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Vanessa Becquet, Rafal Lasota, Eric Pante, Adam Sokolowski, Maciej Wolowicz, Pascale Garcia

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V. Becquet [1,3], R. Lasota [2,4], E. Pante [1,5], A. Sokolowski [2,6], M. Wolowicz [2,7] , P. Garcia [1,8]

Corresponding author: V. Becquet

Tel: 033546507637

Fax: 033546458264

[1] Littoral Environnement et Sociétés, UMR 7266 CNRS, 2 rue Olympe de Gouges, 17000 La Rochelle, France

[2] Department of Marine Ecosystems Functioning, Institute of Oceanography, University of Gdañsk, Al. M. Piłsudskiego 46, 81-378 Gdynia, Poland

[3] E-mail: vbecquet@univ-lr.fr

[4] E-mail: ocerl@ug.edu.pl

[5] E-mail: pante.eric@gmail.com

[6] E-mail: oceas@univ.gda.pl

[7] E-mail: ocemw@ug.edu.pl

[8] E-mail: pgarciam@univ-lr.fr

Abstract

It is increasingly recognized that populations of marine organisms with potential for large-scale dispersal may exhibit fine-scale genetic structure. The Gulf of Gdañsk (Poland) is an interesting setting to study fine-grained population structure in marine organisms as it is characterized by chronic anthropogenic pollution and strong salinity gradients. We investigated, at two nested spatial scales (35 and 7 km), genetic structure among populations of the infaunal tellinid bivalve *Macoma balthica*. The COI mitochondrial marker revealed a likely mix of evolutionary lineages in the Gulf, with no detectable spatial structure. Seven microsatellite markers detected weak population structure, separating deep and shallow populations within short distances (7 km) and assignment tests suggested asymmetric gene flow among these populations, with no shallow recruits being detected in deep waters. Given the specific environmental conditions encountered at deeper depths in the Gulf (increased salinity, lower temperatures, oxygen depletion, hydrogen sulfide pollution), we suggest that deeper populations may be subjected to local adaptation.

Keywords

Population structure ; Vistula ; Asymmetric gene flow ; Local recruitment ; Depth stratification ; Microsatellites

Introduction

Many marine species are characterized by a high fecundity, large population sizes, long distance dispersal via a planktonic larval stage, and, very often, low geographic differentiation. As a consequence, the extent of small-scale genetic structure has long been neglected for marine organisms. However, many studies have shown that marine populations may exhibit strong genetic structure due to limited gene flow, genetic drift and unequal dispersion, caused by cryptic barriers or local adaptation in a heterogeneous environment (Koehn et al., 1980, Mitton, 1997, Powers & Schulte, 1998, Schmidt & Rand, 2001, Riginos et al., 2002).

The Baltic Sea is a noteworthy system to study the impact of high environmental patchiness on the genetic structure of marine organisms. It is characterized by restricted water exchanges with other oceanic basins, shallow waters, and low salinities (3 to 7 PSU). Temperature and salinity are strongly stratified by depth, water below 20-50 m being colder and saltier (Rheinheimer, 1995). Moreover, oxygen concentration is often reduced, particularly in the bottom zone, due to an eutrophication-induced increase in primary production (Cederwall & Elmgren, 1980) and total particulate matter content (Maksymowska et al., 1997) along with a high sedimentation rate (of up to 1-2 mm yr^{-1}; Witkowski & Pempkowiak, 1995). In addition, the Baltic Sea suffers from chronic anthropogenic pollution. Southern waters are impacted by a constant input of heavy metals from the Vistula river (Gulf of Gdañsk, Sokolowski et al., 1998, 2002, 2007), which carries pollutant runoff from the industrial cities of Katowice, Kracow or Warsaw (Rainbow et al., 2004, Szefer, 2002).

The infaunal bivalve, *Macoma balthica* is a dominant species of the benthic macrofauna in the Baltic Sea (Rumohr et al., 1996), being present from the sub-littoral to the aphotic zone (Väinölä & Varvio, 1989). Populations from the Baltic Sea are genetically distinct from those of the Atlantic and the North Sea, resulting from a mixture of Atlantic and Pacific parental lineages that are maintained by a strong barrier to gene flow in the Kattegat straight (Nikula et al., 2008). Local genetic differentiation can be strong in this species, as evidenced by the sharp differences in allele frequencies recorded along a 60 m-long temperature gradient in the White Sea (allozymic locus Idh; Hummel et al., 1998). The contrasted environmental conditions in the Gulf of Gdañsk represent an ideal place to

study fine-scale genetic structure among *M. balthica* subpopulations. In this work, we therefore used one mitochondrial and seven microsatellite markers (Becquet et al., 2009) to determine whether local genetic structure could be detected in these southern Baltic populations. We aimed at identifying putative barriers to gene flow at two nested geographic scales: a regional one (the Gulf of Gdañsk) and a local one (along a depth gradient, away from the mouth of the Vistula River).

Materials and methods

Adult specimens of *M. balthica* were collected between 1997 and 1999 at 11 locations with various environmental conditions (Fig. 1 and Table 1). Puck lagoon (MW30) is characterized by strong eutrophication and anoxia. Three populations were sampled in the lee of the Hel Peninsula at 45 m (HEL45), 60 m (HEL60) and 85 m (HEL85) depth. GN40 was sampled in the main seaway between the ports of Gdynia and Gdañsk. Finally, 6 populations were sampled at the mouth of the Vistula, along a gradient of increasing depth and various pollutant concentrations (SW5 to SW60). Specimens were stored in 95% ethanol until DNA extraction. Total DNA was purified from <15 mg of muscle using the Dneasy™ Tissue Kit (Qiagen, Germany).

Multiple mitochondrial lineages with distinct colonization histories can co-occur in the Baltic Sea (Luttikhuisen et al., 2003; Nikula et al., 2007, 2008). If specimens from multiple lineages are indiscriminately used, an older historical signal may mask a younger and weaker geographical signal. We therefore first tested for the presence of multiple mitochondrial lineages in our collection. 313 bp of the COI gene were successfully sequenced for 232 individuals, as in Becquet et al. (2012). In addition to the mitochondrial data, seven microsatellite loci (Mb-mac4, Mb-mac19, Mb-mac10, Mb-mac17, Mb-mac40, Mb-mac84 and Mb-mac64) were genotyped for 165 individuals, according to Becquet et al. (2009). We have attempted to genotype all individuals for which a COI sequence was obtained. All individuals for whom more than 3 microsatellite loci failed to amplify were removed from the analysis (Table S1).

Phylogenetic analyses were performed on mitochondrial sequences with the Network software v.4.0.0.0 (Bandelt et al., 1999) that builds haplotype networks based on the median-joining

algorithm (Cassens et al., 2003). Pairwise linkage disequilibrium between microsatellite loci was tested using GENEPOP version 4.0.10 (Rousset, 2008), and the significance of each test was evaluated using a Markov-chain randomization procedure (MCMC) with 1000 dememorization steps, 100 batches and 1000 iterations per batch. The numbers of alleles per population and locus (N_{all}), and the observed (H_o) and expected (H_e) heterozygoties under Hardy-Weinberg equilibrium were calculated using Genetix 4.05.2. (Belkhir et al., 1996-2004). Allelic richness (R_{all}) was computed using FSTAT 2.9.3.2 (Goudet, 2002) to account for differences in sampling sizes across populations. Fixation indices (F_{IS}, F_{IT}, F_{ST}) were calculated using Genetix 4.05.2 according to Weir & Cockerham (1984). The significance of pairwise population differentiation, as measured by F_{ST}, was estimated based on 1000 permutations. Population self-assignment tests were conducted using Geneclass v2.0 (Piry et al., 2004), based on the Bayesian method of Rannala & Mountain (1997), and incorporating the exclusion-simulation significance test of Cornuet et al. (1999). The level of statistical significance was set to $\alpha = 0.05$.

Spatial population structure was further explored using bayesian clustering analyses. These analyses were performed using the BYM admixture model implemented in TESS v.2.3 (Durand et al., 2009), using 12,000 MCMC steps and a burnin period of 2,000 steps. Based on the results of preliminary test runs, the presence of two to 4 genetic clusters was evaluated, with 10 replicate runs of each Kmax (spatial interaction parameter set to 0.6, linear degree of trend). The most likely Kmax was selected based on the Deviance Information Criterion (DIC), by minimizing its value and its variance, and by examining plots of individual membership probabilities. The most likely run among 10 replicates was then selected based on DIC.

Results

A total of 32 COI haplotypes were detected among 232 individuals, 6 of which being putatively endemic to the Baltic Sea. Three closely-related clades were observed: clade 3, clade 4 and clade 8, separated by 1 to 3 mutations along the 313 bp examined (Fig. 1 and Fig. 2). clade 4, characterized by 12 haplotypes, was the most prevalent group, with 123 individuals. clade 3 (12 haplotypes) and clade

8 (6 haplotypes) group 35 and 70 individuals, respectively. There was no spatial or bathymetric trend in the distribution of haplotypes (Fig. 1 and Fig. 2).

A multilocus nuclear genotype was obtained for 165 individuals collected at 11 sampling sites with seven microsatellite markers. No linkage disequilibrium was detected ($p \geq 0.2$). Overall, the number of alleles (N_{all}) ranged from 2.5 (SW40) to 6.8 (HEL60 and MW30) with a total of 11.2 over the whole dataset (Table 2). There was little variation in allele number between populations (mean = 5.1 ± 1.7), as confirmed by the allelic richness analysis (R_{all}); R_{all} varied from 1.5 (SW40) to 1.6 (HEL45) even in the population with small number of individuals (HEL85, SW40, SW60, mean value = 1.6 ± 0.03). The observed heterozygosity (H_o) varied from 0.3 (SW5) to 0.5 (SW30) and the mean value obtained for the 11 populations was 0.4 (standard error = 0.1). The expected heterozygosity (H_e) showed little variation across the whole dataset (mean and standard deviation across the 11 populations equals 0.538 ± 0.048) and the heterozygote deficiency was quite high for all populations (mean F_{IS} value and standard deviation equals 0.334 ± 0.135).

At the regional scale, the overall structure observed (F_{IT} = 0.557) was essentially due to the high heterozygote deficiency within populations (F_{IS} = 0.554) as genetic differentiation between populations was quite low (F_{ST} = 0.007, p = 0.27). Pairwise differentiation between populations was globally non significant (data not shown). Nevertheless, the SW60 population was significantly divergent from HEL60, SW5, SW10, SW20, SW30, GN40 and MW30 ($0.094 < F_{ST} < 0.186$). Bayesian assignment of individuals to populations highlighted a great rate of self-recruitment varying from 30% (MW30) to 87% (SW60) with a mean value of 57% (Fig. 3). Excluding HEL85 (50%), the self-recruitment rate was function of depth (Linear regression test between paired samples, $R^2 = 0.65$, t = 2.45, df = 8, p = 0.039, Fig.4). This result was confirmed for the Vistula populations (SW5 to SW60) for which self-recruitment rate varied from 40% for SW5 to 87% for SW60 (Fig. 3 and Fig. 4). Furthermore, beyond 40 m depth, this rate was higher than 80% (40% < SW5-SW30 < 50% and 80% < SW40-SW60 < 87%). Thus, no individual from the shallowest sites were recruited in populations located between 30 and 60 m depth (Fig. 3).

Bayesian clustering analyses suggested the presence of four clusters among 165 individuals examined. No spatial or bathymetric trend could be detected among these clusters.

Mapping clades 3, 4 and 8 onto these clusters revealed no association between multi-locus genotypes and haplotypes, suggesting strong introgression (Fig. S1).

Discussion

1- Genetic structure at the regional scale: the Gulf of Gdañsk

1-1 Large genetic diversity and depth stratification

Populations in the Gulf of Gdañsk were characterized by a large gene diversity ($0.440 < H_e < 0.591$). These diversity levels were similar to that obtained by Becquet et al. (2012), who observed a mean H_e of 0.63 among 18 European populations sampled from France to Russia. As their European counterparts, Baltic populations were heterozygote deficient ($F_{IS} = 0.55$), as currently observed in marine mollusks (Zouros & Foltz, 1984). However, populations were probably not subjected to inbreeding, given their high genetic diversity. In comparison with European populations (mean Rall = 4.39), the allelic richness in the Gulf of Gdañsk was reduced ($1.53 < Rall < 1.62$). This result might be due to the rarefaction method used to standardize allele counts (El Mousadik & Petit, 1996), which is known to underestimate the allelic richness especially when there is a large difference in the sample size among populations ($5 < N < 30$).

No genetic structure was detected among shallow-water subpopulations. In addition to tests of significance on fixation statistics, TESS runs confirmed the absence of genetic structuring. This result was probably linked to the ability of *M. balthica* larvae to disperse over long distances (e.g. Günther et al., 1998). Private alleles were not correlated with environmental factors (salinity, pollution…), but gene flow was reduced between deep (40-50 m depth) and shallow stations. This pattern, concordant with previous results (Hummel et al., 2000), is probably linked to the thermo-haline stratification of water masses occurring in the Gulf of Gdañsk at around 40 meters (Cyberska et al., 1998, Rheinheimer, 1995, Rumhor et al., 1996).

174 **1-2 Strong self-recruitment**

175 The assignment probability of individuals to each population highlights a great rate of self-recruitment
176 (30 to 87%). It is now well accepted that panmixia is not the rule in the marine environment (Swearer
177 et al., 2002, Hellberg et al., 2002). On the contrary, larvae may recruit locally, because (i) spatio-
178 temporals movements of water masses influence larval trajectories (Gaines et al., 2003, Siegel et al.,
179 2003), (ii) survival conditions vary in space and time (Gaines & Bertness, 1992, Morgan et al., 2000,
180 Ellien et al., 2004) and (iii) reproductive success is random (Hedgecock, 1994a, 1994b). The thermo-
181 halocline present in the Bay of Gdañsk might be a strong vertical barrier to larval exchange and might
182 explain the different self-recruitment rate for populations living above or below this cline. The low
183 self-recruitment rate recorded at HEL85 (50% compared to > 70% at other deep-water locations), does
184 not contradict this hypothesis, and may be explained by the specific sea-floor morphological features
185 found near the Hel Cape. Indeed, there is a relatively steep slope descending to a depth of 50+ m on
186 the open-sea side of the cape (Rucinska-Zjadacz & Rudowski, 2009). Upwelling and downwelling
187 events occur often in this region (Kowalewski & Ostrowski, 2005) and maybe responsible for cross-
188 slope larval transport.

189

190 **2- Genetic structure at the local scale, among Vistula populations**

191

192 **2-1 Asymmetric gene flow**

193 Genetic connectivity among Vistula populations from different depths was asymmetric, as gene flow
194 was stronger from deep to shallow stations (Fig. 3). Even if these populations are geographically close
195 to each other (horizontal distance is about 7 km), they are located along a clear environmental gradient
196 (Table 1). While shallower populations (SW5 and SW10) are directly impacted by the freshwater of
197 the Vistula River, carrying heavy metal pollution (Sokolowski et al., 1998, 2002, 2007), deeper
198 populations (> 20 m) are challenged by saltier waters from the open part of the Gulf of Gdañsk and
199 impacted by the release of metallic elements from the upper sediment layer that might exert toxic
200 effects on bivalves (Janas et al., 2004, Sokolowski et al., 2007). Moreover bivalves leaving in the

201 deeper parts of the Gulf of Gdañsk can be subjected to hypoxia or anoxia and even hydrogen sulphide
202 (Janas et al., 2004), because of the strong eutrophication occurring in this zone (Laine, 2003).
203 These heterogeneous environmental conditions might favor chaotic genetic patchiness (Johnson &
204 Black, 1982). Indeed, we observed a greater genetic heterogeneity between close populations (e.g.
205 Vistula populations), than between more distant populations (e.g at the scale of the entire Gulf of
206 Gdañsk populations, Fig. 3). The asymmetric gene flow in the Gulf of Gdañsk might be the result of
207 natural selection impacting larval and/or recruit survival induced by environmental conditions (e.g.
208 Johnson & Black, 1982, 1984, Watts et al., 1990, Hedgecock, 1994a, Edmands et al., 1996, David et
209 al., 1997).
210 The gene flow from deep (30 to 60 m) to shallow populations is probably due to the specific water
211 mass dynamics in the Vistula Estuary. In this salt wedge estuary, a sharp boundary exists between an
212 upper layer of brackish water and an intruding wedge-shaped bottom layer of denser saltwater. Under
213 the typical circulation for such estuary, low salinity surface water moves toward the open sea (flushing
214 larvae away from the mouth of the estuary), whereas sea bottom water moves toward the land (and
215 transporting larvae from deep to shallow). Moreover, the thermo-halocline may act as a physical
216 barrier to larvae dispersal, leading to genetic differentiation at a microscale, as was already found in
217 many marine organisms (e.g. Torrents et al., 2008, Bongaerts et al., 2010, Mokhtar-Jamai et al., 2011).
218 Depth-dependent timing of gonadal development and reproductive period might also influence genetic
219 differentiation. Between 0 and 25 m depth, spawning is regulated by the warming of surface waters
220 following air temperatures changes (Wenne, 1985 and 1993), while at intermediate depths (25 – 35 m)
221 the spawning period is extended because of the longer persistence of colder water temperatures. In the
222 deeper waters (70 – 75 m), spawning is uncorrelated with surface water temperatures and often
223 depends on food availability (Wenne, 1993 after Pierścieniak et al., 2010).
224
225 **2-2 Are SW60 populations subjected to local adaptation?**
226
227 Microsatellite markers revealed a significant genetic differentiation between the deepest SW
228 population (SW60) and others (except with SW40). Moreover, the SW60 population has the highest

229 self-recruitment rate (87%), which further supports the idea of genetic isolation between this
230 population and shallower ones. The fact that SW60 is also characterized by a great genetic diversity
231 (H_e = 0.52) suggests that this population is not declining, or suffering from source-sink dynamics or
232 inbreeding.
233 The challenging environmental conditions recorded at the SW60 site, associated with the high genetic
234 polymorphism and reduced connectivity observed with other sites, may promote local adaptation via
235 divergent natural selection within this population (Hasting, 1983). However, populations in this region
236 deal with many natural (salinity, temperature) and/or anthropogenic stressors (eutrophication, heavy
237 metals contamination) that may have synergistic effects on population structure. It was therefore
238 difficult to distinguish which processes are directly implicated in structuring this deep population, and
239 a more thorough sampling may address this issue in the future. Thus, given the observed variation in
240 spawning time and the existence of physical barriers to gamete and larval dispersal (limited cross
241 fertilization), SW populations may be far from panmixia.
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243
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Figure
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Fig.1: Sampling locations and haplotype distribution in the Gulf of Gdañsk. HEL populations were collected close to the Hel Peninsula, MW30 in the Puck lagoon, GN40 in the main pathway of marine traffic and SW5 to SW60 in the Vistula mouth. Haplotype groups represents the most common haplotypes and their closest relatives (see haplotype network in Fig.2). Haplotypes H3 and H4 correspond to H3 and H4 of Becquet et al. 2012.

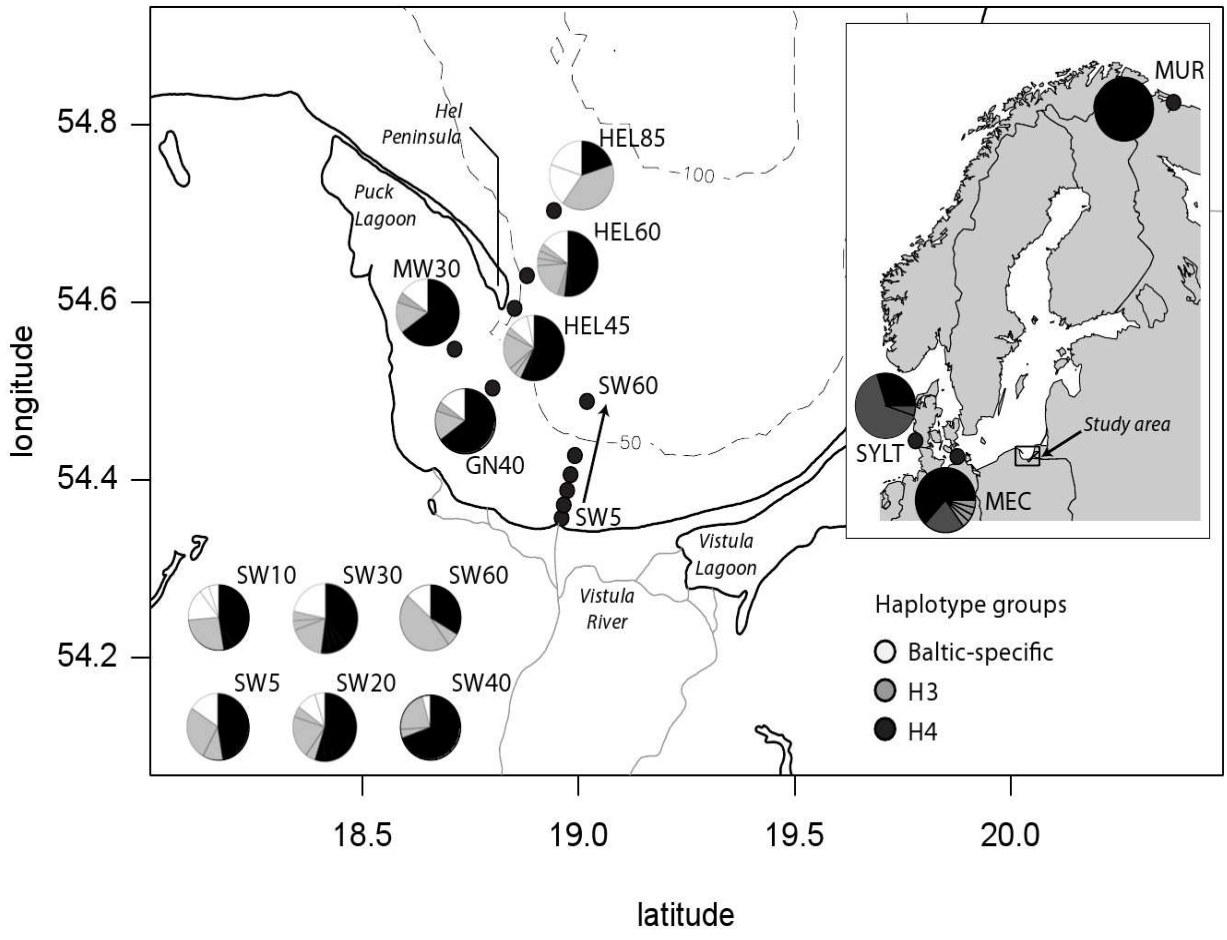


Fig.2: Minimum spanning network displaying mitochondrial variation along a 313 bp of the COI gene. Each circle represents a haplotype. Circle size is proportional to haplotype frequency (n = 30, 52, 106 for H8, H3, H4, respectively; rares haplotypes have a frequency between one and four). Each segment represents a single mutational event. H8 are characteristic of Baltic.

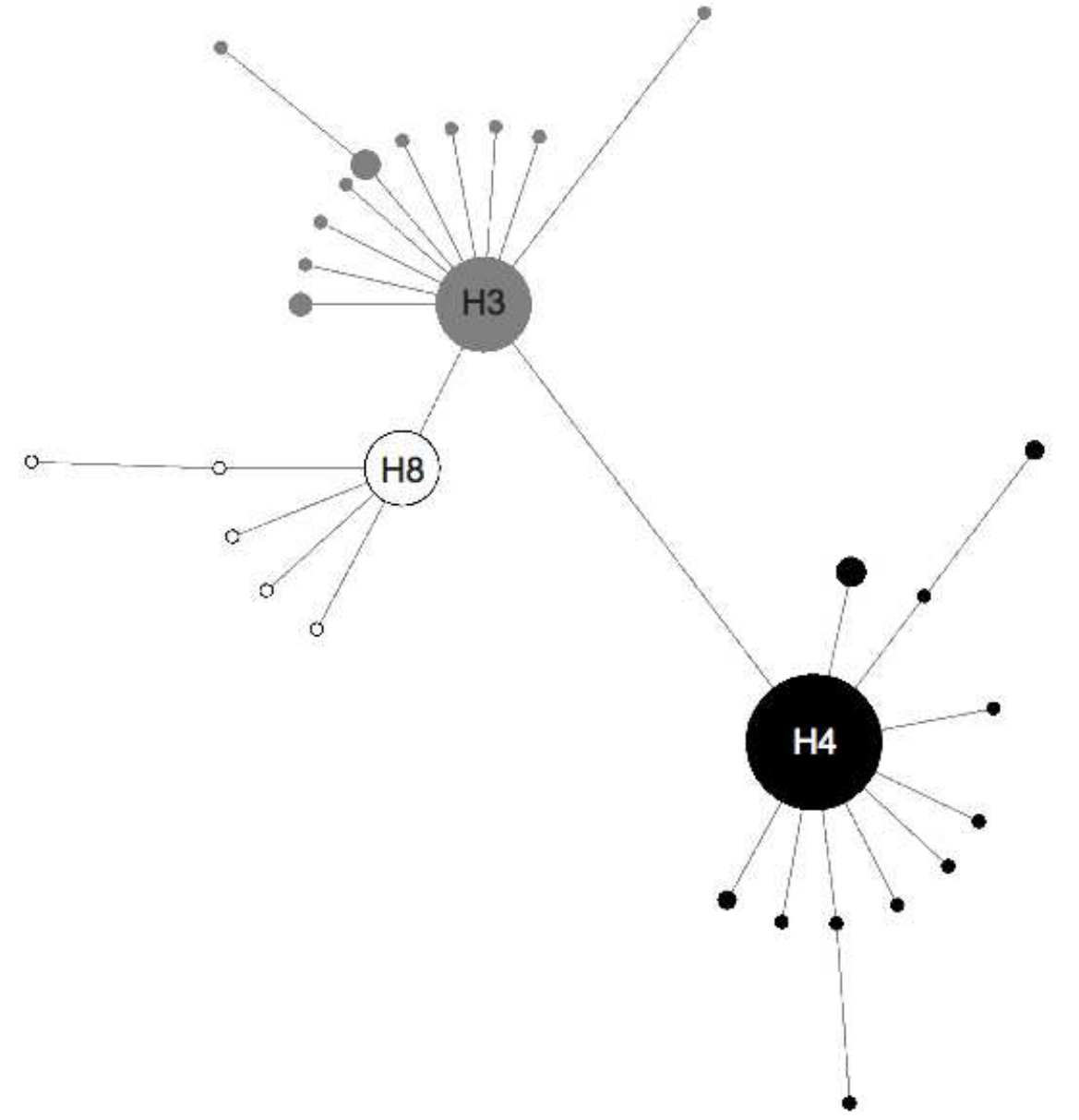


Fig.3: Assignment results. For each population, percentage of individuals was assigned to each potential source (Larval origin) and represented by a square. Square size is a function of a percentage of individuals.

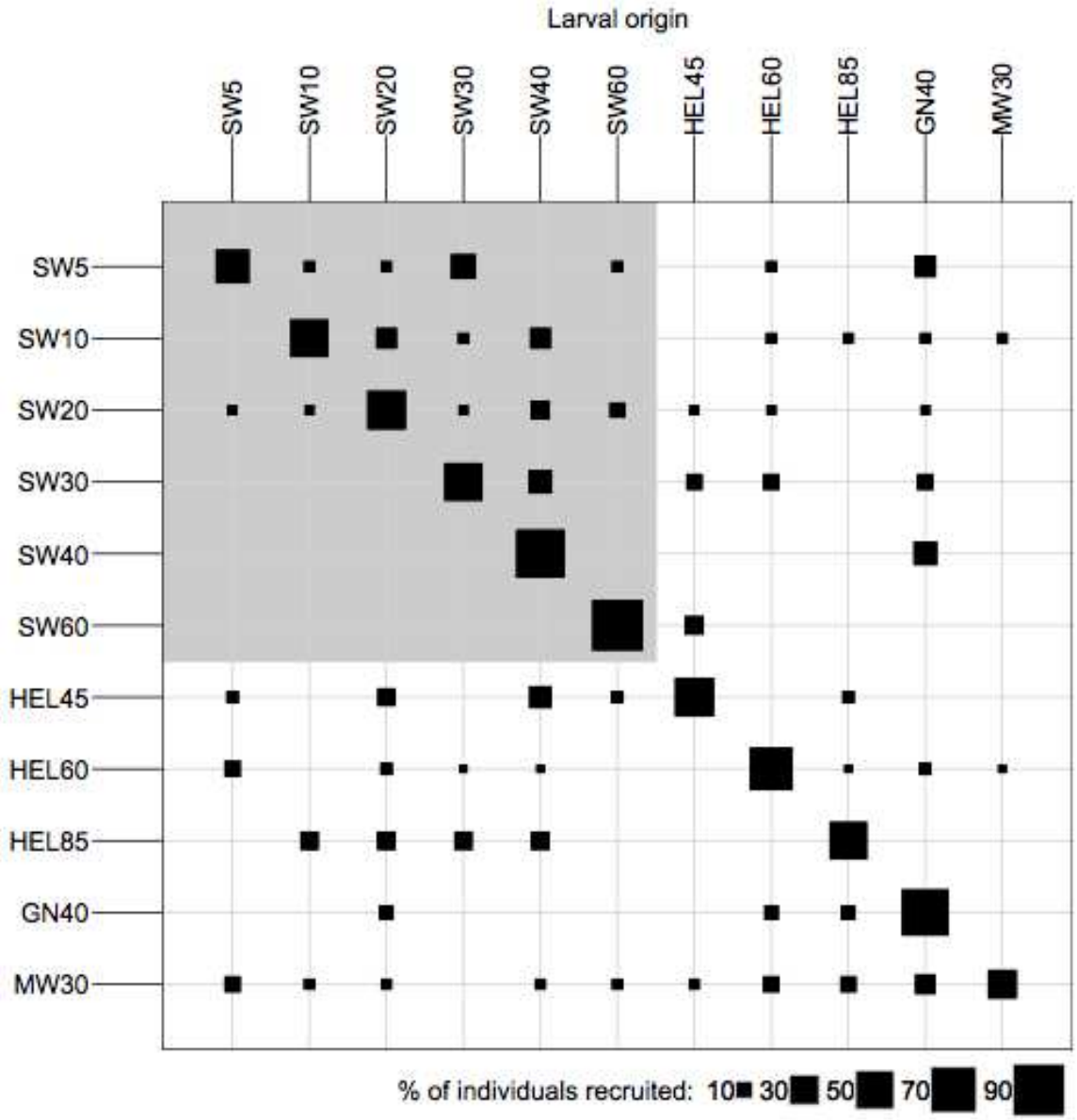
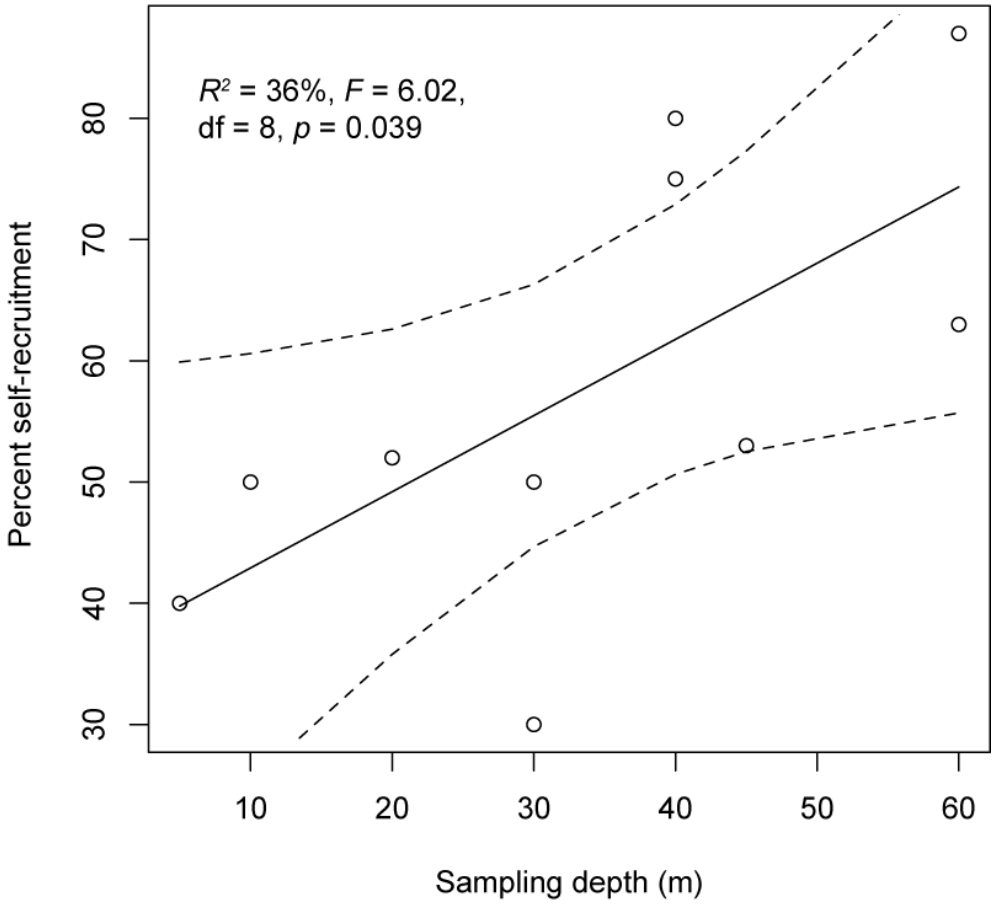


Fig. 4: Relationship between sampling depth and the level of self-recruitment as estimated by GeneClass2. Top left corner: results of the linear regression analysis. R2 is adjusted for the number of variables used in the model.



table
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Table 1: Physico-chemical characteristics of sampling locations in the Vistula river mouth. Data are presented as means. More detailed information about metallic contamination can be found in Sokolowski 1999.

Population Code	Depth (m)	Salinity (PSU)	Temperature (°C)	Specific environmental features
SW60	60	9.6	5.1	Anoxia, hydrogen sulphide contamination, metallic pollutant release from sediments
SW40	40	8.2	13	Thermo-halocline
SW30	30	7.6	14.2	Intermediate zone
SW20	20	7.3	14.2	Vistula inflow
SW10	10	6.7	14.1	Vistula inflow
SW5	5	6.7	14.4	Vistula inflow

Table 2: Nuclear diversity for each sample site. N_{nuc}: number of individuals analyzed for microsatellite markers; N_{all}: allele number; R_{all}: allelic richness; H_o: observed heterozygoty; H_s: gene diversity; F_{IS}: heterozygote deficiency. N_{mito} corresponds to the number of individuals for which a mitochondrial sequence was obtained. See also Table S1.

	N_{nuc}	N_{mito}	N_{all}	R_{all}	H_o	H_s	F_{IS}
HEL85	8	5	4.33	1.58	0.405	0.511	0.328
HEL60	26	28	6.83	1.59	0.408	0.578	0.315
HEL45	17	29	6.16	1.62	0.394	0.591	0.373
GN40	11	24	4.33	1.51	0.313	0.493	0.417
MW30	21	20	6.83	1.61	0.410	0.588	0.382
SW5	18	19	5.14	1.56	0.282	0.534	0.539
SW10	18	20	5.83	1.60	0.461	0.586	0.255
SW20	23	21	5.83	1.58	0.370	0.565	0.373
SW30	10	25	3.83	1.58	0.535	0.519	0.049
SW40	5	23	2.5	1.53	0.447	0.440	0.183
SW60	8	18	4.16	1.55	0.317	0.520	0.464
Total	165	232	11.16	1.61	0.281	0.600	0.558
Mean	15.45	21.09	5.07	1.57	0.394	0.538	0.334
(S.D.)	7.59	6.42	1.36	0.03	0.073	0.048	0.135