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Genetic differentiation of brackish water populations of cod *Gadus morhua* in the southern Baltic, inferred from genotyping using SNP-arrays

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ABSTRACT

The Baltic is a semi-enclosed sea characterised by decreasing salinity in the eastern and northern direction with only the deeper parts of the southern Baltic suitable as spawning grounds for marine species like cod. Baltic cod exhibits various adaptations to brackish water conditions, yet the inflow of salty North Sea water near the bottom remains an influence on the spawning success of the Baltic cod. The eastern Baltic population has been very weakly studied in comparison with the western population.

The aim of this study is to demonstrate for the first time genetic differentiation by the use of a large number of SNPs between eastern and western Baltic populations existing in differentiated salinity conditions. Two cod samples were collected from the Bay of Gdańsk, Poland and one from the Kiel Bight, Germany. Samples were genotyped using a cod derived SNP-array (Illumina) with 10 913 SNPs. A selection of diagnostic SNPs was performed. A set of 7944 validated SNPs were analysed to assess the differentiation of three samples of cod. Results indicated a clear distinctness of the Kiel Bight from the populations of the eastern Baltic. F_{ST} comparison between both eastern samples was non-significant. Clustering analysis, principal coordinates analysis and assignment test clearly indicated that the eastern samples should be considered as one subpopulation, well differentiated from the western subpopulation. With the SNP approach, no differentiation between groups containing 'healthy' and 'non-healthy' cod individuals was observed.

1. Introduction

The Baltic Sea is the world largest brackish sea, with highly variable hydrographic conditions influenced by inflows of high salinity water from the North Sea. This semi-enclosed Baltic Sea, since over 7000 years, is characterised by decreasing surface salinity from the west to the eastern areas which reach almost fresh water conditions to the north east. The vertical salinity stratification is high, especially in the three main deep basins (Bornholm, Gdansk Deep and Gotland), with low salinity at the surface and increased salinity at the halocline and below (Tomkiewicz et al., 1998). The high salinity waters form a layer in the deeper parts of the Baltic Sea and serve as spawning grounds for species requiring higher salinity for egg development (Nissling and Westin, 1997).

One of the main Baltic fishes depending on elevated salinity conditions is cod (*Gadus morhua*, L.) (Nissling and Westin, 1997). Cod is commercially the most important Baltic Sea species with a total catch of the

two managed stocks of about 77 000 tonnes in 2012 (ICES, 2013a, 2013b). Its role as a top predator is fundamental in the ecosystem (Köster and Möllmann, 2000). The Baltic cod has significantly bigger eggs with thinner chorion compared to fish from beyond the Baltic (Nissling et al., 1994) as an assumed result of adaptation to a level of salinity lower than in the ocean. But this species still requires much higher salinity than the average surface salinity in the Baltic Sea for successful reproduction (Westin and Nissling, 1991; Nissling and Westin, 1997). Interrelations between cod breeding places and environmental conditions make the structure of Baltic cod populations very characteristic.

Within Atlantic cod two subspecies have been identified (geographically distinct races): *Gadus morhua morhua* (L. 1758) and *Gadus morhua callarias* (L. 1758). These differ from each other in the range of occurrence, environmental preferences (e.g. salinity, feeding place) and body size and coloration (Westernhagen von, 1970; Cohen et al., 1990; Righton et al., 2001; Hüssy, 2011). Studies of Baltic cod populations have demonstrated their significant distinctiveness from Atlantic

populations (O'Leary et al., 2007) but the present knowledge of local genetic and physiological adaptations to low salinity water of that population is still far from complete. The eastern Baltic population (SD 25 + 26) is genetically insufficiently studied compared to the western population (Nielsen et al., 2003; Antoszek et al., 2011; Kijewska et al., 2011).

One consequence of adaptation to different salinity gradients can also be the different susceptibility of fish to bacterial infections (Moles, 1997; Verma et al., 2011; Bjelland et al., 2012). The health status of Baltic cod populations is very important because it will be strongly affected by projected environmental and climate changes (Hinrichsen et al., 2011). Infections of skin (skin ulcers) are one of the most widespread diseases in cod from the Baltic proper. Mellergaard and Lang (1999) and Podolska et al. (2010) observed skin ulcers in ICES SD25. Wawrzyniak and Grawinski (1991) detected fin erosions in cod in this area and determined in the infected tissues different bacteria: *Aeromonas, Vibrio, Moraxella* and Enterobacteriaceae. Podolska et al. (2010) also identified in ulcers, bacteria belonging to Pseudomonadaceae, Aeromonadaceae, Shewanellaceae and Vibrionaceae. The health status of cod can be influenced by climate changes driving local fluctuations of salinity and oxygenation which in turn affect the susceptibility of fish to bacterial infections (Moles, 1997; Verma et al., 2011; Bjelland et al., 2012).

The variation between samples representing different subpopulations in the Baltic Sea could affect also the genetic profiles of the immune system of each subpopulation. Thus, it is likely that the MHC has evolved in response to local pathogen communities in many species across the vertebrate taxa (Evans and Neff, 2009). The SNP-array results could provide information to help determine if there are differences between populations of healthy and unhealthy cod.

Recently, single nucleotide polymorphisms (SNPs) have demonstrated local directional selection, despite high gene flow, in cod populations in the North Atlantic (Moen et al., 2008; Nielsen et al., 2009; Therkildsen et al., 2013). These results confirm that complete extinction of local populations irreversibly affects the gene pool and reduces the evolutionary potential of cod. Predictions concerning future changes in the Baltic cod populations and their composition need to be based on knowledge of their genetic structure and differentiation. The aim of this study is to demonstrate for the first time genetic differentiation by the use of 10K SNP-array between eastern and western Baltic populations existing in differentiated salinity conditions. Traditionally,

management of eastern and western Baltic cod for fishery purposes has been based on separate geographic and biological populations. The populations have been exploited with different intensity. Studies of genetic differences between these populations will provide further support for their separate management.

2. Materials and methods

2.1. SNP genotyping of cod samples

In total, 95 individuals obtained from 3 sampling sites were analysed: G1 (40 individuals) from the Bay of Gdańsk, International Council for the Exploration of the Sea (ICES) subdivision 26; G2 (29 individuals), ICES SD 25 and KIEL (26 individuals) from Kiel Bight, ICES SD 22 (Fig. 1) during March and June of 2012. Samples were completed from several hauls in the same region to obtain a sufficient number of fish per location. DNA was extracted from fin clip samples using Qiagen DNeasy 96 blood and tissue kit, according to the protocol recommended by the manufacturer, quantified on a NanoDrop device. Concentrations were adjusted to approx. 100 ng/μl with sterile-filtered distilled water. These samples were then genotyped for 10 923 SNPs in the Centre for Integrative Genetics (CIGENE), Norwegian University of Life Sciences, Ås, Norway. The array was developed by a Norwegian consortium composed of four research organisations (CEES, CIGENE, NOFIMA, Havforskningsinstituttet). SNPs were detected by resequencing a number of geographically diverse cod specimens and aligning the reads to the Cod reference genome sequence. SNP quality and validity were assessed by genotyping cod from a breeding programme and wild cod from various locations (approx. 2000 samples). SNP sequences will be submitted by the Norwegian Consortium to dbSNP. Further information on the array can be obtained from Matthew Peter Kent (CIGENE, Norway).

2.2. Selection of diagnostic SNPs

SNP validation revealed that amongst 10 923 SNPs, 2702 did not meet the criteria of classification as normal bi-allelic SNPs. After omitting them, i.e. MSV (multisite variant), SNP-0, Fail, 8221 loci were analysed. 70 SNPs, across total genotyped loci, had a level of missing data higher than the accepted threshold of 80% and they were removed
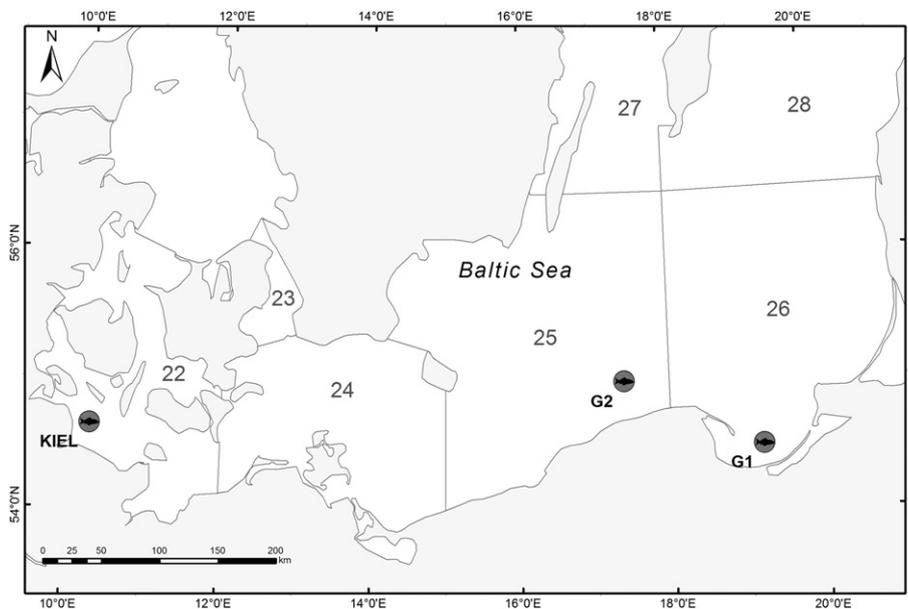

Fig. 1. Map showing the location of the sampled cod populations from Baltic Sea.

from the dataset. The results of Analysis of Molecular Variance (AMOVA) showed that amongst the remaining 8151 loci, 111 loci were monomorphic in all samples and they were excluded from further analysis. Analysis of allele frequency revealed the presence of 96 MAFs below the threshold of 0.01 and these were excluded from further research. Finally, the set of diagnostic SNPs consisted of 7944 markers.

2.3. Data analysis

The deviations between observed (Ho) and expected (He) heterozygosity assuming Hardy–Weinberg equilibrium (HWE) were tested within cod populations using a Markov chain with length = of 1 000 000 and dememorisation steps = of 100 000 using Arlequin software v. 3.5.1.3 (Excoffier and Lischer, 2010). H_o and H_e values for population were calculated using data with p < 0.05. Bonferroni correction was included to adjust P value in multiple tests. AMOVA, with number of permutations = of 10 000, tests of differentiation amongst pairwise F_{ST} estimates, locus-by-locus F_{ST} and F_{IS} estimates, allele frequencies and minor allele frequencies (MAFs) in each population were assessed using Arlequin software.

Structure v. 2.0 (Pritchard et al., 2000) was used to estimate the number of distinct populations (K). Cod individuals were clustered using runs for each value of K from 1 to 5. The admixture model with seven replicates for each number of inferred clusters was employed in order to evaluate the consistency of the results. Each run consisted of 100 000 iterations followed by 200 000 Markov-Chain Monte-Carlo (MCMC) steps.

CLUMPP v.1.1.1 (Jakobsson and Rosenberg, 2007) was applied to average cluster membership using the Large K Greedy algorithm. Output from CLUMPP was visualised in Distruct v. 1.1 (Roseneberg, 2004). With the method of Evanno (Evanno et al., 2005) implemented in the STRUCTURE HARVESTER programme (Earl and vonHoldt, 2012), ΔK was calculated to estimate the appropriate K value. Principal coordinates analysis (PCoA) was conducted in GenAlex (Peakall and Smouse, 2006, 2012) and the neighbour-joining (NJ) phylogenetic tree was constructed based on pairwise F_{ST} distance matrix in Mega 5.2 software (Tamura et al., 2011). Test of assignment was performed with Rannala and Mountain's (1997) Bayesian individual assignment method, implemented in GeneClass (Piry et al., 2004), to determine the most probable sources for all 95 tested individuals (assignment threshold of scores = 0.05).

In order to detect the outlier loci the hierarchical island model with 100 000 simulations implemented in Arlequin was used. For a given value of heterozygozity, loci exhibiting F_{ST} values higher than expected on the basis of neutral variation, and showing F_{ST} out of the 99% quantile based on coalescent simulations, were taken into account as candidates being under selection (Beaumont and Nichols, 1996).

2.4. Methods of analysis of healthy and non-healthy fish

Genetic differences between healthy and infected fish were analysed. Firstly, samples G1 and G2 were pooled and then divided into 2 groups. From infected fish (25% of the total number of individuals), 15 fish with symptoms of diseases other than skin infections were excluded from the analysis. Selected, infected individuals accounted for 42% of analysed fish (19.8% of all collected individuals G1 and G2). Finally, 40 healthy fish and 29 infected individuals were used for AMOVA and structure analysis using Structure v. 2.0 (Pritchard et al., 2000).

The comparison of the genetic differences in two groups of fish, healthy and infected, demonstrated that F_{ST} was very low: 0.00094 and was non-significant. Additionally, structure analysis did not reveal any differentiation between these two groups. No genetic differences were shown even using a set of outlier loci.

3. Results and discussion

3.1. Genetic diversity and MAFs

95 cod individuals from 3 Baltic locations were genotyped using a 10K SNP-array. All analysed loci had two alleles each. The allele frequencies, calculated with a molecular indices parameter implemented in Arlequin software, enabled the classes of minor allele frequency (MAF) to be identified. The mean minor allele frequency amongst polymorphic SNPs was 0.258. Particular classes of MAFs are presented in Fig. 2. A threshold of 0.01 is usually regarded as the minimum acceptable level of polymorphism for population genetic studies (Sladek et al., 2007; Chan et al., 2008) and it was applied in this study. A validated set of SNPs, composed of 7944 polymorphic loci, was used to calculate the diversity indices. The number of polymorphic loci was 7903 in KIEL population, 7771 in G1 population and 7688 in G2 population. A level of genetic variation, indicated by the mean number of alleles (MNA), was similar in all studied populations (MNA was 1.968 in G1, 1.978 in G2 sample and 1.995 in KIEL). For statistically significant loci (p < 0.05), expected heterozygosity (He) ranged from 0.398 in G1 population to 0.407 in G2 population, and observed heterozygosity (Ho) ranged from 0.350 in KIEL to 0.358 in G1 population (Table 1). Prior to Bonferroni, the numbers of loci found in the analysed populations, deviating from Hardy–Weinberg equilibrium (HWE), were 237, 157 and 172 for G1, G2 and KIEL, respectively. After Bonferroni correction for multiple comparisons, the majority of loci were in HWE, except for 11 loci for G1, 7 for G2 and 2 for KIEL. For the three populations, observed heterozygosity (Ho) was slightly lower than expected heterozygosity (He) which could indicate a small excess of homozygotes.

The local inbreeding coefficients (F_{IS}) were very low (−0.007 to 0.005) and statistically insignificant. The low number of loci with significant Hardy–Weinberg disequilibrium and insignificant local inbreeding coefficient in all 3 subpopulations could be a signal that we observe stable subpopulations with a limited number of migrants.

Genetic differentiation of the three cod populations was measured using the fixation index (F_{ST}) (Weir and Cockerham, 1984). Two models of Baltic population structure were tested: first assuming the existence of 3 separate subpopulations and the second where eastern subpopulations were grouped together. In the first model variance amongst populations was low (Va = −0.13%) whilst in the second dataset Va was noticeably higher (Va = 5.49%). The highest amount of variance occurred within individuals (96.75% in 1st and 94.53% in 2nd dataset). Variance amongst individuals within populations was very low (respectively 3.38% and −0.03%).

Pairwise F_{ST} values were calculated between the three cod populations. Significant values were between samples G1 and KIEL and between G2 and KIEL. F_{ST} comparison between both eastern samples, G1 and G2, was non-significant. The level of differentiation between
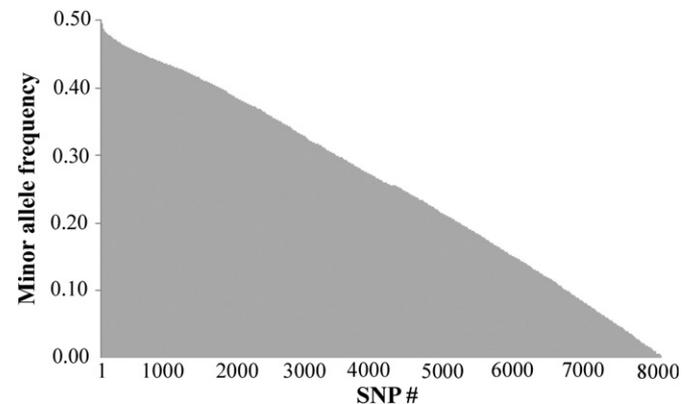

Fig. 2. Allele frequency distribution. 7944 SNPs in 95 cod individuals ranked according to allele frequency.

Table 1
Genetic variability of 7944 SNPs amongst three populations of Baltic cod.

Population name	No. of individuals	No. of polymorphic loci	For all loci		For significant loci (p < 0.05)		HWE departure p < 0.05	After Bonferroni correction
			H_o	H_e	H_o	H_e		
G1	40	7771	0.341	0.343	0.358	0.398	237	11
G2	29	7688	0.348	0.346	0.353	0.407	157	7
KIEL	26	7903	0.367	0.365	0.350	0.405	172	2

eastern samples and western Baltic samples was low and amounted 0.053 and 0.055 independently from the population structures defined for calculations. The global F_{ST} across the three cod samples was 0.034 indicating a low, but significant level of differentiation. According to Ward et al. (1994) a low level of genetic differentiation (F_{ST}) amongst putative populations is a common factor. There are several reasons for this: firstly, amongst marine subpopulations there is higher level of gene flow than in terrestrial populations (Ward et al., 1994). In the case of Baltic cod, due to statistically insignificant, near 0, values of F_{IS}, the hypothesis of high migration between eastern and western subpopulations was rejected. A second reason for low F_{ST} values could be the large effective size of the population (Ward et al., 1994) observed in Baltic subpopulations of cod. Low F_{ST} values could also be an effect of recent origin of the population after postglacial expansion, as suggested by Pampoulie et al. (2008) for Atlantic cod.

291 loci were identified as potential candidates for divergence selection. These were above the 99% quantile of the simulation model. A global F_{ST} calculated for the set of 291 outlier loci increased to 0.078 (for all 7944 polymorphic SNPs it was 0.034). For comparisons between G1-KIEL and G2-KIEL, pairwise F_{ST} values also increased significantly however an F_{ST} pairwise comparison between G1 and G2 remained non significant (Fig. 3).

3.2. Clustering and distance analysis

Genetic structure amongst three cod populations was evaluated with Bayesian clustering methods implemented in Structure 2.3.3. The 7944 SNPs were analysed using an admixture model where each individual had some fraction of the genome from each of the K populations. The results of this analysis indicated that for the 3 analysed populations the most likely number of populations was 2, which was confirmed by

calculation made by Harvester Structure. The maximum value of ΔK was for K = 2 (114.09) (Fig. 4C). At K = 2 the Polish populations G1 and G2 were found to be distinct from the KIEL population. Results obtained from these genetic clusterings were averaged by Clumpp and plots were generated by Distruct. The arithmetic clustering configurations for K = 2 are shown in Fig. 4 where plots depict individual and population levels of stratification (Fig. 4A and B). Results of the clustering of the analysed samples clearly indicated that eastern samples should be considered as one subpopulation, well differentiated from the western subpopulation represented by KIEL sample.

The clear split into two clusters and the population parameters obtained from previous calculations indicated a kind of permanent barrier effected by environmental conditions like salinity gradient and intrinsic factors like spawning time. Petereit et al. (2014) have shown the absence of density layers for successful eastward drift of non-feeding early-life stages from the western cod population. This would explain the strong population divergence (based on passive life-stages only) along the west–east axis.

Principal coordinates analysis (PCoA) enabled further investigation of the genetic relationships amongst populations. The genetic distances amongst individuals were determined by means of Genalex v.6.4. A PCoA showed that analysed cod individuals formed two clusters: the first encompassing the individuals from KIEL population which was well separated from the other, second one where individuals from G1 and G1 populations were poorly separated from each other, with no clear demarcation of populations (Fig. 5). Axes 1 and 2 explain 5.84% and 2.73% of the total genetic variation, respectively.

Phylogenetic relationships between the cod populations were examined with the neighbour-joining (NJ) method. Analysis revealed that the investigated genotypes belonged to two major clusters (Fig. 6). The first cluster encompassed genotypes from the eastern
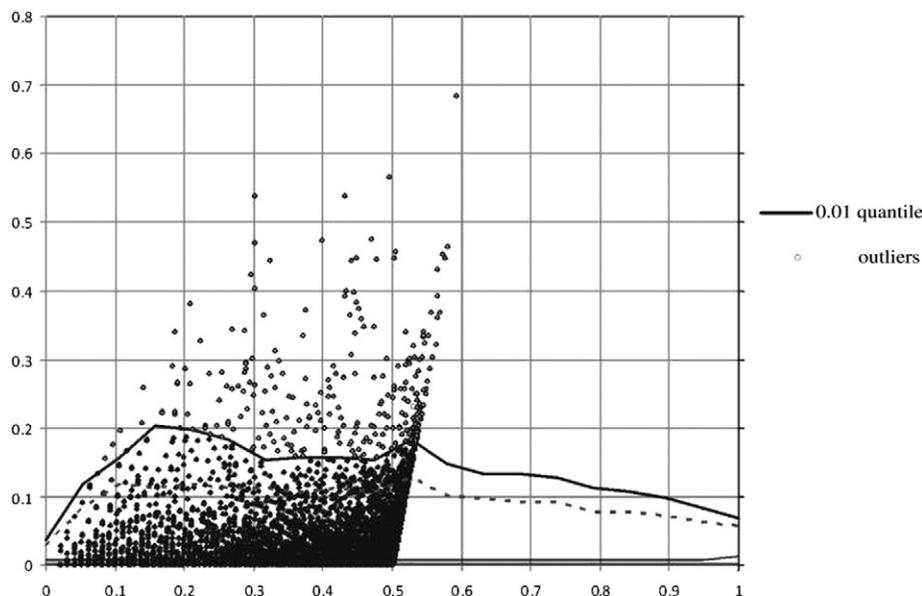

Fig. 3. Detection of loci under selection assuming a hierarchically structurerd population. The solid line indicates the 1% quantile from coalescent simulation. Empty circles indicate the identified outlier loci.
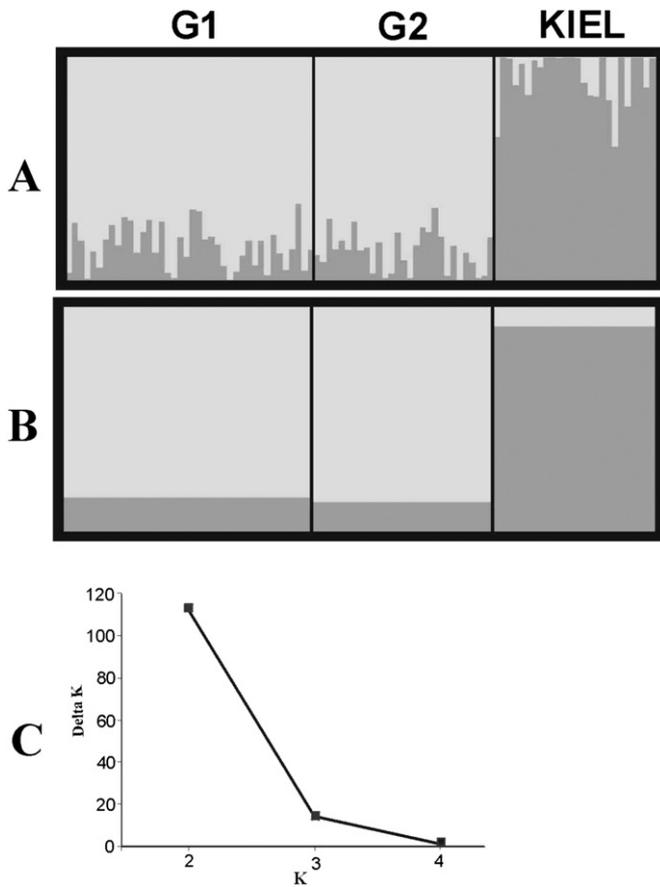
Fig. 4. Genetic structure results for K = 2 amongst cod populations obtained using STRUCTURE on a data set of 95 individuals and 7944 SNP markers, with replicates averaged by CLUMPP. Plots were generated by Distruct. A – individual level. B – population level. C – plot obtained from Harvester for detecting the K number.

subpopulation (G1 and G2) and the second cluster contained genotypes from the KIEL western population.

3.3. Assignment test

The statistical certainty of assignment or exclusion for each individual to the population was conducted using Bayesian method (Pritchard et al., 2000) and by the exclusion-simulation significance test (Cornuet et al., 1999) of the method of Rannala and Mountain (1997) implemented in
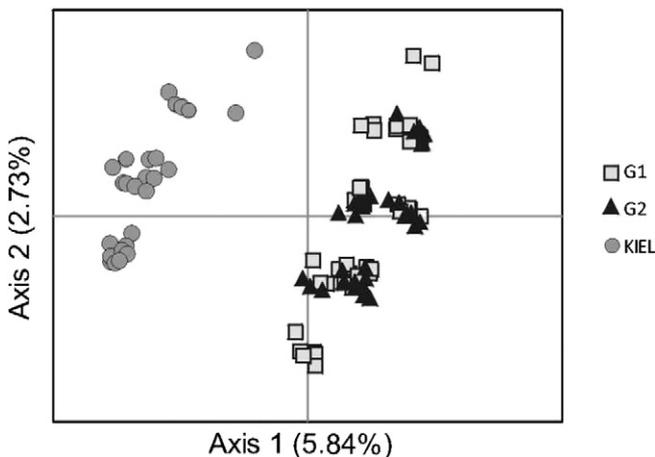
the Geneclass software. All cod individuals were assigned to the populations in which the genotype of the individual was most likely to occur. The median percentage of individuals correctly assigned for all populations was 71.4%. The percentage of correctly assigned individuals from G1 population was 87.5% and 12.5% individuals were assigned to G2 population. Only 26.7% of individuals from G2 population were assigned to G2 and 73.3% to G1 population. Self-assignment of individuals from KIEL population was 100%.

We demonstrated genetic differences between eastern and western Baltic cod populations using large SNP markers for the first time. Recently Hemmer-Hansen et al. (2014) reported differences between these populations using the outlier SNPs in the candidate aromatase gene and suggested occurrence of adaptive divergence which could be related to variable temperature and differences of salinity. A recent study combining experimental and modelling approaches showed that the pelagic egg and early yolk sac larval phase is an important life stage for the western Baltic cod (e.g. from Kiel Bight, in ICES subdivision SD 22) to maintain its spatial local integrity (Petereit et al., 2014). This is the result of the significant difference in egg density (high egg density in the western population compared to lower egg density in the eastern population) restricting dispersal into the deep, central Arkona Sea (SD 24) and thus further east into the Basin of Bornholm (SD 25) or Gdansk Deep (SD 26) (Petereit et al., 2014). The shallow connection between basins causes egg mortality during drift by preventing dense or "salty" enough hydrographic conditions for positive buoyancy (Petereit et al., 2014). The salinity gradient is probably one of the most important factors maintaining population structure of the Baltic cod. A second factor could be due to homing behaviour described for cod from the Danish Straits and western part of the Baltic Sea (Nielsen et al., 2005; Svedäng et al., 2007) which limits the choice of spawning areas.

4. Conclusions

The set of validated 7944 SNPs effectively separated the western Baltic cod sample (KIEL) from the eastern samples (G1 and G2).

The two samples from the eastern Baltic (G1 and G2) were not clearly distinguishable from each other indicating that they belong to the same, eastern subpopulation.

No evidence of inbreeding was found in the analysed samples using F_{IS} statistics.

The observed genetic differences between eastern and western Baltic populations of cod provide further justification for their separate management for fisheries and ecological purposes.
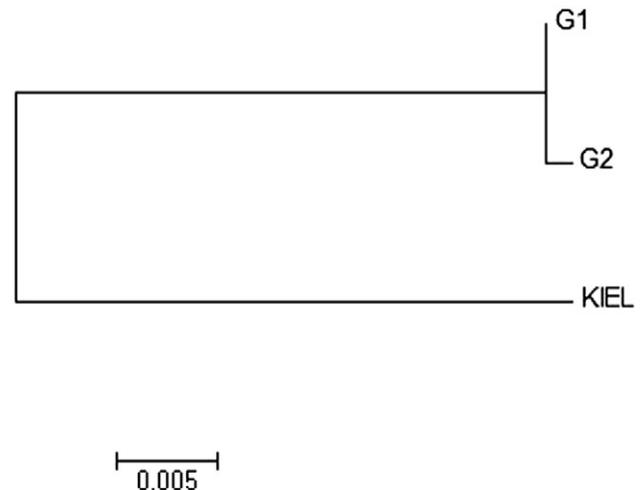

Fig. 5. Principal coordinates analysis (PCoA) using all polymorphic SNP markers. Symbols: grey square – G1, black triangle – G1, dark grey circle – KIEL.

Fig. 6. Distance based, neighbour joining tree calculated from frequencies of 7944 SNP in three cod populations.

Acknowledgements

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