some differences in alleles between Irish- and North Sea no subdivision |
|  | Ward \& Beardmore 1977 | allozymes <br> 5 loci | $\begin{aligned} & 3 \\ & (\mathrm{~N}=?) \end{aligned}$ | 0.118 (high) | cites Purdom \& Thompson 1976 | not quantified | Bristol Channel, North Wales, North Sea (location not specified) |  |
|  | Purdom \& Thompson $1976$ | $\begin{aligned} & \text { allozymes } \\ & 5 \text { loci } \\ & \hline \end{aligned}$ | $\begin{aligned} & 3 \\ & (\mathrm{~N}=?) \\ & \hline \end{aligned}$ |  | mentioned for some loci. | not quantified | Western English Channel, Irish Sea, North Sea |  |
| Turbot |  |  |  |  |  |  |  |  |
| Scophthalmus maximus | $\begin{aligned} & \hline \text { Coughlan et al. } \\ & 1998 \end{aligned}$ | 3 loci  <br> (mean Na /locus $=7)$ $(\mathrm{N}=50 /$ pop $)$ |  | 0.66-0.77 (high) | ns | 0.009 ns | Ireland and Norway could not be distinguished | no subdivision |
|  |  | allozymes 14 loci | $\begin{aligned} & 3 \\ & (\mathrm{~N}=13-50) \end{aligned}$ | 0.029 (low) | HWE "assumed" | 0.012 ns | Galician coast | no subdivision |
|  | Blanquer et al. $1992$ | $\begin{aligned} & \text { allozymes } \\ & 17 \text { loci } \\ & \hline \end{aligned}$ | 10 | 0.02 (low) | ns | 0.000 ns | Mediterranean to Kattegat | Aegean was the only one different from all others |
| Flounder |  |  |  |  |  |  |  |  |
| Paralichthys dentatus | Jones \& Quattro 1999 | mtDNA haplotypes | $\begin{aligned} & 10 \\ & (\mathrm{~N}=10-25) \end{aligned}$ | -- |  | 0.004 ns | East coast USA (Cape Cod to South Carolina) | MA/RI were different from the rest. Cape Hatteras not a barrier |
| Platichthys flesus/stellatus | Borsa et al. 1997 | allozymes <br> 31 loci mtDNA haplotypes | 8 regions <br> 7 within North Atlantic | 0.095 | ns | $\begin{aligned} & 0.039^{*} \\ & \text { (north and south North Sea) } \end{aligned}$ | Baltic, North Sea, SW Britain, Brittany not different | significant among 'regions' but not within regions. North Sea was single exception |
| Sole |  |  |  |  |  |  |  |  |
| Solea vulgaris $[=S$. solea] Solea solea | Kotoulas et al. 1995 <br> Exadactylos et al. 1998 | allozymes <br> 12 loci <br> allozymes <br> 27 loci | $\begin{aligned} & 27 \\ & (\mathrm{~N}=10-120 / \text { pop }) \\ & 7 \\ & (\mathrm{~N}=18-73 / \text { pop }) \end{aligned}$ | 0.170 (high) Atlantic region $0.034-0.141$ <br> (related to latitude) | some locations, some loci some locations, some loci (Irish, German Bight) | $0.0147^{* * *}$ for all regions Atlantic only 0.005 ns 0.0262 ns (no pairwise comparisons made) | Med to Normandy only (no North Sea samples) Mediterranean to Normandy (3 North Sea) | IBD across regions but not within regions no differentiation detectable |
| Halibut |  |  |  |  |  |  |  |  |
| Hippoglossus hippoglossus | $\begin{aligned} & \text { Foss et al. } \\ & 1998 \end{aligned}$ | allozymes <br> 4 loci | $\begin{aligned} & 6 \\ & (\mathrm{~N}=33-137 / \mathrm{pop}) \end{aligned}$ | ? | HWE assumed | not given Nei distance tree | Norway, Iceland, Faeroes, Greenland | east-west axis <br> Norway apart for the three others |
| Brill |  |  |  |  |  |  |  |  |
| Scophthalmus. rhombus | Blanquer et al. 1992 | $\begin{aligned} & \hline \text { allozymes } \\ & 17 \text { loci } \\ & \hline \end{aligned}$ | $\begin{aligned} & 5 \\ & (\mathrm{~N}=5-55 / \mathrm{pop}) \end{aligned}$ | 0.11 (high) | ns | not given <br> Nei distance tree only | Mediterranean/Portugal/ Britt-Kattegat | three regional groups identified |

## Materials and methods

## Sampling

In total, 480 individuals were sampled $(\approx 50 /$ location $)$ from 11 locations (Figure 1 and Table 2). Adults were obtained by trawl from feeding ground locations LO, TR, FA, IS and FE; and spawning ground TB. Juveniles were collected with small beam trawls towed by hand or from a rubber dinghy. All juveniles were offspring of the year "0-group" and collected from locations ic, ob, bv, ba and am. Blood samples were obtained from 240 live, adult fish and muscle samples were obtained from 240 preserved ( $70 \%$ ethanol) juvenile fish. Both tissue types were stored in $70 \%$ ethanol.

Adult plaice were sampled across a large part of the species' biogeographic range $(\approx 500-1400 \mathrm{~km})$ to include both feeding and spawning grounds. 0-group juveniles were sampled from the nursery grounds. Nursery grounds are generally located adjacent to spawning grounds and were hypothesised to retain cohorts. 0-class juveniles were further subgrouped by size as a proxy for age. Sampling of eggs directly from the spawning grounds was not possible.


Fig. 1. Sampling locations of Pleuronectes platessa. Triangles indicate adult populations. Circles indicate juvenile populations. The grey line delimits the $200-\mathrm{m}$ contour of the continental shelf. See Table 2 for abbreviations.

Table 2. Pleuronectes platessa. Sampling site and its code, number of individuals per sampling site ( $N$ ), life stage, type of sampling site, tissue used for DNA extraction and sampling date

| Location | Code | N | Life stage | Type | Tissue | Sampling date |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Lofoten, Norway | LO | 24 | Adults | Feeding ground | Blood | 01 Aug 99 |
| Trondheim fjord, <br> Norway | TR | 48 | Adults | Feeding ground | Blood | Sep 00 |
| Faroe's Plateau | FA | 26 | Adults | Feeding ground | Blood | Summer 99 |
| Irish Sea, England | IS | 44 | Adults | Feeding ground | Blood | 07 Sep 99 |
| Terschellinger <br> Bank, Netherlands <br> Femer Bælt, | TB | 49 | Adults | Spawning <br> ground | Blood | 15 Feb 00 |
| Denmark <br> Alftanes, Iceland | FE | ic | 49 | Adults | Feeding ground | Blood |
| Oban, Scotland Sep 99 | ob | 48 | Juveniles | Nursery ground | Muscle | 13 Jul 00 |
| Amrum, Germany <br> Balgzand, | am | 48 | Juveniles | Nursery ground | Muscle | 16 May 00 ground |
| Netherlands <br> Bay of Vilaine, <br> France | ba | 48 | Juveniles | Nursery ground | Muscle | 18 Jun 00 May 00 |

## DNA extraction

Total genomic DNA was extracted using a modified CTAB protocol (De Jong et al. 1998). Prior to extraction, the ethanol was evaporated from the blood or muscle sample. Approximately $100 \mu \mathrm{l}$ of blood or $5 \mathrm{~mm}^{3}$ of muscle tissue was incubated for 45 min at $60^{\circ} \mathrm{C}$ in $800 \mu \mathrm{l}$ extraction buffer $(100 \mathrm{mM}$ Tris- $\mathrm{HCl}, 1.4 \mathrm{NaCl}, 20 \mathrm{mM}$ EDTA, 2\% CTAB), $2 \mu \mathrm{l} \beta$-mercaptoethanol, and 6 U proteinase K (Promega, Madison, USA), followed by two chloroform-isoamyl alcohol (24:1) separations. DNA was precipitated with $500 \mu \mathrm{l}$ of ice-cold isopropanol, followed by storage at minus $20^{\circ} \mathrm{C}$ for 45 min , and centrifugation ( 14000 rpm ) for 20 min at $4^{\circ} \mathrm{C}$. The DNA pellet was rinsed with $80 \%$ ethanol, washed with $70 \%$ ethanol, air dried, and resuspended in $150 \mu$ l of sterile filtered $\mathrm{dH}_{2} \mathrm{O}$.

## Microsatellites amplification and genotyping

Samples were genotyped for six loci, two of which (List1001 and List1003) were developed by Watts et al. (1999) and four of which (PL09, PL92, PL115, and PL142) were developed by us (Hoarau et al. 2002).

Prior to PCR, the DNA samples were diluted $1 / 10$. PCR amplification was carried out in $10-\mu \mathrm{l}$ volumes containing $1 \mu \mathrm{l}$ of $1 / 10$ diluted DNA, 1X reaction buffer (Promega), 0.2 mM of each dNTP, $2 \mathrm{mM} \mathrm{MgCl}_{2}, 0.25 \mathrm{U}$ Taq DNA polymerase (Promega) and each primer at the concentration indicated in Table 3. One primer of each pair was end-labelled with a fluorescent dye. PCR amplification was performed either in a PTC100 (MJ Research) or a Mastercycler gradient cycler (Eppendorf). The reaction profile was as follows: initial denaturation at $94{ }^{\circ} \mathrm{C}$ for 1 m ; 3 cycles of denaturation at $94^{\circ} \mathrm{C}$ for 1 m , annealing at $48-58^{\circ} \mathrm{C}$ for 1 m , and extension at $72^{\circ} \mathrm{C}$ for 30 s ; followed by 30 cycles of denaturation at $94^{\circ} \mathrm{C}$ for 20 s , annealing at $48-58^{\circ} \mathrm{C}$ for 15 s , and extension at $72^{\circ} \mathrm{C}$ for 12 s ; and a final extension at $72^{\circ} \mathrm{C}$ for 10 m . See Hoarau et al. (2002) for further details.

Table 3. Pleuronectes platessa. Annealing temperature and primers concentration for six microsatellite loci

| Locus | Annealing temperature | Primer concentration (each) |
| :--- | :--- | :--- |
| PL09 | $50^{\circ} \mathrm{C}$ | $0.5 \mu \mathrm{M}$ |
| PL92 | $48^{\circ} \mathrm{C}$ | $0.1 \mu \mathrm{M}$ |
| PL115 | $53^{\circ} \mathrm{C}$ | $0.2 \mu \mathrm{M}$ |
| PL142 | $51.5^{\circ} \mathrm{C}$ | $0.2 \mu \mathrm{M}$ |
| LIST1001 | $52^{\circ} \mathrm{C}$ | $0.14 \mu \mathrm{M}$ |
| LIST1003 | $50^{\circ} \mathrm{C}$ | $0.14 \mu \mathrm{M}$ |

Microsatellite loci were visualised on an ABI Prism-377 automatic sequencer (Applied Biosystems), using an internal lane standard and Genescan ${ }^{\text {TM }}$ software for determination of allele size. To ensure reliability of allelic scoring, a set of reference samples was rerun on each gel.

## Data analysis

All computations were performed using the program packages GENETIX v. 4.01 (Belkhir et al. 2000), GENEPOP v. 3.2 (Raymond and Rousset 1995) and PHYLIP (Felsentein 1995). Observed and unbiased expected heterozygosity (Nei 1978) and allelic richness were computed for each locus individually and as a multi-locus estimate for each of the eleven locations (Table 4). Single and multilocus $F_{\text {IS }}$ were estimated using Weir and Cockerham's $f$ (Weir and Cockerham 1984). Linkage
disequilibrium was tested among all pairs of loci and for all locations using the LinkDis procedure (Black and Krafsur 1985).

Population differentiation was analysed using Wright's $F_{\mathrm{ST}}$ (Wright 1969) rather than Slatkin's $R_{\mathrm{ST}}$ (Slatkin 1995b) because, $F_{\mathrm{ST}}$-based estimates of differentiation are considered more reliable when $\leq 20$ loci are used (Gaggiotti et al. 1999).

Global $F_{\text {ST }}$ and pairwise $F_{\text {ST }}$ were estimated using Weir and Cockerham's $\theta$ (Weir and Cockerham 1984). Significance of all $f$ and $\theta$ estimates was tested using permutation ( $\mathrm{N}=2000$ ). Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied where necessary.

Exact tests of population differentiation (Raymond and Rousset 1995) were also performed for each locus and for each pair of samples using GENEPOP. This approach gives more weight to rare alleles and can, therefore, be more sensitive to the detection of weak population differentiation.

A matrix of Reynolds' genetic distances (Reynolds et al. 1983) was computed using the GENEDIST procedure of the PHYLIP software, and was used in the NEIGHBOR procedure to infer a neighbour-joining tree (Saitou and Nei 1987). Robustness of the topology was evaluated by 1000 bootstrap resamplings using the SEQBOOT procedure in PHYLIP.

Isolation by distance was examined by plotting the pairwise $\theta /(1-\theta)$ values against the Log of the geographic distances (Rousset 1997) and tested using the Mantel test (Mantel 1967) with permutations as implemented in GENETIX.

The frequency of expected null alleles and the corresponding expected number of failed amplifications in our data set were calculated according to the method of Brookfield (1996).

## Results

Genetic variation was moderate to high (Table 4). The total number of alleles per locus ranged from 7 to $44(\mathrm{~N}=460)$. The mean number of alleles per locus was 13.9 and ranged from 5.37 to 20.4. There was no evidence for private alleles based on the distribution of allelic frequancies. The two loci, List1001 and List1003 (Watts et al. 1999), displayed the lowest levels of polymorphism as compared to our loci by a factor of 2-4. Mean expected heterozygosities ( $H_{\text {exp }}$ ) were uniformly high, ranging
from 0.72 to 0.77 . Despite similar $H_{\text {exp }}$, the mean number of alleles/location was lowest in Iceland ( $\mathrm{N}=48$ ), Faroe $(\mathrm{N}=26)$ and Lofoten $(\mathrm{N}=24)$, all of which represent populations in the most northern part of the species' range. While the lower number of alleles found in the Lofoten and Faeroe locations may be an artefact of smaller sample sizes as compared with all others $(\mathrm{N}=48)$, this was not the case for Iceland. Continental-shelf ( $200-\mathrm{m}$ contour line) samples exhibited a mean of 14.6 alleles/loci as compared with a mean of 10.9 alleles/locus for non-shelf samples.

There was no significant difference in the number of alleles associated with adult $v s$. juvenile populations ( t -test, $\mathrm{p}=0.589$ ).

All locations showed a significant departure from Hardy Weinberg equilibrium (HWE) with a significant multi-locus heterozygote deficiency (Table 4). Although PL09 contributed the highest $f$-values, even if excluded from the multi-locus estimate, $f$ remained significant.
Null alleles are a common problem with microsatellite loci that can lead to high heterozygote deficiencies (Callen et al. 1993, Hare et al. 1996, O’Connell and Wright 1997). They arise when one allele is not amplified due to mutations in one of the primer, and/or when technical problems associated with amplification and scoring arise. In the present data set, amplification failure rate was zero in five of the six loci. For locus PL09 9/480 (2\%) individuals failed to amplify. In all cases, reamplifications of homozygous individuals (or failed amplifications) under less stringent reaction conditions did not alter the results. Incorrect scoring of bands was also an unlikely factor, as the use of an automatic sequencer with internal lane standards allowed for very high resolution and direct reading of allele sizes. As a final diagnostic on the potential null-allele problem, we used the method of Brookfield (1996) to calculate the expected frequency $r$ of null alleles per locus from our total sample of 480 individuals. For locus PL109, $0.1571<r<0.3205$, corresponded to 23 expected non-amplifications which is far greater than the actual number observed (9/480). For the remaining loci $r<0.11$ which corresponded to 5.8 expected nulls. These estimates suggest that that null alleles are unlikely to be the cause of the observed heterozygote deficiency in our data. We will return to other causes of heterozygote deficiency in the Discussion.

Pairwise comparisons among the six loci and for all 11 locations revealed no linkage disequlibrium ( $p>0.1$ in every case).

Table 4. Pleuronectes platessa. Summary of genetic variation at six microsatellite loci at 11 locations (Table 2). $N_{\mathrm{A}}=$ number of alleles, $H_{\text {exp }}=$ unbiased expected heterozygosity (Nei 1978), $H_{\mathrm{obs}}=$ observed heterozygosity, and $f=$ inbreeding coefficient. Significant values (bold) have been applied after sequential Bonferroni corrections (Rice 1989) (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ ).

| Locus/ Location | LO | TR | FA | IS | TB | FE | ic | ob | am | ba | bv | mean <br> Na /locus |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| List1001 | 5 | 5 | 4 | 5 | 5 | 7 | 5 | 4 | 6 | 6 | 6 | 5.27 |
|  | 0.610 | 0.634 | 0.698 | 0.608 | 0.650 | 0.674 | 0.725 | 0.622 | 0.656 | 0.616 | 0.655 |  |
|  | 0.542 | 0.625 | 0.577 | 0.636 | 0.571 | 0.735 | 0.708 | 0.688 | 0.583 | 0.617 | 0.750 |  |
|  | 0.114 | 0.015 | 0.177 | -0.047 | 0.122 | -0.091 | 0.023 | -0.106 | 0.111 | -0.002 | -0.148 |  |
| PL115 | 11 | 13 | 10 | 17 | 14 | 16 | 11 | 13 | 14 | 15 | 14 | 13.45 |
|  | 0.780 | 0.775 | 0.686 | 0.667 | 0.748 | 0.732 | 0.749 | 0.747 | 0.781 | 0.687 | 0.759 |  |
|  | 0.792 | 0.833 | 0.615 | 0.659 | 0.674 | 0.694 | 0.729 | 0.809 | 0.792 | 0.604 | 0.792 |  |
|  | -0.015 | -0.076 | 0.105 | 0.015 | 0.101 | 0.053 | 0.027 | -0.083 | -0.014 | 0.121 | -0.044 |  |
| PL92 | 14 | 23 | 11 | 19 | 20 | 16 | 15 | 20 | 15 | 15 | 18 | 16.9 |
|  | 0.901 | 0.883 | 0.847 | 0.889 | 0.902 | 0.885 | 0.885 | 0.874 | 0.881 | 0.848 | 0.889 |  |
|  | 0.792 | 0.729 | 0.880 | 0.773 | 0.792 | 0.775 | 0.702 | 0.804 | 0.750 | 0.617 | $\begin{aligned} & 0.696 \\ & 0.220 \end{aligned}$ |  |
|  | 0.123 | 0.176 | -0.040 | 0.132 | 0.164 | 0.125 | 0.203 | 0.080 | 0.150 | 0.274 |  |  |
| PL09 | 16 | 21 | 12 | 20 | 22 | 24 | 15 | 25 | 25 | 23 | 22 | 20.45 |
|  | 0.90 | 0.812 | 0.825 | 0.880 | 0.861 | 0.900 | 0.595 | 0.883 | 0.889 | 0.872 | 0.874 |  |
|  | 0.375 | 0.417 | 0.240 | 0.429 | 0.449 | 0.592 | 0.239 | 0.587 | 0.478 | 0.413 | 0.521 |  |
|  | 0.590 | 0.430 | 0.713 | 0.516 | 0.431 | 0.345 | 0.660 | 0.338 | 0.4465 | 0.529 | 0.408 |  |
| List1003 | 5 | 8 | 6 | 8 | 9 | 7 | 8 | 8 | 10 | 8 | 7 | 7.63 |
|  | 0.368 | 0.622 | 0.582 | 0.475 | 0.404 | 0.403 | 0.624 | 0.472 | 0.530 | 0.485 | 0.448 |  |
|  | 0.333 | 0.625 | 0.539 | 0.477 | 0.388 | 0.347 | 0.479 | 0.458 | 0.500 | 0.500 | 0.417 |  |
|  | 0.096 | -0.004 | 0.077 | -0.004 | 0.041 | 0.140 | 0.234 | 0.030 | 0.056 | -0.031 | 0.071 |  |
| PL142 | 14 | 21 | 15 | 18 | 21 | 25 | 18 | 25 | 20 | 23 | 18 | 19.8 |
|  | 0.833 | 0.843 | 0.857 | 0.804 | 0.857 | 0.871 | 0.972 | 0.877 | 0.883 | 0.850 | 0.805 |  |
|  | 0.625 | 0.708 | 0.769 | 0.683 | 0.792 | 0.771 | 0.833 | 0.787 | 0.813 | 0.833 | 0.667 |  |
|  | 0.254 | 0.161 | 0.104 | 0.152 | 0.077 | 0.116 | 0.102 | 0.104 | 0.030 | 0.020 | 0.173 |  |
| Mean $N_{\mathrm{A}} /$ location | 10.8 | 15.2 | 9.7 | 14.5 | 15.2 | 15.8 | 12.0 | 15.8 | 15.0 | 15.0 | 14.2 | 13.9 |
| $\text { Mean } H_{\exp }$ | 0.733 | 0.762 | 0.749 | 0.721 | 0.737 | 0.744 | 0.751 | 0.746 | 0.770 | 0.726 | 0.738 |  |
| $\text { Mean } H_{\mathrm{obs}}$ | 0.576 | 0.656 | 0.603 | 0.610 | 0.605 | 0.652 | 0.615 | 0.689 | 0.653 | 0.597 | 0.640 |  |
| Multilocus $F_{\text {IS }}$ |  |  |  |  |  |  |  |  |  |  |  |  |
| All loci <br> All loci excl. | $0.217^{* * *}$ | 0.140 *** | $0.198 * * *$ | 0.156** | $0.181 * * *$ | 0.125*** | $0.182^{* * *}$ | $0.078{ }^{* * *}$ | $0.154^{* * *}$ | $0.179^{* * *}$ | $0.187^{* * *}$ |  |
| PL09 | $0.119^{* * *}$ | 0.064** | 0.080** | 0.064** | $0.108 * * *$ | 0.069** | $0.119^{* * *}$ | 0.013* | $0.079 * *$ | $0.091{ }^{\text {*** }}$ | $0.067^{* * *}$ |  |

Consistencies of single-locus as well as multi-locus estimates of $\theta$ for all samples pooled together are compared in Table 5. All show significant differentiation although loci PL09 and PL142 contribute more. These two loci also have the highest number of alleles.

Table 5. Pleuronectes platessa. Global $\theta$ (Weir and Cockerham 1984). Continental shelf: all samples except those from Iceland and Faroe ( ${ }^{*} P<0.05$, ${ }^{* *} P<0.01$, *** $P<0.001$ ).

|  |  |  |  |  |  |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Locus | List1001 | PL115 | PL92 | PL09 | List1003 | PL142 | Multi-locus |
| All samples | $0.0090^{*}$ | $0.0040^{*}$ | $0.0060^{* *}$ | $0.0146^{* * *}$ | $0.0080^{*}$ | $0.0115^{* * *}$ | $0.0086^{* * *}$ |
| Continental shelf only | 0.0004 | 0.0033 | 0.0000 | 0.0014 | 0.0012 | 0.0023 | 0.0014 |

Population differentiation was estimated using both $\theta$ and exact tests. Pairwise comparisons of multi-locus $\theta$ (Table 6) showed that only Iceland (ic) was significantly different from all other locations. When pairwise comparisons of $\theta$ were made on a single-locus basis, only one comparison (TB and FA) was found to be significant and this involved Locus PL09. However, when an exact test was applied for each locus (a procedure which places more emphasis on rare alleles), 19 comparisons were significant for locus PL09 (Table 6). Most of these values occurred in the comparisons involving southern Brittany (bv) and Trondheim (TR). No distinctions were found among nursery ground, spawning ground or feeding ground locations.

A plot of isolation by distance (IBD) (Fig. 2) revealed a lack of significant correlation among continental shelf locations ( $p=0.31$ ). When Iceland was included, a significant IBD was found ( $p=0.02$ ) but this is probably spurious because the slope is based on only one, short-distance comparison (TB vs. ba) against all of the Iceland comparisons.

Relationships among the locations were also explored using Reynold's genetic distances (Fig. 3). Iceland and Faeroe formed a distinct, long-branched clade separated from a dense, continental-shelf cluster. Although bootstrap support is low, the topology suggests that both Iceland and Faroe are separate from the continentalshelf cluster.

Table 6. Population differentiation in Pleuronectes platessa. Upper right of matrix: pairwise, multilocus $\theta$ (Weir and Cockerham 1984) values tested by permutation and sequential Bonferroni corrections (Rice 1989) (* $P<0.05,{ }^{* * * P<0.001) \text {. Lower left of matrix: The values on the left are the }}$ number of individual loci that showed a significant $\theta$. The values to the right of the slash represents the number of individual loci that showed significant heterogeneity of allelic frequencies using an exact test of population differentiation. Excluding Iceland, this always involved Locus PL09. For abbreviations of sampling sites see Table 2.

| sampling site | FE | TB | FA | LO | IS | TR | bv | am | ba | ob | ic |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| FE | - | -0.0032 | 0.0033 | -0.0037 | -0.0001 | 0.0060 | 0.0004 | -0.0018 | -0.0038 | 0.0009 | $0,0373^{* * *}$ |
| TB | $0 / 1$ | - | 0.0069 | 0.0003 | -0.0022 | 0.0053 | -0.0004 | 0.0003 | -0.0009 | 0.0026 | $0,0339^{* * *}$ |
| FA | $0 / 0$ | $1 / 1$ | - | 0.0059 | 0.0009 | 0.0082 | 0.0131 | 0.0037 | 0.0072 | 0.0092 | $0,0290^{*}$ |
| LO | $0 / 0$ | $0 / 0$ | $0 / 1$ | - | -0.0010 | 0.0027 | 0.0012 | -0.0015 | -0.0011 | 0.0010 | $0,0429^{* * *}$ |
| IS | $0 / 1$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | - | 0.0058 | 0.0029 | 0.0015 | -0.0005 | 0.0036 | $0,0456^{* * *}$ |
| TR | $0 / 1$ | $0 / 1$ | $0 / 1$ | $0 / 0$ | $0 / 1$ | - | 0.0035 | 0.0011 | 0.0050 | 0.0075 | $0,0345^{* * *}$ |
| fb | $0 / 1$ | $0 / 1$ | $0 / 2$ | $0 / 1$ | $0 / 1$ | $0 / 1$ | - | 0.0034 | 0.0020 | 0.0014 | $0,0393^{* * *}$ |
| am | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | - | 0.0000 | 0.0008 | $0,0320^{* * *}$ |
| fba | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 1$ | $0 / 1$ | $0 / 1$ | $0 / 0$ | - | 0.0031 | $0,0377^{* * *}$ |
| ob | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 0$ | $0 / 1$ | $0 / 0$ | $0 / 1$ | - | $0,0315^{* * *}$ |
| jc | $4 / 3$ | $3 / 3$ | $2 / 4$ | $3 / 2$ | $4 / 4$ | $4 / 4$ | $4 / 3$ | $4 / 4$ | $4 / 3$ | $4 / 4$ | - |



Fig. 2. Global isolation by distance for Pleuronectes platessa. Pairwise comparisons involving Iceland (triangles), Faeroe (black circles), and all other continental-shelf comparisons (open circles) are plotted as shown. There is no significant isolation by distance (see text).


Fig. 3. On-shelf:off-shelf clusters of Pleuronectes platessa based on unrooted neighbour-joining tree inferred from Reynolds genetic distances. The circle surrounds the continental-shelf samples. Iceland and Faeroe are significantly different from one another (Table 6). Only bootstrap values above $50 \%$ are shown.

## Discussion

## Heterozygote deficiency: fact or artefact?

Strong heterozygote deficiencies have been reported for invertebrates (Zouros and Foltz 1984, Shaw et al. 1999a, Huang et al. 2000) and many fish species (Waldman and McKinnon 1993) including flatfish (Purdom et al. 1976, Ward and Beardmore 1977, Blanquer et al. 1992, Kotoulas et al. 1995. They can be the result of null alleles, a Wahlund effect, or to processes such as inbreeding and selection. As shown in the Results, technical artifacts due to null alleles are an unlikely cause of heterozygote deficiency in the present data set. Therefore, other explanations must be sought, i.e., inbreeding, Wahlund effects or selection.

Inbreeding remains an unlikely explanation for the observed heterozygote deficiency in fish with large census populations such as plaice. Reduced effective population sizes have been suggested for plaice based on higher mortality rates among males (thus affecting the sex ratio) combined with younger age/size of first maturity (Rijnsdorp 1994). However, even if combined with high reproductive variance among families (Hedgecock 1994) caused by random fluctuations in seasonal and/or annual hydrographic regimes and temperature (Van der Veer et al. 2000) reduction in effective population sizes $\left(N_{\mathrm{e}}\right)$, to the point where inbreeding would become significant remains unlikely unless it could be shown that there was strong assortative mating.

Wahlund effects are the most frequent explanation given for observed heterozygote deficiencies. This occurs when two or more subpopulations, are inadvertently sampled as a single subpopulation. For a Wahlund effect to be observed, a change in allele frequencies must occur within the geographic scale of the sampling. If, for example, differences in the timing of spawning or strong selection in time/space affect whole clusters of larvae, these initially separated cohorts could be mixed by currents or other phenomena. Ruzzante et al (1996) demonstrated that when a larval cod aggregation displaying significant heterozygous deficiency was divided into cohorts on the basis of length (as a proxy for age), no departure from Hardy Weinberg expectation (HWE) was observed in each cohort (temporal Wahlund Effect). When 0-class, juvenile samples of plaice were divided into two sub-samples based on length, $F_{\text {IS }}$ values remained positive but differed in


Fig. 4. Change in heterozygote deficiency ( $F_{\text {IS }}$ ) with fish size. Samples from each of the five nursery ground locations were divided into a small (S) and large (L) subsample. The line connecting the small and large subsamples represents time. All $F_{\text {IS }}$ values were significant except for the smallest size class at Location am.
magnitude (Fig. 4). Larger fish had higher $F_{\text {is }}$ values than smaller fish (except Iceland). Since length is a proxy for age and because the probability of cohorts coming into contact with one another will increase with time, the most probable explanation for the observation is a temporal Wahlund effect, although an alternative explanation could be selection against heterozygotes (discussed below). It is noteworthy that the $F_{\text {is }}$ value for the smallest size class at Location ob is not significant. This may reflect a single cohort or subpopulation. In conclusion, if heterozygote deficiencies are due to a Wahlund effect, then mixing occurs at a very early stage, possibly on the spawning grounds themselves, and definitely on the nursery grounds. Proof of a temporal Wahlund effect will require an analysis based on eggs collected from the spawning grounds combined with a fine-scale time series of 0-class juveniles taken from the adjacent nursery grounds. In the latter case, parallel quantification of meristic-phenotypic characters including numbers of vertebrae, dorsal- and anal-fin rays reflect water temperature during the pelagic stage (Van der Veer et al. 2000) may help to clarify the dynamic links between spawning and nursery grounds-at least in the southern North Sea.

Is selection a possible explanation for the observed heterozygote deficiencies in plaice? We have observed that the inbreeding coefficient for plaice are lowest in early juvenile stages and that it increases with maturity. Age related changes were also reported for plaice by Beardmore and Ward (1977) and by Kotoulas et al. (1995) in sole. If a Wahlund effect can be excluded, then selection against heterozygotes during early stages of development could be an explanation. This could result from outbreeding depression in which mating between populations that
have been geographically separated in the past, produce outbreed progeny with lower fitness. This type of population-level hybridisation has been shown in copepods (Edmands 1999). Although speculative at the moment, old refugial populations of plaice may very well have been brought into contact when presentday continental shelf seas were formed at the end of the last glacial maximum. Shallow genetic architectures combined with low levels of population differentiation and heterozygote deficiencies have been documented in sardines and anchovies (Grant and Bowen 1998); again in relation to extinction and recolonization throughout the North Atlantic since the last glacial maximum. In these kinds of climate change scenarios, the northern Atlantic gene pool may actually exist as an admixture of old populations-producing a kind of vestigial Wahlund effect maintained by selection. To prove this would require demonstration of, e.g., separate mitochrondrial lineages in plaice. Taken a step further, hybridization between undetected sibling species (Knowlton 1993), a common occurrence in marine organisms, could also produce outbreeding depression.

## Population differentiation on and off the continental shelf

Our data show that northern European plaice consist of a least two genetically distinct entities: continental shelf and Iceland. This separation is highly significant (Table 6) and supported by four of the six loci. The genetic differentiation of Icelandic plaice is probably shaped by bathymetry as deep water between the Icelandic plateau and the continental shelf restricts adult migration of this shallowwater species ( $<100 \mathrm{~m}$ ) (Nielsen 1986). Even so, the degree of differentiation is not very strong, which suggests that exchange occurs or has occurred in the past. Similar deep-water separations (Table 1) have been found for halibut (Hippoglossus hippoglossus) across the North Atlantic and Foss et al. (1998) distinguished Norway from Iceland-Faeroe-Greenland based on allozymes and Nei's genetic distances. Using microsatellites, Shaw et al. (1999b) found significant differentiation between Icelandic and Norwegian herring (Clupea harengus), with $\theta$ values close to the values we estimated in plaice.

Iceland, though geographically isolated, has comparable $H_{\text {exp }}$ with continental shelf samples. The lower number of alleles observed in the Iceland population could reflect a recent bottleneck, as loss of alleles is generally faster relative to a reduction
in $H_{\text {exp }}$ (Maruyama and Fuerst 1985), but may also reflect being at the northern edge of the species' range.

The apparent lack of differentiation among northern European continental-shelf populations based on multi-locus $\theta$ (Table 6) was unexpected, especially between the North and Irish Seas. However, when individual loci were analyzed using an exact test, locus PL09 did show significant heterogeneity of allelic distribution between several of the continental shelf samples. The discrepancy between permutation and exact tests is related to the relative importance assigned to common vs. rare alleles between the two methods. While this result is interesting, its biological meaning remains questionable. We emphasize this because two recent studies that have examined North Sea populations (usually two or three locations only) of Dover sole (Exadactylos et al. 1998) and whiting (Rico et al. 1997), have reported population differentiation (e.g., North and South sides of Dogger Bank) based on one or two individual allozyme or microsatellite loci. The accuracy of such estimates based on such minimal data should be regarded with extreme caution. Waples (1998) reviewed 57 studies of population differentiation in high gene flow species of fish. $F_{\text {st }}$ values averaged 0.062 with a median of 0.020 . Given that differentiation is naturally weak, the importance of both adequate sample size and number of loci take on added importance in minimising the signal:noise ratio.

## Rethinking population structure in plaice

The identification of discrete spawning grounds, larval retention areas and strong annual migratory behaviour (De Veen 1978, Rijnsdorp and Pastoors 1995) in the life cycle of plaice (see Rijnsdorp et al. 1995), has led to the idea of relatively discrete ecological stocks in plaice which may correspond to different genetic stocks. Reproductive isolation, in this view, would be maintained by strong philopatry to the natal (or at least one) spawning site throughout the life of the fish. Although completely distinct populations would not be expected, a continuum of populations with some isolation by distance might be postulated as a variant from panmixia. Tagging experiments suggest that the vast majority of plaice belonging to a particular stock probably do return to the same spawning ground year after year. However, immigration in to the stock as well as emigration out of a stock will also occur. For example, young plaice leaving the nursery grounds each year must join established groups that are not necessarily related. In this model, homing behaviour
is innate but the actual spawning ground used is thought to be a learned behaviour (reviewed in McQuinn 1997) which may change during the life of the fish. In addition, loss of adult fish through fishing mortality may also iteratively affect the annual migratory cycle to the spawning grounds via Allee effects in which adult fish become lost unless they are able to join new groups (McQuinn 1997). Such behaviour has been documented in North Sea herring, where following the fisheries collapse in the 1960s, a number of former spawning grounds were never recolonized after populations recovered (Corten 1993). While there is no direct evidence for this in plaice, relaxed philopatry is probably the rule in which exchange of individuals from different areas will occur on the spawning grounds.

In conclusion, matching the appropriate population model to the species is still a challenge, precisely because subtle differentiation seems to be the rule and thus lends itself to multiple interpretations. Over the past few years, a number of somewhat contradictory population models have been proposed for marine fishes more generally e.g., metapopulation, source-sink, discrete, panmixic (reviewed in Hansen 2001). For plaice, it appears that the North Sea Basin constitutes a panmictic or nearly panmictic unit with high gene flow among geographically recognizable stocks. However, evidence for temporal Wahlund effects (and possibly selection) also suggests that an underlying genetic substructure does exist. Capturing the structure, however, is going to require short-interval, time-series sampling of the spawning and nursery grounds.

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## Chapter 4

# POPULATION STRUCTURE OF PLAICE (Pleuronectes platessa L.) IN NORTHERN EUROPE: A COMPARISON OF RESOLVING POWER BETWEEN MICROSATELLITES AND MITOCHONDRIAL DNA DATA 

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#### Abstract

We used Single Strand Conformation Polymorphism (SSCP) of mtDNA control region to assess the population structure of the flatfish Pleuronectes platessa (plaice), to compare these data with a previous study based on microsatellite loci, and to test for possible sex-biased dispersal. From 461 individuals, 163 haplotypes were identified across 11 locations. Diversity was higher with mtDNA ( $h=0.776$ to $0.981 ; \pi=0.0178$ to 0.0298 ) as compared to microsatellite loci using the same samples $\left(H_{\mathrm{e}}=0.721\right.$ to 0.77$)$. Genetic diversity was lower in samples from Iceland and Faroe, as compared to the continental shelf samples. Although both classes of markers revealed a relatively strong differentiation between shelf and off-shelf populations ( $\theta=0.1015$ and $\theta=0.0351$, respectively), only the mtDNA data was able to detect differentiation within the continental shelf, i.e., a North Sea-Irish Sea group which was weakly distinguishable from Norway $(\theta=0.0046)$, the Baltic ( $\theta=$ $0.0136)$ and the Bay of Biscay $(\theta=0.0162)$. No evidence was obtained for isolation by distance, nor for sex-biased dispersal.This study demonstrates the importance of using more than one class of markers, especially for species like plaice, with large populations, high dispersal and recent colonization histories.


## Introduction

The European plaice (Pleuronectes platessa L.) is an important flatfish for Northern Europe fisheries. It is widely distributed in shallow European waters $(<100 \mathrm{~m})$ from the Western Mediterranean to Iceland and the White Sea (Wimpenny 1953). Reproduction takes place offshore during winter and the eggs/larvae are pelagic for about three to four months (Harding et al. 1978) after which they settle in shallow coastal waters where they metamorphose (Zijlstra 1972). Tagging data have shown that adults exhibit seasonal migration patterns from spawning grounds to feeding grounds (de Veen 1978) and that regional scale population structure of plaice appears to be mainly shaped by oceanographic features such as deep water, the occurrence of retention areas and suitable nursery grounds (Rijnsdorp and Grift, in press). This has suggested the possibility of philopatry which could lead to strong population differentiation despite a high dispersal potential via the long pelagic phase of eggs or migration of adults.

A recent population genetic study of plaice based on six microsatellite loci revealed high genetic homogeneity among continental shelf populations from Norway to southern Brittany (Hoarau et al. 2002a) regardless of whether individuals were collected from feeding, spawning or nursery grounds. Only the samples from Iceland and Faroe, that are separated from the continental shelf by deep ocean channels, showed significant population differentiation. These results were unexpected and suggested significant mixing among spawning grounds on the continental shelf.

Microsatellite loci are considered to be the marker of choice for many population genetic studies, mainly because of their high level of polymorphism (Jarne and Lagoda 1996) and ability to detect subtle population differentiation (e.g. Shaw et al. 1999), although in a few cases they have been found to produce similar (e.g. Buonaccorsi et al.2001) or even lower resolution than mitochondrial DNA (mtDNA) (e.g. Bérubé et al. 1998). Direct comparison between microsatellite and mtDNA loci can be very informative, as evolutionary forces will differentially affect each class of marker. In animals, nuclear DNA is usually diploid and biparentally inherited whereas mtDNA is haploid and, with a few exceptions (e.g. Gyllensten et al. 1991), maternally inherited. Because of these differences in ploidy and inheritance, the mitochondrial effective population size $\left(N_{\mathrm{e}(\mathrm{mt})}\right)$ is four times smaller than for nuclear
loci (Birky et al. 1989), and thus more susceptible to the effects of genetic drift. Consequently, estimates of population differentiation are expected to be higher for mitochondrial markers (see discussion in Crochet 2000, but also Birky et al. 1989 for possible exceptions). This is of interest for populations with presumptively large effective population sizes $\left(N_{\mathrm{e}}\right)$ (such as plaice) where drift might be too small to be detected with nuclear markers. Another advantage of mtDNA is that it will reach migration-drift equilibrium sooner than nuclear DNA (Birky et al. 1989). Migrationdrift equilibrium is the equilibrium between migration, which homogenises populations, and genetic drift which increases inter-population differentiation. Attainment of this equilibrium is relevant for species that are known to be part of the recent recolonization of the North Atlantic following the last glacial maximum (last $18-10,000$ years). The use of mtDNA will, therefore, increase the chance of detecting population differentiation.

The possibility of sex-biased dispersal has been raised for plaice as a result of the recent finding of inbreeding (Hoarau et al., in prep). This is an important issue because inbreeding is generally not considered in species with very large populations. Even where the effective population size has been shown to be several orders of magnitude smaller than the census size in marine fish (Hauser et al. 2002, Turner et al. 2002), the number of individuals is still very large. The presence/absence of inbreeding avoidance mechanisms in plaice, therefore, need to studied and sex-biased dispersal provides a potential mechanism for inbreeding avoidance (Pusey and Wolf 1996). The availability of comparative data from mitochondrial and nuclear genomes makes it potentially possible to test for sexbiased dispersal. This approach has been successfully used in several species, mainly mammals and birds (Pusey 1987). Very few studies have looked at sex-biased dispersal in fish. No sex-biased dispersal was found in blue marlin (Buonaccorsi et al. 2001) whereas male-biased dispersal has been shown in brook trout (Hutchings and Gerber 2002) and white sharks (Pardini et al. 2001). In both of these cases, males dispersed more frequently and over longer distances.

The objective of the present study was therefore to: 1) re-examine the genetic structure of plaice based on the mtDNA control region; 2) compare these results with a previous analysis of the same individuals based on six microsatellite loci; and 3) test for sex-biased dispersal.

## Material and Methods

## Sampling

We used the same 480 individuals as Hoarau et al. (2002a). The samples consisted of 240 adults and 240 offspring of the year ( 0 -group), collected from 11 locations (Fig.1).


Fig. 1. Sampling locations and sample sizes of Pleuronectes platessa. The grey line delimits the $200-\mathrm{m}$ contour of the continental shelf. Adults samples ( $\boldsymbol{\wedge}$ ): LO Lofoten, Norway (N=24); FA Faroe's Plateau (26); TR Trondheim fjord, Norway (48); IS Irish Sea, England (44);TB Terschellinger Bank, Netherlands (49); FE Femer Bælt, Denmark (49). 0-group samples (•): ic Alftanes, Iceland (48); ob Oban, Scotland (48); ba Balgzand, Netherlands (48); am Amrum, Germany (48); bv Bay of Vilaine, France (48). Continental shelf samples: all except Iceland and Faroe. (From Hoarau et al. 2002a)

## DNA extraction and PCR-SSCP

DNA was extracted according to Hoarau et al. (2002a). A $\approx 150 \mathrm{bp}$ fragment of the mtDNA control region was amplified by PCR using the primers : DLF 5'-CCA CCT CTA ACT CCC AAA GC-3') ( $5^{\prime}$ fluorescently labelled with 6-FAM) and DLR (5'-TGA AGG GAT TTT GAG TCT TGG -3') (5' fluorescently labelled with NED). All PCR amplifications were carried out in $10-\mu \mathrm{l}$ volumes containing $1 \mu \mathrm{l}$ of $1 / 10$ diluted DNA, 1 X reaction buffer (Promega), 0.2 mM of each dNTP, 2 mM $\mathrm{MgCl}_{2}, 0.30$ U Taq DNA polymerase (Promega) and $0.1 \mu \mathrm{M}$ of each primer. PCR was performed in either a PTC100 (MJ Research) or a MasterCycler gradient cycler (Eppendorf). The reaction profile was: $90^{\circ} \mathrm{C}$ for $1 \mathrm{~m} ; 30$ cycles of $94^{\circ} \mathrm{C}$ for 30 s , $50^{\circ} \mathrm{C}$ for $30 \mathrm{~s}, 72^{\circ} \mathrm{C}$ for 1 m ; and $72^{\circ} \mathrm{C}$ for 10 m .

Single strand conformation polymorphism (SSCP) (Orita et al. 1989, Sunnucks et al. 2000) was used to detect sequence variation in the mtDNA control region. Mutations that affect the conformation of single strand DNA are revealed by migration in non-denaturing polyacryalamide gels. This technique is very sensitive and provides a quick and relative inexpensive way to screen a large number of samples for sequence variation. SSCP gels were run on an ABI Prism-377 automatic sequencer (Applied Biosystems) as described in Coyer et al. (2002), except that 5\% glycerol was added to the gels. The use of an automatic sequencer together with different labelling of the primers allows quicker and more accurate scoring of the haplotypes.

## Sequence

All unique haplotypes detected by SSCP were subsequently sequenced and frequent haplotypes were sequenced for at least two individuals each. PCR products were cleaned using Sigmaspin (Sigma) purification columns. Both strands were cycle-sequenced using the dGTP Big Dye Terminator Kit (Applied Biosystems) and run on an ABI Prism-377 automatic sequencer (Applied Biosystems). Sequences were edited and aligned manually using BioEdit v.5.0.9 (Hall 1999).

## Microsatellites

All microsatellite data are from Hoarau et al. (2002a). Six microsatellite loci were used : PL09, PL92, PL115, PL142, LIST1001 and LIST1003.

## Data analyses

Statistical parsimony was use to analyze the intraspecific phylogeny of mtDNA haplotypes using the software TCS v.1.13 (Clement et al. 2000).

Haplotype ( $h$, Nei 1987) and nucleotide ( $\pi$, Nei 1987) diversities were estimated from the mtDNA data using DNASP v.3.53 (Rozas and Rozas 1999) ; gene diversity ( $H_{\mathrm{e}}$, expected multilocus heterozygosity, Nei 1987) was estimated for the six microsatellites loci using Genetix v.4.03 (Belkhir et al. 2002).

Population differentiation was analysed using Wright's $F_{\mathrm{ST}}$ (Wright 1969); global $F_{\mathrm{ST}}$ and pairwise $F_{\mathrm{ST}}$ were estimated using Weir and Cockerham's $\theta$ (Weir and Cockerham 1984). Significance of all $\theta$ estimates was tested with GENETIX using 2000 permutations. Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied where necessary.

Isolation by distance was examined by plotting the pairwise $\theta /(1-\theta)$ estimates against the Log of the geographic distances (Rousset 1997) and tested using the Mantel test (Mantel 1967) with 2000 permutations as implemented in Genetix.

Sex-biased dispersal was tested by comparing $\theta_{\text {mitochondrial }}\left(\theta_{\text {mito }}\right)$ with $\theta_{\text {microsatellites }}$ ( $\theta_{\text {msat }}$ ). The null hypothesis of no sex-biased dispersal is based on the equation, $\theta_{\text {mito }}$ $=4 \theta_{\text {msat }} /\left[1+3 \theta_{\text {msat }}\right.$ (Crochet 2000) which assumes equilibrium conditions, random mating, a 1:1 sex ratio, a Poisson-distributed reproductive success, and an infiniteisland model. Departures from this theoretical relationship, therefore, indicate sexbiased dispersal; male biased if the data points are above this plot and female biased if they are below. The correlation between the two pairwise $\theta$ matrices was tested using the Mantel test in Genetix with 2000 permutations. The software Prism v.3.02 (GraphPad Software, www.graphpad.com) was used to fit the theoretical curve to the data and to compute the non-linear $R^{2}$ (analogue to the correlation coefficient in linear regression) as well as the distribution of the residuals from the regression.

## Results

## Genetic diversity

The mtDNA diversity was extremely high, with 167 mtDNA haplotypes recovered (GenBank accession number AY442522-AY442688) from 461
individuals. Of these, $104 / 167(62 \%)$ were unique. The most common haplotype was present in $10 \%$ of the samples. An attempt to establish a haplotype network was unsuccessful owing to the number of reticulations in which no clear-cut pattern could be established (Fig 2).


Fig. 2. Statistical parsimony network of Pleuronectes platessa mtDNA haplotypes

Genetic diversity was lower in the Icelandic population, as compared to the continental populations (Table 1). Comparison of the two approaches, showed that mtDNA diversity was higher than microsatellite diversity (Table 1)

## Population differentiation

Significant population differentiation was detected in both data sets between continental shelf populations and those from Iceland and the Faeroe Plateau area. MtDNA, however, provided up to 10 -fold higher resolution among continental-shelf populations. In the centre of the European continental shelf, a large undifferentiated group was found (North Sea and Irish Sea). This North Sea-Irish Sea group showed significant differentiation from Norway, the Baltic and the Bay of Biscay. As for microsatellite data (Hoarau et al. 2002a), no pattern of isolation by distance was detected among continental-shelf locations based on the mitochondrial data $(Z=5.975, P=0.10)$. No isolation by distance could be demonstrated either between on- and off-shelf populations, or among the on-shelf populations.

Table 1. Genetic diversity and population differentiation in Pleuronectes platessa. $N=$ sample size; mitochondrial DNA (mtDNA) data: $N_{\mathrm{h}}=$ number of haplotype, $h=$ haplotype diversity, $\pi=$ nucleotide diversity; microsatellite (msat) data: $N_{\mathrm{A}}=$ number of allele $H_{\mathrm{e}}=$ expected heterozygosity. ns $=$ not significant, $*=P<0.05, * *=P<0.01, * * *=P<0.001$

| Location | Code | $N$ | Genetic diversity |  |  |  |  | Population differentiation |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  | mtDNA |  |  | msat |  | mtDNA | msat |
|  |  |  | $N_{\text {h }}$ | $h$ | $\pi$ | $N_{\text {A }}$ | $H_{\text {e }}$ | $\theta$ | $\theta$ |
| Northern Europe : | - | - | - |  | - |  |  | $0.0383^{\text {** }}$ | $0,0086^{* \pi}$ |
| Alftanes, Iceland | ic | 48 | 17 | 0.776 | 0.0178 | 12.0 | 0.751 | - | - |
| Faero's Plateau | FA | 25 | 12 | 0.900 | 0.0191 | 9.7 | 0.749 | - | - |
| Trondheim fjord, Norway | TR | 46 | 23 | 0.943 | 0.0267 | 15.2 | 0.762 | - | - |
| Lofoten, Norway | LO | 23 | 14 | 0.929 | 0.0196 | 10.8 | 0.733 | - | - |
| Irish Sea, UK | IS | 44 | 26 | 0.953 | 0.0272 | 14.5 | 0.721 | - | - |
| Oban, Scotland | ob | 46 | 33 | 0.980 | 0.0280 | 15.8 | 0.746 | - | - |
| Terschellinger Bank, | TB | 47 | 34 | 0.939 | 0.0249 | 15.2 | 0.737 | - | - |
| Netherlands |  |  |  |  |  |  |  |  |  |
| Balgzand, Netherlands | ba | 48 | 30 | 0.970 | 0.0279 | 15.0 | 0.726 | - | - |
| Amrum, Germany | am | 47 | 34 | 0.981 | 0.0302 | 15.0 | 0.770 | - | - |
| Femer Bæılt, Denmark | FE | 48 | 28 | 0.942 | 0.0294 | 15.8 | 0.744 | - | - |
| Bay of Vilaine, France | bv | 39 | 26 | 0.976 | 0.0298 | 14.2 | 0.738 | ${ }^{-}{ }^{* * *}$ | - |
| Continental Shelf : <br> LO, TR, IS, TB, FE, ob, ba, am, bv | - | - | - | - | - | - | - | $0.0132^{* * *}$ | $0.0014^{\text {ns }}$ |
| North Sea \& Irish Sea : IS, TB, ob, ba, am | - | - | - | - | - | - | - | $0.0061{ }^{\text {ns }}$ | $0.0008^{\text {ns }}$ |
| Norway: LO, TR | - | - | - | - | - | - | - | $0.0035{ }^{\text {ns }}$ | $0.0027^{\text {ns }}$ |
| North Sea \& Irish Sea versus | - | - | - | - | - | - | - | $0.0136^{* *}$ | $0.0021^{\text {ns }}$ |
| Baltic : <br> IS, TB, ob, ba, am vs. FE |  |  |  |  |  |  |  |  |  |
| North Sea \& Irish Sea vs. | - | - | - | - | - | - | - | $0.0046{ }^{*}$ | $0.0023{ }^{\text {ns }}$ |
| Norway : <br> IS, TB, ob, ba, am, vs. LO, TR |  |  |  |  |  |  |  |  |  |
| North Sea \& Irish Sea vs. Bay of Biscay : <br> IS, TB, ob, ba, am vs. bv | - | - | - | - | - | - | - | $0.0162^{* *}$ | $0.0014^{\text {ns }}$ |
| Iceland vs. Continental Shelf | - | - | - | - | - | - | - | $0.1015^{* * *}$ | $0.0351^{* * *}$ |

## Sex-biased dispersal

A strong correlation was found for the population differentiation $(\theta)$ matrix $(Z=0.1, P<0.01)$ between the mitochondrial and microsatellite estimates. Fitting the theoretical curve of no sex-biased dispersal to the observed data gave a non-linear correlation coefficient $R^{2}=0.7977$ (Fig. 3) and the normal distribution of the residuals from the regression was not rejected (Normality Test, $P>0.1$ ). Therefore, our data did not significantly deviate from the expected curve and suggests that there
is no sex-biased dispersal in plaice. Separate analysis of the "low" and "high" differentiation clouds (Fig. 3) showed similar results, i.e., no deviation from the null hypothesis of "no sex-biased dispersal". Despite this clear-cut result, violations of the underlying assumptions of the model lead us to accept this result with caution (see Discussion).


Fig. 3. Test for sex-biased dispersal in Pleuronectes platessa. Pairwise $\theta_{\text {mitochondrial }}$ versus $\theta_{\text {microsatellites }}$ (Weir and Cockerham 1984). The plot represents the theoretical $\theta_{\text {mito }}$ $=4 \theta_{\text {msat }} /\left[\begin{array}{lll}1+ & 3 & \theta_{\text {msat }}\end{array}\right]$ indicative of no sex-biased dispersal. Our data ( $\bullet$ ) did not deviate from this theoretical expectation

## Discussion

Significant population substructure was detected on the continental shelf using the mtDNA data which was not the case with the microsatellite data. The mtDNA results are more consistent with ecological and fisheries observations which include differences in demographic parameters such as fecundity, maturation, and growth (Rijnsdorp 1989, 1991). The inability to detect seemingly distinct stocks with microsatellite data is, in this case, most likely related to the extremely shallow population history in the Northern North Atlantic. Plaice have only repopulated the North Atlantic and the North Sea within approximately the past 10,000 years. MtDNA, with its smaller effective population size, faster genetic drift and haploid maternal inheritance can actually provide a better view through this extremely short timeframe.

The mtDNA diversity in plaice is high but comparable to what has been found in other flatfish (Jones and Quattro 1999). The higher diversity in mtDNA as compared to microsatellites observed in the present study, is most likely to be related to a
larger female effective population size. A larger female effective population size may be expected because males show both a higher natural mortality as well as a higher fishing mortality rate (Beverton 1964, Rijnsdorp 1993, Solmundsson et al. 2003), which may lead to the development of a skew in the sex ratio (Rijnsdorp 1994). Furthermore, variance in female reproductive success is expected to be lower than in males because even a few males can fertilise many females. Finally, longer female life span with overlapping generations acts as a buffer against fluctuating population size (Gaggiotti and Vetter 1999), thus also increasing $N_{\mathrm{e}}(\mathrm{f})$. Taken together, these factors will increase $N_{\mathrm{e}}(\mathrm{f})$ relative to $N_{\mathrm{e}}(\mathrm{m})$ which is what is being detected with the mtDNA data.

The lower diversities associated with the Icelandic population are qualitatively similar to those observed with the microsatellite data (only the number of alleles differed). There are two potential explanations for this, which are not mutually exclusive. First, the lower diversity of the Icelandic population may be due to a lower overall population size as well as a lower effective population size. The census population size of Icelandic plaice is estimated to be an order of magnitude smaller than that found in the North Sea (ICES-www.ices.dk). Second, the geographic position of Iceland at the edge of the distributional range of plaice (Nielssen 1986) may also account for the lower diversity, as lower genetic diversity is typical of edge populations in general (Hewitt 2000, Comps et al. 2001, Coyer et al. 2003).

The reticulations in the haplotype network (Fig. 2) are due to homoplasy, which is the result of multiple substitutions at a given site. Such a large amount of homoplasy can only be explained by the very high mutation rate in this region and/or to recombination. High mutation rates are a natural feature of the mtDNA control region. To date, recombination has only been shown in flounder (Hoarau et al. 2002b). The structure of the mtDNA control region in flatfish seems to promote heteroplasmy and recombination (Hoarau G. unpublished data). At present, these observations need further investigation. Such a process has implications for genealogical comparisons but not for $F_{\mathrm{ST}}$-based studies which rely on unordered characters.

Although population structure is governed by both demographic and genetic processes, oceanographic features are also crucial. Deep water is a major barrier as shown by: 1) the relatively strong differentiation between shelf and off-shelf
populations in both mtDNA and microsatellite data sets; and 2) between the North Sea and Norway which are separated by the tongue of the Norwegian Deep. Currents (and salinity fluctuations) also play a role in separating Baltic and North Sea populations in the Skagerrak-Kattegat region. A similar restriction of gene flow has been observed in cod (Nielsen et al. 2003). With respect to the Bay of Vilaine (Fr), it seems most likely that these populations represent the northern range of a more southern stock within the Bay of Biscay. There was no significant relationship detected between population differentiation $(\theta)$ and geographic distance regardless of inclusion or exclusion of the off-shelf populations. This is again consistent with recent population history combined with high dispersal and migration of adult populations.

The ability to demonstrate sex-biased dispersal depends upon how well the underlying assumptions of the model are met. In the present case at least two (and possibly three) of these underlying assumption are not met. First equilibrium conditions almost certainly do not exist given the short history of the North Atlantic over the past 10,000 years; and second, there is at least some evidence for a sex skew in plaice. Finally, the apparent absence of sex-biased dispersal in plaice is further supported by independent tagging data (L Bolle unpublished data) in which no difference in male/female movement was detected. If our analysis is correct, then the absence this inbreeding avoidance mechanism is an important issue in our understanding of inbreeding in plaice.

## Conclusion

The present study demonstrates the value mtDNA as an addition to nuclear microsatellite loci in detecting genetic stock structure and gene flow in a species with a large population size, high dispersal capacity and shallow population history. The pattern of genetic differentiation depicted by the combined nuclear-mtDNA data sets is in good agreement with other biological and tagging data.

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## Chapter 5

# EVIDENCE FOR INBREEDING IN JUVENILE PLAICE, Pleuronectes platessa L. (TELEOSTEI: PLEURONECTIDAE) 

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Manuscript


#### Abstract

Heterozygote deficiencies have been consistently observed in plaice. To investigate possible causes of this result, we first ruled out the possibility of genotyping artefacts. We then compared temporal changes in $F_{\text {IS }}$ estimates by genotyping sequential cohorts of individuals collected on a biweekly basis from two genetically distinct nursery grounds in the Dutch Wadden Sea and in Iceland. Strongly positive and highly significant $F_{\text {IS }}$ estimates $(0.151-0.233)$ were found in all ten cohorts based on 8 -locus, microsatellite genotypes. Six of the eight loci showed significant single-locus $F_{\text {IS }}$ estimates in $>80 \%$ of the cohorts. No evidence was found for a temporal Wahlund effect and a plot of multilocus $F_{\text {IS }}$ against age suggested no evidence for selection. The frequency of lower individual-multilocus-heterozygosity (MLH)-classes was higher than expected under random mating ( $P<0.001$ ) indicating the presence of inbred individuals. Relatedness $(I)$, was also found in two cohorts. We discuss how low effective population size, in combination with fisheries related depletion of kin-structured spawning aggregations, may have led to inbreeding in plaice. We further recommend that the finding of heterozygote deficiencies -which are common in fish population genetic surveys- be thoroughly investigated before assuming that it is due to technical artefacts as opposed to biological processes.


## Introduction

Plaice (Pleuronectes platessa L.) is a common flatfish species inhabiting northern European coastal waters and is an important target species in the North Sea fisheries (Rijnsdorp and Millner 1996). In a recent microsatellite-based genetic survey of plaice populations throughout the species' range (Hoarau et al. 2002a), large-scale genetic homogeneity was found among continental-shelf populations consistent with the recency of recolonisation of the North Atlantic and North Sea region since the last glacial maximum ( $18-10,000 \mathrm{yr}$ BP). Unexpectedly, significant heterozygote deficiencies were found in both young-of-the-year juveniles and adults (Hoarau et al. 2002a).

Although heterozygote deficiencies have been commonly observed in marine bivalves (Zouros and Foltz 1984) and many fish species (reviewed by Waldman and McKinnon 1993), most explanations have been attributed to genotyping (null alleles and/or large allele dropout ) or sampling (Wahlund effect) artefacts. Null alleles (Callen et al. 1993) are caused by PCR amplification failure mainly due to point mutations within the primer site. Large allele dropout is due to preferential amplification of shorter alleles. The presence of null alleles and/or large allele dropout in a data set creates false homozygotes that, in turn, produce an artificial heterozygote deficiency.

Wahlund effects occur when two or more genetically differentiated subpopulations are inadvertently sampled as a single population. A temporal Wahlund effect can occur as a result of an admixture between different cohorts sequentially arriving on their nursery ground over time. This has been previously documented within larval aggregations of cod (Ruzzante et al. 1996) and hypothesised for plaice (Hoarau et al. 2002a). A variant of the Wahlund effect can also occur if different groups of genetically related individuals are sampled, thereby creating a 'family' Wahlund effect (Castric et al. 2002).

If technical and sampling artefacts can be ruled out as a cause of the heterozygote deficiency, then biological explanations such as selection or inbreeding must be considered. Selection against heterozygotes can occur as a consequence of outbreeding depression (Waldman and McKinnon 1993) in which populations that have been separated (usually for a long period of time) are brought into secondary contact, resulting in matings that produce less fit inter-populational hybrids
(Edmands 1999). Interbreeding between different refugial populations of plaice at the end of the last glacial maximum, could have, in principle produced such an effect. However, no evidence has been found for this, based either on microsatellite data (Hoarau et al. 2002a) or on mitochondrial data (Hoarau et al. 2004). Another explanation is partial inbreeding resulting from non-random mating between genetically related individuals. Heterozygote deficiencies caused by partial inbreeding have rarely been considered in marine fish populations characterised by pelagic spawning and wide distributional ranges because it is assumed that large population sizes ( $10^{6}-10^{9}$ individuals), high fecundity, and putatively random mating, would preclude this possibility. A growing body of evidence, however, suggests that effective population sizes ( Ne ) in many fish species are 3-5 orders of magnitude smaller (Hauser et al. 2002, Turner et al. 2002, Hutchinson et al. 2003). We recently obtained similar results for plaice (chapter 6). Ne was estimated at $\approx 2,000$ individuals in Iceland and $\approx 20,000$ in the North Sea based on a temporal ML analysis (Wang 2001) of archived, plaice otoliths collected between 1924 and 2002 (Chapter 6). Although these numbers are still moderately large, in combination with complex social and mating behaviour, involving kin aggregations (Pouyaud et al. 1999), it is possible that inbreeding could occur. The goal of the present study was, therefore to systematically eliminate competing causes for the observed heterozygote deficiency in plaice.

## Materials and Methods

## Sampling

Young-of-the-year juvenile plaice were collected from two nursery grounds in the North Sea at Balgzand in the Dutch Wadden Sea $\left(52^{\circ} 53^{\prime}-52^{\circ} 59^{\prime} \mathrm{N}, 4^{\circ} 48^{\prime}-\right.$ $\left.4^{\circ} 55^{\prime} \mathrm{E}\right)$ and at Alftanes on the west coast of Iceland $\left(64^{\circ} 06^{\prime} \mathrm{N}, 22^{\circ} 02^{\prime} \mathrm{W}\right)$. The two locations were selected because they are genetically isolated (Hoarau et al. 2002a), thus representing two independent samples.

Biweekly sampling was performed in both areas during the settlement season of juvenile plaice using the same type of beam trawl ( 5 mm mesh). Sampling (referred to as Week 1) began on 26 Feb 2002 at Balgzand and 22 Jun 2002 at Alftanes. Samples collected from weeks 1, 3, 7 and 9 in the North Sea and weeks 1, 5, 7 and

11 off Iceland were designated as cohorts (Table 1). Sampling from week 9 in the North Sea was divided into two sub-samples (designated cohorts 4 and 5) based on differences in the size of the individuals (Table 1). The number of individuals per cohort ranged from 37 to 64 . Each cohort was collected in one or two hauls along a 100 m transect.

Whole individuals were immediately preserved in $70 \%$ ethanol and subsequently measured to the nearest mm (TL). In addition to the juvenile cohorts (total $\mathrm{n}=477$ ), fertilised eggs ( $\mathrm{n}=64$, designated cohort 0) were collected in January 2002 in the southern North Sea using a single haul of high-speed plankton net called a gulf torpedo (Zijlstra 1970).

## Microsatellites, DNA extraction and genotyping

Eight microsatellite loci were used (Table 1 \& 2) (Watts et al. 1999, Hoarau et al. 2002a, b). DNA was extracted from juveniles using a CTAB protocol on muscle tissue (Hoarau et al. 2002a) and from eggs using the following Chelex protocol. Eggs were air dried to remove the remaining ethanol, incubated at $55^{\circ} \mathrm{C}$ in $50 \mu \mathrm{l}$ of a $6 \%$ Chelex 100 (BioRad) solution with 1.2 U proteinase K (Promega) for 2 h , then at $100^{\circ} \mathrm{C}$ for 10 min . Samples were centrifuged ( 2 min at $20,000 \mathrm{~g}$ ) and the crude DNA supernatant was used for PCR reactions as reported elsewhere (Hoarau et al. 2002a, b). Genotyping was done with an ABI377 automatic sequencer (Applied Biosystems), using internal lane standards and the software GENESCAN ${ }^{\text {TM }}$ to determine allele sizes.

## Genotyping artefacts

The possibility of genotyping artefacts was examined in several ways. First, a set of homozygous individuals was reamplified for each of the eight loci under relaxed annealing temperatures $\left(4^{\circ} \mathrm{C}\right.$ lower) to verify the absence of additional alleles. Second, the expected frequency of null alleles ( $r$ ) (Brookfield 1996, Equation 4) was used to calculate the expected frequency of null homozygotes $\left(r^{2}\right)$. Expected counts were then compared with observed counts in the data set. If the observed were less than the expected, it was concluded that null alleles were not a factor. Third, we used the software MICROCHECKER (Van Oosterhout et al. 2003) to test for stuttering and large allele drop-out. These tests are based on the size distribution of the genotypes. Fourth, for two loci, PL142 and PL167, new primers were designed
(PL142newF: 5’-GCCTCATTTTCACACTGTTACC-3', PL142newR: 5’-GGGCAATT-ACTTGAGATGAAAAAG-3', PL167newF : 5’-GGGAATACACCAGACAAAATG-3', PL167newR: 5'-GCACATGTCAAGCTGCAGTCCC-3'). These two loci were chosen because of their higher amount of stuttering. All homozygotes were re-scored using these new primer pairs.

## Genetic diversity

All of the following computations were performed using GENETIX 4.04 (Belkhir et al. 2003). Observed and unbiased expected heterozygosity (Nei 1978) were computed for each locus individually and as a multilocus estimate for each of the cohorts. Single and multilocus $F_{\text {IS }}$ were estimated using Weir and Cockerham's $f$ (Weir and Cockerham 1984). Heterogeneity of heterozygote deficiency among loci was tested according to Gaffney et al. (1990).

## Cohort differentiation

Population differentiation was analysed with Wright's $F_{\text {ST }}$ (Wright 1969) using Weir and Cockerham's estimator, $\theta$ (Weir and Cockerham 1984). Global $F_{\text {ST }}$ was used to detect differentiation between cohorts within each nursery. Significance of all $f$ and $\theta$ estimates was tested using permutation ( $\mathrm{N}=2000$ ). Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied where necessary.

## Wahlund effect

Tests for Wahlund effects were performed using PartitionML (Belkhir and Bonhomme 2002). The method searches for the best way to partition the samples into two (or more) panmictic clusters and provides the likelihood for such a partition. Significance was tested using permutations (Castric et al. 2002). In order to test if the partition into two clusters was more likely than no subdivision, we generated the null distribution of the likelihood in 100 panmictic pseudo-samples and compared them with the observed likelihood. The panmictic pseudo-samples were generated by permuting the monolocus genotypes.

## Inbreeding

We tested for inbreeding by comparing the distribution of individual Multi Locus Heterozygosity (number of heterozygous loci per individual) (MLH) with the
expected distribution under random mating (Castric et al. 2002). Inbred individuals are expected to be more homozygous over all loci, leading to lower MLH values than expected and a shift in the distribution towards lower MLH values. The expected MLH distribution ( $\mathrm{MLH}_{\text {exp }}$ ) was generated by permutation, 1000 pseudosamples were generated by permuting alleles and the difference between the observed $\left(\mathrm{MLH}_{\mathrm{obs}}\right)$ and expected $\left(\mathrm{MLH}_{\mathrm{exp}}\right)$ frequencies was computed for each MLH class. Significance was tested by comparing the mean observed MLH with the means of the 1000 pseudo-samples (F. Bonhomme pers. comm.).

## Testing for related individuals

The possibility of related individuals being present within each cohort, as estimated by the pairwise identity coefficient (I) (Mathieu et al. 1990), was tested using the software IDENTIX (Belkhir et al. 2002). Identity was used because it has a smaller variance as compared with other estimators of relatedness (Belkhir et al. 2002). The mean and the variance of $I$ were estimated for each cohort and compared with their expected distribution under the null hypothesis of no relatedness. These expected distributions were generated by permutation of genotypes ( $\mathrm{n}=1000$ ). Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied where necessary.

## Selection against heterozygotes

Selection against heterozygotes was tested by plotting the multilocus $F_{\text {IS }}$ against the mean body-size found in each cohort. If selection was a factor, we predicted an increase in the heterozygote deficiency with size which can serve as a proxy for age.

## Results

## Data quality

All microsatellite loci were extensively tested for genotyping artefacts (Table 1). Scoring errors were reduced by the use of an automatic sequencer with an internal lane standard for allele sizing. Amplification failure rates were $<1 \%$ for six of the eight loci and 4\% for loci PL06 and PL09. All loci displayed clean signals with

Table 1．Microsatellite data quality（n．t．：not tested ）．

|  | Microsatellite loci |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | PL06 | PL09 | PL92 | PL115 | PL142 | PL167 | List1001 | List1003 |
| Amplification failure | 4\％ | 4\％ | $<1 \%$ | 0 | 0 | $<1 \%$ | $<1 \%$ | 0 |
| Stuttering | no | no | no | slight | moderate | moderate | no | no |
| Brookfield test <br> Presence of null alleles | yes | yes | no | no | no | no | no | no |
| Effect of relaxed annealing temperature． | $\begin{aligned} & \text { 己 } \\ & \frac{0}{\sigma} \end{aligned}$ | $\underset{\underset{\sim}{0}}{\stackrel{0}{0}}$ | none | none | none | none | none | none |
| Large allele drop－out | .ヨ | -ヨ | no | no | no | no | no | no |
| New PCR primers designed | 0 | $\cdots$ | no | no | yes | yes | no | no |
| Effect of new primers on the number of homozygotes | $\begin{aligned} & \overrightarrow{0} \\ & 0 \\ & \end{aligned}$ | $\begin{aligned} & \overrightarrow{0} \\ & 0 \\ & \end{aligned}$ | n．t | n．t | none | none | n．t | n．t |



Fig．1．Microsatellite data quality ：fluorograms．
limited stuttering (Fig. 1). For loci PL142 and PL167 a post-PCR treatment with T4polymerase (Ginot et al. 1996) was used to reduce the stuttering. New PCR primer pairs were also designed for these two loci in order to further minimise stuttering and to test for null alleles. There was no change in the observed number of homozygotes. An amplification test using relaxed annealing temperatures for all loci did not decrease the number of observed homozygotes.

Following Brookfield's Equation $4\left(N \times r^{2}\right)$ (Brookfield 1996), the expected number of null homozygotes (12) was greater than the actual number observed (4) in six of the eight loci (Table 1) allowing us to reject the possibility of nulls. For loci PL06 and PL09, null alleles could not be rejected and these loci were subsequently eliminated from further analyses. Using Microchecker (Van Oosterhout et al. 2003), no large allele drop-out was found for any of the loci. We, therefore conclude that genotyping artefacts cannot explain the departure from HWE in our data set..

## Genetic diversity

Genetic variation was moderate to high with 5 to 40 alleles per locus per cohort and $H_{\text {exp }}$ ranged from 0.3182 to 0.9678 (Table 2). Icelandic samples were less diverse genetically than North Sea samples.

A significant, multilocus heterozygote deficiency was found in all cohorts with and without loci PL06 and PL09 (Table 2). A test for homogeneity among the remaining six loci could not be rejected ( $\chi^{2}=54.9$, d.f=59) despite the absence of significant heterozygote deficiencies for List1001 and List 1003 (which might be attributable to their low variation). Homogeneity across loci therefore supports the inbreeding hypothesis.

## Cohort differentiation

No genetic differentiation was detected among cohorts from the same location (Balgzand: $\theta=0.0021, P=0.06$; Alftanes: $\theta=0.0018, P=0.14$ ). When samples from the same locations were pooled, the two biogeographically-distant nurseries differed significantly ( $\theta=0.0207, P<0.001$ ).

Table 2. Summary of genetic variation at eight microsatellite loci. $N=$ sample size, $N_{\mathrm{A}}=$ number of alleles, $H_{\text {exp }}=$ unbiased expected heterozygosity (Nei 1978), $H_{\text {obs }}=$ observed heterozygosity, and $f=$ inbreeding coefficient (Weir and Cockerham 1984). * $P<0.05$, ** $P$ $<0.01, * * * P<0.001$. $P$-values are corrected for multiple tests.

|  | Cohort | Balgzand (The Netherlands) |  |  |  |  |  | Alftanes (Iceland) |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | 0 (eggs) | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 |
| Sampling week <br> Mean length (mm) |  | - | 1 | 3 | 7 | 9 | 9 | 1 | 5 | 7 | 11 |
|  |  | - | 14.6 | 14.5 | 13.3 | 29.2 | 13.1 | 15.5 | 15 | 28.5 | 53 |
|  | $N$ | 64 | 64 | 64 | 61 | 48 | 48 | 37 | 52 | 52 | 51 |
| Locus |  |  |  |  |  |  |  |  |  |  |  |
| PL06 | $N_{\text {A }}$ | 29 | 31 | 27 | 21 | 24 | 28 | 25 | 26 | 25 | 25 |
|  | $H_{\text {exp }}$ | 0.9503 | 0.9630 | 0.9206 | 0.9524 | 0.9595 | 0.9640 | 0.9288 | 0.9123 | 0.9108 | 0.9349 |
|  | $H_{\text {obs }}$ | 0.5806 | 0.5918 | 0.667 | 0.3182 | 0.5455 | 0.6000 | 0.5143 | 0.3750 | 0.3617 | 0.5600 |
|  | $f$ | $0.3909{ }^{* * *}$ | $0.3879{ }^{* * *}$ | 0.2775*** | $0.6711^{* * *}$ | $0.4375 * * *$ | 0.3810*** | $0.4499 * * *$ | $0.5915{ }^{* * *}$ | 0.6055*** | 0.4035*** |
| PL09 | $N_{\text {A }}$ | 24 | 28 | 29 | 29 | 27 | 25 | 14 | 17 | 19 | 12 |
|  | $H_{\text {exp }}$ | 0.9416 | 0.8516 | 0.9128 | 0.9102 | 0.9517 | 0.9397 | 0.5542 | 0.6154 | 0.6892 | 0.5797 |
|  | $H_{\text {obs }}$ | 0.5882 | 0.4375 | 0.5000 | 0.5667 | 0.4894 | 0.5000 | 0.4000 | 0.4490 | 0.5882 | 0.5882 |
|  | $f$ | $0.3788^{* * *}$ | 0.4882*** | 0.4543*** | $0.3794 * *$ | 0.4885*** | 0.4708*** | 0.2817*** | 0.2725*** | 0.1477* | -0.0149 |
| PL92 | $N_{\text {A }}$ | 22 | 28 | 25 | 25 | 20 | 28 | 15 | 18 | 15 | 15 |
|  | $H_{\text {exp }}$ | 0.9023 | 0.9248 | 0.9010 | 0.8964 | 0.9129 | 0.9092 | 0.9143 | 0.8951 | 0.8910 | 0.8604 |
|  | $H_{\text {obs }}$ | 0.7500 | 0.8906 | 0.8281 | 0.8197 | 0.8958 | 0.8723 | 0.7222 | 0.8077 | 0.7692 | 0.8627 |
|  | $f$ | $0.1699 * * *$ | 0.0373 | $0.0814^{*}$ | 0.0862** | 0.0189 | 0.0409 | $\mathbf{0 . 2 1 2 5 * * *}$ | 0.0985 | $0.1378 * *$ | -0.0027 |
| PL115 | $N_{\text {A }}$ | 15 | 17 | 19 | 16 | 21 | 17 | 12 | 12 | 15 | 12 |
|  | $H_{\text {exp }}$ | 0.7158 | 0.7307 | 0.7575 | 0.8025 | 0.7618 | 0.7580 | 0.6964 | 0.7644 | 0.8083 | 0.7449 |
|  | $H_{\text {obs }}$ | 0.5556 | 0.6557 | 0.7969 | 0.6066 | 0.7500 | 0.5957 | 0.5278 | 0.5385 | 0.7885 | 0.6078 |
|  | $f$ | $\mathbf{0 . 2 2 5 3}{ }^{* * *}$ | 0.1033* | -0.0524 | $\mathbf{0 . 2 4 5 7 * * *}$ | 0.01571 | $0.2158{ }^{* * *}$ | $0.2448{ }^{* * *}$ | $0.2976{ }^{* * *}$ | 0.0247 | 0.1855*** |
| PL142 | $N_{\text {A }}$ | 24 | 29 | 25 | 25 | 22 | 21 | 18 | 21 | 20 | 18 |
|  | $H_{\text {exp }}$ | 0.8984 | 0.8970 | 0.8625 | 0.8724 | 0.9075 | 0.8463 | 0.8878 | 0.9108 | 0.8956 | 0.8790 |
|  | $H_{\text {obs }}$ | 0.8281 | 0.7031 | 0.8095 | 0.8361 | 0.6875 | 0.5208 | 0.7838 | 0.8846 | 0.8462 | 0.5800 |
|  | $f$ | 0.0788** | $\mathbf{0 . 2 1 7 5 * * *}$ | 0.0619 | 0.0419 | $0.2443 * * *$ | $0.3871{ }^{* * *}$ | 0.1186* | 0.0290 | 0.0558 | $0.3424^{* * *}$ |
| PL167 | $N_{\text {A }}$ | 32 | 35 | 31 | 37 | 32 | 40 | 24 | 28 | 26 | 27 |
|  | $H_{\text {exp }}$ | 0.9563 | 0.9856 | 0.9451 | 0.9553 | 0.9638 | 0.9678 | 0.9354 | 0.9390 | 0.9341 | 0.9321 |
|  | $H_{\text {obs }}$ | 0.6271 | 0.8906 | 0.7031 | 0.7869 | 0.6250 | 0.8750 | 0.6944 | 0.6863 | 0.7500 | 0.8039 |
|  | $f$ | $\mathbf{0 . 3 4 6 1 * * * ~}$ | 0.0782** | $\mathbf{0 . 2 5 7 6 * * * ~}$ | $0.1775{ }^{* * *}$ | $\mathbf{0 . 3 5 3 9}{ }^{* * *}$ | $0.0968{ }^{* * *}$ | 0.2604*** | 0.2711*** | $\mathbf{0 . 1 9 8 6 * * * ~}$ | 0.1387*** |

Chapter 5

Table 2. Continued

| Cohort | Balgzand (The Netherlands) |  |  |  |  |  | Alftanes (Iceland) |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 0 (eggs) | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 |
| Sampling week | - | 1 | 3 | 7 | 9 | 9 | 1 | 5 | 7 | 11 |
| Mean length (mm) | - | 14.6 | 14.5 | 13.3 | 29.2 | 13.1 | 15.5 | 15 | 28.5 | 53 |
| $N$ | 64 | 64 | 64 | 61 | 48 | 48 | 37 | 52 | 52 | 51 |
| Locus |  |  |  |  |  |  |  |  |  |  |
| List1001 $N_{\text {A }}$ | 5 | 7 | 5 | 6 | 6 | 6 | 5 | 8 | 5 | 5 |
| $H_{\text {exp }}$ | 0.5863 | 0.5687 | 0.6654 | 0.5789 | 0.6147 | 0.6504 | 0.7693 | 0.7562 | 0.7397 | 0.7290 |
| $H_{\text {obs }}$ | 0.6032 | 0.5938 | 0.7031 | 0.5000 | 0.5417 | 0.6042 | 0.7297 | 0.7308 | 0.8077 | 0.7451 |
| $f$ | -0.0290 | -0.0445 | -0.0572 | 0.1372 | 0.1193 | 0.0718 | 0.0522 | 0.0339 | -0.0929 | -0.0223 |
| List1003 $N_{\text {A }}$ | 9 | 9 | 7 | 12 | 11 | 7 | 6 | 7 | 6 | 5 |
| $H_{\text {exp }}$ | 0.3878 | 0.4599 | 0.4401 | 0.4364 | 0.5548 | 0.4406 | 0.3580 | 0.3182 | 0.4757 | 0.4122 |
| $H_{\text {obs }}$ | 0.4127 | 0.4688 | 0.4219 | 0.4590 | 0.5625 | 0.4375 | 0.3514 | 0.2941 | 0.5000 | 0.4118 |
| $f$ | -0.0647 | -0.0194 | 0.0417 | -0.0523 | -0.0139 | 0.0070 | 0.0189 | 0.0764 | -0.0516 | 0.0010 |
| Multilocus $f$ |  |  |  |  |  |  |  |  |  |  |
| Multilocus $f$, excluding PL06 \& PL09 | 0.1518*** | $0.0762^{* *}$ | 0.0680** | $0.1183{ }^{* * *}$ | $0.1397^{* * *}$ | $0.1471{ }^{* * *}$ | $0.1668{ }^{* * *}$ | 0.1412*** | 0.0602** | $0.1209^{* * *}$ |

## Wahlund effect

A temporal Wahlund effect could be rejected for all cohorts ( $P \geq 0.11$ ) as none of the PartitionML log-likelihood values departed from the null distribution of a homogeneous population (data not shown).

## Inbreeding

The frequency of low MLH classes was higher then expected under random mating at both locations ( $P<0.001$ ) (Fig 2). Consequently, individuals were more homozygous over all six loci than expected under random mating, indicating that more inbred fish than expected were present in all of the cohorts. The same analysis performed on each cohort separately (not shown) also showed a significant shift towards low MLH classes ( $P \leq 0.004$ ) in every case.



Fig. 2. Distribution of $\left[\mathrm{MLH}_{\mathrm{obs}}-\mathrm{MLH}_{\text {exp }}\right]$ frequencies (loci PL06 and PL09 excluded). A: all cohorts from Balgzand (Dutch Wadden Sea), B: all cohorts from Alftanes (Iceland).

## Relatedness

Evidence for relatedness was found for two of the ten cohorts (Table 3). The mean and the variance of pairwise $I$ departed significantly from the expected distribution for cohort 3 at Balgzand (mean $I=0.2040, P<0.05$ ) and cohort 1 at Alftanes (var. $I=0.1229, P<0.05$ ). Evidence for relatedness was also found in adults samples (data from Hoarau et al. 2002a). The mean and the variance of pairwise $I$ departed significantly from the expected distribution for samples from Terschellinger Bank (North Sea, spawning ground) (mean $\mathrm{I}=0.2102, P<0.05$ ) and for samples from the Irish Sea (var. $I=0.1459, P<0.05$ ).

Table 3. Mean and variance of pairwise identity coefficient (I). $P$-values were calculated using 1000 permutations. Significant values ( $P<0.05$ ) are boldface. Loci PL06 and PL09 were excluded.

| Location Cohort | Balgzand (Wadden Sea) |  |  |  |  |  | Alftanes (Iceland) |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | 0 (eggs) | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 |
| Mean I | 0.1907 | 0.1824 | 0.2224 | 0.2040 | 0.1671 | 0.1813 | 0.2112 | 0.2110 | 0.2245 | 0.2424 |
| Variance (I) | 0.1241 | 0.1128 | 0.1313 | 0.1283 | 0.1167 | 0.1203 | 0.1229 | 0.1264 | 0.1295 | 0.1374 |

## Selection

The relationship between multilocus $F_{\text {IS }}$ and mean fish size within each cohort (mm) appears to decrease with age. Although suggestive of selection against homozygotes (Fig. 3), the trend was not significant.


Fig. 3. Changes in multilocus $F_{\text {IS }}$ (loci PL06 and PL09 excluded) with mean fish size within cohort (mm). • = Balgzand (Dutch Wadden Sea), $\square$ Alftanes (Iceland); trend lines: $\ldots \ldots . .=$ Balgzand, $\ldots \ldots=$ Alftanes, $\quad$ = all. The trends are not significant.

## Discussion

Heterozygote deficiencies observed in plaice are most probably related to inbreeding and kinship; and not to genotyping artefacts, Wahlund effects (either spatial or temporal), or selection against heterozygotes. While it is hard to imagine how inbreeding could be a factor when plaice populations in the North Sea are estimated at $10^{9}$ adults (ICES www.ices.dk), moderately low $N$ e in combination with social structure (which naturally involves kinship) may make plaice exceptionally vulnerable to potential inbreeding when exploitation levels exceed a relatively low threshold. Below we discuss how low effective population size, in combination with fisheries related depletion of kin-structured spawning aggregations, could result in the observed heterozygote deficiencies.

## Low effective population size, social structure and mating behaviour

Effective population size $(\mathrm{Ne})$ reflects the number of mating individuals that contribute to the next generation and is typically much smaller than the census size (Frankham 1995). Differences between census and effective population sizes of three to five orders of magnitude have been documented for New Zealand snapper (Hauser et al. 2002), red drum (Turner et al. 2002) and cod (Hutchinson et al. 2003). In a recent study of plaice, $N \mathrm{e}$ was estimated to be $2000-20,000$ individuals based on temporal fluctuation in allele frequency (Chapter 6). From a theoretical standpoint, however, $N e$ estimates of thousands of individuals would still be far too large for the effects of genetic drift, loss of variability and inbreeding to play a significant role under the assumption of random mating. But what if mating is not random?

Previous generalisations about the apparent lack of social structure in plaice have been challenged by a recent study which directly observed evidence for social structure in two other temperate species of Pleuronectes (Gomelyuk and Shchetkov 1999). Furthermore, indirect evidence for group living in the form of kin aggregation is at least suggested from our demonstration of kin structure (relatedness) among juvenile plaice in two of the ten cohorts sampled (Table 3), as well as among adults sampled on feeding and spawning grounds. Thus, observational and inferential evidence implies that subtle structure may be maintained throughout the plaice life cycle. Kin structure has also been found in several other fishes including perch (Gerlach et al. 2001), rainbowfish (Arnold 2000) and tilapia (Pouyaud et al. 1999); the latter also revealing inbreeding.

Another link to be laid is between kin structure and the documented fidelity of plaice to their spawning grounds (De Veen 1978, Hunter et al. 2003). How do plaice know how to find their spawning ground given that spawning larvae are quickly advected from their offshore natal site? One possibility is through larval attachment (natal philopatry), which should promote strong population differentiation. This can be rejected, however, because recent genetic studies on plaice indicate that spawning populations are only weakly differentiated and strongly connected by gene flow (Hoarau et al. 2002a, 2004). A second possibility suggested by Arnold and Metcalfe (1995) is that immature plaice accompany mature fish to the spawning ground in order to learn the location, and to associate some feature of the mature spawning fish (e.g., pheromone) with physical features of the sea bed. Learning behaviour of this
type is considered to be the most plausible explanation in herring (reviewed in Corten 2002), a species that shares many life history traits with plaice.

The apparent contradiction of simultaneously finding kin-structure (small scall genetic heterogeneity) (the present study) and large-scale genetic homogeneity (Hoarau et al. 2002a, 2004) can be explained by the philopatric behaviour of plaice. On the one hand plaice show high spawning site fidelity, between $72 \%$ and $94 \%$ in Iceland (Solmundsson et al. in prep). Such high fidelity could be responsible for a extended family structure. On the other hand $6 \%$ to $28 \%$ of "unfaithful" individuals are also enough to homogenise allelic frequencies among spawning grounds. The two observations are not therefore mutually exclusive.

On the spawning grounds, aggregations of males are visited by gravid females (Rijnsdorp 1989). Courting behaviour of plaice (Forster 1953) and flounder (Stoner et al. 1999) involves circling and ventral pairing. In the tropical flatfish, Bothus podas, strong territorial behaviour, and strong dominance hierarchies in the mating behaviour have also been reported (Carvalho et al. 2003). For plaice, sex-specific behaviour has been hypothesised to explain the greater catchability of spawning males (Solmundsson et al. 2003). If it turns out that female choice is an important factor in plaice mating, then anything that disrupts this process could have strong effects on fertilisation success and population fitness for the local aggregation.

We hypothesise that if groups of genetically related plaice stay together from the juvenile stage, individuals will learn the same migrating route and share the same spawning ground, ultimately leading to an extended family structure. As long as spawning aggregations remain large, inbreeding will remain unlikely; but if effective population sizes are reduced, directly and/or as a consequence of behavioural disruptions, then inbreeding may emerge.

## Effects of heavy fishing pressure

Clearly, fishing directly reduces population size and, by extension, effective population size. Plaice is the main target of the intensive North Sea beam trawl fishery with catches increasing from $55,000 \mathrm{t}$ in the 1940 s to $170,000 \mathrm{t}$ in the 1980 s . In subsequent years, however, the fishery collapsed to 90.000 t in the late 1990 s (Rijnsdorp and Millner 1996, ICES www.ices.dk). Furthermore, Icelandic stocks declined by more than 50\% between 1991 and 2000 (Anon. 2003). Therefore, both census and effective population sizes have been greatly reduced.

Second, fishing on the spawning grounds can indirectly cause functional reductions in effective population size by disrupting dominance hierarchies and courtship behaviours that are necessary for successful mating. The effects of noise and vibration from fishing gear has been shown to affect mating in several fish species, including cod, which utilise squeaks and grunts to locate, warn and attract one another (Rowe and Hutchings 2003). Trawling intensity over the spawning grounds in the North Sea has been estimated to be more than four times per year (Rijnsdorp et al. 1998). During the spawning season, such a disturbance on social and behavioural mechanisms in plaice is likely to be severe.

The potential for inbreeding is inherent in the life history of plaice but would only become an issue under severe exploitation. Our results are further supported by a recently completed temporal comparison of inbreeding coefficients in plaice covering an $80-\mathrm{yr}$ period from 1924 to 2002 . DNA analysis of archived otolith samples from pre-industrial fishing of plaice (i.e., pre-1970) revealed no evidence for inbreeding $\leq 1950$ (Chapter 6).

## Potential consequences of inbreeding

Inbreeding can lead to decline and extinction of natural populations (Saccheri et al. 1998, Brook et al. 2002) as a consequence of lower fitness. The negative, albeit non-significant, trendline between $F_{\text {IS }}$ and size (Fig 1) suggests that inbred plaice juveniles may be experiencing inbreeding depression, (lower fitness) which can be caused by deleterious recessive alleles (dominance hypothesis) (Charlesworth and Charlesworth 1987). The presence of inbreeding depression suggests that insufficient time has elapsed for selection to purge deleterious alleles. In plaice we know that inbreeding is $<10$ generations old (Chapter 6). Plaice is therefore likely to suffer from inbreeding depression. In order to precisely determine the effect of inbreeding a minimum of a thousand individuals must be analysed for several fitness measures in order to gain sufficient statistical power (David 1998), a laborious but worthwhile endeavour.

## Conclusions

Inbreeding has been overlooked in the management of marine fisheries because of traditional assumptions that commercial fish species, characterised by large populations and free spawning would preclude such processes. However, it is now recognised that even in fish with $10^{9}$ individuals, effective population sizes can be on the order of only $1,000-10,000$ individuals and family structured spawning aggregation can lead to a departure from random mating. We contend that overexploitation has both directly and indirectly reduced effective population sizes to the point that inbreeding has become detectable. Inbreeding is likely, on the long term, to further reduce the viability of the population.

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## Chapter 6

# LOW EFFECTIVE POPULATION SIZE AND INBREEDING IN PLAICE (Pleuronectes platessa): AN ABUNDANT BUT OVEREXPLOITED FLATFISH 

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## Manuscript


#### Abstract

We used DNA from archived otoliths to estimate effective population size $(\mathrm{Ne})$ in plaice (Pleuronectes platessa) based temporal fluctuations in allele frequencies from samples collected between 1924 and 2002. Our results show that plaice $N e$ is five orders of magnitude smaller than the estimated census sizes, i.e., at 20,000 in the North Sea and 2,000 in Iceland. Populations examined between 1924 and ca 1950 were in Hardy Weinberg Equilibrium. The emergence of heterozygote deficiencies from ca. 1970 (that are not attributable to sampling or technical artefacts, but attributable to inbreeding) coincide with the beginning of post-WWII, industrialscale beam-trawling on the spawning grounds of the North Sea and around Iceland. We hypothesise that fishing-mediated reduction in both census and effective population sizes of plaice, in combination with disruption of mating behaviour at the spawning grounds has further reduced the effective population size thus tipping the balance and allowing inbreeding to emerge. Although the mechanism remains speculative, this is the first demonstration of probable fisheries-induced inbreeding and signals the need for understanding the social and mating behaviour in these fish.


## Introduction

The over-exploitation and subsequent collapse of most major fisheries in the world have emphasised the point that marine stocks are not inexhaustible. In 2002, $75 \%$ of the world's marine stocks were fully exploited or overexploited (FAO, www.fao.org). Even after the collapse of major fisheries such as cod and herring (Hutchings 2000), the perception has remained that marine fishes are fundamentally resilient to large population reductions.

The fact that a 'collapsed' stock may still consist of millions of individuals, has led to complacency with regard to the potential effects of fisheries on the genetic health of affected species. The problem is that the census size of a population $(N)$ is not the relevant measurement; it's effective population size ( Ne ) that counts. Ne refers to the number of individuals contributing to the next generation and can be much smaller than the census population size (Franckham 1995). In marine fish, Ne is often several orders of magnitude smaller than $N$, producing $N \mathrm{e} / N$ ratios varying from $10^{-5}$ to $10^{-3}$ (Turner et al. 2002, Hauser et al. 2002, Hutchinson et al. 2003). With such small $N e / N$ ratios, even species with a very large $N$ can suffer loss of genetic diversity due to fishing pressure as it has been shown for the New Zealand snapper (Hauser et al. 2002), the red drum (Turner et al. 2002) or the North Sea cod (Hutchinson et al. 2003).

Plaice is a common flatfish species inhabiting Northern European coastal waters and a main target of the North Sea beam-trawl fisheries (Rijnsdorp and Millner 1996). North Sea catches last century increased from a stable $55,000 \mathrm{t}$ in the mid 1940s to a record $170,000 \mathrm{t}$ in 1989 , after which fisheries collapsed to $82,000 \mathrm{t}$ in 2001 (Rijnsdorp and Millner 1996, ICES www.ices.dk). In Iceland a 50\% decline of the stock has been observed this last 10 years (Anno. 2003).

Two recent genetic surveys of plaice populations in the Northern East Atlantic based on microsatellite loci and mitochondrial DNA, revealed weak but significant genetic differentiation (Hoarau et al. 2002, Hoarau et al. 2004) consistent with large scale homogeneity and recency of recolonisation of the North Atlantic over the past 15,000 years. Significant heterozygote deficiencies were also found in these surveys suggesting that mating was not random and that inbreeding might be a factor. Because genotyping (null alleles and large allele drop-out) and sampling artefacts (Wahlund effects) can often produce heterozygote deficiencies, extensive testing has
been performed in order to rule out these possibilities (Chapter 5). Testing for genotyping artefacts included reamplification of loci at lower temperatures, redesign of flanking primers for two of the six loci and an assessment of the proportion of expected null alleles using Brookfield's method (1996). Size distribution of the genotypes was analysed using MiCROCHEKER (van Oosterhout et al. 2003) to rule out the possibility of large allele drop-out (Chapter 5). Testing for sampling artefacts associated with a possible temporal Wahlund effect included a analysis of juveniles from ten separate cohorts. Even in this sampling regime, the finding of heterozygote deficiency persisted where no Wahlund effect was possible. The conclusion from all of these tests was that none of these factors could explain the observed excess of homozygotes.

Evidence for partial inbreeding was also the result of the cohort analyses (Chapter 5). Genetic evidence for kin structure, a prerequisite for inbreeding, was also shown in some of the cohorts. These results imply that social structure may exist and that effective population size in plaice must be small.

The aim of the present study was to estimate effective population size $(\mathrm{Ne})$ in plaice, look for a possible reduction in genetic diversity $\left(H_{\mathrm{e}}\right)$ through time, and to determine whether inbreeding have only recently emerged. We use DNA extracted from archived otolith samples collected between 1924 and 2002 and six microsatellite loci.

## Materials and methods

## Sampling and DNA extraction

Two plaice populations were chosen-one from Iceland; and one from the North Sea. These two populations are genetically isolated (Hoarau et al 2002a, Hoarau et $a l$. in press) and thus represent two independent samples.

Otolith samples from Iceland ( 64 samples from 1924, 1948 and 1972 each) and the North Sea (1950 and 1970, with $\mathrm{n}=50$ and $\mathrm{n}=64$ each) were obtained from the HAFRO (Reykjavik, Iceland) and RIVO (IJmuiden, The Netherlands) fisheries institutes. Data from 2002, (North Sea, n=336; Iceland, n=186), are from a previous study (Chapter 5).

Historical DNA was extracted from the dry tissues surrounding the otolith (Fig. 1) (Hutchinson et al. 1999) in a laboratory where no DNA-work had ever been done before. DNA-free pipettes and filtertips were also used to avoid contamination. Negative controls were used at every step from extraction to genotyping. Extracted DNA was of good quality with an overall amplification failure of only $1.4 \%$, a level comparable to DNA extracted from muscle tissue (ca. $1 \%$ amplification failure).


Fig. 1. Paper envelope and plaice otoliths.

## Microsatellite loci

Six microsatellite loci (PL92, PL115, PL142, PL167, List1001, List1003 were genotyped as previously described (Hoarau et al. 2002). As previously explained, these loci have been extensively tested for null alleles and large-allele drop-out (Chapter 5).

## Estimates of Ne

Variance effective population sizes $(\mathrm{Ne})$ were estimated using a likelihood based temporal method developed by Wang (2001). The variance effective population size is the size of an ideal population (i.e., one with no selection, random mating, and a Poisson-distributed reproductive success) that has the same properties with respect to allele frequency variance (genetic drift) as the actual population. An MLapproach is superior to those based an $F$-statistics because it can utilise more of the information in the data, i.e., the presence of many low-frequencies alleles, which are typical for microsatellite loci. We used the software MNE 1.0 (Wang 2001) with four points in time for the Iceland samples (i.e. 1924, 1948, 1972 and 2002) and three
points in time for the North Sea samples (i.e. 1950, 1970 and 2002). The accuracy of the $N e$ estimate increases with the number of alleles, the sample size and the interval of sampling (Wang 2001). Based on the analysis of a real data set, Wang (2001) found that optimal accuracy was achieved when at least three time intervals (representing ca. 12 generations in this case) were used and 400 individuals. Two loci with 35 alleles in total were found to be adequate. Because sample size, number of alleles and sampling interval can compensate for one another to a certain extent, there is no absolute minimum value for any one parameter. In our case, the use of three to four time points covering 75 years and perhaps 20 generations, in combination with 828 individuals, six loci with 353 alleles gives us confidence that the estimates of Ne for plaice are reasonably accurate.

## Diversity and departures from HWE

Genetic diversity $\left(H_{\mathrm{e}}\right)$ was estimated using Genetix 4.04 (Belkhir et al. 2003). The occurrence of inbreeding was tested by comparing the mean observed Multi Locus Heterozygosity (number of heterozygous loci per individual) $\left(\mathrm{MLH}_{\mathrm{obs}}\right)$ in the samples with the mean expected MLH ( $\mathrm{MLH}_{\text {exp }}$ ) under random mating (Chapter 5) and by computing the excess of homozygotes: $\left(\mathrm{MLH}_{\text {obs }}-\mathrm{MLH}_{\text {exp }}\right) / \mathrm{MLH}_{\text {exp }}$. Significance level were determined using 1000 permutations (Chapter 5).

## Results and Discussion

## Effective population size

The ML-estimated Ne for Iceland is 1733 individuals [1063; 3598 at $95 \% \mathrm{CI}]$ and for the North Sea 19,535 individuals [3435; 70,000 at $95 \%$ CI]. Adult ( $>3$ years old) census sizes $(N)$ are estimated to be $\sim 10^{8}$ for Iceland (J Palsson, personal communication) and $\sim 10^{9}$ for the North Sea (AD Rijnsdorp, personal communication).

The $N \mathrm{e} / \mathrm{N}$ ratios for both populations are similar (ca. $2 \times 10^{-5}$ ). Although this ratio is very small, it is within the range found for other marine fish with a similar life history (Hauser et al. 2002, Hutchinson et al. 2003, Turner et al. 2002). Thus, very low $N \mathrm{e} / \mathrm{N}$ ratios appear to be common in marine fish characterised by high fecundity and high juvenile mortality (type III survivorship curves). Both of these factors can
lead to high variance in individual reproductive success and subsequently, to large discrepancies between $N e$ and $N$ (Hedgecock 1994).

A high level of reproductive variance is assumed for plaice, with high levels of female fecundity ( $20,000-600,000$ eggs•female ${ }^{-1}$ ) (Rijnsdorp 1991) offset by high levels of daily mortality for eggs (up to 20\%) (Rijnsdorp and Jaworski 1990) and juveniles (up to 4\%) (Van der Veer et al. 1990). Therefore, it is likely that variance in plaice reproductive success is the main factor for the small $\mathrm{Ne} / \mathrm{N}$ ratio we observed.

Social structure and mating behaviour also increase the variance in reproductive success. The importance of mating behaviour is often overlooked in these types of commercial fish species mainly due to a lack of data. For plaice the commonly accepted model of free spawning in large, seasonal aggregations suggests little or no courting behaviour. In fact, very little is known about plaice mating behaviour in the wild due to technical unfeasibility of observation. However, recent observations of flatfish more generally, indicate complex mating behaviours including the possibility of female choice (Stoner et al. 1999, Carvalho et al. 2003).

Finally, as there is sexual dimorphism in maturation and growth, as well as a difference in the natural mortality and vulnerability to fishing, the heavy fishing pressure in the North Sea has removed the larger adult males specimens more than females. Consequently the sex ratio in plaice is slightly skewed towards females (Rijnsdorp 1994), which would further reduce of Ne .

## Genetic diversity through the $20^{\text {th }}$ century

Genetic diversity $\left(H_{\mathrm{e}}\right)$ has remained constant for both the Icelandic and North Sea populations (Fig. 1) despite increased fishing pressure on plaice over the last century. This was unexpected, as decreases in genetic diversity have been recorded for the overexploited New Zealand snapper and North Sea cod populations (Hauser et al. 2002, Hutchinson et al. 2003). For plaice, Ne is probably still too large to detect any effect of exploitation on the genetic diversity. Indeed, plaice is still relatively abundant in the Northeast Atlantic and even though our estimates of Ne are small, they are still one to two orders of magnitude higher than the $N$ e estimated for the New Zealand snapper or the North Sea cod populations (Hauser et al. 2002, Hutchinson et al. 2003). Theoretically, a Ne of 50 is all that is required to maintain short term genetic diversity and a Ne of 500 individuals to maintain longer-term
stability. Our $N e$ estimates for plaice are above these threshold values suggesting that plaice is in no immediate danger of losing genetic diversity. However this conclusion should be taken with caution as a very recent bottleneck may not have had the time to lead to a detectable reduction of diversity.

## Inbreeding through the $20^{\text {th }}$ century

There is no evidence for a departure from HWE in either the North Sea or Icelandic populations in assays from samples taken $\leq 1950$. The individual MLH distributions did not differ significantly from those expected under random mating. However, the finding of significant homozygote excesses ( $P<0.001$ ), indicative of inbreeding in samples from 1970 onward in both North Sea and Icelandic populations (Fig. 2) is particularly alarming.


Fig. 2. Temporal changes in genetic diversity $\left(H_{\mathrm{e}}\right)$ : dashed line; and inbreeding (define as excess of homozygotes: $\left.\left(\mathrm{MLH}_{\text {exp }}-\mathrm{MLH}_{\mathrm{obs}}\right) / \mathrm{MLH}_{\text {exp }}\right)$ ): solid line, open circle: non significant, filled circle: $P<0.001$.

The apparent emergence of inbreeding coincides with the introduction of the beam trawl in the beginning of the 1960s. Although plaice has been heavily exploited for centuries (Van Neer et al. 2002), past fishing activities targeted mainly juveniles on the nursery grounds. Expansions of the fishery after WWII led to a greater focus on fishing mature individuals from spawning grounds. It is likely that increased fishing pressure on spawning aggregations, together with more efficient fishing gear and techniques, have led to unavoidable inbreeding in plaice, by reducing the size of spawning aggregations. If, as we hypothesise, kinship-based social structure is associated with the spawning grounds (Chapter 5), then fishingmediated reductions in clan-sizes in combination with fishing-mediated disruptions of behaviour during the mating season, may force non-random matings to occur. Consequently, the rise of inbreeding after the 1950s is most likely related to the 3 fold increase in landings of plaice in the last fifty years, especially as fisheries activities account for $80 \%$ of plaice mortality (Rijnsdorp and Millner 1996).

Although our proposed mechanism for inbreeding remains speculative, the consequences of inbreeding for plaice populations are likely to be severe, potentially leading to population decline and eventual extinction (Saccheri et al. 1998). As inbreeding in plaice is less than ten generations old, inbreeding depression or a reduced fitness of inbred individuals could result (Charlesworth and Charlesworth 1987). The effects of inbreeding depression are expected to be most severe just after the onset of inbreeding notably as deleterious recessive alleles would not yet be purged by selection. Furthermore, previous results suggest inbreeding depression, is occurring (Chapter 5). An increase in the landings of "abnormal" fish (RIVO unpublished data) may also be indicative of possible inbreeding depression. A rigorous test is necessary but logistically difficult, as sufficient statistical power can be attained only by estimating several fitness components in thousands of individuals (David 1998).

## Conclusion

We have shown that effective population sizes in plaice are small and have provided evidence for the emergence of inbreeding between 1950 and 1970. Although the mechanism underlying the observed results remains speculative, it is
clear that plaice stocks are not in Hardy-Weinberg Equilibrium and that a strong link exists between the onset of industrial scale fishing on the spawning grounds in the 1960s. Under the precautionary principle, we recommend that fishing on the spawning grounds be stopped.

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## Chapter 7

# HETEROPLASMY AND EVIDENCE FOR RECOMBINATION IN THE MITOCHONDRIAL CONTROL REGION OF THE FLATFISH 

## Platichthys flesus

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#### Abstract

The general assumption that mitochondrial DNA (mtDNA) does not undergo recombination has been challenged recently in invertebrates. Here we present the first direct evidence for recombination in the mtDNA of a vertebrate, the flounder Platichthys flesus. The control region in the mtDNA of this flatfish is characterized by the presence of a VNTR and a high level of heteroplasmy. Two types of repeats were recognized, differing by two $\mathrm{C} / \mathrm{T}$ point mutations. Most individuals carry a pure "C" or a pure " T " array, but one individual showed a compound "CT" array. Such a compound array is evidence for recombination in the mtDNA control region from the flounder.


## Introduction

It has generally been assumed that animal mitochondrial DNA (mtDNA) does not undergo recombination. This assumption has come from indirect evidence such as failure to detect excision repair activity and crossover products (Clayton, Doda and Friedberg 1975), failure to detect mtDNA recombination in interspecific somatic cell hybrids (Zuckerman et al. 1984), clustering of mtDNA molecules preventing physical contact (Satoh and Kuriowa 1991), and high mutation rate (Howell 1997). However, two recent studies have directly demonstrated mtDNA recombination in two invertebrates, a nematode Meloidogyne javanica (Lunt and Hyman 1997) and the mussel Mytilus galloprovincialis (Ladoukakis and Zouros 2001). With respect to vertebrates, only indirect evidence for mtDNA recombination has been found in the form of high frequencies of homoplasies (Eyre-Walker, Smith and Maynard Smith 1999) and lower levels of linkage disequilibrium between more distance polymorphic sites (Awadalla, Eyre-Walker and Maynard Smith 1999).

Here, we provide the first direct evidence of recombination in the mtDNA of a vertebrate, the flounder Platichthys flesus (Teleostei : Pleuronectiformes [flatfish]). In flounder, as in many other fishes (Nesbo, Arab and Jakobsen 1998, Ludwig et al. 2000), the 5 '-end of the mitochondrial control region is characterized by the presence of a Variable Number of Tandem Repeats (VNTRs) and a high level of length heteroplasmy for this region. The cause of variation in the repeat number is not fully understood but is variously attributed to slipped strand mispairing, illegitimate elongation and termination associated sequence (TAS) -based replication (Ludwig et al. 2000). It may also be caused by mtDNA recombination, although this is more difficult to demonstrate when VNTRs consist of perfect repeats.

## Material and Methods

A total of 168 individuals of flounder collected from 5 locations in the northern Atlantic and the Mediterranean were used to analyze the variation of the VNTRs and the extent of heteroplasmy (Table 1). Total genomic DNA was extracted using a CTAB protocol (Hoarau et al 2002). PCR amplification of the 5'-end of the mitochondrial control region was carried out in $10 \mu \mathrm{l}$ volumes containing $1 \mu \mathrm{l}$ of
$1 / 10$ diluted DNA, 1 X reaction buffer, 0.2 mM of each dNTP, $2 \mathrm{mM} \mathrm{MgCl} 2,0.25 \mathrm{U}$ Taq DNA polymerase (Promega) and $0.5 \mu \mathrm{M}$ of each of the following primers, DLF (5’-CCA CCT CTA ACT CCC AAA GC-3') (modified from A, Lee et al. 1995) and DLR3 ( ${ }^{\prime}$ '-GGT TAT TAT ACA TGA ATG TCC T-3'). For fragment analysis, primer DLF was fluorescently labeled with the 6-FAM (Carboxyfluorescein) dye label (Applied Biosystem). Amplifications were performed in either a PTC100 (MJ Research) or a Mastercycler gradient cycler (Eppendorf). The reaction profile was as follows: initial denaturation at $94^{\circ} \mathrm{C}$ for $3 \mathrm{~min} ; 30$ cycles of denaturation at $94^{\circ} \mathrm{C}$ for 30 s , annealing at $50^{\circ} \mathrm{C}$ for 30 s , and extension at $72^{\circ} \mathrm{C}$ for 1 min ; and a final extension at $72^{\circ} \mathrm{C}$ for 10 min . Length variation was visualized on an ABI Prism-377 automatic sequencer (Applied Biosystems) using a $4.5 \%$ denaturing polyacrylamide gel with an internal lane standard and Genescan ${ }^{\mathrm{TM}}$ software for determination of the fragment size. PCR products from 18 individuals were ligated into pGEM-T Easy plasmid (Promega) and cloned in Escherichia coli JM109 (Promega) according to the manufacturer's instructions. PCR amplification of 6 to 48 clones per individual ( 288 clones in total) was performed as described above and 3-6 clones per individual ( 84 clones in total) were sequenced using a BigDye Cycle Sequencing kit (Applied Biosystem) and an ABI Prism-377 automatic sequencer (Applied Biosystems). Negative controls were used at every step of the experiment.

## Results and Discussion

Nearly all (165/168) individuals were heteroplasmic (Table 1), containing 3 to 9 length variants per individual.

Table 1. Heteroplasmy in Platichthys flesus. $N$ : number of individuals screened for length polymorphism, $H: \%$ of individuals showing heteroplasmy, $N_{\mathrm{IC}}$ : number of individuals cloned and sequenced, $N_{\mathrm{TC}}$ : total number of clones sequenced.

| Location | $N$ | $H$ | $N_{\text {IC }}$ | $N_{\text {TC }}$ | Array type |
| :--- | ---: | ---: | ---: | ---: | :--- |
| Vacares (France) | 2 | 50 | 2 | 6 | C |
| Bay of Biscay (France) | 10 | 100 | 4 | 14 | C |
| Terschellinger Bank (The Netherlands) | 49 | 100 | 4 | 32 | $\mathrm{C}, \mathrm{T}, \mathrm{CT}$ |
| Dollard Estuary (The Netherlands) | 73 | 100 | 4 | 16 | $\mathrm{C}, \mathrm{T}$ |
| Lofoten (Norway) | 34 | 97 | 4 | 16 | $\mathrm{C}, \mathrm{T}$ |

The basic repeat unit is 19 bp long and the number of repeats ranges from 1 to 10. From the total set of 168 individuals, 18 individuals were chosen for further characterization. First, PCR products were cloned from these individuals and between 6 and 48 clones/individual (total of 288) were then reamplified and run on a gel to confirm that only one band was present as expected from individual clones. This was an important check because PCR artifacts (resulting in multiple bands) associated with these types of repeat motifs have been reported by Campbell et al. (2001). We found single sharp bands in 274 clones, whereas 14 amplifications produced double bands. Products involving more than two bands were never observed. Thus, although artifacts accounted for $4.8 \%$ of the observations, the high level of heteroplasmy observed is real though perhaps slightly overestimated.

Again from the same set of 18 individuals, 3-6 clones per individual were sequenced (total of 84) in order to characterize the array motifs themselves and their number. Two types of core repeats were detected, a "C" type and a "T" type, which differed by two point mutations (Fig. 1). Among the 18 individuals, 13 contained only the pure "C" type array, 4 only the pure "T" type array and one individual a compound array of "C" and "T" (Fig. 1, A, B and C respectively). The compound array is indicative of mtDNA recombination. It is highly unlikely that the compound array arose by mutation as the differences between the " C " type and the " $T$ " type involve two independent mutations and neither seems to be associated with secondary structure such as hairpin (no palindrome in the sequence of the repeat unit). Moreover these two positions are highly conserved in the closely related species, Pleuronectes platessa (Hoarau G. unpublished data).

```
A Ind1 CGATGC ATGTACAATGAAGGCTTTC ATGTACAATGAAGGCTTTC ATGTACAATGAAGGCTTTC ATGTACAATGAAGGCTTTC ATGTACAT
B Ind2 CGATGC ATGTACAATGAAGGTTTTT ATGTACAATGAAGGTTTTT ATGTACAATGAAGGTTTTT ---------------------------
C Ind3 CGATGC ATGTACAATGAAGGCTTTC ATGTACAATGAAGGCTTTC ATGTACAATGAAGGTTTTT ATGTACAATGAAGGTTTTT ATGTACAT
```

Fig. 1. Alignment of the VNTR of mtDNA control region in Platichthys flesus. $\quad$ C type repeat, - T type repeat. The two point mutations are boldface. Ind1 shows a pure "C" type array, Ind2 a pure "T" type array and Ind3 a "CT" compound array. GenBank AY143162 to AY143164.

Once again, however, the issue of possible PCR artifacts arises because Taq polymerase can produce chimeric DNA molecules (i.e., PCR jumping sensu Paabo, Irvin and Wilson 1990) when damaged fragments of a mixed template of DNAs are joined during the amplification process. Mixed templates of undamaged fragments do not result in PCR jumping (Ladoukakis and Zouros 2001). In order to check for this possibility, equal amounts of DNA from pure " C " and pure " T " individuals were mixed in the same PCR reaction in an effort to create a chimeric product. The PCR product was cloned and 16 clones were sequenced. Ten were pure " $C$ " and 6 were pure "T". There were no "CT" arrays detected. Thus a PCR artifact is unlikely.

Further support for true recombination comes from the fact that the recombinant "CT" array was found in each of 3 independent DNA extractions, PCR and sequencing reactions from the recombinant individual. In addition, the entire DNA extraction, amplification, cloning and sequencing procedure was conducted in an independent laboratory where no studies on fish have ever been conducted. Twelve clones were sequenced and recombinant "CT" arrays were found in 7 clones and a parental " $T$ " was found in the 5 other clones (Table 2). Interestingly, only one of the "parental" types (" T ") is found together with recombinant arrays. This suggests that recombination took place at least a generation ago and that the other "parental" type ("C") was lost by random drift during ontogenesis or gametogenesis (Chinnery et al. 2000). Variation in the number of "C" and/or "T" repeats in recombinant arrays further suggests that other mechanisms, such as slipped strand mispairing, besides (or in addition to) recombination are involved in the evolution of the number of repeats.

Table 2. Type of repeat arrays found in the recombinant individual.

| Array | Number of clones |
| :--- | :--- |
| T-T-T | 5 |
| C-T-T-T | 1 |
| C-C-T-T | 2 |
| C-C-T-T-T- | 2 |
| C-C-C-T-T | 2 |

Recombination can explain the evolution of mtDNA VNTRs, but it is usually difficult to trace when VNTRs exist as perfect arrays. Because flounder VNTRs are imperfect and the two different types of repeat unit are found, the demonstration of mtDNA recombination is straightforward.

In principle, an alternative hypothesis to recombination could be that the compound array is the ancestral form and that the derived type of array we have observed evolved by a series of duplication-and-loss events. In this scenario, the two mutations (i.e., switching between the C to T type and vice versa) occurred in a duplicated region, such that both it and the ancestral form would be retained in the same individual. The different number of basic repeats could then be accounted for by a history of duplication and loss. Given the range in the number of repeat regions found (1-10), such duplications and losses might occur fairly regularly. However, we do not favor this alternative hypothesis because: 1) If the "CT" arrays are indeed the ancestral form, then they should have been found more frequently in $P$. flesus and in closely related flatfish such as Pleuronectes platessa (=plaice) - plaice contains only one repeat, suggesting that the ancestral form was one repeat; and 2) this hypothesis requires that the two mutations be retained in the same individual, whereas the recombination scenario requires only that the two types be present in the same population.

The mtDNA recombination we found between " C " and " T " type arrays in flounder has several implications for vertebrate mtDNA in general. Before such recombination is possible, the " C " and " T " type mitochondria must be present in the same cell and their DNA must be coupled. This implies paternal leakage followed by fusion of the mitochondria. Paternal leakage has been reported for mice (Gyllensten et al. 1991) and anchovies (Magoulas and Zouros 1993), whereas fusion of mitochondria has been demonstrated in Drosophila (Yaffe 1999), and the enzymes necessary for recombination have been found in human mitochondria (Thyagarajan, Padua and Campbell 1996). It therefore appears that all of these properties are present in flounder and in vertebrates more generally.

The importance of recombination in vertebrate mitochondria has broad implications across several fields, ranging from human mitochondrial diseases (Schon 2000) to the compromise of phylogenetic and population studies that assume strict clonal inheritance of mtDNA (Schierup and Hein 2000). In the case of human mitochondrial diseases, mtDNA recombination will greatly change the mode and
patterns of inheritance, which in turn may affect current diagnostic methods. Recombination can also affect the accuracy of phylogenetic reconstruction (Posada and Crandall 2002), inferences related to demographic history, and the application of molecular clocks (Schierup and Hein 2000).

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Chapter 8
SUMMARY


#### Abstract

Summary The central aim of this thesis was to investigate the genetic structure of plaice, a commercially important flatfish characterised by a pelagic larval phase and adult homing behaviour.


The European plaice (Pleuronectes platessa L.) is an important flatfish for Northern Europe fisheries. Reproduction takes place offshore during winter and the eggs/larvae are pelagic for about three to four months after which they settle in shallow coastal waters (nurseries) where they metamorphose. Tagging data have shown that adults exhibit seasonal migration patterns from spawning grounds to feeding grounds and that regional scale population structure of plaice appears to be mainly shaped by oceanographic features such as deep water, the occurrence of retention areas and suitable nursery grounds. This has suggested the possibility of philopatry, which could lead to strong population differentiation despite a high dispersal potential via the long pelagic phase of eggs or migration of adults. This led to an initial investigation of the following questions:

1. What is the spatial scale of population genetic structure in plaice throughout its range in Northern European waters?
2. To what extent are spawning grounds connected to specific nursery grounds?
3. To what extent is there a mismatch between the spatial scale of genetic stock and management of those stocks?

Circa 50 plaice were collected from each of 11 locations ranging from Iceland to France. Samples were obtained from feeding, spawning and nursery grounds. Population structure was analysed using six microsatellite loci developed in our laboratory (Chapters 2 and 3) and using the mitochrondrial control region (Chapter 4). Although both classes of markers revealed a relatively strong differentiation between continental shelf and off-shelf populations $(\theta=0.0351$ and $\theta=0.1015$, respectively), only the mtDNA data was able to detect differentiation within the continental shelf, i.e., a North Sea-Irish Sea region, which was weakly distinguishable from Norway $(\theta=0.0046)$, the Baltic $(\theta=0.0136)$ and the Bay of Biscay $(\theta=0.0162)$. No evidence was obtained for isolation by distance, nor for sex-biased dispersal. Due to the weak population differentiation it was not possible
to infer the connections between spawning grounds and specific nurseries; and finally, there is apparently no mismatch between the scale of genetic structure and the scale of the management unit as the different stocks we have identified are already managed separately following International Council for the Exploration of the Sea (ICES) guidelines.

The next question we asked was:

## 4. What insights about the mating system (especially philopatry) of plaice can be gleaned from the genetic data?

Heterozygote deficiencies (significantly positive $F_{\text {IS }}$ estimates) were consistently observed in all populations, which could not be attributed to sampling or genotyping artefacts, including Wahlund effects and null alleles respectively. The causes for the departure from Hardy-Weinberg Equilibrium were further investigated by redesigning two of the PCR primers at existing loci to absolutely confirm that null alleles were not a factor and the development of two new loci (bringing the total to eight). As a next step we then compared temporal changes in $F_{\text {IS }}$ with cohort age. This was done by harvesting juvenile plaice from genetically distinct nursery grounds in the Dutch Wadden Sea and Iceland (Chapter 5). If inbreeding is present, it is expected that the individual multi-locus-heterozygosity (MLH) will be shifted towards homozygotes. Using six loci, the frequency of lower individual MLH classes, i.e., those with more homozygotes was significantly greater than expected under random mating ( $P<0.001$ ) indicating the presence of inbred individuals. In addition, relatedness or kinship was demonstrable in $2 / 10$ cohorts as well as in adults samples. Collectively, these results suggest that inbreeding may very well be occurring in plaice and that present assumptions about the life history, social structure and mating behaviour have been overly simplified. In any case, dismissal of the inbreeding result on the grounds of technical artefacts must be rejected. Although inbreeding seems extraordinarily unlikely in fish with large populations, it is clear that the concept needs to be critically re-evaluated. We hypothesise that under normal conditions, plaice sharing the same spawning grounds form loose extended families or clans, which would not be inbred. However, under increased fishing pressure in which both population size and behaviour are disrupted
(especially on the spawning grounds), unavoidable inbreeding may occur. In order to examine this possibility further we need another piece of the puzzle. This led to the final set of questions:

## 5. What is effective population size ( Ne )?

6. Is there evidence for fisheries effects on genetic diversity and genetic structure?

Commercially exploited marine fish are usually considered resistant to inbreeding and loss of genetic diversity because their populations remain very large from the genetic point of view, even in the face of overexploitation. In other words, the population size would never become small enough for inbreeding to play a role. This widely held view for pelagic fisheries is now being challenged. While census population sizes $(N)$ may be large, the number of individuals actually contributing to the next generation ( Ne )-the breeding population-can be very small. Estimation of Ne is technically difficult. At present, the best way is the temporal ML method in which the same area/stock is sampled over a long period of time. The changes in allelic frequencies over time can be used to estimate the genetic drift and consequently Ne . We used DNA from archived otolith samples collected over the period from 1924 through 2002 (Chapter 6). Our results show that plaice effective population size is five orders of magnitude smaller than the estimated census size of $10^{8}$ to $10^{9}$. This corresponds to a $N e$ of ca. 2000 in Iceland and a $N e$ of ca 20,000 in the North Sea. Although no reduction of genetic diversity (i.e. allelic richness and heterozygosity) could be detected over the last century, a dramatic increase in inbreeding was found in samples after 1950, which coincides with post-WWII industrial-scale fishing practices and explicit fishing on the spawning grounds.

In parallel with the basic research questions, the development of suitable molecular markers was (and remains) an ongoing process. In the course of screening for variation at a number of mitochondrial loci, evidence for heteroplasmy and mitochondrial recombination was discovered in flounder (Platichthys flesus L). This was the first documented case of recombination in a vertebrate mitochondrial genome (Chapter 7) and has obvious implications for phylogenetic and population genetic studies.

## Concluding remarks and challenges

## Management

Despite the apparent match between the spatial scales of genetic stock structure and management area, the finding of small effective population size and inbreeding after 1950 can no longer be dismissed from the discussion of scientifically based management and conservation of demersal fish stocks-even though the exact behavioural mechanism is still unclear. Applying the precautionary principle, it is probable that other species of flatfish within the mixed flatfish fishery are also affected. At the most conservative and practical levels, therefore, we recommend that fishing on the spawning grounds be prohibited. This would reduce disturbance of the spawning aggregations thereby increasing the chances for larger numbers of breeding individuals to successfully mate. Since the best (though politically untenable) solution would actually be a ban on flatfish fishing altogether, the proposed recommendation above provides a reasonable step towards sustainability at the very least.

However the question remains: how bad is inbreeding for the fitness of plaice and how can this be explored further? A rigorous test of inbreeding depression is obviously necessary but will be technically demanding. To attain sufficient statistical power it will be necessary to estimate several fitness components such as growth and parasite loads for thousands individuals. But even with this knowledge confirmed, the issue of timely decision making remains. In the case of plaice, it may already be too late. The recent EU decision to only slightly reduce plaice quotas as opposed to the advised $40 \%$ is not encouraging.

Finally, the lack of basic knowledge about the social and reproductive behaviour of plaice needs to be redressed. Again, this will be technically challenging because in situ observations are difficult. Recent studies in other pelagic fisheries species have repeatedly shown, however, that audio and olfactory communications, especially on the spawning grounds, are severely disrupted by the physical disturbance and noise of fishing gear. These aspects can no longer be overlooked as a part of fisheries "best practices" which intercalate with our recommendation to halt fishing on the spawning grounds.

## Flatfish evolution

Pleuronectiformes are an ancient teleost lineage that is still poorly known despite two recent molecular phylogenetic studies. The problem lies in the fact that among the $>500$ described species, the vast majority of which are tropical, both studies focused almost exclusively on the 20 or so most-important, temperate, commercial species. Such an obvious sampling bias does little to advance our knowledge of the evolution of this order. Limited fossil evidence in combination with molecular phylogenetic data suggest a rapid radiation of the extant taxa. The degree to which this reflects a sampling bias or a real phenomenon needs to be distinguished.

New phylogenies that include tropical sampling and employ molecular markers with different evolutionary rates are required. In particular, we would like to know more about speciation rates at the genetic level, the genetic basis for the development of such extreme morphological asymmetry, and why mitochondrial heteroplasmy is so common in many flatfish. With the coming availability of several whole-genome sequences for a number of teleost fish, the possibility to explore the evolutionary development of plaice and other related species is now on the horizon.

## SAMENVATTING

Het hoofddoel van dit proefschrift was het bestuderen van de genetische structuur van de schol, een commercieel belangrijke platvis die gekarakteriseerd wordt door een pelagische ("openzee") larvale fase en (voor volwassenen) een instinct om naar paargronden terug te keren (homing behaviour).

De Europese schol (Pleuronectes platessa L.) is een belangrijke platvis voor de Noord - Europese visserij. De voortplanting vindt ver van de kust in de winter plaats en de eieren/larven verblijven daar drie tot vier maanden om vervolgens in de ondiepe kustwateren (kweekkamers) de metamorfose te ondergaan. Experimenten met gemerkte vissen hebben aangetoond dat volwassen vissen seizoensgebonden migratiepatronen van voortplantings- naar foerageergebieden laten zien en dat de populatiestructuur van schol op regionale schaal gevormd lijkt te zijn door oceanografische factoren, zoals diep water, aanwezigheid van retentiegebieden en geschikte kweekkamers. Dit duidt op een mogelijke aanwezigheid van philopatry (homing behaviour), die kan leiden tot een sterke populatie-differentiatie, dit ondanks een hoog verspreidingspotentieel via de lange pelagische fase van de eieren en/of de migratie van de volwassen vissen.

Dit bracht ons tot een initieel onderzoek van de onderstaande vragen:

1. Wat is de ruimtelijke schaal van de populati- genetische structuur van de schol in de Noord - Europese wateren?
2. In hoeverre zijn de paargronden verbonden met bepaalde kweekamers?
3. In hoeverre is er een discrepantie tussen de ruimtelijke schaal van het genetische visbestand en het beheer van dit bestand?

Op elf verschillende locaties, variërend van IJsland tot Frankrijk, werden ongeveer 50 schollen per locatie verzameld. Het monsteren vond plaats in foerageeren broedgebieden en in kweekkamers. De populatiestructuur is geanalyseerd m.b.v. zes in ons laboratorium ontwikkelde microsatellieten (Hoofdstuk 2 en 3) en de mitochondriële control region (Hoofdstuk 4). Hoewel er met beide typen merkers relatief sterke differentiatie tussen populaties op het continentale plat en populaties daarbuiten is aangetoond $\theta=0.0351$ voor de microsatellieten en $\theta=0.1015$ voor de mitochondriële control region), hebben alleen de mitochondriële gegevens
differentiatie binnen populaties op het continentale plat kunnen detecteren, d.w.z. het Noordzee-Ierse Zee gebied was zwak te onderscheiden van Noorwegen $(\theta=$ $0.0046)$, de Baltische Zee $(\theta=0.0136)$ en de Golf van Biskaje $(\theta=0.0162)$. Er is geen bewijs gevonden voor isolation by distance (bij een toenemende geografische afstand tussen populaties neemt de genetische differentiatie tussen populaties toe), of een verspreiding beïnvloed door geslacht. Door de zwakke populatiedifferentiatie was het niet mogelijk om een verband tussen de paargronden en de kweekkamers te vinden. Tenslotte is er blijkbaar geen discrepantie tussen de ruimtelijke schaal van de genetische structuur van het visbestand en het beheer van dit bestand, aangezien de door ons geïdentificeerde verschillende visbestanden al afzonderlijk beheerd worden volgens de richtlijnen van het ICES (International Counsel for the Exploration of the Sea).

De volgende vraag die we ons gesteld hebben was:

## 4. Welke inzichten over het paringssysteem (voornamelijk philopatry) van de schol kunnen uit de genetische gegevens afgeleid worden?

In alle populaties is consistent een tekort aan heterozygoten (significant positieve $F_{\text {IS }}$ schattingen) gevonden, die niet toegeschreven konden worden aan bemonsteringsfouten, zoals Wahlund effecten, of genotyperings fouten, door b.v. nul-allelen. De oorzaken voor deze afwijking van Hardy-Weinberg Equilibrium zijn verder onderzocht door twee van de PCR primers van bestaande loci opnieuw te ontwerpen om daarmee onomstotelijk aan te tonen dat nul allelen geen factor vormen, en door het ontwikkelen van twee nieuwe loci (wat het totaal op acht bracht). Vervolgens hebben we de tijdelijke veranderingen in $F_{\text {IS }}$ vergeleken met de leeftijd van de verschillende cohorten van juveniele schollen. Dit is gedaan door juveniele schollen uit verschillende kweekkamers in de Wadden Zee en IJsland te verzamelen (Hoofdstuk 5). Als er sprake is van inteelt dan wordt een verschuiving van de individuele multi-locus-heterozygosity (MLH) in de richting van de homozygoten verwacht. Met gebruikmaking van zes loci was de frequentie van de lagere individuele MLH klassen, dit zijn de klassen met meer homozygoten, significant hoger dan verwacht onder willekeurige paring ( $P<0.001$ ). Dit duidt op de aanwezigheid van inteelt. Bovendien was er bewijs voor bloedverwantschap in twee
van de tien juveniele cohorten en in de monsters van de volwassen schollen. Gezamenlijk suggereren deze resultaten dat inteelt in schollen wel degelijk tot de mogelijkheden behoort en dat de huidige veronderstellingen over de levensgeschiedenis, sociale structuur en paringsgedrag te simpel zijn voorgesteld. Verwerpen van de inteeltresultaten op grond van technische fouten moet in elk geval worden afgewezen. Hoewel inteelt bij vissen met grote populaties uiterst onwaarschijnlijk lijkt, is het wel duidelijk dat dit concept kritisch gerevalueerd moet worden. Onze hypothese is dat onder normale condities schollen die dezelfde voortplantingsgebieden delen losse uitgebreide families of clans vormen, die geen inteelt vertonen. Echter, onder toenemende visserijdruk, waardoor zowel populatiegrootte als gedrag verstoord worden (voornamelijk in de voortplantingsgebieden), kan de onvermijdelijke inteelt zich manifesteren. Om deze mogelijkheid nader te bestuderen hebben we nog een stukje van de puzzel nodig. Dit heeft geleid tot het laatste stel vragen:

## 5. Wat is de effectieve populatie grootte ( Ne )?

6. Is er bewijs voor visserijeffecten op de genetische diversiteit en genetische structuur?

Commercieel geëxploiteerde zeevissen worden over het algemeen bestand geacht tegen inteelt en tegen een verlies van genetische diversiteit, omdat de populaties zelfs bij overbevissing vanuit een genetisch standpunt nog steeds erg groot blijven. In andere woorden, de populatiegrootte kan nooit klein genoeg worden om inteelt een rol te laten spelen. Dit algemeen geaccepteerde standpunt voor openzeevisserij wordt nu aan de kaak gesteld. Terwijl de census populatiegrootte ( $N$ ) groot kan zijn, is het mogelijk dat de hoeveelheid individuen die daadwerkelijk bijdragen aan de volgende generatie ( Ne ) - de effectieve populatiegrootte - zeer klein is. Het is technisch gezien moeilijk een schatting te maken van $N \mathrm{e}$. Op dit moment is de beste benadering de temporal ML methode waarin eenzelfde gebied of bestand gedurende lange tijd bemonsterd wordt. De veranderingen in allelfrequenties door de tijd kunnen worden gebruikt om genetische drift te berekenen en vervolgens ook de Ne . Wij hebben gebruik gemaakt van DNA uit gearchiveerde otolieten, die werden verzameld over de tijdspanne van 1924 tot 2002 (Hoofdstuk 6). Onze resultaten laten zien dat de effectieve populatiegrootte van schol vijf ordes van grootte kleiner
is dan de geschatte census grootte van 108 tot 109. Dit komt overeen met een $N e$ van ca. 2000 in IJsland en ca. 20.000 in de Noordzee. Ook al kon er geen waarneembare afname van de genetische diversiteit (d.w.z. allelic richness en heterozygotie) over de laatste eeuw ontdekt worden, er vond wel een dramatische toename in inteelt in de monsters van ná 1950 plaats. Deze toename valt samen met zowel een toename van visserij op industriële schaal na de $2^{\text {de }}$ Wereldoorlog, als met een toename in het expliciet bevissen van de paargronden.

Tegelijk met deze fundamentele onderzoeksvragen blijft het ontwikkelen van geschikte moleculaire merkers een doorlopend proces. Tijdens het zoeken naar variatie binnen een aantal mitochondriële loci werd bewijs gevonden voor heteroplasmie en mitochondriële recombinatie in de bot (Platichthys flesus L.). Dit was de eerste vermelding van recombinatie in het mitochondriële genoom van een gewerveld dier (Hoofdstuk 7), en heeft ook duidelijke implicaties voor fylogenetische en populatie genetische studies.

## Afsluitende opmerkingen en aanbevelingen

## Beheer

Ondanks het ogenschijnlijke verband tussen de ruimtelijke schaal van de genetische populatiestructuur en het te beheren gebied, kunnen de consequenties van de in dit proefschrift aangetoonde kleine effectieve populatiegrootte en inteelt na 1950 niet langer worden genegeerd in de discussie over - wetenschappelijk - beheer en het behoud van demersale visbestanden - ook al is het precieze gedragsmechanisme nog niet duidelijk. Als we het voorzorgsbeginsel toepassen, dan lijkt het waarschijnlijk dat ook populaties van andere soorten platvissen in deze gemengde visserij al zijn getroffen. De meest behoudende en praktische aanbeveling die wij dan ook maken is om het bevissen van platvissen in hun voortplantingsgebieden te verbieden. Dit zal verstoring van de voortplantingsaggregaties verminderen en op die manier meer vissen een grotere kans bieden om zich succesvol voort te planten. Aangezien de beste (hoewel politiek onmogelijke) oplossing zou zijn de visserij op platvis helemaal te verbieden, biedt de bovenstaande aanbeveling een redelijke stap in de goede richting: die van een duurzame visserij.

Echter, een vraag blijft vooralsnog onbeantwoord: hoe slecht is inteelt voor de fitness van schol en op welke manier kan dit verder onderzocht worden? Een zorgvuldige toets op inbreeding depression is zonder meer noodzakelijk, maar zal in de praktijk een uitdaging zijn. Om voldoende statistische resolutie te krijgen zal het noodzakelijk zijn verscheidene fitnesscomponenten, zoals groei en infectiegraad met parasieten, voor duizenden individuen te bepalen. Maar zelfs als we deze inbreeding depression kunnen bevestigen, dan blijft het van groot belang op tijd beslissingen te nemen. In het geval van de schol kan het al te laat zijn. De recente EU-beslissing om de quota voor schol minimaal te verlagen, in plaats van met de geadviseerde $40 \%$, is weinig hoopgevend.

Tenslotte dient de lacune in de basiskennis over het sociale- en voortplantingsgedrag van de schol te worden opgevuld. Ook hier geldt echter dat dit in de praktijk moeilijk zal zijn, aangezien in situ observaties erg lastig te realiseren zijn. Recente studies van andere commercieel belangrijke pelagische vissoorten hebben echter herhaaldelijk aangetoond dat communicatie met behulp van geluid en geur ernstig belemmerd wordt door de fysische verstoring door, en het geluid van vistuig. Deze aspecten kunnen niet langer opzij worden geschoven als een minimaal randeffect van de visserij, maar sterken onze aanbeveling om het vissen in voortplantingsgebieden te verbieden.

## Evolutie van platvissen

De Pleuronectiformes zijn een zeer oude groep binnen de Teleostei, de beenvissen. Er is weinig bekend over de afkomst van deze groep, ondanks twee recente moleculair-fylogenetische studies. Beide studies beperkten zich vrijwel uitsluitend tot de ongeveer 20 meest commercieel belangrijke soorten uit gematigde gebieden; dat is slechts een klein deel van de meer dan 500 beschreven soorten platvissen, waarvan het merendeel in tropische wateren voorkomt. Deze overduidelijke sampling bias biedt geen bijdrage aan de kennis over de evolutie van deze orde. Beperkte fossiele informatie, in combinatie met de moleculairfylogenetische gegevens, suggereert dat er een snelle radiatie is geweest van de bestaande taxa. De mate waarin dit veroorzaakt is door een sampling bias of door echte processen valt nog te bezien.

Nieuwe fylogenieën, waarin ook tropische monsters worden opgenomen en moleculaire markers worden gebruikt met verschillende evolutionaire snelheden,
zijn noodzakelijk. We willen in het bijzonder graag meer weten over snelheden van soortvorming op genetisch niveau, de genetische basis voor de ontwikkeling van een dergelijke extreme morfologische asymmetrie, en waarom mitochondriële heteroplasmie zo algemeen is bij veel platvissen. Met de toekomstige beschikbaarheid van verscheidene complete sequenties van het genoom van een aantal beenvissen, gloort de mogelijkheid om de evolutionaire ontwikkeling van de schol en andere gerelateerde soorten te achterhalen aan de horizon.

## BIO(GEO)GRAPHY

I was born on the $16^{\text {th }}$ of October 1976 in a hippy clinic just outside of Paris. My mother was a burgeoning painter and art teacher; my father a French teacher. Until I was nine, we mainly lived in Paris-except for a year spent in Madras, India where my father started to work for the French Foreign Office. Shortly after, my mother and I moved to the south of France where I attended high school. During the holidays I visited my father and relatives in such exotic locations as the Philippines, Réunion Island and Tanzania. During this time there were plenty of opportunities for visits to the tropical seaside where I developed a fascination for diving and marine life.

I began my study in Biology at the University of Montpellier in 1994. In 1997, I spent my summer working in François Bonhomme's lab at the Sète Marine Station, on the Mediterranean coast, where he convinced me to specialize in Evolutionary Biology and Population Genetics of marine organisms for my Master's degree. I then worked for my Maîtrise with Philippe Borsa on the genetics of a deep sea fish, Beryx splendens. In 1998 I was invited to participate in an IFREMER field trip to collect samples of the invasive alga, Caulerpa taxifolia, along 250 km of the Italian and French Riviera. It was during this trip that I met Jeanine Olsen and Wytze Stam. During my $5^{\text {th }}$ year of study, I moved to the University of Paris-Orsay but still worked with Philippe in Sète. I graduated in 1999 with a Diplôme d'études approfondies in Evolutionary Biology.

Despite my love for the tropical Pacific, I was convinced to move north to start my Ph.D on plaice population genetics in the Department of Marine Biology at the University of Groningen. My project started in February with a three-week cruise on the North Sea with the Dutch Fisheries Institute! All I can say is that, "if you don't get sea sick under those conditions, you are safe for the rest of your life". Life in Northern Europe is cold and damp. Luckily, however, my mother- an accomplished diver- took a teaching position in New Caledonia-one of those delightful tropical places in the middle of nowhere. That at least, enabled me to escape dark Dutch winters, work on my tan and cuddle some sharks.

Having now come to the end of my PhD , as evidenced by this thesis, I will continue working on a number of ongoing projects related to plaice and Sardinella. In November 2004, I will start a post-doc position within the EU Network of Excellence Marine Genomics Europe, where I will be working on a major EST project for fucoid seaweeds. Though based in Groningen, I will have some opportunity to escape to sunny Portugal. I have also just learned that my mother will soon be moving from New Caledonia to Martinique, French Antilles, so I am sure to keep my tan.

## RESEARCH INTERESTS

I am interested in molecular evolution and population genetics of marine organisms, focusing on fish and macro-algae. At present I am studying the impact of fisheries on the genetic diversity and structure of commercial species with small effective population sizes. I am also interested in speciation mechanisms, marine hybrids zones and their maintenance

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# Population genetics of plaice (Pleuronectes platessa L.) in Northern Europe 

## Galice Hoarau

1. The population structure of Pleuronectes platessa in the Eastern Atlantic appears to be mainly shaped by the bathymetry.
Dit proefschrift
2. Effective population size of marine fish can be several order of magnitude smaller than the census size.
Hauser et al., PNAS 2002, dit proefschrift
3. Overexploitation and fisheries mismanagement can lead to inbreeding in an albeit abundant species.
Dit proefschrift
4. Probably all of the great sea fisheries are inexhaustible, that is to say nothing we do seriously affects the numbers of fish
Thomas Huxley, president of the Royal Society of London 1883
5. Contrarily to the dogma, animal mitochondrial DNA recombines

Rokas et al., Trends in Ecology and Evolution 2003, dit proefschrift
6. Nothing in biology makes sense except in the light of evolution

Theodosius Dobzhansky
7. Anything more complicated than a screwdriver (a PCR machine?) has a soul and therefore is inherently evil.
Zimmerman, $20^{\text {th }}$ century
8. Any sufficiently advanced technology is indistinguishable from magic.

Arthur C. Clark, The Lost Worlds of 2001
9. Any technology distinguishable from magic is insufficiently advanced. Gregory Benford, Foundation's Fear 1997
10. When a distinguished but elderly scientist states that something is possible, he is almost certainly right. When he states that something is impossible, he is very probably wrong.
Arthur C. Clark, Hazards of Prophecy: The Failure of Imagination 1962
11. Four stages of acceptance:
i) this is worthless nonsense;
ii) this is an interesting, but perverse, point of view;
iii) this is true, but quite unimportant;
iv) I always said so.
J.B.S Haldane, Journal of Genetics 1963
12. It is thanks to the wonders of genetic engineering that soon there will be an end to hunger, disease, pollution, even war. I have created things that will change the world for the better. For instance, here is a monkey with four asses.
Dr. Moreau, South Park Genetic Engineering Ranch
13. English is the common language in sciences.


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