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Differential modes of selection on the rhodopsin gene in coastal Baltic and North Sea populations of the sand goby, *Pomatoschistus minutus*

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Abstract

An excellent model to elucidate the mechanisms and importance of evolution in the marine environment is the spectral tuning mechanism of the visual pigment in vertebrates. In the sand goby *Pomatoschistus minutus* (Teleostei; Gobiidae), a distribution-wide study showed that spatial variation at the rhodopsin gene (*RH1*) matches the characteristics of specific light environments. This match suggests that populations are locally adapted to selective light regimes targeting the *RH1* gene. If so, then the direction of selection should depend on the regional spatial and temporal stability of the light conditions. We tested this prediction by comparing goby populations from two regions: the Baltic Sea, characterized by divergent, but temporally stable light conditions, and the North Sea, characterized by locally heterogeneous and temporally variable light conditions. *RH1* sequences of 491 *Pomatoschistus minutus* individuals from 15 locations were analysed. We found that variation at the *RH1* gene in the Baltic populations showed signatures of diversifying selection, whereas the *RH1* gene in the North Sea showed signatures of stabilizing selection. These different modes of selection are consistent with the regional light conditions and hence support our predictions, but may also be influenced by migration between the open sea and more turbid estuarine environments. An interesting observation is that within one gene, synonymous and non-synonymous SNPs show a totally different pattern between populations. Population differentiation based on non-synonymous SNPs of the *RH1* gene correlated with spectral variation of the local environment of the sand goby populations. In contrast, the differentiation based on synonymous SNPs of *RH1* reflects more the neutral historical pattern of the species.

Keywords: adaptive evolution, candidate genes, *de novo* mutation, Gobiidae, marine fish, vision

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Introduction

Understanding the genetic basis of local adaptation is of prime interest in biology as it involves the role of natural selection in promoting evolutionary change (Mayr 1963). The common belief was that local adaptation may be rare or absent in marine organisms because gene flow is expected to slow down adaptive population divergence (Hemmer-Hansen *et al.* 2007). Local adaptation in marine organisms has become increasingly documented, indicating that natural selection may be a potent evolutionary force in the 'open' ocean (Canino *et al.* 2005; Pampoulie *et al.* 2006; Hemmer-Hansen *et al.* 2007; Sherman & Ayre 2008). Nevertheless, knowledge of the spatial and temporal scale of adaptive

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genetic variation in marine systems remains scant, yet crucial to improve our understanding of how evolution operates in the ocean (Conover *et al.* 2006; Zane 2007).

Adaptive evolution in the ocean remains poorly known because of the scarcity of genetic systems to evaluate natural selection (Yokoyama 2002). One of the few well known models to elucidate the mechanism and importance of selection as evolutionary force is the spectral tuning mechanism of the visual pigment (VP) in marine vertebrates (Yokoyama 2000). This model can identify specific amino acid (AA) changes that are responsible for the adaptation of organisms to specific environments. VP molecules in vertebrates are bound in dense membrane stacks in retinal photoreceptors to mediate vision. The VP protein moiety is opsin, a G protein linked receptor bound to a light-sensitive chromophore (Park *et al.* 2008). Each pigment shows a characteristic peak of maximal absorbance (λ_{max}), its precise location depending on the interactions between the chromophore and the opsin protein. VP may evolve different λ_{max} values through functional mutations in the opsin DNA (Bowmaker 2008). Evolutionary adaptation of VP genes has mainly been established in phylogenetic studies of marine taxa (Hunt *et al.* 2001; Yokoyama & Takenaka 2004; Larmuseau *et al.* 2010a) and recently also in a population genetics analysis on the marine sand goby *Pomatoschistus minutus* (Teleostei, Gobiidae) (Larmuseau *et al.* 2009a).

The sand goby *P. minutus* is a relatively small demersal species that is abundant along European coasts and estuaries (Bouchereau & Guelorget 1998). It forms an important ecological link between benthic invertebrates and larger predatory fish such as cod and whiting (Maes *et al.* 2003). The sand goby is a visual predator with mostly nocturnal activity (Healey 1971; Aarnio & Bonsdorff 1993). Its nocturnal foraging has advantages for approaching prey and avoiding predators (Ehrenberg & Ejdung 2008). Therefore this lifestyle requires good sight in very dim light (Thetmeyer 1997). However, the geographical distribution of the sand goby encompasses a wide range of photic environments, varying in turbidity, colour and brightness. A recent study on the sand goby investigated the intraspecific variation of the rhodopsin (*RH1*) gene, which is crucial for dim light vision in vertebrates (Larmuseau *et al.* 2009a). The study revealed a strong, distribution-wide correlation between the *RH1* gene's spectral tuning sites and dim light environments, quantified as the 'wavelength of the maximum transmitted light' (WMTL). Specifically, sand gobies of the Iberian Peninsula, the Bay of Biscay and Irish Sea were adapted to mainly blue light conditions at the *RH1* gene; individuals of the southern North Sea were adapted to greener water; and sand gobies of the Baltic Sea and the Mediterranean lagoons were locally adapted to water with relatively high WMTL values. The population differentiation of *P. minutus* based on *RH1* variation was significantly in strong discrepancy with the neutral differentiation based on nuclear microsatellites and mitochondrial data. Using Mantel tests, Procrustes analyses and F_{ST} outlier methods, random processes were ruled out to explain the functional differentiation on the *RH1* gene between sand goby populations. Furthermore, codon-based models of molecular evolution detected significant signals of positive Darwinian selection on the *RH1* gene and identified three individual sites as probable targets of selection causing a shift of λ_{max} of VPs (Larmuseau *et al.* 2009a). The study on the sand goby was the first population genetic analysis of intraspecific variation in an opsin gene in natural populations and provided evidence of selection acting on spectral sensitivity (Ebert & Rose 2009).

The possibility of local adaptation at the *RH1* gene of the sand goby was assessed at a distribution-wide scale, including populations showing deep divergence at the mtDNA level (Larmuseau *et al.* 2009b). However, the processes generating adaptive divergence likely act at smaller geographical and temporal scales. Therefore, the spectral tuning mechanism on the rhodopsin of *P. minutus* is as well an optimal genetic system to evaluate natural selection on regional spatial and temporal scale. If the match between WMTL and *RH1* gene variants suggests that populations are locally adapted to selective light regimes targeting the *RH1* gene, then the direction of selection should depend on the degree of spectral heterogeneity at the regional level, including the spatial and temporal stability of the light conditions. In particular, among populations from divergent, but temporally stable light environments, functional variation at the *RH1* gene should show signals of diversifying selection, which may lead to local adaptation (Nielsen 2005). In contrast, functional variation at the *RH1* gene among populations from locally heterogeneous and temporally variable light environments should show signals of stabilizing selection and the absence of local adaptation (Nielsen 2005; Charlesworth 2006).

The aim of Larmuseau *et al.* (2009a) was to assess whether variation in dim light vision in sand gobies has a genetic basis on the rhodopsin gene (*RH1*), and hence whether they are evolutionary adapted to local photic environments. In this study, we want to go further by documenting the differences in mode of selection at the *RH1* gene between the sand goby populations within different marine systems due to regional spectral heterogeneity. This should provide a more mechanistic understanding of natural selection and local adaptation in the marine environment. We compare goby populations from two regions: the Baltic Sea, characterized by divergent, temporally stable light conditions, and the

North Sea, characterized by locally heterogeneous and temporally variable light conditions. The Baltic Sea is highly turbid and is therefore characterized by much higher WMTL values than along other European coasts. Within the Baltic Sea, there are local differences in WMTL with a general trend of higher turbidity in the Northern part than in the South (Jerlov 1976; Lindström 2000; Audzijonyte *et al.* 2005). This spectral pattern in the Baltic is temporally stable and does not fluctuate significantly during seasons (Jerlov 1976; Larmuseau *et al.* 2009a). In contrast, the WMTL-values in the North Sea area with its highly turbid estuaries change considerably during seasons. However, these circumstances are not highly different between several locations within the North Sea (Jerlov 1976; Larmuseau *et al.* 2009a). Therefore, we will test in this study the particular hypothesis that sand goby populations in the Baltic Sea are specifically adapted on the *RH1* gene to the local photic environment under diversifying selection, in contrast to the North Sea populations that are not differentiated and maintain a high level of polymorphism on *RH1* as expected in the context of balancing selection.

Materials and methods

Sampling and species identification

A total of 491 *Pomatoschistus minutus* individuals were caught at 15 locations along the coasts and estuaries of the Baltic-North Sea region (Table 1; Fig. 1). Samples were taken either by fyke, hand net, beam trawl or from the cooling-water intake screens of nuclear power plants (Borssele and Doel). Samples TBS (northern Baltic Sea) and PBS (southern Baltic Sea) were included in Larmuseau *et al.* (2009a). Sand gobies were distinguished from other cryptic *Pomatoschistus* species morphologically based on the dermal head papillae (Miller 1986) and pigmentation pattern (Hamerlynck 1990), and genetically, based on a PCR-RFLP species-specific identification protocol (Larmuseau *et al.* 2008).

Gene amplification and sequencing

Genomic DNA was extracted from fin clips, stored in 100% ethanol, using the NucleoSpin Extraction Kit (Machery-Nagel GmBH, Düren, Germany). A 548 bp fragment of the *RH1* gene was amplified in polymerase chain reactions (PCR) with the forward primer LMCercleBrF GTCCTGGCTGTTGAGAGGTG and the reverse primer LMCercleBrR TGCTTGTTCATGCAGATGTAG. The primers were designed using the PRIMER 3 program (Rozen & Skaletsky 1998) on conserved regions of the alignment of *RH1* gene sequences from *P. minutus* (GenBank acc. no. X62405), *Gobius niger* (Y18675), *Zeus faber* (Y14484), *Sargocentron diadema* (U57537) and *S. microstoma* (U57542). The amplified fragment of the *RH1* gene contains all non-synonymous mutations and seven of the eight polymorphic synonymous SNPs that were observed in *P. minutus* individuals covering the full species distribution (Larmuseau *et al.* 2009a). PCR reactions were carried out on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl, containing 1 μl of genomic DNA, 1 X PCR buffer, 0.2 mM dNTPs, 0.8 μM of each primer, 2.0 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Silverstar, Eurogentec, Seraing, Belgium) and mQ–H_2O. The PCR

Table 1 Overview of the *Pomatoschistus minutus* samples collected at 15 sampling sites along the coasts of the North Sea and Baltic Sea

Code	Area	Country	Location	Date	Coordinates	*N*
NOS	Bothnian Sea	Sweden	Nordmaling	July 2008	63°24′ N-19°45′ E	28
TBS	Northern Baltic Sea	Finland	Tvärminne, Vargskar island	July 2006	59°50′ N-23°12′ E	20
HBS	Northern Baltic Sea	Sweden	Nasaäng	October 2005	58°59′ N-17°27′ E	26
PBS	Southern Baltic Sea	Poland	Sopot, Bay of Gdańsk	February 2007	54°27′ N-18°36′ E	10
WCS	Kattegat	Sweden	Bökevik Bay, Skaftö island	July 2006	58°14′ N-11°26′ E	30
BEN	Northern North Sea	Norway	Bergen	July 2008	60°16′ N-04°59′ E	20
NNS	Southern North Sea	Netherlands	Stuifdijk	March 2003	52°58′ N-04°42′ E	36
BAH	Southern North Sea	Netherlands	Balgzand	August 2007	52°56′ N-04°53′ E	40
DOM	Southern North Sea	Netherlands	Domburg	March 2004	51°34′ N-03°29′ E	42
OSO	Oosterschelde	Netherlands	Kattendijke	April 2004	51°33′ N-03°58′ E	42
BOR	Scheldt Estuary	Netherlands	Borssele	March 2004	51°24′ N-03°43′ E	43
DOE	Scheldt Estuary	Belgium	Doel	March 2004	51°18′ N-04°16′ E	39
OOS	Southern North Sea	Belgium	Oostende	March 2005	51°15′ N-02°55′ E	42
NBF	Southern North Sea	Belgium	Oostduinkerke	August 2008	51°08′ N-02°39′ E	39
DEP	Southern North Sea	Belgium	De Panne	March 2004	51°06′ N-02°34′ E	34

N, sample size. Sampling sites TBS and PBS are included in Larmuseau *et al.* (2009).

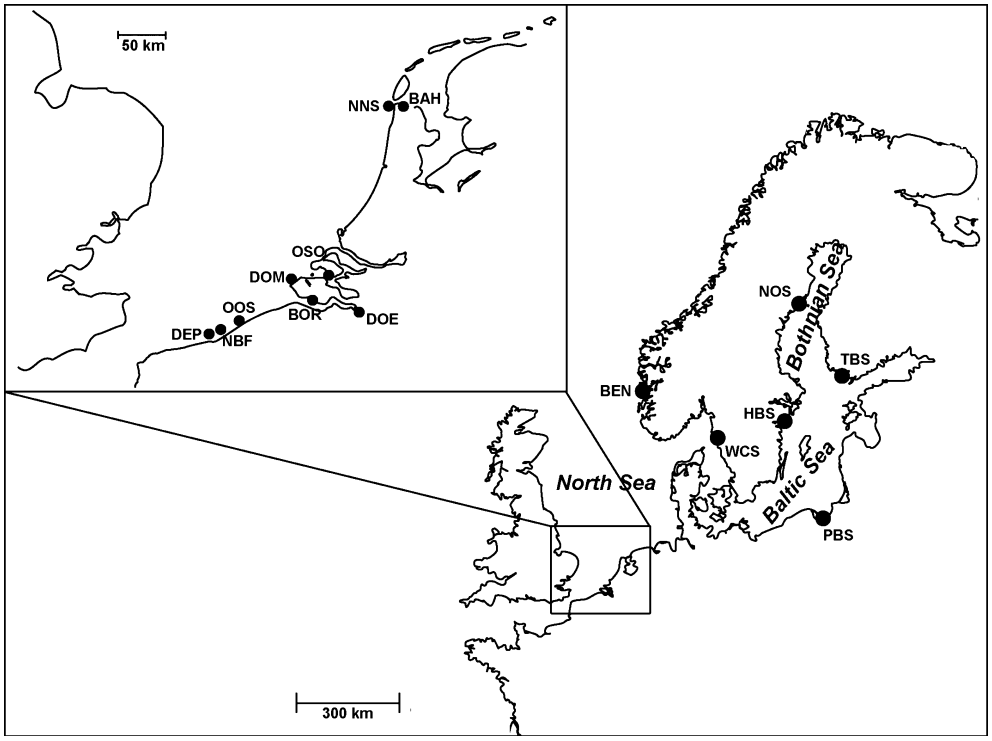

Fig. 1 Geographical distribution of 15 sampling locations in the Baltic-North Sea region for the sand goby, *Pomatoschistus minutus*. See Table 1 for specifications of the sampling locations.

profile was: 4 min at 94 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 56 °C and 1 min at 72 °C; with a final 10 min extension at 72 °C. To avoid contamination, separate pipettes, aerosol barrier tips and sections of the laboratory were used for pre- and post-PCR work. Every 15th individual (corresponding with one every two rows of a PCR-plate) a negative control was inserted to detect contamination. No contamination occurred during the screening procedures. All PCR products were visualized on agarose gel with ethidium bromide. After purification with the 'GFX PCR DNA and Gel Band Purification kit' (GE Healthcare, Piscataway, NJ, USA), the PCR products were sequenced in both directions using the BigDye Terminator v. 3.1 Cycle Sequencing Kit on an ABI 3130 automated capillary DNA sequencer (Applied Biosystems). Sequences were checked and aligned to each other with SEQSCAPE v. 2.1 (Applied Biosystems). Automated detection of point mutations was realized with the GAP4 subprogram embedded in the STADEN package v. 1.7 (http://sourceforge.net/projects/staden) and rechecked manually by eye.

Haplotypes were inferred from the genotypes using the Bayesian statistical methods in the program PHASE v. 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). Runs were conducted separately for each population, with known haplotype information being included as prior information. Ten independent runs per population were conducted, each with a burn-in-period of 1000 followed by 10 000 iterations with a thinning interval of 100 steps. The estimates and the goodness-of-fit values were consistent among runs, as recommended by the instructions for PHASE, indicating that the run lengths were sufficient.

Population diversity and tests for differing modes of selection

The number of segregating sites (S), the mean number of pairwise differences (k) and estimates of nucleotide polymorphism (π, θ) were calculated for the haplotype data of the *RH1* gene using DnaSP v. 4.10.9 (Rozas *et al.* 2003). Three different methods were used using DnaSP to test the null hypothesis that mutational patterns in the *RH1* gene were as expected under a model of neutral evolution: Tajima's D (Tajima 1989), Fu & Li's D^* and Fu & Li's F^* (Fu & Li 1993). Tests for Hardy–Weinberg equilibrium (HWE) were calculated for each SNP with FSTAT v. 2.9.3 (Goudet 2001).

Genetic differentiation

Four different methods were used to compare population genetic substructure between the *P. minutus* samples from the marine systems. Each analysis was

performed on all synonymous and non-synonymous SNPs of the *RH1* fragment together and separately. First, global and pairwise population differentiation was quantified in GENETIX v. 4.05 (Belkhir *et al.* 2004) using the standardized allelic variation F_{ST}, estimated as θ (Weir & Cockerham 1984). Pairwise genetic distances were calculated according to Cavalli-Sforza & Edwards (1967) (D_{CE}) with GENETIX. F_{ST}s and D_{CE}s were tested for significance against 10^4 random permutations of the data in GENETIX. Second, pairwise genetic distances were used in a non-metric multidimensional scaling analysis (NMDS) to reveal group structure in STATISTICA v. 6.0 (StatSoft, Inc., Tulsa, OK, USA). Third, the correlation between the genetic and geographic distances among all 15 samples of the dataset and among the samples from populations of the southern North Sea was tested using a Mantel test with 1000 permutations in GENETIX. Geographical distances were measured as the shortest coastal distances between sites using the electronic atlas Google Earth (http://earth.google.com). Fourth, the potential geographical zones associated with genetic discontinuities across the total region were further investigated using Monmonier's algorithm of maximum differences implemented in the program BARRIERS v. 2.2. (Manni *et al.* 2004). These landscape genetics analyses were run on 100 resampled bootstrapped matrices of the pairwise F_{ST} data to assess the robustness of the barriers.

Results

Nucleotide diversity and neutrality tests of the RH1 gene

The sequences matched the general properties of the *Pomatoschistus minutus RH1* gene (X62405) (Archer *et al.* 1992). In total, 19 segregating sites or SNPs appeared among genotypes (Table S1; acc. nr. HM037280–HM037341); 14 SNPs had already been observed for the sand goby and are labelled following Larmuseau *et al.* (2009a). Five synonymous SNPs (SNP 13b, 13t, 13q, 17b, 17t) were observed for the first time but they were not polymorphic according to the 99% criterion. The alignment in AAs showed five polymorphic AA, four located in the transmembrane helices and one in the C-II loop of the RH1 opsin. In total, there were six non-synonymous SNPs because two of them are part of the same codon (Table 2). PHASE analysis confidently resolved 35 haplotypes (Table S2), which were called 'haplogroups' to distinguish them from the longer haplotypes of Larmuseau *et al.* (2009a).

The lowest *RH1* nucleotide diversity (π) values were found in samples from the Bothnian and Baltic seas (0.00131–0.00267), the highest values were found in samples from the North Sea and Kattegat (0.00487–0.00719). The value for DOE (Scheldt estuary) was much lower than the values for the other samples from

Table 2 Diversity indices and neutrality tests for the 15 samples of *Pomatoschistus minutus* in the Baltic-North Sea region based on haplotype data of the rhodopsin gene.

		No. of haplogroups								Neutrality tests statistics		
Population	*N*	Total	Private	Sn	Ss	*k*	π	θ	F_{IS}	Tajima's *D*	Fu & Li's *D**	Fu & Li's *F**
NOS	28	6	1	3	1	0.719	0.001 ± 0.0003	0.002	−0.087	−0.38475	0.99343	0.66240
TBS	20	8	2	5	3	1.463	0.003 ± 0.0004	0.003	0.050	−0.63384	−2.08730*	−0.91090
HBS	26	5	1	3	2	0.864	0.002 ± 0.0002	0.002	−0.004	−0.52622	−0.84487	−0.87245
PBS	10	4	1	4	2	0.895	0.002 ± 0.0006	0.003	0.265	−1.50085	−2.25841*	−2.36203*
WCS	30	10	1	5	5	2.842	0.005 ± 0.0004	0.004	−0.009	0.90129	0.75443	0.94909
BEN	20	10	3	4	5	2.745	0.005 ± 0.0006	0.003	−0.277*	1.31037	1.31111	1.53719
NNS	36	6	1	5	6	3.642	0.007 ± 0.0006	0.004	0.009	1.65708	0.18772	0.82011
BAH	40	6	0	4	5	3.155	0.006 ± 0.0007	0.003	−0.265*	1.90112*	1.33093*	1.80436*
DOM	42	9	1	5	7	3.939	0.007 ± 0.0005	0.004	0.109	1.74758*	−0.34694	0.46099
OSO	42	7	0	4	5	3.826	0.007 ± 0.0005	0.003	−0.086	2.88740**	1.32832	2.20271**
BOR	43	8	1	4	6	3.818	0.007 ± 0.0005	0.004	0.011	2.40176*	0.69434	1.52244*
DOE	39	8	3	4	8	2.670	0.005 ± 0.0008	0.004	0.077	0.26527	−0.31851	−0.13636
OOS	42	7	0	5	5	3.781	0.007 ± 0.0005	0.004	0.038	2.33820*	0.69829	1.49788*
NBF	39	5	0	4	5	3.716	0.007 ± 0.0006	0.003	0.092	2.68186**	1.33225*	2.11796**
DEP	34	3	0	4	5	3.604	0.007 ± 0.0007	0.003	0.022	2.43457*	1.33907*	2.01612**

N, number of individuals surveyed; Sn, number of non-synonymous segregating sites; Ss, number of synonymous segregating sites; *k*, mean number of pairwise differences; π, average number of nucleotide differences per site; θ, theta value per site; F_{IS}, intropulation fixation index. For site abbreviations see Table 1. *p < 0.05, **p < 0.01

the North Sea (Table 2). As shown in Table 2, almost all the neutrality statistic were negative for the samples from the Bothnian and Baltic seas, a pattern consistent with positive selection, a population expansion or purifying selection on slightly deleterious alleles. Almost all the neutrality tests were positive for the samples of the North Sea suggesting balancing selection on the *RH1* gene or the presence of genetic structuring in the analysed samples. No significant deviation from HWE was found for each SNP after correction for multiple testing.

Genetic differentiation

Non-synonymous SNPs showed strong shifts in allele frequencies, and hence AAs, between the Baltic and North Sea samples, especially for AA151, AA261 and AA299 (Table 3). Within the Baltic Sea, strong shifts in AAs were detected between samples, especially on AA217 and AA261. Within the North Sea and Kattegat, only small shifts in allele frequencies were observed. The most divergent sample in the North Sea was DOE (Scheldt estuary), which had a much higher frequency for alanine on AA214 and AA299 than samples from the North Sea (Table 3). Pearson's chi-square tests confirmed this trend for both AA sites between the sample DOE and the other North Sea samples ($\chi^2 = 0.917$, df = 2, $p < 0.05$ for AA214; $\chi^2 = 1.182$, df = 2, $p < 0.05$ for AA299).

Four statistical methods were used to compare *RH1*-based population structure between and within both marine systems. First, the highest pairwise F_{ST}-values based on all SNPs were shared between the Baltic and North Sea samples (Table S3). Remarkably, pairwise F_{ST}-values between Baltic and North Sea samples were twice as large based on non-synonymous SNPs as for synonymous SNPs (Table S4). Pairwise F_{ST}-values between samples of the Baltic and Bothnian Sea were significant for all polymorphic sites (mean F_{ST}-value = 0.142; $p < 0.05$) and for only the six non-synonymous SNPs (mean F_{ST}-value = 0.165; $p < 0.05$) but not for only the synonymous SNPs (mean F_{ST}-value = −0.020; $p > 0.05$). Pairwise F_{ST}-values between North Sea samples revealed two clusters of samples based on all SNPs of *RH1*: a northern North Sea group including the samples of Bergen (BER) and Kattegat (WCS), and the southern North Sea group with all samples from Belgium and The Netherlands (NNS, BAH, DOM, OSO, BOR, DOE, OOS, NBF and DEP) (Table S3). Pairwise F_{ST}-values between samples from the northern North Sea and southern North Sea were significant based on synonymous SNPs (mean F_{ST}-value = 0.131; $p < 0.05$), but not on non-synonymous SNPs (mean F_{ST}-value = 0.011; p-value > 0.05) (Table S4). None of the pairwise F_{ST}-values in the southern North Sea based on the three categories of SNPs were significant, except for several sample pairs including sample DOE (Scheldt estuary) (Table S3).

Second, the NMDS plots based on D_{CE} distances of all SNPs revealed three groups: the Baltic group with the samples from the Baltic and Bothnian seas (NOS, TBS, HBS and PBS), the northern North Sea group with the samples from Bergen (BER) and Kattegat (WCS), and the southern North Sea group with samples from Belgium and The Netherlands (NNS, BAH, DOM, OSO, BOR, DOE, OOS, NBF and DEP) (Fig. 2a). The NMDS analysis also confirmed that the group with the Baltic

Table 3 Frequency of amino acid substitutions detected at the rhodopsin gene of sand gobies from 15 sampling sites

	Population NOS	TBS	HBS	PBS	WCS	BEN	NNS	BAH	DOM	OSO	BOR	DOE	OOS	NBF	DEP
AA151 (or SNP4)															
Asn	0.077	0.125	0.019	0.250	**0.875**	**0.675**	**0.917**	**0.936**	**0.842**	**0.905**	**0.833**	**0.885**	**0.881**	**0.842**	**0.845**
Thr	**0.923**	**0.875**	**0.981**	**0.750**	0.125	0.325	0.083	0.064	0.158	0.095	0.017	0.115	0.119	0.158	0.155
AA214 (or SNP9_10_11)															
Ala	**1**	**0.975**	**1**	**0.950**	**0.714**	**0.700**	**0.694**	**0.750**	**0.655**	**0.655**	**0.674**	**0.821**	**0.655**	**0.692**	**0.697**
Ile	0	0.025	0	0.050	0.286	0.300	0.306	0.250	0.345	0.345	0.326	0.179	0.345	0.308	0.303
AA217 (or SNP 12)															
Ile	**0.893**	**0.625**	**0.923**	**0.950**	**0.965**	**1**	**0.986**	**1**	**0.988**	**1**	**1**	**1**	**0.988**	**1**	**1**
Thr	0.107	0.375	0.077	0.050	0.035	0	0.014	0	0.012	0	0	0	0.012	0	0
AA261 (or SNP 14)															
Phe	**0.893**	0.450	**0.596**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Tyr	0.107	**0.550**	0.404	0	0	0	0	0	0	0	0	0	0	0	0
AA299 (or SNP 19)															
Ala	**1**	**1**	**1**	**1**	**0.672**	**0.675**	**0.700**	**0.756**	**0.707**	**0.647**	**0.683**	**0.820**	**0.655**	**0.684**	**0.697**
Ser	0	0	0	0	0.328	0.325	0.300	0.245	0.293	0.354	0.317	0.180	0.345	0.316	0.300

The highest frequency at a sampling site is given in bold for each amino acid. For site abbreviations see Table 1.

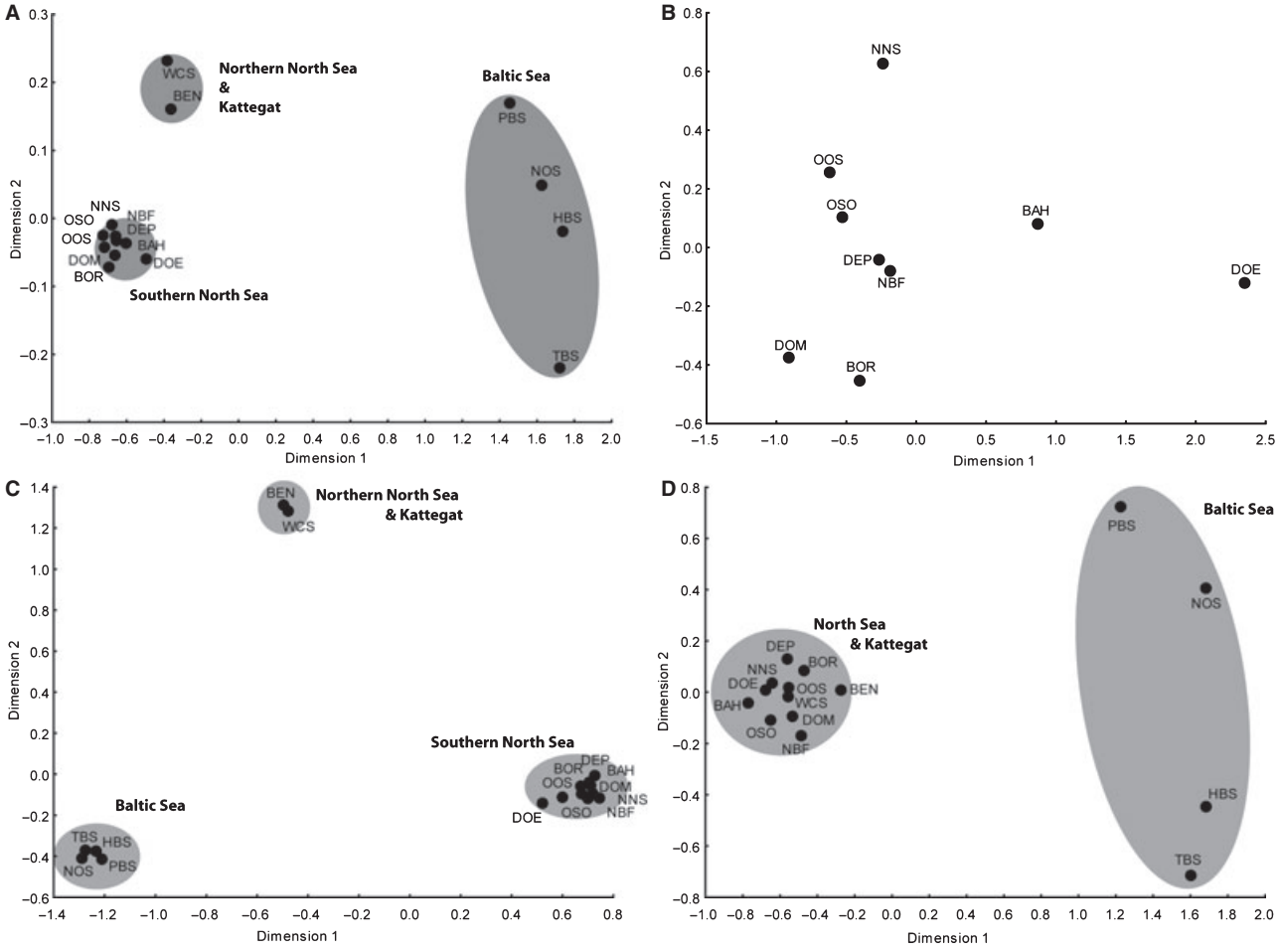

Fig. 2 NMDS plots of sand goby populations based on genetic distances of the rhodopsin variation (Cavalli-Sforza & Edwards 1967); (a) NMDS plot based on the full rhodopsin dataset of *Pomatoschistus minutus* from the Baltic and North Sea, (b) NMDS plot based on the rhodopsin dataset of the southern North Sea group, (c) NMDS plot based on the synonymous SNPs of the *RH1* gene of *P. minutus* from the Baltic and North Sea and (d) NMDS plot based on the non-synonymous SNPs of the *RH1* gene of the *P. minutus* from the Baltic and North Sea. For site abbreviations see Table 1.

and Bothnian samples was more variable than the group with samples from the southern North Sea (Fig. 2a). The NMDS plot based on synonymous SNPs showed three clearly differentiated groups; the four Baltic and Bothnian Sea samples, the southern North Sea samples, and the northern North Sea and Kattegat samples (Fig. 2c). This contrasts with the NMDS plot based on non-synonymous SNP data (Fig. 2d). In the latter, only two groups appeared; the North Sea and Kattegat samples, and the Baltic and Bothnian Sea samples. Differentiation between the four Baltic and Bothnian Sea samples is much larger than between the North Sea and Kattegat samples. NMDS analysis on only the samples of the southern North Sea alone showed no clustering, but confirmed that the sample DOE (Scheldt estuary) was the most divergent population (Fig. 2b). The NMDS plots had stress values below 0.20, suggesting interpretable information concerning inter-sample relationships.

Third, the global Mantel test revealed a significant isolation by distance pattern with D_{CE} ($r = 0.916$, $p < 0.01$). The Mantel tests based on the synonymous and non-synonymous SNPs both revealed a significant isolation by distance pattern ($r = 0.921$ with $p < 0.01$ versus $r = 0.835$ with $p < 0.01$, respectively).

Finally, the results for BARRIER also provided support for a partitioning of the samples into distinct groups similar to the NMDS plots. For all synonymous and non-synonymous SNPs, three barriers were identified with the 100 bootstrapped data sets. The strongest genetic separation was between the Baltic and North Sea samples. The second barrier was positioned between the Bothnian sample and the Baltic samples; the third one was placed between the two northern Baltic samples (TBS, HBS) and the southern Baltic sample (PBS). The BARRIER analysis based on synonymous sites found two significant barriers: again between the Baltic and North Sea samples; and

a second barrier between the northern and southern North Sea samples. Finally in the landscape genetics analysis based on non-synonymous SNPs, three barriers were found. The strongest genetic barrier was again situated between the Baltic and North Sea samples. The second barrier was positioned between the two northern Baltic Sea (TBS, HBS) and the southern Baltic sample (PBS); the third one was placed between the Bothnian sample and the Baltic samples.

Discussion

A previous study on intraspecific sequence variation in the rhodopsin gene of *Pomatoschistus minutus* detected statistical evidence for natural selection using population genetics and molecular evolution approaches (Larmuseau *et al.* 2009a). In this study, we want to go further by documenting the differences in mode of selection at the *RH1* gene between the sand goby populations within different marine systems due to regional spectral heterogeneity. Therefore, the *RH1* gene variation between sand goby populations from two regions was compared with each other: the Baltic Sea, characterised by divergent, temporally stable light conditions, and the North Sea, characterized by locally heterogeneous and temporally variable light conditions. Before comparing the patterns on the rhodopsin gene within both marine systems, we have to discuss first the relationship and the differentiation of *RH1* between the sand goby populations in the Baltic and North Sea.

Differentiation at the RH1 gene between the Baltic and North seas

The largest degree of differentiation and the strongest genetic barrier based on the frequency of *RH1* gene alleles were found between populations inhabiting the Baltic and North seas. Differentiation was mainly based on non-synonymous variation of *RH1*, which points to diversifying selection towards the light regime of the local marine system instead of neutral processes. The Baltic environment, which has high turbidity values, is characterised by an on average higher WMTL in comparison with the North Sea (Jerlov 1976), which has less turbid waters. The distribution of the variation on the known spectral tuning sites of the *RH1* gene matches these environmental differences. The best known spectral tuning sites of aquatic vertebrates are AA261 and AA299, from where the effect for AA-substitutions on the maximum absorbance of rhodopsin has been estimated (Yokoyama *et al.* 2007). The North Sea populations appear to be fixed for the blue-shifted mutation at AA261 (Y261F) and populations in the Baltic and Bothnian seas are fixed for the red-shifted allele at AA299 (S299A). Differentiation between the two systems was also observed at other polymorphic AAs, but understanding the effect of AA-substitutions awaits testing on rod opsins by mutagenic experiments (Yokoyama 2000). For example AA214 is well known to function as a spectral tuning site on cone opsins (Asenjo *et al.* 1994); it is almost fixed for alanine in Baltic Sea populations in contrast to North Sea populations.

Differentiation between Baltic and North Sea sand gobies is largely based on functional mutations at spectral tuning sites and not by silent mutations at random sites. It confirms that the *RH1* pattern is mainly influenced by diversifying selection, rather than neutral processes or relaxation of stabilizing selection after a recent bottleneck (Hughes 2007). Moreover, allele frequencies of those spectral tuning sites are similar between sand gobies in the Baltic Sea and the highly turbid lagoons in the Mediterranean (Larmuseau *et al.* 2009a). Mediterranean and Baltic sand gobies have apparently employed the same strategy to adapt to a more reddish light environment independently from each other, based on the phylogeographical pattern of the sand goby (Larmuseau *et al.* 2009b). Convergent evolution provides additional support for diversifying selection on *RH1*.

Most marine organisms, including gobies, immigrated into the Baltic Sea during the last marine (Littorina) phase, starting some 8000 years ago and gradually evolving into the present-day brackish water condition (Björck 1995). Despite its geological history, a growing list of populations inhabiting the estuarine Baltic Sea have evolved substantially from populations of the fully marine North Sea, e.g. herring *Clupea harengus* (Jørgensen *et al.* 2005), turbot *Scophthalmus maximus* (Nielsen *et al.* 2004) and the green alga *Cladophora rupestris* (Johansson *et al.* 2003). Genetic differences between marine and estuarine areas are most likely a consequence of isolation and bottlenecks, as well as adaptation (Johannesson & André 2006). The short transition period suggests that all these organisms adapted to the changing environmental conditions by selection on pre-existing genetic variants rather than on new mutations. Standing variation can lead to rapid evolution in novel environments because it is available immediately, whereas more time is required for a new beneficial mutation to arise (Barrett & Schluter 2008). All AAs of the *RH1* gene of the sand goby, except AA261, were polymorphic in North Sea populations, suggesting an adaptive shift from North Sea to Baltic Sea light conditions by selection on standing variation. Remarkably, mutation F261Y, which is known to have a strong phenotypic effect on the λ_{max} of the rod opsins (Yokoyama *et al.* 1995), is observed only in the northern Baltic and Bothnian seas. Therefore, the scenario that might explain the origin of this important functional mutation,

either from *de novo* or standing variation, remains unknown (Barrett & Schluter 2008). The absence of F261Y in all populations of the NA-Group outside the northern Baltic supports selection on a *de novo* mutation. Among all 417 individuals from the southern Baltic Sea, Kattegat and the North Sea, the allele with a red-shifted effect on the λ_{max} of rod opsins was not observed, although sampling included regions with high local WMTL values. Nevertheless, the mutation might be present in the source population at a frequency below the detection threshold (< 0.12%). Finally, the F261Y mutation may also have been introduced through hybridization and introgression following backcrossing with other species (Gibson & Dworkin 2004). *Pomatoschistus minutus* hybridizes with *P. lozanoi* (Wallis & Beardmore 1980) but there are no observations for such specific substitution in *P. lozanoi* (Larmuseau *et al.* 2010a). Based on these observations, the most likely hypothesis for the occurrence of F261Y is *de novo* mutation in the Baltic Sea since the colonization of this region less than 8000 years ago. The observation that the only potential *de novo* mutation in the Baltic Sea is the one with a high phenotypic effect on rod opsins, underlines once more that selection rather than neutral processes contributed to *RH1* gene variation in the Baltic Sea (Hughes 2007).

Differentiation at the RH1 gene within the Baltic Sea and North Sea

RH1 variation in the Baltic and Bothnian seas differed significantly from each other. Remarkably, the F_{ST}-values between Baltic and Bothnian Sea samples were only significant based on frequencies at the six non-synonymous SNPs and not for the synonymous SNPs. Apparently, a much higher differentiation is realized on non-synonymous than on synonymous SNPs over the 8000 years since the colonization of the Baltic Sea. This difference suggests strong diversifying selection on the non-synonymous variation at the rhodopsin gene. There was indeed a strong correlation between the allele frequency of the known spectral tuning sites, the effects of those alleles on the characteristic peak of maximal absorbance (λ_{max}) of rhodopsin and the WMTL values of the sampling locations. The WMTL value in the northern part of the Baltic Sea (samples TBS and HBS) is higher than in the Bothnian Sea and much higher than in the southern Baltic Sea. This pattern is completely congruent with the allele frequency on AA261, the spectral tuning site with a high known effect on the λ_{max} of rhodopsin in Teleostei (Yokoyama *et al.* 1995). The red-shifted allele F261Y had a higher frequency in the northern Baltic Sea than in the Bothnian Sea and was absent from the southern Baltic Sea.

Sand goby populations within the North Sea maintained a high level of polymorphism of the rhodopsin phenotypes. One striking observation was the relatively stable frequencies of the various alleles at the polymorphic AAs of the RH1 in all North Sea samples. No differentiation was observed among the North Sea samples based on AA variation at the *RH1* gene despite the well known population structure within the North Sea based on catchment data (Guelinckx 2008) and on microsatellite and mtDNA data (Pampoulie *et al.* 2004; Larmuseau *et al.* 2009b, 2010b;). Remarkably, in contrast to the lack of non-synonymous differentiation, two groups were significantly differentiated from each other based on the synonymous SNPs of *RH1* (F_{ST}-value = ~0.10); a northern North Sea group including the samples from Bergen and Kattegat, and a southern North Sea group with Belgian and Dutch samples. The discrepancy between the population differentiation based on synonymous and non-synonymous SNPs was confirmed by MDS and Barrier analyses. The synonymous differentiation was congruent with the pattern of mitochondrial DNA and nuclear microsatellite markers variation, which separated sand gobies of Kattegat and the Southern North Sea (Larmuseau *et al.* 2009a, 2009b). Although all SNPs of the *RH1* are linked and hence represent a single haplotype, only the synonymous SNPs revealed a population structure matching historical patterns.

The strong difference in the pattern of population differentiation of the *RH1* gene between the Baltic and North Sea populations suggests differential modes of selection on this gene within both marine systems. The photic regimes of the Baltic and Bothnian seas have a stable temporal pattern based on WMTL values (Jerlov 1976; Lindström 2000). This stable environmental structure provides an opportunity for local populations to adapt environmentally. The same suggestion has been made to explain the differentiation in absorbance of VP opsins of *Mysis relicta* between several Baltic Sea sites (Jokela-Määttä *et al.* 2005). Therefore, the results for the Baltic Sea suggest population-specific diversifying selection on the *RH1* (Nielsen 2005). The robust WMTL pattern observed in the Baltic strongly deviates from temporal heterogeneity in light regimes along the North Sea coastline (Jerlov 1976; Larmuseau *et al.* 2009a). Similar selective pressures shape the non-synonymous *RH1* variation in the entire North Sea, which fits with the similar light regimes at each location sampled. Therefore, the maintenance of a high level of polymorphism of rhodopsin phenotypes and the lack of differentiation on the non-synonymous SNPs of the *RH1* gene suggests balancing selection (Charlesworth 2006). Although neutrality tests can only give indications about the mode of selection on a particular locus due to the influence of

demographical events on most tests of selective neutrality (Excoffier *et al.* 2009), the results of the neutrality tests on *RH1* were consistent with our conclusion of a difference of selection regimes in the two regions. The negative values for the neutrality tests for Baltic and Bothnian sand goby populations suggest the possibility that positive selection has modelled the pattern of variation found in the *RH1* gene. In contrast, the positive values for the neutrality statistics for the North Sea sand goby populations can indicate balancing selection on the rhodopsin gene (Wayne & Simonsen 1998).

Sand gobies of the North Sea maintain high levels of AA polymorphism at the *RH1* gene, most likely because of environmental heterogeneity in the light regime of the North Sea. However, similar to many marine fish, juvenile sand gobies grow up in the turbid waters of the North Atlantic estuaries and coastal surf zones (Healey 1971; Fonds 1973; Maes *et al.* 1998) in contrast to the sedentary juvenile sand gobies of the Baltic Sea. Therefore, sand gobies of the North Sea encounter spectrally heterogeneous habitats, and this offers an alternative explanation for the maintenance of the polymorphism at *RH1* in this area. The sample from the Scheldt estuary (sample DOE) indeed showed a higher frequency of the red-shifted allele at AA299 and of the allele at AA214, which is fixed in sand goby populations from other turbid environments (Larmuseau *et al.* 2009a). Therefore, estuarine sand gobies do not represent just a random group of marine residents. Gobies with red-shift AA-substitutions at the *RH1* gene may prefer to migrate deeper into the estuary, which has a more reddish light environment in comparison to the open sea. Based on the isotope composition and dynamics of muscle and liver tissue (Guelinckx *et al.* 2008) and on the elemental composition of otoliths (Guelinckx 2008), sand gobies make facultatively use of the estuary. These findings support the hypothesis that turbidity shapes estuarine habitat use by marine fish (Benaka 1999; Maes *et al.* 2005). Nevertheless, because just a single sample from the Scheldt River was analysed, confirmation is awaiting of the importance of adaptative variation at the *RH1* gene and the facultative use of estuaries by sand gobies and by so many other marine fishes.

Conclusion

We improved our mechanistic understanding of how selection on visual genes operates in marine environments. As predicted, signals of diversifying selection at the *RH1* gene of *Pomatoschistus minutus* appeared among populations in divergent, but temporally stable light environments. In contrast, the *RH1* gene showed signals of stabilizing selection in populations inhabiting locally heterogeneous and temporally variable light environments. However, functional variation at the *RH1* gene may be also influenced by migration patterns between the open sea and more turbid estuarine environments, suggesting that the visual capacity of fish with various RH1 variants influences life histories.

An interesting observation is that within the *RH1* gene, synonymous and non-synonymous SNPs show different patterns between populations. Population differentiation based on non-synonymous SNPs of the *RH1* gene was correlated with the spectral variation of the local environment. In contrast, differentiation based on synonymous SNPs of *RH1* was correlated with patterns of historical dispersal and colonization inferred by neutral microsatellites and mtDNA. These results are comparable with previous studies on other genes under influence of natural selection, which showed that the candidate gene approach is useful to characterize the modes of selection on particular genomic regions (Berry & Kreitman 1993; Ingvarsson *et al.* 2006). Therefore, searching for concrete candidate genes to study local adaptation remains a complementary tool in molecular ecology, in addition to more advanced genome-wide approaches.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Nucleotide polymorphisms at the rhodopsin gene in the sand goby of 15 sampling sites. Dots indicate homology with the reference sequence (Acc. nr. X62405). AA numbers are listed for the non-synonymous mutations, which are listed in bold. For site abbreviations see Table 1

Table S2 Rhodopsin haplogroups and their geographical distribution in *Pomatoschistus minutus*. Dots indicate homology with the reference gene (Acc. nr. X62405) of sand goby. Non-synonymous SNPs are listed in bold. For site abbreviations see Table 1

Table S3 Pairwise F_{ST} estimates based on the rhodopsin gene (above diagonal) and pairwise geographical distances in km (below diagonal) between sand goby populations. For site abbreviations see Table 1. Within-region comparisons are highlighted in grey. *Significant ($p < 0.05$); **remains significant after Bonferroni correction (Rice, 1989)

Table S4 Pairwise F_{ST} estimates based on the non-synonymous SNPs of the rhodopsin gene (above diagonal) and based on the synonymous SNPs of the rhodopsin gene (below diagonal) between sand goby populations. The within-region comparisons were highlighted in grey. For site abbreviations see Table 1. *Significant ($p < 0.05$); **remains significant after Bonferroni correction (Rice, 1989)

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