Heredity (2008) 101, 27–38

www.nature.com/hdy

ORIGINAL ARTICLE

Population structure of flounder (*Platichthys flesus*) in the Baltic Sea: differences among demersal and pelagic spawners

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We found significant population structure and isolation by distance among samples of flounder (*Platichthys flesus*) in the Baltic, Kattegat and Skagerrak seas using microsatellite genetic markers. This pattern was almost entirely due to a difference between flounder that have demersal spawning in the northern Baltic, as compared to pelagic spawners in the southern Baltic and on the west coast of Sweden. Among demersal spawners we found neither genetic differentiation nor any isolation by distance among sampling sites. We speculate that demersal flounder are descendants of a population that colonized the Baltic previous to pelagic spawners. The demersal flounder may thus have had longer time to adapt to the low salinity in the Baltic, and accordingly display egg characteristics that make it possible to reproduce at the low salinity levels in the northern Baltic. Among pelagic spawners significant isolation by distance was detected. Pelagic spawners have previously been shown to display clinal variation in egg size, which allows them to float also at the moderate salinity levels up to the region north of the island Bornholm. Management units for harvesting should ideally be based on true biological populations, and for the commercially important flounder up to 15 different management stocks in the Baltic have been suggested. We could not find a population genetic foundation for such a high number of management units, and our data suggest three management units: the northern Baltic (demersal populations), southern Baltic with the Öresund straits and the most northwestern sampling sites (Skagerrak, Kattegat and North Sea).
Heredity (2008) **101,** 27–38; doi:10.1038/hdy.2008.22; published online 7 May 2008

Keywords: population structure; *Platichthys flesus*; Baltic Sea; local adaptation; reproduction; microsatellites

Introduction

The Baltic Sea, being the largest brackish water area in the world, offers a challenge for both marine and freshwater species to live and prosper in (Voipio, 1981; Ojaveer and Lehtonen, 2001; Johannesson and André, 2006). The main limitation for marine species in the Baltic is the decreasing salinity with distance from the Öresund straits, whereas for freshwater species the problem is reversed. This is the main reason why the number of species is so low in the Baltic compared to adjacent areas. For marine fish, less than half of the approximately 120 species present in the North Sea thrive in the central Baltic Sea and in the Gulf of Bothnia and Gulf of Finland less than 20 marine fish species occur regularly (Voipio, 1981). Low salinity can be a problem for successful reproduction in marine teleosts. Low salinity immobilizes sperm, rendering many of the eggs unfertilized, and also diminishes egg survival (Holliday, 1969; Nissling *et al*., 2002, 2006). Another problem with the low salinity is the reduced buoyancy of eggs leading to pelagic eggs sinking into the more oxygen-depleted water where successful development is not possible. Nevertheless, many marine species, including fishes, have adapted to the low salinity in the Baltic. Several species have solved the problem with buoyancy by development of larger eggs, with less density (Mielck and Künne, 1932; Lönning and Solemdal, 1979; Thorsen *et al*., 1996; Nissling and Westin, 1997; Nissling *et al*., 2002). Recent studies have suggested that some of these adaptations may have resulted in genetic differentiation between the Baltic populations of a species and its marine counterpart (Nielsen *et al*., 2003, 2004; Jørgensen *et al*., 2005; Johannesson and André, 2006; but see Florin and Höglund, 2007).

Fish stocks are today mainly managed in traditional, political and practical management units. It is often not known, however, whether these management units also reflect biological stock units, that is if the harvest stock also represents a true population in the biological sense, with discrete population dynamics, or even an evolutionary significant unit (Begon *et al*., 1990; Carvalho and Hauser, 1994; Fraser and Bernatchez, 2001). The management of fish stocks would be more effective if it were actually based on true biological stocks rather than arbitrary defined stocks (Carvalho and Hauser, 1994), and there could be severe effects of lumping together populations that have separate population genetics and population dynamics when managing species (Ryman *et al*., 1995; Bailey, 1997; Frank and Brickman, 2001;

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Received 6 December 2006; revised 10 January 2008; accepted 11 March 2008; published online 7 May 2008

Laikre *et al*., 2005). In the northeast Atlantic, assessment and management are often made according to the subdivisions (SD) determined by the International Council for the Exploration of the Sea (ICES) (Figure 1).

The European flounder (*Platichthys flesus*) is the most commercially important flatfish in the Baltic Sea with yearly international landings of 15 000 tonnes. The flounder is assumed to be divided into several local populations, but few genetic or ecological studies have confirmed this idea. The flounder is distributed from the Skagerrak far up into the Baltic Sea. It is less frequently observed north of the Sea of Åland, and rarely north of the Northern Quark (Curry-Lindahl, 1985; Bagge and Steffensen, 1989; Muus *et al*., 1999; Voigt, 2002; Florin, 2005). Flounder migrate into less saline waters, and closer to the shore in shallower water, than other flatfish (Molander, 1964; Voigt, 2002; Florin, 2005).

The flounder could thus be considered both a coastal species and a migratory species. In general, flounder feed in shallow waters and migrate to spawn in deep waters; however, in the central and northern Baltic, flounder both spawn and feed in shallow water (Ehrenbaum, 1909; Molander, 1923, 1925, 1964; Aro, 1989; Florin, 2005). Tagging of adult flounders has revealed that migration in the southern Baltic is not so substantial, hence it is highly plausible that the flounder could be divided into local populations (Otterlind, 1967). The same was shown in the central Baltic (Otterlind, 1966), in this case however, single individuals did undertake substantial movement (hundreds of km).

On the basis of tagging, several harvest stocks and potential biological populations of flounder have been identified in the Baltic. In the management units SD 22 and 23 (Figure 1) three and one local stocks were identified, respectively (Bagge, 1966; Bagge and Steffensen, 1989). Further tagging studies in SD 24 and 25 indicated that each region supported a distinct stock (Otterlind, 1967). Tagging experiments in SD 26 and 28 (Cieglewicz, 1947, 1961, 1963; Otterlind, 1967; Vitinš, 1972; Bagge and Steffensen, 1989) suggested that there

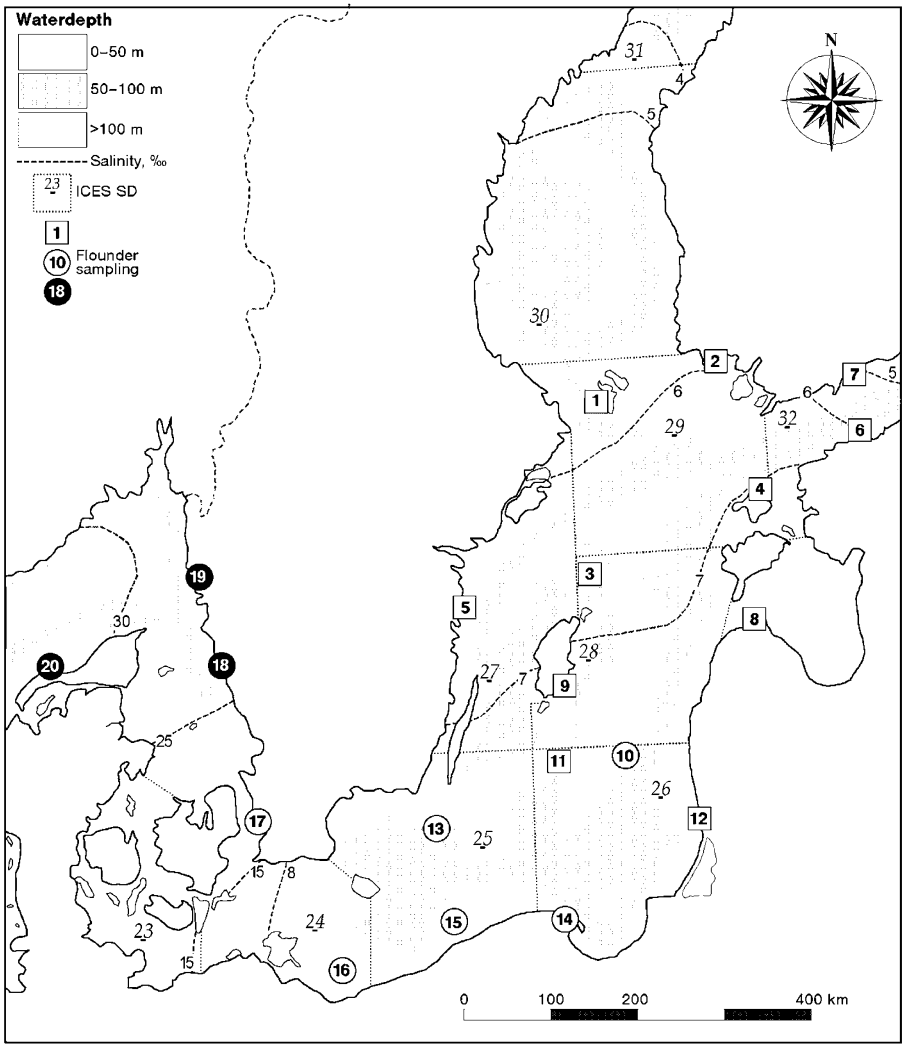

Figure 1 Map of sampling locations, International Council for the Exploration of the Sea (ICES) subdivisions (22–32, salinity and water depth (data from Seifert *et al*., 2001). (1) Finbo, (2) Åbo, (3) Gotska Sandön, (4) Hiiumaa, (5) Kvädöfjärden, (6) Muuga, (7) Helsinki, (8) Irbe, (9) Gotland, (10) Latvian Sea, (11) Hoburgsbank, (12) Smiltyne, (13) Bornholm, (14) Gdynia, (15) Dabki, (16) Oderbank, (17) Barsebäck, (18) Kungsbackafjorden, (19) Gullmaren and (20) Thyborön. Circles correspond to putative pelagic whereas squares represent putative demersal samples. Filled and open circles correspond to the suggested management units in the discussion.

were two stocks in each SD. The Gotland basin, with low oxygen content, seems to prevent flounder from crossing over and may act as an east–west barrier (Aro, 1989; Bagge and Steffensen, 1989). It is unclear if SD 27 supports one (Aro, 1989) or two (Bagge and Steffensen, 1989) stocks of flounder. Tagging experiments in SD 29, 30 and 32 (reviewed in Aro, 1989) suggested that there was one stock of flounder in SD 29 and 30 and a separate stock in SD 32. Ojaveer *et al*. (1985) further speculated that flounders in SD 32 are divided into two stocks—one along the Finnish coast and one along the coast of Estonia. This gives in total 15 potential stocks of flounder in the Baltic Sea. It remains, however, to be discerned whether these are true biological, genetically different, stocks or 'merely' harvest stocks.

The development of larger eggs, with lower specific gravity, as an adaptation to less saline water is seen in flounder (Mielck and Künne, 1932; Lönning and Solemdal, 1979; Nissling *et al*., 2002). Although some of the response may be due to plasticity, transplantation experiments suggest that flounders, like cod, have distinct populations with distinct innate egg properties, and a limited ability to acclimatize to new salinities (Solemdal, 1967, 1971, 1973; Thorsen *et al*., 1996; Nissling and Westin, 1997; Nissling *et al*., 2002). The maximum size of flounder eggs (that is those with minimum specific gravity) is found in waters of 10–12‰ salinity. This may indicate that eggs cannot be buoyant in water of lower salinity. Supporting this conclusion, Mielck (1926) found no floating flounder eggs above 40 m depth to the north and west of Bornholm, nor above 50 m in the deep of the Bornholm Basin nor above 100 m in the deep area in the Gdansk basin and east of Gotland. This corresponds to a 10–11‰ isohaline. For flounder in SD 24 and 25 the appropriate habitat for successful reproduction has a minimum salinity of approximately 12‰ and minimum oxygen concentration of $2\,ml\,l^{-1}$ (ACFM, 2005). This means that the recruitment success fluctuates depending on the hydrological conditions on the spawning ground. According to calculations by Nissling *et al*. (2002), successful reproduction for pelagic flounder may occur regularly in the Sound, the Arkona and the Bornholm basins and, during favourable conditions, in the Gdansk and Gotland basins. The same authors also found a significant difference in the salinity required for neutral egg buoyance for flounders collected from the Sound, compared to those from the Arcona or Bornholm basin. There was also a significant increase in mean egg size from 1.12 mm in the Sound to 1.34 mm in the Arcona basin and finally to 1.43 mm in the Bornholm basin (Nissling *et al*., 2002).

Reproductive populations of flounders do, however, also exist on the shallow central banks and in the eastern part of the Baltic with water of only 5–7‰ salinity. Eggs from these areas are smaller and heavier and develop at the bottom (Sandman, 1906; Mielck, 1926; Mielck and Künne, 1932; Solemdal, 1967, 1971; Lönning and Solemdal, 1979; Bonsdorff and Norkko, 1994; Nissling *et al*., 2002). According to Nissling *et al*. (2002) the mean egg size in the Eastern Gotland basin was only 0.99 mm. Presumably, selection has favoured tougher, heavier eggs that are better at resisting the mechanical forces acting at the bottom (Solemdal, 1967, 1971). The existence of two separate reproductive patterns in the Baltic is further supported by Mielck (1926) and Mielck and Künne (1932) who caught ripe female species at Oderbank and Mittelbank, locations with 6–7‰ salinity. Some of the female species had normal, small 'bank' type of eggs, but also a few were found with large 'pelagic' eggs. It is uncertain if individual flounders can change spawning behaviour and type of eggs between years, or if there are truly two different, genetically distinct stocks of flounder. The demersal spawning flounder, presumably constituting one distinct stock with respect to salinity requirements for reproduction may spawn successfully as far north as the southern Gulf of Bothnia and the Gulf of Finland (Nissling *et al*., 2002).

In this study we used microsatellite DNA to map the genetic population structure of flounder in the Baltic and in the Skagerrak/Kattegat area. We were in particular interested to test the hypothesis that flounder form different populations, and if so whether they correspond to demersal and pelagic flounder and whether the clinal variation in egg characteristics and spawning behaviour correspond with genetical differences at neutral microsatellite loci.

Materials and methods

Sampling

In spring and early summer 2003, tissue samples from the base of the pectoral fin were taken from approximately 50 adult fish from 20 localities. Effort was made to sample as close to spawning time as possible. Flounder spawn between February and April in the North Sea, Skagerrak and Kattegat. In the Baltic spawning is delayed eastwards and northwards, so that around Gotland spawning takes place in April–June and in the Gulf of Finland in May–June (Molander, 1964; Curry-Lindahl, 1985). For practical and jurisdictional reasons, however, some locations were sampled prior to or just after the spawning period. The different sampled areas ranged from the Åland Sea to the North Sea, and were Finbo, Åbo, Gotska Sandön, Hiiumaa, Kvädöfjärden, Muuga, Helsinki, Irbe, Gotland, Latvian Sea, Hoburgsbank, Smiltyne, Bornholm, Gdynia, Dabki, Oderbank, Barsebäck, Kungsbackafjorden, Gullmaren and Thyborön. Sample statistics and locations are shown in Table 1 and Figure 1, respectively.

Molecular methods

DNA was extracted using 500 µl 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) solution with $4\,\mu l$ $10\,mg\,ml^{-1}$ proteinase K. Samples were incubated at 56 °C on a rocking platform for 2 h, vortexed for 15 s, heated to 96 °C for 15 min in temperature blocks and finally vortexed again for 15 s. Samples were centrifuged at 13 000 r.p.m. for 5 min and the supernatant was taken for use in PCR.

Seven microsatellite loci (Table 2, sequences obtained from GenBank, submitted by TJ Dixon 2001) were typed for all individuals. The loci AJ315971 and AJ315975 were amplified together in a multiplex PCR and the same was true for AJ315970 and AJ315974. PCR reactions (10 µl) were set up in 96-well PCR plates using the final concentrations: $1 \times NH_4$ reaction buffer (BioLine, London, UK), 1 mM $MgCl_2$, 0.13 mM of each of the four deoxyribonucleotide triphosphates, 1 µM of the reversed primer, 0.5 µM labelled forward primer, 0.5 µM unlabelled

Table 1 Summary statistics of samples of flounder (*P. flesus*) used for genetic analysis

No.	*Name*	*Water basin*	*ICES SD*	*Position*	*Sampling date (median and range)*	*No. of individuals*	*% S*	*% R*	*Length (mm)*
1	Finbo	Åland archipelago	29	60.09N, 19.42E	30 May (30.05–31.06)	53	92	60	310 (27)
2	Åbo	Archipelago Sea	29	60.26N, 22.05E	8 May (3.05–13.05)	52	90	82	273 (34)
3	Gotska Sandön	Northern Gotland basin	28	58.22N, 19.15E	3 June (02.06–13.06)	48	15	88	247 (16)
4	Hiuma	Northern Gotland basin	29	58.58N, 22.43E	1 June (28.05–4.06)[a]	50	68	84	226 (37)
5	Kvädöfjärden	Western Gotland basin	27	58.01N, 16.46E	4 May	48	75	85	272 (33)
6	Muuga	S. Gulf of Finland	32	59.32N, 24.52E	20 May (20.05–04.06)	44	82	73	254 (27)
7	Helsinki	N. Gulf of Finland	32	60.15N, 25.30E	26 May (21.05–27.05)	60	14	?[b]	251 (36)
8	Irbe	Gulf of Riga	28	57.44N, 22.22E	14 May	48	71	?	274 (34)
9	Gotland	West part of Eastern Gotland basin	28	57.11N, 18.38E	8 May	46	100	87	306 (30)
10	Latvian Sea	East part of Eastern Gotland basin	28	56.25N, 19.44E	15 April (01.04–30.04)	47	62	?	262 (27)
11	Hoburgsbank	Central part of Eastern Gotland basin	26	56.24N, 18.31E	12 March	48	100	83	293 (46)
12	Smiltyne	East part of Eastern Gotland basin	26	55.43N, 21.05E	30 July (29.07–01–08)	50	?	?	200 (35)
13	Bornholm	Bornholm basin	25	55.42N, 16.11E	10 March	48	100	77	282 (39)
14	Gdynia	Bay of Gdansk	26	54.45N, 18.30E	20 May	61	13	81	268 (35)
15	Dabki	Bornholm basin	25	54.45N, 16.30E	26 May (22.05–30.05)	59	12	75	304 (43)
16	Oderbank	Arcona basin	24	54.15N, 14.30E	16 June	48	0	85	290 (32)
17	Barsebäck	Oresund straits	23	55.45N, 12.53E	10 April (02.04–30.04)	32	22	69	255 (27)
18				Kungsbackafjorden	Kattegat	20	57.23N, 12.02E	19 March (19.03–21.03)	48
21	71	243 (37)							
19	Gullmaren	Skagerrak	21	58.17N, 11.30E	3 April	48	46	69	303 (33)
20	Thyborön	North Sea	IV	56.45N, 08.30E	15 February (01.02–28.02)	50	76	77	299(37)

Abbreviations: ICES, International Council for the Exploration of the Sea; %S, percentage spawning; SD, subdivisions; %R, percentage rightsided.
Sample number corresponds to location in Figure 1.
Length, mean total length and SD.
[a]7 individuals in sample 4 were taken at 28 April.
[b]Parameter not noted.

forward primer 0.25 units Taq polymerase (BioLine, Biotaq, London, UK) and 1 µl DNA (10–100 ng). The amplifications were run on an Eppendorf Mastercycler Gradient or a Geneamp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) using the following temperature cycle: initial denaturing at 96 °C for 3 min followed by 32 cycles of 50 s at 95 °C, 50 s T_a °C and 70 s at 72 °C and finally there was a 4 °C hold. Annealing temperatures (T_a) are given in Table 2.

Fragment sizes were analysed using an ABI Prism 377 DNA sequencer according to the manufacturers' protocol. Two to five different loci were run together in a single line on the sequencer. A mix of samples was made by taking 2 µl of each PCR reaction and diluting with 5 µl dd H_2O. This mix (1 µl) was loaded onto the gel together with 0.29 µl TAMRA 500 size standard and 0.71 µl formamid loading dye. Gel images were analysed using the ABI Prism 377 DNA sequencer data collection software GeneScan 2.1.1.

Statistical analyses

Observed and Nei's unbiased expected heterozygosity were estimated for each population and locus using FSTAT 2.9.3 (Goudet, 1995, 2001). Using the same software, deviations from Hardy–Weinberg (HW) equilibrium were tested by 2800 permutations and the independence of loci was tested with G-statistics and 8400 permutations. Both global and pair-wise F_{ST} (Weir and Cockerham, 1984) were calculated using FSTAT 2.9.3 that also provided confidence interval for the global F_{ST} using bootstrapping over loci (15 000 replacements) and

Table 2 Microsatellite loci for *P. flesus*

Locus	*Primer sequence*	T_a (°C)	*5′ mod.*	*Motif*
AJ315970	F: CATCAAAGCATGAAACCC R: CTGGCCCAAGTGGAGCAT	56.5	FAM	$(CTAT)_{10}(CTCT)_2$
AJ315971	F: GTCAAATTAGGGAGGGCAGTG R: CTCTGACCTTGCACAGATAAAT	60	FAM	$(CCAT)_2CCACCATC(CTAT)_4$ $(CCAT)_3CTAT(CCAT)_4$
AJ315972	F: GAGAGAGAGAAGAAAGGAAACAAAG R: TCTGTGGACCATTGGGTA	60	HEX	$(GATA)_{31}$
AJ315973	F: ATGAGGACGTGGATGTTCTTC R: CCCCTATCTCTGCTTAATGTTCAC	54	HEX	$(CT)_{18}$
AJ315974	F: TGCTGCCGAGCTGGGCTTATTATC R: CACACGGCATCCCAACTGTCACAT	56.5	TET (HEX)	$(GT)_5TA(GT)_{10}$
AJ315975	F: ACCCGATCAAGTTGTAGTCAT R: CATTTCTCCTCTCGGCGTGTT	60.0	HEX	$(CTGT)_7$
AJ538314	F: CTTTAATTGCGCCAGACTGACAG R: CCCTCCGGGGATGAATAAAGT	54.7	TET (HEX)	$(GATA)_9GTTA(GATA)_{10}$

Sequences obtained from GenBank (submitted by TJ Dixon 2001). Names of loci correspond to their GenBank accession numbers.

significance testing of pair-wise F_{ST}:s by G-statistics and 3800 permutations. FSTAT 2.9.3 was further used to analyse isolation by distance (Mantel tests with 10 000 permutations) and perform partial Mantel tests (10 000 randomizations). GENEPOP 3.4 (Raymond and Rousset, 1995) was used to calculate *P*-values for single loci F_{ST} that were combined according to Fisher's method (Sokal and Rohlf, 1995) to provide a *P*-value for the global F_{ST}. GENETIX 4.05 (Belkhir, 2004) was used to calculate pair-wise genetic distances *sensu* (Nei, 1972). The latter was further analysed using multidimensional scaling (MDS) with an alternating least-squares algorithm in SPSS 12.0 (SPSS Inc.) and a Young's S-stress convergence limit of 0.001. Tests for the presence of null alleles and scoring errors were made with MICRO-CHECKER 2.2 (Van Oosterhout *et al*., 2004).

Geographical statistics and calculations of distance between sampling areas were made with ARCGIS 9.1 (ESRI). Inference of number of populations and locations of possible genetic boundaries without assuming any *a priori* population structure was made using the R package GENELAND (Guillot *et al*, 2005b). By implementation of a Bayesian cluster model into a Markov Chain Monte Carlo algorithm each individual was probabilistically assigned to a population (Pritchard *et al*., 2000). We used 100 000 iterations, an uncertainty of coordinates of 1 km, and possible number of populations between 1 and 20. The model was re-run with 50 000 iterations, a burn-in period of 50 iterations and the estimated number of populations from the first run fixed. Voronoi tessellation of observed genetic data resulted in maps of the posterior probability of belonging to a certain population (see Guillot *et al*, 2005a, b for a more extensive description of the technique).

Environmental information about the sampling locations, that is temperature and salinity, was extracted from the oceanographic surface database of ICES, the oceanographic database of the Swedish Meteorological and Hydrological Institute and the monitory fishing database of the Institute of Coastal Research at the Swedish Board of Fisheries. Data were restricted to less than 10 m depth and the summertime (May–August) during the time period 1993–2003. Observations within a 30-km radius from the sampling location and mean values for temperature and salinity were used.

Results

To test for laboratory artefacts one sample (Oderbank) was run twice. We obtained very similar results, with 95% of allele sizes being the same, and the same qualitative conclusions regardless of run used. Total allele numbers ranged between 8 and 41 per locus and between 3 and 34 within samples. The mean observed heterozygosity within samples varied between 0.65 and 0.83, whereas the observed heterozygosity per locus per sample varied between 0.50 and 0.97 (see appendix for details). Tests for linkage disequilibrium were nonsignificant after Bonferroni correction but two samples (Åbo and Gdynia) showed deviations from HW expectations at the AJ315972 locus. Furthermore, MICRO-CHECKER suggested that there could be a problem with null alleles at some loci in some populations, although the estimated frequencies of null alleles were always <0.1. Therefore, in addition to using the original data, all statistical analyses were also made excluding the most problematic locus (AJ315972), which had an estimated mean null allele frequency above 0.05 in six of the samples. However, including or excluding this locus did not qualitative change the outcome of these tests and hence only results from using all loci are presented.

Genetic differentiation

The global F_{ST} (Weir and Cockerham, 1984) including all samples was 0.012 (Fisher's method of test combination, $\chi^2 = 152$, d.f. $= 14$, $P < 0.001$; 95% CI 0.008–0.016). Total 58 of 190 pair-wise estimates of F_{ST} were significant after Bonferroni correction. Ten of these involved either one of the most separate samples; Finbo or Thyborön (Table 3). The highest pair-wise F_{ST} was 0.048 between the Gotland and the Dabki samples.

Using only the samples from the Baltic Sea (including Öresund but excluding Kattegat) resulted in a global F_{ST} of 0.011 (95% CI 0.007–0.016). The samples later identified as demersal (see below: 1–9, 11 and 12) had a nonsignificant global F_{ST} although there was a global F_{ST} of 0.006 (95% CI 0.003–0.011) among the putative pelagic samples.

Table 3 Pair-wise F_{ST} values and significance levels after 3800 permutations

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*
2	0.0055**																		
3	0.0007	0.0021																	
4	**0.0061***	0.0023	0.0016*																
5	0.0060	−0.0025	0.0022	0.0022*															
6	−0.0015	0.0048	0.0045	0.0046	0.0040														
7	0.0052**	−0.003	0.0053	0.0057*	0.0045	0.0029													
8	0.0017**	0.0002	0.0035*	0.0027*	0.0030	0.0023	−0.0006												
9	0.0071**	0.0038	0.0080	0.0087**	−0.0016	0.0013	0.0033	0.0051*											
10	**0.0185***	**0.0133***	**0.0125***	**0.0139***	**0.0195***	**0.0199***	**0.0115***	0.0019**	**0.0232***										
11	0.0038**	0.0025	0.0010	0.0050**	−0.0004	0.0024	0.0045	−0.0009	0.0034	0.0113***									
12	0.0051*	−0.0020	0.0029	0.0023	0.0012	0.0045	−0.0020	−0.0008	0.0030	0.0058**	0.0043*								
13	**0.0179***	0.0134*	0.0114**	0.0185***	0.0161*	0.0150**	0.0119**	0.0043	0.0222**	0.0033*	0.0093	0.0088							
14	**0.0327***	**0.0316***	**0.0300***	**0.0370***	**0.0351***	**0.0339***	**0.0341***	0.0181**	**0.0481***	0.0138**	0.0222**	**0.0253***	0.0066*						
15	**0.0120***	0.0130**	0.0073**	**0.0151***	**0.0143***	**0.0128***	0.0170***	0.0057	**0.0236***	0.0063	0.0048*	0.0101**	−0.0001	0.0029					
16	**0.0261***	**0.0207***	0.0213***	**0.0272***	0.0265	0.0232*	0.0179**	0.0099	0.0339**	0.0055	0.0179	0.0126	0.0008	0.0036	0.0066				
17	**0.0188***	0.0164**	0.0214**	**0.0275***	0.0264***	**0.0233***	0.0164***	0.0086**	**0.0326***	0.0071*	0.0190**	0.0134**	−0.0000	0.0134**	0.0086*	0.0065			
18	**0.0192***	**0.0193***	0.0095***	**0.0168***	**0.0248***	**0.0208***	**0.0166***	**0.0121***	**0.0333***	0.0075**	**0.0143***	0.0153***	0.0030	**0.0153***	0.0030*	0.0083	0.0061		
19	**0.0203***	**0.0212***	0.0097***	**0.0204***	**0.0256***	**0.0228***	**0.0233***	**0.0158***	**0.0338***	0.0113**	0.0123***	**0.0180***	0.0039	0.0107**	0.0001	0.0115	0.0067	−0.0030	
20	**0.0197***	**0.0196***	0.0063***	**0.0192***	**0.0246***	**0.0239***	**0.0212***	**0.0167***	**0.0341***	0.0090	**0.0183***	**0.0160***	0.0077	0.0164*	0.0037	0.0141	0.0129*	−0.0028	−0.0010

***$P<0.001$, **$P<0.01$, *$P<0.05$.
Bold values are significant at the 0.05 level after Bonferroni correction.

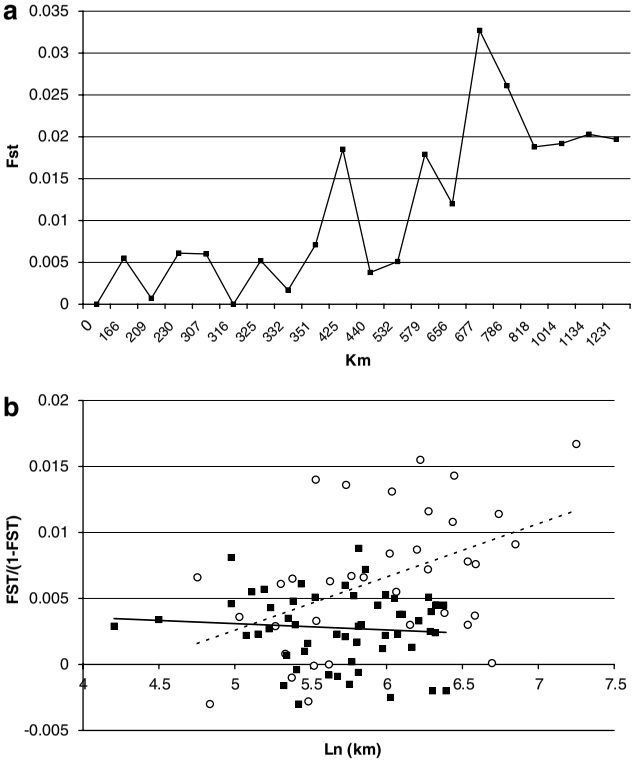

Figure 2 (**a**) Relationship between genetic distance (F_{ST}) and geographic distance from the northernmost sample at Åland. (**b**) Relationship between linearized genetic distance ($F_{ST}/(1-F_{ST})$) and the logarithm of geographic distance (filled squares, putative demersal spawners; open circles, putative pelagic spawners).

Isolation by distance

There was a significant correlation between nearest waterway distance (in km) and genetic distance ($F_{ST}/(1-F_{ST})$) (Mantel test, $R^2=0.23$, $P=0.0001$). A partial mantel test, with the ecological indicator variables salinity and temperature together with geographical distance, showed that only geographic distance was correlated to genetic distance.

A plot of genetic distance and geographic distance showed that samples 2–9, 11 and 12 were more alike the Finbo (1) sample than the rest (Figure 2a). The most striking deviation from the genetic–geographic distance relationship was shown by sample 10 (Latvian Sea). If samples were divided in potential demersal and pelagic stocks, according to the MDS plot (see below), it was revealed that the isolation by distance pattern was driven by the pelagic spawners ($R^2=0.25$, $P=0.002$), but not the demersal ($R^2=0.007$, $P=0.54$; Figure 2b).

Number of populations

The most likely number of populations was two (GENELAND; Figure 3a). A map of posterior probabilities showed that the northern Baltic samples were more likely to belong to one population, whereas the southern Baltic samples and the Kattegat, Skagerrak and North Sea samples belonged to another (Figure 3b).

Multidimensional scaling

An MDS analysis of pair-wise Nei's genetic distances (Nei, 1972) showed that the first dimension explained

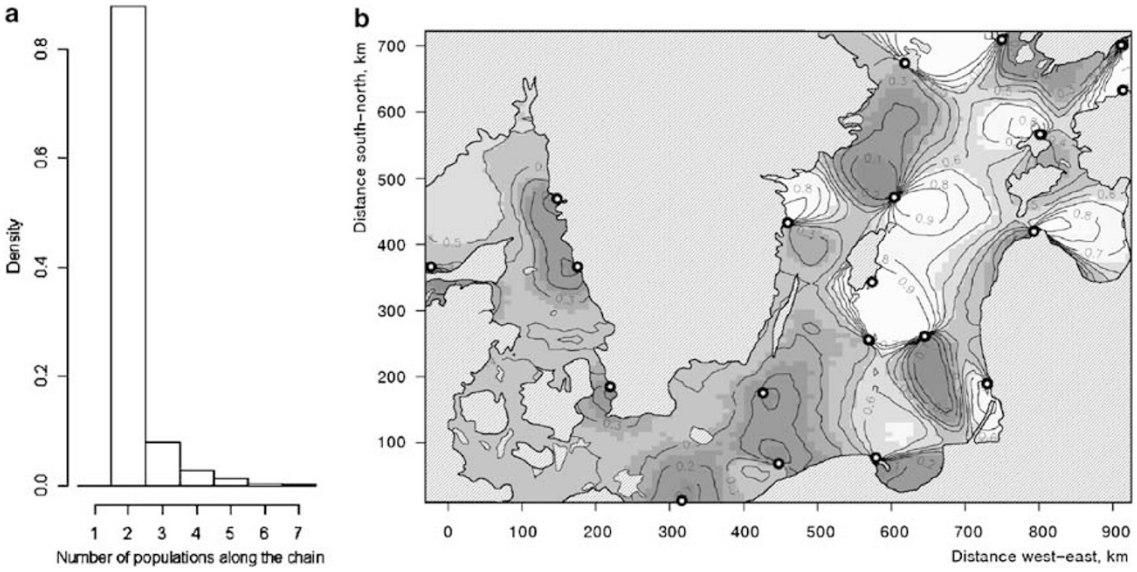

Figure 3 (**a**) Posterior distribution of the estimated number of populations in 100 000 MCMC iterations in GENELAND. (**b**) Map of posterior probabilities of population membership (number of populations = 2 and 50 000 iterations). Lighter areas correspond to higher probability to belong to the demersal population, sampling locations are indicated with white dots.

81% of the variance (one-dimensional $r^2 = 0.81$). The second dimension explained an additional 8% (two-dimensional $r^2 = 0.89$) and the third 4% (three-dimensional $r^2 = 0.93$). There was no clear geographic trend correlated to dimension 1, but a general east/west division could be detected (Figure 4a), and the North Sea, Skagerrak and Kattegat samples were clustered together.

An MDS plot of potential demersal samples showed that there was no pattern among these (Figure 4b). However, among the pelagic samples the Skagerrak, Kattegat and North Sea samples were clustered together (Figure 4c)

Discussion

In accordance with many population genetic studies of organisms inhabiting both the North Sea and the Baltic (reviewed in Johannesson and André, 2006), the flounder show clinal variation in allele frequencies from the west coast of Sweden to the northern areas of the Baltic. However, not all studies have revealed this pattern. In a study of turbot (*Psetta maxima*, Florin and Höglund, 2007) we found no evidence of isolation by distance or population structure, despite the fact that previous works (Nielsen *et al*., 2004) have suggested differences between the North Sea and the Baltic for this species. We attributed the absence of population structure in our study to admixture during major salt-water influxes in to the Baltic; events that occur stochastically with approximately 10-year intervals (Fonselius and Valderrama, 2003).

We found a clear pattern of isolation by distance in the present study of the flounder, and a closer examination of the map of posterior probabilities of population membership, as well as the isolation by distance plots, reveals that the pattern to a large extent is generated by a stepped cline situated somewhere in the region of the island of Bornholm. This stepped cline coincides with a difference in spawning behaviour in the flounder. North of Bornholm, the flounder is largely resident and spawns at shallow waters with demersal eggs. These demersal eggs also show evidence of adaptations to lower salinity by having a thicker chorion and being more robust (Lönning and Solemdal, 1979). South of Bornholm, the flounder is migratory and spawns in deeper waters with pelagic eggs.

The evidence for the existence of a genetically distinct demersal type of flounder is indirect, but fits with our observation of a stepped cline in allele frequencies, as well as the map of posterior probabilities of population memberships. Although tagging experiments (reviewed in Aro and Sjöblom, 1983; Florin, 2005) have revealed extensive migratory behaviour of flounder there are no direct estimates of migration between demersal and pelagic populations. Extensive gene flow between populations in the Baltic would counteract any local adaptations to salinity. However, stepped clines can evolve despite gene flow, provided that local selection pressures are strong enough (Endler, 1977, but see Vasemägi, 2006).

Within samples taken from demersal populations we could find no evidence of population structure, suggesting that gene flow is extensive among these populations. However, among pelagic samples, we found both a significant isolation by distance pattern, and that the most northwestern samples (the North Sea, Skagerrak and Kattegat) were differentiated from the rest of the pelagic samples. These observations argue that there is indeed a true isolation by distance pattern within pelagic spawners. This is in agreement with the observation of significantly different egg characteristics that has been demonstrated between flounders from the Sound and the southern Baltic Sea (Nissling *et al*., 2002). We speculate that the difference between demersal and pelagic spawners may be of a more ancient origin than the population structure observed within pelagic spawners. Such a scenario may also explain why we observe population structure in flounder, but not in turbot (Florin and Höglund, 2007). In flounder, the demersal spawners

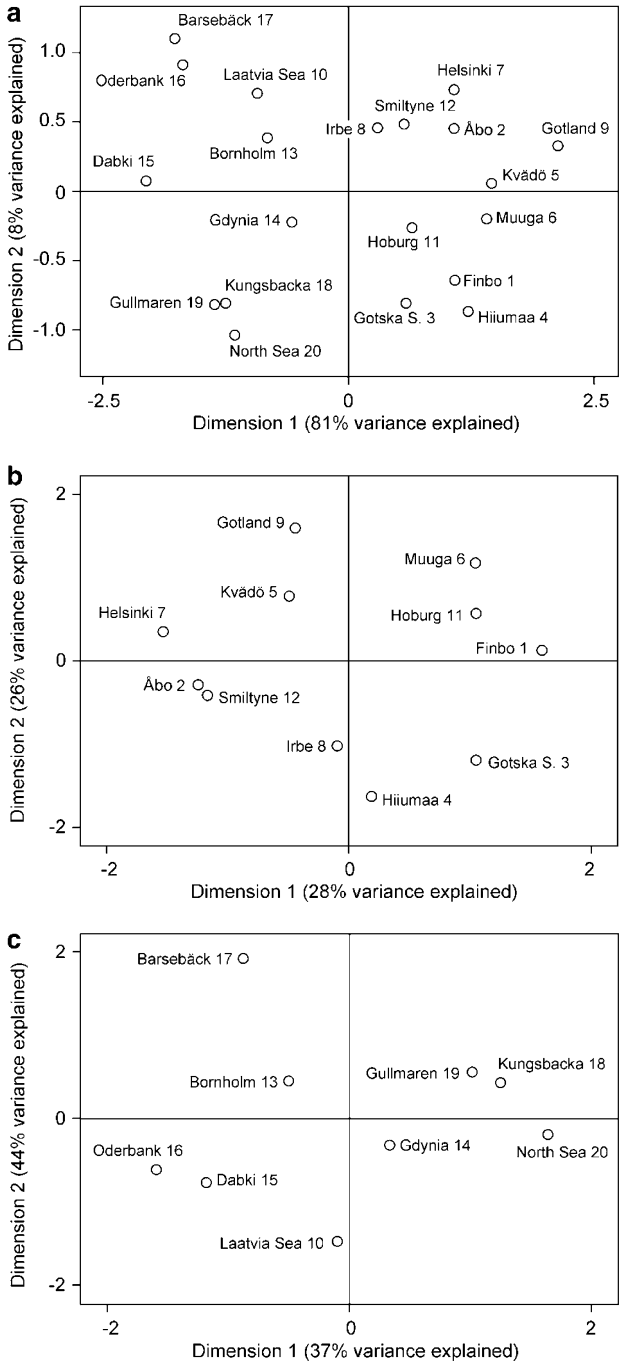

Figure 4 (**a**) Multidimensional scaling (MDS) plot of Nei's distance among samples, one-dimensional $r^2 = 0.81$, two-dimensional $r^2 = 0.89$. (**b**) MDS plot among potential demersal samples, one-dimensional $r^2 = 0.28$, two-dimensional $r^2 = 0.54$. (**c**) MDS plot of Nei's genetic distances among pelagic spawners, one-dimensional $r^2 = 0.37$; two-dimensional $r^2 = 0.81$.

may be descended from a population that colonized the Baltic previous to present pelagic flounder populations and any population of turbot. This demersal stock of flounder may thus have had longer time to adapt to the brackish salinity found in the northern Baltic. One such key adaptation may be their thicker shelled eggs, a feature that may be essential for survival at low salinity. No such clear differentiation in egg characteristics can be observed in turbot.

Management

When considering management units it seems clear that from a strict population genetic perspective, on the basis of neutral (or at least nearly neutral) microsatellite markers, there are no arguments for more than three management units. These are the northern Baltic (demersal populations); southern Baltic with the Öresund straits and the most northwestern sampling sites (Skagerrak, Kattegat and North Sea), respectively. There is no population genetic foundation for the many SDs currently implemented. Furthermore, our data reveal that some of the SDs harbour flounder of both types. Unfortunately, from a management point of view, no clear geographic boundary between the demersal and pelagic flounder can be given. Even if, during spawning, they divide into shallower and deeper areas, respectively, they probably mix in the feeding season. The distribution of the pelagic type is most probably highly affected by the changing salinity due to shifting hydrological conditions. However, decisions about management units should best be based on more than one type of genetic marker, and also other relevant biological criteria such as ecological differences between populations (Ruzzante *et al*., 2006). Although our data strongly argue for three management units, we argue for further studies using more markers and ecological data to further strengthen the conclusions from this study. Genetic studies could be based on mitochondrial markers that give information on historical population divergence between different colonization events of the Baltic (cf. *Macoma Baltica*, Luttikhuizen *et al*., 2003), and single nucleotide polymorphisms reflecting adaptive differences among populations. Ecological studies could be based on tagging, differences in spawning behaviour and egg characteristics.

Acknowledgements

The collection of genetic material would not have been possible without Kaj Ådjers at the Fisheries Division in the Provincial Government of Åland Islands, Claus-Christian Friees at the Institute for Baltic Sea Fisheries in Rostock, Didziz Ustup at Latvian Fish Resource Agency, Timo Myllelä and Eero Aro at the Finnish Game and Fisheries Research Institute, Tenno Drevs at the Estonian Marine Institute, Iwona Psuty-Lipska at the Sea Fisheries Institute in Gdynia, Jacob-Hemmer Hansen at the Danish Institute for Fisheries Research and the Fisheries Research Laboratory in Lithuania. Within Sweden the sampling was made with help of Anna Lingman, Fredrik Franzen, Mikael Petterson and Björn Fagerholm and Kurt Torildsson at the Institute of Coastal Research in Simpevarp and Ringhals, respectively and Anders Nissling at the Gotland University. We also want to thank Robert Ekblom and Gunilla Engström at the Department of Population Biology, Uppsala University for the molecular work and Carl André and one anonymous referee for valuable comments on the chapter.

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Appendix 1

Summary of basic genetic data per sample and locus: number of scored individuals (*N*), number of alleles, allelic richness in a sample of 17 individuals (*r*(17)), observed (Hobs) and expected (Hexp) heterozygosity and *P*-values for deviation from Hardy–Weinberg equilibrium (HW). HW per locus per sample is based on 2800 randomizations. Adjusted 5% level is 0.00036.

Sample	*Variable*	*AJ315970*	*AJ315971*	*AJ315972*	*AJ315973*	*AJ315974*	*AJ315975*	*AJ38314*	*Overall*
1	*N*	49	53	51	45	51	51	50	
	No. of alleles	16	4	23	11	6	6	15	11.57
	r(17)	11.53	3.98	15.57	7.86	4.82	5.07	9.82	
	Hobs	0.90	0.57	0.92	0.82	0.67	0.76	0.78	0.77
	Hexp	0.88	0.61	0.93	0.78	0.72	0.73	0.80	0.78
	HW	0.71	0.31	0.44	0.82	0.25	0.79	0.39	0.43
2	*N*	47	44	35	50	49	44	42	
	No. of alleles	13	8	22	12	7	8	14	11.43
	r(17)	12.01	3.91	17.54	8.13	5.6	4.30	9.68	
	Hobs	0.85	0.62	0.71	0.76	0.53	0.66	0.81	0.71
	Hexp	0.84	0.65	0.92	0.78	0.69	0.76	0.83	0.78
	HW	0.64	0.75	0.0004	0.43	0.01	0.07	0.41	0.0014
3	*N*	36	48	41	46	42	44	29	
	No. of alleles	18	5	25	9	4	8	12	11.57
	r(17)	12.45	4.29	16.44	7.04	3.40	5.98	9.37	
	Hobs	0.78	0.52	0.88	0.74	0.5	0.73	0.72	0.70
	Hexp	0.83	0.60	0.94	0.78	0.67	0.75	0.74	0.76
	HW	0.23	0.11	0.11	0.27	0.02	0.42	0.49	0.0018
4	*N*	48	49	47	48	49	48	47	
	No. of alleles	13	4	23	10	5	6	11	10.29
	r(17)	10.36	3.93	16.48	7.32	3.74	4.60	8.72	
	Hobs	0.81	0.62	0.96	0.79	0.65	0.60	0.74	0.74
	Hexp	0.84	0.64	0.95	0.76	0.68	0.79	0.78	0.78
	HW	0.36	0.36	0.70	0.79	0.40	0.004	0.28	0.033
5	*N*	35	45	42	45	47	44	41	
	No. of alleles	14	4	23	10	4	5	12	10.29
	r(17)	10.39	3.96	17.54	7.02	3.85	4.73	7.97	
	Hobs	0.74	0.56	0.88	0.71	0.53	0.70	0.68	0.69
	Hexp	0.80	0.66	0.94	0.73	0.66	0.71	0.77	0.75
	HW	0.22	0.07	0.10	0.46	0.04	0.51	0.10	0.0014
6	*N*	41	42	40	40	43	42	35	
	No. of alleles	18	4	24	10	8	5	13	11.71
	r(17)	11.54	3.99	17.01	7.4	4.59	5.06	11.03	
	Hobs	0.90	0.67	0.95	0.8	0.70	0.83	0.94	0.83
	Hexp	0.86	0.61	0.95	0.82	0.72	0.69	0.83	0.78
	HW	0.88	0.89	0.63	0.46	0.40	0.98	0.99	0.98
7	*N*	40	41	47	44	41	38	34	
	No. of alleles	16	4	27	8	5	6	16	11.71
	r(17)	10.47	3.79	18.11	9.48	3.59	4.44	11.41	
	Hobs	0.93	0.73	0.91	0.84	0.66	0.63	0.82	0.79
	Hexp	0.89	0.66	0.96	0.81	0.66	0.71	0.88	0.80
	HW	0.84	0.89	0.15	0.77	0.53	0.17	0.20	0.39

Continued

Sample	Variable	AJ315970	AJ315971	AJ315972	AJ315973	AJ315974	AJ315975	AJ38314	Overall
8	*N*	41	45	42	40	44	44	46	
	No. of alleles	13	6	25	13	7	6	13	11.86
	r(17)	10.03	4.36	19.92	9.88	3.00	4.563	11.00	
	Hobs	0.56	0.67	0.90	0.62	0.84	0.51	0.87	0.72
	Hexp	0.81	0.65	0.95	0.78	0.68	0.78	0.84	0.78
	HW	0.0004	0.62	0.15	0.005	0.99	0.004	0.81	0.0011
9	*N*	43	46	42	44	46	46	44	
	No. of alleles	14	4	25	8	4	6	12	10.43
	r(17)	11.01	4.49	18.65	12.25	3.98	4.55	7.99	
	Hobs	0.88	0.70	0.95	0.79	0.67	0.76	0.70	0.78
	Hexp	0.84	0.65	0.94	0.79	0.66	0.67	0.78	0.76
	HW	0.88	0.82	0.68	0.631	0.60	0.95	0.14	0.81
10	*N*	45	43	46	47	46	43	39	
	No. of alleles	18	4	27	17	3	7	12	12.57
	r(17)	11.24	4.64	19.14	11.82	3.00	4.87	9.95	
	Hobs	0.71	0.77	0.93	0.81	0.63	0.72	0.80	0.77
	Hexp	0.79	0.69	0.95	0.82	0.60	0.78	0.82	0.78
	HW	0.084	0.92	0.42	0.44	0.69	0.22	0.36	0.24
11	*N*	42	44	41	45	46	45	39	
	No. of alleles	16	4	23	12	6	6	15	11.71
	r(17)	10.89	3.95	19.30	8.25	4.38	4.79	7.60	
	Hobs	0.83	0.57	0.95	0.8	0.63	0.67	0.67	0.73
	Hexp	0.83	0.62	0.94	0.74	0.67	0.72	0.84	0.77
	HW	0.57	0.28	0.65	0.92	0.32	0.22	0.002	0.049
12	*N*	46	47	46	45	49	48	44	
	No. of alleles	17	4	30	9	6	6	15	12.43
	r(17)	11.03	3.97	20.07	9.53	3.00	4.88	8.09	
	Hobs	0.74	0.68	0.89	0.62	0.69	0.81	0.80	0.75
	Hexp	0.85	0.68	0.95	0.75	0.68	0.76	0.82	0.78
	HW	0.01	0.54	0.06	0.02	0.65	0.86	0.40	0.035
13	*N*	38	42	38	44	46	48	38	
	No. of alleles	15	4	24	13	5	6	14	11.57
	r(17)	11.75	3.96	18.40	7.35	3.83	4.73	11.89	
	Hobs	0.68	0.57	0.82	0.75	0.70	0.73	0.79	0.72
	Hexp	0.79	0.59	0.96	0.80	0.54	0.69	0.88	0.75
	HW	0.04	0.45	0.0007	0.20	0.99	0.79	0.11	0.071
14	*N*	47	56	51	55	60	45	51	
	No. of alleles	18	4	28	14	9	5	11	12.71
	r(17)	11.30	3.94	19.65	6.64	3.58	4.34	9.44	
	Hobs	0.77	0.62	0.82	0.76	0.7	0.51	0.84	0.72
	Hexp	0.79	0.57	0.95	0.70	0.65	0.69	0.80	0.74
	HW	0.34	0.91	0.0004	0.95	0.85	0.009	0.84	0.16
15	*N*	56	56	55	41	58	46	52	
	No. of alleles	23	4	34	8	5	6	12	13.14
	r(17)	11.05	3.93	18.76	9.00	5.11	4.23	8.45	
	Hobs	0.73	0.71	0.84	0.44	0.55	0.5	0.81	0.65
	Hexp	0.73	0.63	0.96	0.60	0.57	0.67	0.85	0.71
	HW	0.62	0.96	0.0007	0.003	0.42	0.009	0.27	0.0007
16	*N*	40	46	40	44	45	45	17	
	No. of alleles	16	5	29	13	3	5	11	11.71
	r(17)	10.23	3.94	15.94	8.68	4.62	6.22	10.18	
	Hobs	0.67	0.78	0.98	0.80	0.6	0.67	0.76	0.75
	Hexp	0.73	0.70	0.96	0.77	0.60	0.68	0.90	0.76
	HW	0.19	0.93	0.83	0.76	0.59	0.50	0.06	0.29
17	*N*	29	30	25	19	30	28	29	
	No. of alleles	13	5	22	13	4	5	10	10.29
	r(17)	10.27	4.72	18.04	8.90	4.78	5.80	9.33	
	Hobs	0.83	0.6	0.84	0.79	0.6	0.54	0.83	0.73
	Hexp	0.84	0.66	0.96	0.82	0.66	0.64	0.79	0.77
	HW	0.48	0.61	0.02	0.45	0.29	0.12	0.75	0.056
18	*N*	40	41	39	40	39	36	43	
	No. of alleles	16	5	27	16	3	6	13	12.29
	r(17)	12.10	3.98	18.85	7.29	4.65	5.59	9.86	
	Hobs	0.90	0.63	0.95	0.77	0.62	0.64	0.79	0.76
	Hexp	0.84	0.63	0.96	0.85	0.60	0.68	0.80	0.76
	HW	0.92	0.60	0.50	0.11	0.65	0.35	0.51	0.37
19	*N*	43	44	44	34	41	44	45	
	No. of alleles	16	4	28	9	5	6	11	11.29
	r(17)	10.01	3.97	17.06	7.91	3.92	5.56	8.58	
	Hobs	0.81	0.52	0.89	0.76	0.51	0.73	0.76	0.71
	Hexp	0.83	0.56	0.96	0.75	0.63	0.7	0.73	0.74
	HW	0.44	0.30	0.03	0.69	0.05	0.72	0.74	0.12

Continued

Sample	*Variable*	*AJ315970*	*AJ315971*	*AJ315972*	*AJ315973*	*AJ315974*	*AJ315975*	*AJ38314*	*Overall*
20	*N*	44	48	44	47	50	46	46	
	No. of alleles	15	4	30	13	3	6	11	11.71
	r(17)	10.95	3.99	18.03	11.44	2.99	5.96	9.34	
	Hobs	0.80	0.65	0.82	0.81	0.58	0.70	0.59	0.70
	Hexp	0.83	0.64	0.96	0.81	0.59	0.69	0.69	0.74
	HW	0.32	0.57	0.0007	0.55	0.50	0.58	0.03	0.02
	Total No. of alleles	41	8	42	27	13	11	32	
Means	No. of alleles	15.9	4.3	25.95	11.4	5.1	6	12.65	
	r(17)	11.60	4.08	18.43	8.83	4.22	5.34	9.82	
	Hobs	0.79	0.64	0.89	0.75	0.623	0.67	0.78	
	Hexp	0.82	0.64	0.95	0.77	0.65	0.72	0.81	