Population structure of plaice (Pleuronectes platessa L.) in northern Europe: microsatellites revealed large-scale spatial and temporal homogeneity

G. HOARAU,* A. D. RIJNSDORP,† H. W. VAN DER VEER,‡ W. T. STAM and J. L. OLSEN*

*Department of Marine Biology, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands, †Netherlands Institute for Fisheries Research, PO Box 68 1970 AB IJmuiden, The Netherlands, ‡Netherlands Institute for Sea Research, PO Box 59 1790 AB Den Burg Texel, the Netherlands

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 to 20.4, with a mean of 13.9. Expected heterozygosity was uniformly high across all locations (multilocus $H_{exp} = 0.744 ± 0.02$). Pairwise comparisons of $\theta$ among all 11 locations revealed significant differentiation between Iceland and all other locations ($\theta = 0.0290^{***}$ 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 geographical stocks is discussed in the context of high gene flow.

Keywords: flatfish, genetic structure, microsatellite, plaice, Pleuronectes platessa

Received 7 November 2001; revision received 15 March 2002; accepted 15 March 2002

Introduction

The spatial and temporal scales over which subpopulations of a species can be identified and the strength of gene flow among them are of fundamental importance to the conservation and management of marine fisheries (Carvalho & Hauser 1994; Ward & Grewe 1994). Subpopulations or stocks, may possess novel characteristics that promote differences in growth rate, fecundity and disease resistance which, 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 actually to 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 & 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 a 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 1980s to 90,000 t in the late 1990s (Rijnsdorp & Millner 1996; ICES 2000). Concern for the 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 20,000–600,000 eggs per female (Rijnsdorp 1991). The duration of the pelagic egg and larval phase typically lasts about 3–4 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 10–15 mm in shallow coastal areas (Edwards & Steele 1968; Zijlstra 1972). They remain for about 1–2 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 & 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 km/day have been recorded for some individuals using electronic data storage tags (McCalfe & Arnold 1997). Despite the roaming lifestyle of adults across the feeding grounds, 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 & Healey 1994; shad, Waters et al. 2000; sturgeon, Stabile et al. 1996) and freshwater species (e.g. walleye, Stepien & 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 Jarne & 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 (Rizzante 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 geographical differentiation have been performed on commercially important flatfish (Table 1). For flounder (Platichthys flesus/lstellatus) 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 hippoglossus), 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 (Merlangius merlangus) (Rico et al. 1997). Older studies in plaice (Purdom et al. 1976; Ward & 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 geographical 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 compared
Table 1 Comparisons of genetic structure studies based on allozymes, mtDNA haplotypes and microsatellites in flatfish along the North Atlantic continental shelf

<table>
<thead>
<tr>
<th>Fish</th>
<th>Author</th>
<th>Marker</th>
<th>No. locations sampled</th>
<th>Genetic diversity</th>
<th>Departure from HWE</th>
<th>Mean pop. differentiation</th>
<th>Locations</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaice</td>
<td>Hoarau et al. (this study)</td>
<td>msats 6 loci</td>
<td>11 (N = 48/pop)</td>
<td>0.743 (high)</td>
<td>all pop., all loci</td>
<td>0.0086*** for all pop. 0.0014 ns for shelf pop.</td>
<td>see text</td>
<td>subdivision only between continental shelf and non-shelf populations</td>
</tr>
<tr>
<td></td>
<td>Ward &amp; Beardmore (1977)</td>
<td>allozymes 5 loci</td>
<td>3 (N = ?)</td>
<td>0.118 (high)</td>
<td>cites Purdom &amp; Thompson 1976</td>
<td>not quantified</td>
<td>Bristol Channel, North Wales, North Sea (location not specified)</td>
<td>Western English Channel, Irish Sea, North Sea no subdivision</td>
</tr>
<tr>
<td></td>
<td>Purdom et al. (1976)</td>
<td>allozymes 5 loci</td>
<td>3 (N = ?)</td>
<td></td>
<td></td>
<td>0.000ns</td>
<td>Mediterranean Sea to Kattegat</td>
<td>no subdivision</td>
</tr>
<tr>
<td>Turbot</td>
<td>Coughlan et al. (1998)</td>
<td>msats 3 loci</td>
<td>4 (N = 50/pop)</td>
<td>0.66–0.77 (high)</td>
<td>ns</td>
<td>0.009ns</td>
<td>Ireland and Norway could not be distinguished</td>
<td>no subdivision</td>
</tr>
<tr>
<td></td>
<td>Bouza et al. (1997)</td>
<td>allozymes 14 loci</td>
<td>3 (N = 13–50)</td>
<td>0.029 (low)</td>
<td>HWE 'assumed'</td>
<td>0.12ns</td>
<td>Galician coast</td>
<td>no subdivision</td>
</tr>
<tr>
<td></td>
<td>Blanquer et al. (1992)</td>
<td>allozymes 17 loci</td>
<td>10</td>
<td>0.02 (low)</td>
<td>ns</td>
<td>0.000ns</td>
<td>Mediterranean to Kattegat</td>
<td>Aegean was the only one different from all others</td>
</tr>
<tr>
<td>Flounder</td>
<td>Jones &amp; Quattro (1999)</td>
<td>mtDNA haplotypes</td>
<td>10 (N = 10–25)</td>
<td>0.095</td>
<td>ns</td>
<td>0.004ns</td>
<td>East coast USA (Cape Cod to South Carolina)</td>
<td>MA/RI were different from the rest. Cape Hatteras not a barrier significant among 'regions' but not within regions. North Sea was single exception</td>
</tr>
<tr>
<td>Platicthys flesus/stellatus</td>
<td>Borsa et al. (1997)</td>
<td>allozymes 31 loci</td>
<td>8 regions (N = 10–25)</td>
<td>0.039* (north and South Sea)</td>
<td>ns</td>
<td>0.039*</td>
<td>Baltic, Normandy, southwest Britain, Brittany not different</td>
<td>no differentiation detectable (no pairwise comparisons made)</td>
</tr>
<tr>
<td>Sole</td>
<td>Kotoulas et al. (1995)</td>
<td>allozymes 12 loci</td>
<td>27 (N = 10–120/pop)</td>
<td>0.170 (high)</td>
<td>some locations</td>
<td>0.0147*** for all regions Atlantic only 0.005ns</td>
<td>Med to Normandy only (no North Sea samples)</td>
<td>IBD across regions but not within regions</td>
</tr>
<tr>
<td></td>
<td>Exadaktylos et al. (1998)</td>
<td>allozymes 27 loci</td>
<td>7 (N = 18–73/pop)</td>
<td>0.034–0.141 (related to latitude)</td>
<td>some locations</td>
<td>0.0262ns</td>
<td>Mediterranean to Normandy (3 North Sea)</td>
<td>no differentiation detectable</td>
</tr>
<tr>
<td>Halibut</td>
<td>Foss et al. (1998)</td>
<td>allozymes 4 loci</td>
<td>6 (N = 33–137/pop)</td>
<td>HWE assumed</td>
<td>Nei distance tree</td>
<td>not given</td>
<td>Norway, Iceland Faeroes, Greenland</td>
<td>east–west axis Norway apart from the three others</td>
</tr>
<tr>
<td>Brill</td>
<td>Blanquer et al. (1992)</td>
<td>allozymes 17 loci</td>
<td>5 (N = 5–55/pop)</td>
<td>0.11 (high)</td>
<td>ns</td>
<td>not given</td>
<td>Mediterranean/Portugal Britt-Kattegat</td>
<td>three regional groups identified</td>
</tr>
</tbody>
</table>

*P < 0.05, ***P < 0.001, ns = not significant, pop = population.
Materials and methods

Sampling

In total, 480 individuals were sampled (approximately 50 per location) from 11 locations (Fig. 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 (≈500–1400 km) to include both feeding and spawning grounds. The 0-group juveniles were sampled from the nursery grounds. Nursery grounds are generally located adjacent to spawning grounds and were hypothesized to retain cohorts. The 0-class juveniles were further subgrouped by size as a proxy for age. Sampling of eggs directly from the spawning grounds was not possible.

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 µL of blood or 5 mm³ of muscle tissue was incubated for 45 min at 60 °C in 800 µL extraction buffer (100 mm Tris HCl, 1.4 m NaCl, 20 mm ethylenediaminetetraacetic acid, 2% CTAB), 2 µL β-mercaptoethanol, and 6 U proteinase K (Promega), followed by two chloroform–isoamyl alcohol (24 : 1) separations. DNA was precipitated with 500 µL of ice-cold isopropanol, followed by storage at −20 °C for 45 min, and centrifugation (20 000 g) for 20 min at 4 °C. The DNA pellet was rinsed with 80% ethanol, washed with 70% ethanol, air-dried and resuspended in 150 µL of sterile-filtered distilled H₂O.

Microsatellites amplification and genotyping

Samples were genotyped for six loci, two of which (List1001 and List1003) were developed by Watts et al.

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

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>N</th>
<th>Life stage</th>
<th>Type</th>
<th>Tissue</th>
<th>Sampling date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lofoten, Norway</td>
<td>LO</td>
<td>24</td>
<td>Adults</td>
<td>Feeding ground</td>
<td>Blood</td>
<td>1 August 1999</td>
</tr>
<tr>
<td>Trondheim fjord, Norway</td>
<td>TR</td>
<td>48</td>
<td>Adults</td>
<td>Feeding ground</td>
<td>Blood</td>
<td>September 2000</td>
</tr>
<tr>
<td>Faeroe’s Plateau</td>
<td>FA</td>
<td>26</td>
<td>Adults</td>
<td>Feeding ground</td>
<td>Blood</td>
<td>Summer 1999</td>
</tr>
<tr>
<td>Irish Sea, UK</td>
<td>IS</td>
<td>44</td>
<td>Adults</td>
<td>Feeding ground</td>
<td>Blood</td>
<td>7 September 1999</td>
</tr>
<tr>
<td>Terschelling Bank, the Netherlands</td>
<td>TB</td>
<td>49</td>
<td>Adults</td>
<td>Spawning ground</td>
<td>Blood</td>
<td>15 February 2000</td>
</tr>
<tr>
<td>Fenmer Bælt, Denmark</td>
<td>FE</td>
<td>49</td>
<td>Adults</td>
<td>Feeding ground</td>
<td>Blood</td>
<td>11 September 1999</td>
</tr>
<tr>
<td>Alftanes, Iceland</td>
<td>ic</td>
<td>48</td>
<td>Juveniles</td>
<td>Nursery ground</td>
<td>Muscle</td>
<td>13 July 2000</td>
</tr>
<tr>
<td>Oban, UK</td>
<td>ob</td>
<td>48</td>
<td>Juveniles</td>
<td>Nursery ground</td>
<td>Muscle</td>
<td>16 May 2000</td>
</tr>
<tr>
<td>Amrum, Germany</td>
<td>am</td>
<td>48</td>
<td>Juveniles</td>
<td>Nursery ground</td>
<td>Muscle</td>
<td>18 June 2000</td>
</tr>
<tr>
<td>Balgzand, the Netherlands</td>
<td>ba</td>
<td>48</td>
<td>Juveniles</td>
<td>Nursery ground</td>
<td>Muscle</td>
<td>14 May 2000</td>
</tr>
<tr>
<td>Bay of Vilaine, France</td>
<td>bv</td>
<td>48</td>
<td>Juveniles</td>
<td>Nursery ground</td>
<td>Muscle</td>
<td>14 June 2000</td>
</tr>
</tbody>
</table>
Further details are given in Hoarau (1999) and four of which (PL09, PL92, PL115 and PL142) were developed by us (Hoarau et al. 2002).

Prior to polymerase chain reaction (PCR), the DNA samples were diluted 1 : 10. PCR amplification was carried out in 10-µL volumes containing 1 µL of 1 : 10-diluted DNA, 1× reaction buffer (Promega), 0.2 mM of each dNTP, 2 mM MgCl₂, 0.25 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 °C for 1 min; three cycles of denaturation at 94 °C for 1 min, annealing at 48–58 °C for 1 min, and extension at 72 °C for 30 s; followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 48–58 °C for 15 s, and extension at 72 °C for 12 s; and a final extension at 72 °C for 10 min. Further details are given in Hoarau et al. (2002).

Microsatellite loci were visualized on an ABI Prism-377 automatic sequencer (Applied Biosystems), using an internal lane standard and GENESCAN™ 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 version 4.01 (Belkhir et al. 2000), GENEPOP version 3.2 (Raymond & Rousset 1995) and PHYLIP (Felsenstein 1995). Observed and unbiased expected heterozygosities (Nei 1978) and allelic richness were computed for each locus individually and as a multilocus estimate for each of the 11 locations (Table 4). Single and multilocus \( F_{ST} \) were estimated using Weir and Cockerham’s \( f \) (Weir & Cockerham 1984). Linkage disequilibrium was tested among all pairs of loci and for all locations using the LinkDis procedure (Black & Krafsur 1985).

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

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

Exact tests of population differentiation (Raymonds et al. 1983) 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 GENEDEIST procedure of the PHYLIP software, and was used in the NEIGHBOR procedure to infer a neighbour-joining tree (Saitou & Nei 1987). Robustness of the topology was evaluated by 1000 bootstrap re-samplings using the SEQBOOT procedure in PHYLIP.

Isolation by distance was examined by plotting the pairwise \( \theta/(1 – \theta) \) values against the log of the geographical distances (Rousset 1997) and was 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 (\( N = 460 \)). 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 frequencies. 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 (\( H_{exp} \)) were uniformly high, ranging from 0.72 to 0.77. Despite similar \( H_{exp} \), the mean number of alleles/location was lowest in Iceland (\( N = 48 \)), Faeroe (\( N = 26 \)) and Lofoten (\( N = 24 \)), four 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 (\( N = 48 \)), this was not the case for Iceland. Continental-shelf samples (200-m contour line) exhibited a mean of 14.6 alleles/loci as compared with a mean of 10.9 alleles/locus for nonshelf samples.

There was no significant difference in the number of alleles associated with adult vs. juvenile populations (\( t\)-test, \( P = 0.589 \)).

All locations showed a significant departure from Hardy–Weinberg equilibrium, with a significant multilocus heterozygote deficiency (Table 4). Although PL09 contributed the highest \( f \)-values, even if excluded from the multilocus estimate, \( f \) remained significant.

© 2002 Blackwell Science Ltd, Molecular Ecology, 11, 1165–1176
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 & Wright 1997). They arise when one allele is not amplified due to mutations in one of the primers, and/or when technical problems associated with amplification and scoring arise. In the present data set, the amplification failure rate was zero in five of the six loci. For locus PL09 nine of 480 (2%) individuals failed to amplify. In all cases, re-amplifications 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 PL09, 0.1571 < r < 0.3205 corresponded to 23 expected nonamplifications 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 section.

Pairwise comparisons among the six loci and for all 11 locations revealed no linkage disequilibrium (P > 0.1 in every case).
The consistencies of single-locus as well as multilocus 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.

Population differentiation was estimated using both $\theta$ and exact tests. Pairwise comparisons of multilocus $\theta$ (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.

Table 5 Values of global $\theta$ for Pleuronectes platessa

<table>
<thead>
<tr>
<th>Locus</th>
<th>List 1001</th>
<th>PL115</th>
<th>PL92</th>
<th>PL09</th>
<th>List1003</th>
<th>PL142</th>
<th>Multi-locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples</td>
<td>0.0090*</td>
<td>0.0040*</td>
<td>0.0060**</td>
<td>0.0146***</td>
<td>0.0080*</td>
<td>0.0115***</td>
<td>0.0086***</td>
</tr>
<tr>
<td>Continental shelf only</td>
<td>0.0004</td>
<td>0.0033</td>
<td>0.0000</td>
<td>0.0014</td>
<td>0.0012</td>
<td>0.0023</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

Global $\theta$ from Weir and Cockerham (1984).
Continental shelf: all samples except those from Iceland and Faeroe ($P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 6 Population differentiation in Pleuronectes platessa

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Sampling site</th>
<th>FE</th>
<th>TB</th>
<th>FA</th>
<th>LO</th>
<th>IS</th>
<th>TR</th>
<th>bv</th>
<th>am</th>
<th>ba</th>
<th>ob</th>
<th>ic</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE</td>
<td>−</td>
<td>0.0033</td>
<td>−0.0037</td>
<td>−0.0001</td>
<td>0.0060</td>
<td>0.0004</td>
<td>−0.0018</td>
<td>−0.0038</td>
<td>0.0009</td>
<td>0.0373***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td>0/1</td>
<td>−</td>
<td>0.0069</td>
<td>0.0003</td>
<td>−0.0022</td>
<td>0.0053</td>
<td>0.0004</td>
<td>−0.0009</td>
<td>0.0003</td>
<td>0.026</td>
<td>0.0339***</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>0/0</td>
<td>1/1</td>
<td>−</td>
<td>0.0059</td>
<td>0.0009</td>
<td>0.0082</td>
<td>0.0131</td>
<td>0.0037</td>
<td>0.0072</td>
<td>0.0092</td>
<td>0.0290*</td>
<td></td>
</tr>
<tr>
<td>LO</td>
<td>0/0</td>
<td>0/0</td>
<td>0/1</td>
<td>−</td>
<td>−0.0010</td>
<td>0.0027</td>
<td>0.0012</td>
<td>−0.0015</td>
<td>−0.0011</td>
<td>0.0010</td>
<td>0.0429***</td>
<td></td>
</tr>
<tr>
<td>IS</td>
<td>0/1</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>−</td>
<td>0.0058</td>
<td>0.0029</td>
<td>0.0015</td>
<td>−0.0005</td>
<td>0.0036</td>
<td>0.0456***</td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/0</td>
<td>0/1</td>
<td>−</td>
<td>0.0035</td>
<td>0.0011</td>
<td>0.0050</td>
<td>0.0075</td>
<td>0.0345***</td>
<td></td>
</tr>
<tr>
<td>bv</td>
<td>0/1</td>
<td>0/2</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>−</td>
<td>0.0034</td>
<td>0.0020</td>
<td>0.0014</td>
<td>0.0393***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>am</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>−</td>
<td>0.0000</td>
<td>0.0008</td>
<td>0.0000</td>
<td>0.0031</td>
<td>0.0377***</td>
<td></td>
</tr>
<tr>
<td>ba</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>−</td>
<td>0.0010</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0.0315***</td>
<td></td>
</tr>
<tr>
<td>ob</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>−</td>
<td>0.0000</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0.0320***</td>
<td></td>
</tr>
</tbody>
</table>

Upper right of matrix: pairwise, multi-locus $\theta$ (Weir and Cockerham 1984) values tested by permutation and sequential Bonferroni corrections (Rice 1989) ($P < 0.05$, ***$P < 0.001$). 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 represent 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.

The consistencies of single-locus as well as multilocus 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.

Population differentiation was estimated using both $\theta$ and exact tests. Pairwise comparisons of multilocus $\theta$ (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 Faeroe are separate from the continental-shelf cluster.

Discussion

Heterozygote deficiency: fact or artefact?

Strong heterozygote deficiencies have been reported for invertebrates (Zouros & Foltz 1984; Shaw et al. 1999a; Huang et al. 2000) and many fish species (Waldman & McKinnon 1993) including flatfish (Purdom et al. 1976; Ward & Beardmore 1977; Blanquer et al. 1992; Kotoulas et al. 1995). They can be the result of null alleles, a Wahlund effect, or of processes such as inbreeding and selection. As shown in the Results, technical artefacts 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 (Ne), 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 geographical 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 was observed in each cohort (temporal Wahlund effect). When 0-class, juvenile samples of plaice were divided into two subsamples based on length, $F_{IS}$ values remained positive but differed in magnitude (Fig. 4). Larger fish had higher $F_{IS}$ 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 $F_{IS}$ 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 the numbers of vertebrae, dorsal-fin rays 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 is lowest in the early juvenile stages and that it increases with maturity. Age-related changes were also reported for plaice by Beardmore & 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 outbred progeny with lower fitness. This type of population-level hybridization 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 present-day 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 & Bowen 1998); again in relation to extinction and recolonization throughout the North Atlantic since the last glacial maximum. In these types 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, for example, separate mitochondrial 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 shallow-water species (<100 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 θ-values close to the values we estimated in plaice.

Iceland, though geographically isolated, has comparable Hexp 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 Hexp (Maruyama & 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 multilocus θ (Table 6) was unexpected, especially between the North and Irish Seas. However, when individual loci were analysed 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. FST 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 minimizing 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 & 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) that 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, panmictic (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.

Acknowledgements

We thank J. Coyer, R. Lescasse and E.-J. Molenaar for discussions, comments and corrections on the early versions manuscript. We also thank N. Bierre, F. Bonhomme, P. Borsa, W. Kooistra, M. Waycott and E. Zouros for useful discussions; S. Boele-Bos, T. Homan and J. Veldsink for laboratory assistance; and S. Flatman, J. Reinert, A. Geffen, K. Korsbekke, H. Degel and P. Ernst for providing samples. We also thank Trondheim Marine System Research Institute for collections from Borgenford, Trondheim (HPRI-1999-CT-00060) and the European Commission’s Training and Mobility of Researchers programme for their support in getting Access to the Trondheim Marine Systems Research. This research was supported by NWO-Prioriteit Programma ‘Sustainable Use of Marine Natural Resources’ Project No. 885–10.101-P. This is NIOZ Publication No. 3683.

References


Black WC, Krafsur ES (1985) A FORTRAN program for the calculation and analysis of two-locus linkage disequilibrium coefficients. Theoretical and Applied Genetic, 70, 491–496.


Maruyama T, Fuerst PA (1985) Population bottlenecks and non-equilibrium models in population genetics. II. Number of alleles in a small population that was formed by a recent bottleneck. Genetics, 111, 675–689.


Shaw PW, Pierce GJ, Boyle PR (1999a) Subtle population structuring within a highly vagile marine invertebrate, the veined squid Loligo forbesi, demonstrated with microsatellite DNA markers. Molecular Ecology, 8, 407–417.

Shaw PW, Turan C, Wright JM, O’Connell M, Carvalho GR (1999b) Microsatellite DNA analysis of population structure in Atlantic herring (Clupea harengus), with direct comparison to allozyme and mtDNA RFLP analyses. Heredity, 83, 490–499.


polymorphism and sequence analyses of mitochondrial DNA. *Genetics, 144*, 768–775.


Galice Hoarau is a PhD student in marine biology interested in population genetics and phylogenetics of fish. Jeanine Olsen is a molecular ecologist interested in the dispersal, phylogeography and life history evolution of marine organisms. Wytze Stam is a molecular geneticist interested in phylogenetics. Adriaan Rijnsdorp has a long-standing interest in the population dynamics of flatfish. Henk van der Veer studies flatfish ecology.