Molecular Ecology (2002) **11**, 1165–1176

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 to 20.4, with a mean of 13.9. Expected heterozygosity was uniformly high across all locations (multilocus $H_{exp} = 0.744 \pm 0.02$). Pairwise comparisons of θ 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

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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 (Metcalfe & 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 (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 geographical differentiation have been performed 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 *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

Fish	Author	Marker	No. locations sampled	Genetic diversity H or H_{exp}	Departure from HWE	Mean pop. differentiation $\theta/G_{ST}/\Phi_{ST}$	Locations	Conclusion
Plaice								
Pleuronectes platessa	Hoarau *et al.* this study	msats 6 loci (mean Na/locus = 13.9)	11 (N = 48/pop)	0.743 (high)	all pop, all loci	0.0086*** for all pop. 0.0014 ns for shelf pop.	see text	subdivision only between continental shelf and non-shelf populations
	Ward & Beardmore (1977)	allozymes 5 loci	3 (N = ?)	0.118 (high)	cites Purdom & Thompson 1976	not quantified	Bristol Channel, North Wales, North Sea (location not specified)	no subdivision. Mentions some differences in alleles between Irish and North Seas
	Purdom *et al.* (1976)	allozymes 5 loci	3 (N = ?)		mentioned for loci	not quantified	Western English Channel, Irish Sea, North Sea	no subdivision
Turbot								
Scophthalmus maximus	Coughlan *et al.* (1998)	msats 3 loci (mean Na/locus = 7)	4 (N = 50/pop)	0.66–0.77 (high)	ns	0.009ns	Ireland and Norway could not be distinguished	no subdivision
	Bouza *et al.* (1997)	allozymes 14 loci	3 (N = 13–50)	0.029 (low)	HWE 'assumed'	0.12ns	Galician coast	no subdivision
	Blanquer *et al.* (1992)	allozymes 17 loci	10	0.02 (low)	ns	0.000ns	Mediterranean to Kattegat	Aegean was the only one different from all others
Flounder								
Paralichthys dentatus	Jones & Quattro (1999)	mtDNA haplotypes	10 (N = 10–25)	—		0.004ns	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 31 loci	8 regions 7 within North Atlantic	0.095	ns	0.039* (north and south North Sea)	Baltic, North Sea, southwest Britain, Brittany not different	significant among 'regions' but not within regions. North Sea was single exception
Sole								
Solea vulgaris [=*S. solea*]	Kotoulas *et al.* (1995)	allozymes 12 loci	27 (N = 10–120/pop)	0.170 (high) Atlantic regions	some locations some loci	0.0147*** for all regions Atlantic only 0.005ns	Med to Normandy only (no North Sea samples)	IBD across regions but not within regions
Solea solea	Exadactylos *et al.* (1998)	allozymes 27 loci	7 (N = 18–73/pop)	0.034–0.141 (related to latitude)	some locations some loci (Irish German Bight)	0.0262ns (no pairwise comparisons made)	Mediterranean to Normandy (3 North Sea)	no differentiation detectable
Halibut								
Hippoglossus hippoglossus	Foss *et al.* (1998)	allozymes 4 loci	6 (N = 33–137/pop)	?	HWE assumed	not given Nei distance tree	Norway, Iceland Faeroes, Greenland	east–west axis Norway apart from the three others
Brill								
Scophthalmus rhombus	Blanquer *et al.* (1992)	allozymes 17 loci	5 (N = 5–55/pop)	0.11 (high)	ns	not given	Mediterranean/Portugal Britt-Kattegat	three regional groups identified

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

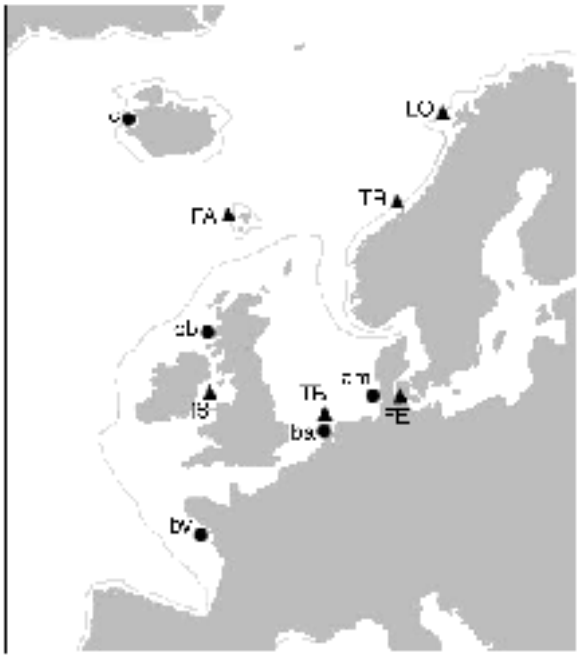

Fig. 1 Sampling locations of *Pleuronectes platessa*: ▲ indicate adult populations, ● indicate juvenile populations. The grey line delimits the 200-m contour of the continental shelf. See Table 2 for abbreviations.

differentiation between spawning, nursery and feeding ground locations.

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 mm^3 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 H_2O.

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

Location	Code	*N*	Life stage	Type	Tissue	Sampling date
Lofoten, Norway	LO	24	Adults	Feeding ground	Blood	1 August 1999
Trondheim fjord, Norway	TR	48	Adults	Feeding ground	Blood	September 2000
Faeroe's Plateau	FA	26	Adults	Feeding ground	Blood	Summer 1999
Irish Sea, UK	IS	44	Adults	Feeding ground	Blood	7 September 1999
Terschellinger Bank, the Netherlands	TB	49	Adults	Spawning ground	Blood	15 February 2000
Femer Bælt, Denmark	FE	49	Adults	Feeding ground	Blood	11 September 1999
Alftanes, Iceland	ic	48	Juveniles	Nursery ground	Muscle	13 July 2000
Oban, UK	ob	48	Juveniles	Nursery ground	Muscle	16 May 2000
Amrum, Germany	am	48	Juveniles	Nursery ground	Muscle	18 June 2000
Balgzand, the Netherlands	ba	48	Juveniles	Nursery ground	Muscle	14 May 2000
Bay of Vilaine, France	bv	48	Juveniles	Nursery ground	Muscle	14 June 2000

Table 3 Annealing temperature and primer concentration for six microsatellite loci on *Pleuronectes platessa*

Locus	Annealing temp. (°C)	Primer concn (each) (μM)
PL09	50	0.5
PL92	48	0.1
PL115	53	0.2
PL142	51.5	0.2
LIST1001	52	0.14
LIST1003	50	0.14

(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 $MgCl_2$, 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 heterozygosity (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_{IS} were estimated using Weir and Cockerham's f (Weir & Cockerham 1984). Linkage disequlibrium 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 θ (Weir & Cockerham 1984). The significance of all f and θ estimates was tested using permutation (N = 2000). Sequential Bonferroni corrections (Rice 1989) for multiple comparisons were applied where necessary.

Exact tests of population differentiation (Raymond & 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 & 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), 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 (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.

Table 4 Summary of genetic variation at six microsatellite loci at 11 locations for *Pleuronectes platessa* (Table 2)

Locus		Location											Mean *Na*/locus
		LO	TR	FA	IS	TB	FE	ic	ob	am	ba	bv	
List1001	N_A	5	5	4	5	5	7	5	4	6	6	6	5.27
	H_{exp}	0.610	0.634	0.698	0.608	0.650	0.674	0.725	0.622	0.656	0.616	0.655	
	H_{obs}	0.542	0.625	0.577	0.636	0.571	0.735	0.708	0.688	0.583	0.617	0.750	
	f	0.114	0.015	0.177	–0.047	0.122	–0.091	0.023	–0.106	0.111	–0.002	–0.148	
PL115	N_A	11	13	10	17	14	16	11	13	14	15	14	13.45
	H_{exp}	0.780	0.775	0.686	0.667	0.748	0.732	0.749	0.747	0.781	0.687	0.759	
	H_{obs}	0.792	0.833	0.615	0.659	0.674	0.694	0.729	0.809	0.792	0.604	0.792	
	f	–0.015	–0.076	0.105	0.015	**0.101**	0.053	0.027	–0.083	–0.014	**0.121**	–0.044	
PL92	N_A	14	23	11	19	20	16	15	20	15	15	18	16.9
	H_{exp}	0.901	0.883	0.847	0.889	0.902	0.885	0.885	0.874	0.881	0.848	0.889	
	H_{obs}	0.792	0.729	0.880	0.773	0.792	0.775	0.702	0.804	0.750	0.617	0.696	
	f	**0.123**	**0.176**	–0.040	**0.132**	**0.164**	**0.125**	**0.203**	0.080	**0.150**	**0.274**	**0.220**	
PL09	N_A	16	21	12	20	22	24	15	25	25	23	22	20.45
	H_{exp}	0.90	0.812	0.825	0.880	0.861	0.900	0.595	0.883	0.889	0.872	0.874	
	H_{obs}	0.375	0.417	0.240	0.429	0.449	0.592	0.239	0.587	0.478	0.413	0.521	
	f	**0.590**	**0.430**	**0.713**	**0.516**	**0.431**	**0.345**	**0.660**	**0.338**	**0.446**	**0.529**	**0.408**	
List1003	N_A	5	8	6	8	9	7	8	8	10	8	7	7.63
	H_{exp}	0.368	0.622	0.582	0.475	0.404	0.403	0.624	0.472	0.530	0.485	0.448	
	H_{obs}	0.333	0.625	0.539	0.477	0.388	0.347	0.479	0.458	0.500	0.500	0.417	
	f	0.096	–0.004	0.077	–0.004	0.041	**0.140**	**0.234**	0.030	0.056	–0.031	0.071	
PL142	N_A	14	21	15	18	21	25	18	25	20	23	18	19.8
	H_{exp}	0.833	0.843	0.857	0.804	0.857	0.871	0.972	0.877	0.883	0.850	0.805	
	H_{obs}	0.625	0.708	0.769	0.683	0.792	0.771	0.833	0.787	0.813	0.833	0.667	
	f	**0.254**	**0.161**	0.104	**0.152**	0.077	**0.116**	**0.102**	0.104	0.030	0.020	**0.173**	
Mean N_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	
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		
Mean H_{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_{IS}													
all loci	**0.217*****	**0.140*****	**0.198*****	**0.156*****	**0.181*****	**0.125*****	**0.182*****	**0.078*****	**0.154*****	**0.179*****	**0.187*****		
all loci excl. PL09	0.119***	0.064**	0.080**	0.064**	0.108***	0.069**	0.119***	0.013*	0.079**	0.091***	0.067***		

N_A = number of alleles, H_{exp} = unbiased expected heterozygosity (Nei 1978), H_{obs} = observed heterozygosity, and f = inbreeding coefficient. Significant values (bold) have been applied after sequential Bonferroni corrections (Rice 1989) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

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 PL109, $0.1571 < r < 0.3205$ 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 disequlibrium ($P > 0.1$ in every case).

Table 5 Values of global θ for *Pleuronectes platessa*

	Locus						
	List 1001	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

Global θ 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*

Sampling	Sampling site										
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***
bv	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***
ba	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***
ic	4/3	3/3	2/4	3/2	4/4	4/4	4/3	4/4	4/3	4/4	—

Upper right of matrix: pairwise, multi-locus θ (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 θ. 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 θ 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 θ and exact tests. Pairwise comparisons of multilocus θ (Table 6) showed that only Iceland (ic) was significantly different from all other locations. When pairwise comparisons of θ 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

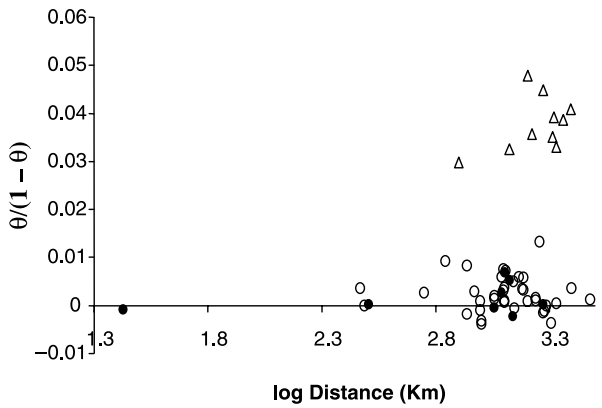

Fig. 2 Global isolation by distance for *Pleuronectes platessa*. Pairwise comparisons involving Iceland (△), Faeroe (●), and all other continental-shelf comparisons (○) are plotted as shown. There is no significant isolation by distance (see text).

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

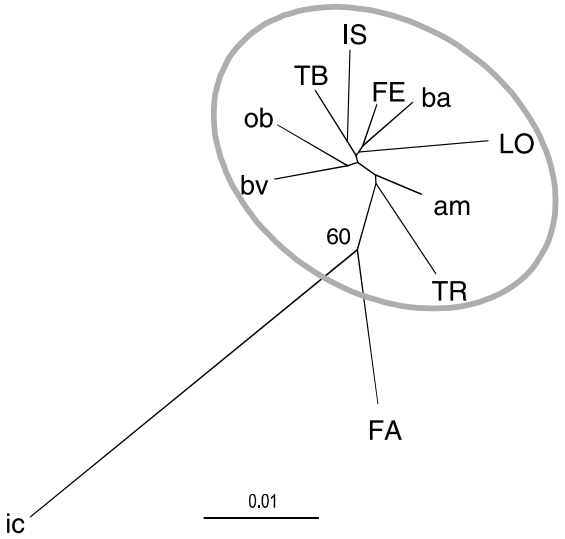

Fig. 3 On-shelf : off-shelf clusters of *Pleuronectes platessa* based on unrooted neighbour-joining tree inferred from Reynolds genetic distances. The circle surrounds the continental-shelf samples. Iceland and Faeroe are significantly different from one another (Table 6). Only bootstrap values above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

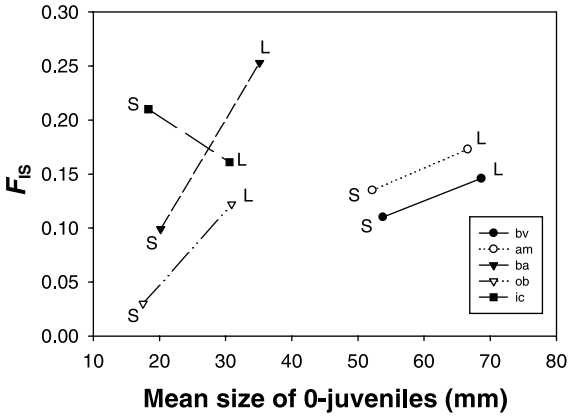

Fig. 4 Change in heterozygote deficiency (F_{IS}) with fish size. Samples from each of the five nursery ground locations were divided into a *small* (S) and *large* (L) subsample. The line connecting the small and large subsamples represents time. All F_{IS} values were significant except for the smallest size class at location am.

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 (N_e), 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 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 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 H_{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_{exp} (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. F_{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 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, 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.

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. Bierne, 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 Borgenfjord, 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.

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