

To see in different seas: spatial variation in the rhodopsin gene of the sand goby (*Pomatoschistus minutus*)

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Abstract

Aquatic organisms living in a range of photic environments require specific mechanisms to tune their visual pigments. Maximum absorbance (λ_{\max}) of retinal rods in populations of the marine demersal sand goby, (*Pomatoschistus minutus*; Gobiidae, Teleostei) correlates with the local optic environment. It has been shown that this is not regulated through a physiological response by exchanging the rhodopsin chromophore. To test for evolutionary adaptation, the sequence of the rhodopsin (*RH1*) gene was analysed in 165 *Pomatoschistus minutus* individuals from seven populations across its distribution range. Analysis showed a high level of intraspecific polymorphism at the *RH1* gene, including nonsynonymous mutations on amino acids, known as spectral tuning sites. Population differentiation at these sites was in agreement with the observed differentiation in λ_{\max} values. Analyses of d_N/d_S substitution rate ratios and likelihood ratio tests under site-specific models detected a significant signal of positive Darwinian selection on the *RH1* gene. A strong discrepancy in differentiation was noticed between *RH1* gene variation and the presumably neutral microsatellites and mitochondrial data. Samples did not cluster according to geographical or historical proximity with regards to *RH1*, but according to the general photic conditions of the habitat environment of the sand goby. This study highlights the usefulness of sensory genes, like rhodopsin, for studying the characteristics of local adaptation in marine nonmodel organisms.

Keywords: adaptive evolution, candidate gene, Gobiidae, marine fish, photoreceptor, remote sensing, vision

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Introduction

For too long, the genetic structure of marine organisms has been thought to be homogeneous because of the lack of obvious barriers to gene flow in the environment. As gene flow is expected to hamper adaptive population divergence, the traditional idea was that local adaptation may be rare or absent in marine fishes (Hemmer-Hansen *et al.* 2007). Lately, however, an

increasing number of population genetic studies have described complex genetic structures in several marine species (Knutsen *et al.* 2003; Pampoulie *et al.* 2008). One major factor responsible for the present genetic structure of marine species is the geological and climatological history during the Pleistocene glaciations (Debes *et al.* 2008; Luttikhuisen *et al.* 2008). Also contemporary factors maintain and promote genetic differentiation among marine populations on various geographical scales. The marine environment shows heterogeneity in response to climate, hydrodynamics and topography (Cowen *et al.* 2000), and biological traits, such as

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sex-dependent migration, site philopatry and assortative mating enhance genetic structuring (Ruzzante *et al.* 1998). Stable neutral genetic structuring among populations may indicate that local selection is overriding the effects of drift and gene flow, resulting in adaptive divergence. Local adaptation in marine organisms has become increasingly documented, indicating that selection is also a potent evolutionary force in the marine environment (Canino *et al.* 2005; Hemmer-Hansen *et al.* 2007; Zane 2007; Sherman & Ayre 2008). Nevertheless, knowledge of the spatial and temporal scale of adaptive genetic variation in marine systems remains scant, yet crucial to improve our understanding of how evolution operates in the ocean (Conover *et al.* 2006).

The sand goby, *Pomatoschistus minutus* (Pallas 1770; Gobiidae, Teleostei) is a common small marine demer-

sal fish inhabiting the shallow waters along European coasts (Miller 1986; Fig. 1). A recent study has shown relatively low levels of gene flow and high genetic structuring in this species compared with other marine fish species (Larmuseau *et al.* 2009). Therefore, it is a suitable model for studying the characteristics of local adaptation in the marine environment. Middle Pleistocene glaciations yielded three isolated and differently evolving sets of sand goby populations. Reciprocal mitogenic monophyly was observed between a Mediterranean Sea (MS) and an Atlantic Ocean (AO) Clade (Larmuseau *et al.* 2009). The AO-Clade contains two major phylogeographic groups: the Iberian Peninsula (IB) group and the North Atlantic (NA) group. For the NA-Group, there is evidence for geographic sorting of the ancestral mitochondrial DNA (mtDNA) haplotypes

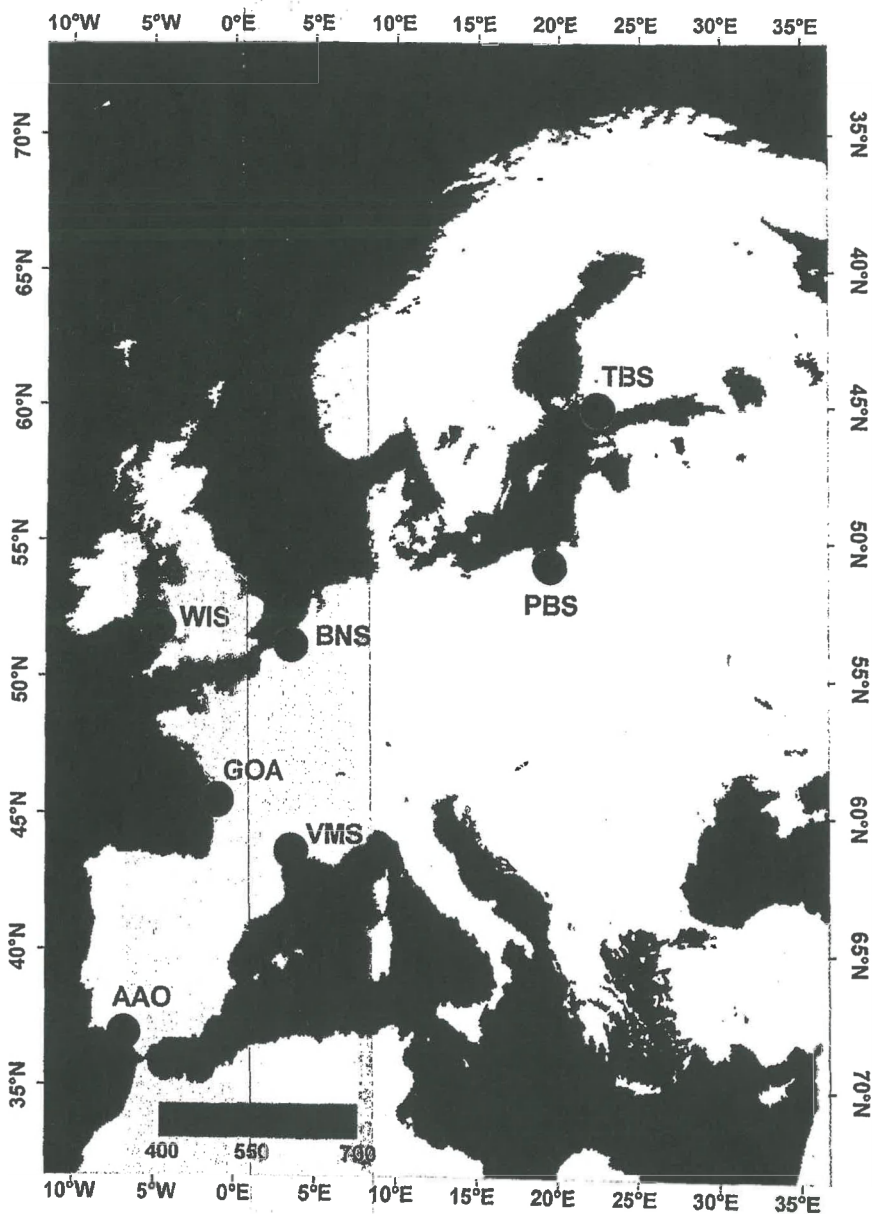


Fig. 1 Geographical distribution of the seven sampling locations in seven European marine systems for sand goby, *Pomatoschistus minutus*. The wavelength (nm) of maximally transmitted light estimated from the MODIS annual composite radiance data of 2007 is shown for all European seas. Discrete colours correspond to the MODIS wavelengths 412 nm (deep blue), 443 nm (blue), 488 nm (pale blue), 531 nm (bright green), 551 nm (dark green), 667 nm (red) and the default value 615 nm (orange) substituted in case of saturated data at 531, 555 or 667 nm.

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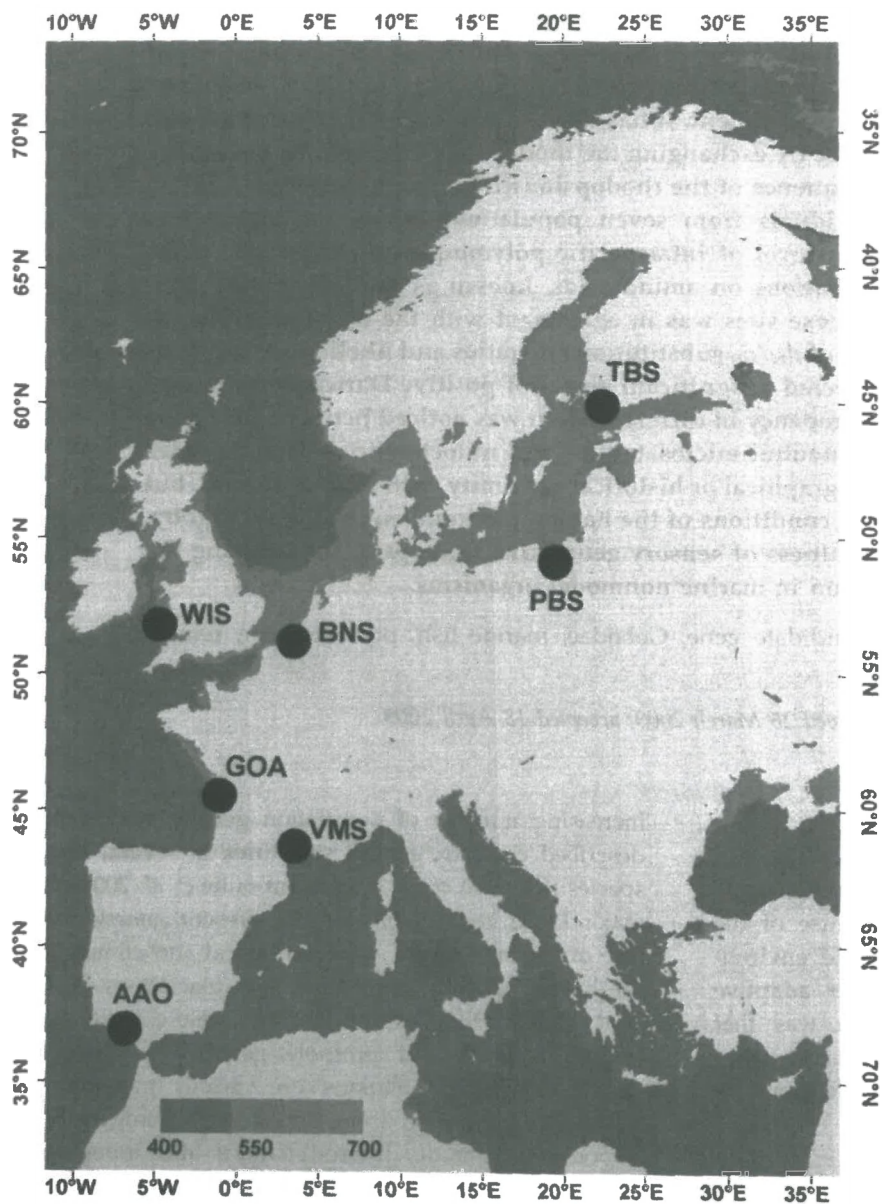


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with recent radiations in the Baltic Sea, Irish Sea, North Sea and Bay of Biscay. Northern Baltic Sea sand gobies are considered to belong to an isolated population with clear evidence for founder effects (Larmuseau *et al.* 2009). Allozyme and microsatellite analyses largely corroborated this phylogeographic pattern (Stefanni *et al.* 2003; M. H. D. Larmuseau, unpublished data).

Sand gobies are visual feeders (Healey 1971; Aarnio & Bonsdorff 1993) and mostly nocturnal. Patterns of activity are largely influenced by tides and light intensity (Ehrenberg & Ejdung 2008). For *P. minutus*, nocturnal foraging is advantageous in approaching prey and in avoiding predators (Thetmeyer 1997). Relative to body size, the protruding eyes are large and might be capable of detecting prey organisms in very dim light (Thetmeyer 1997). The geographical distribution of the sand goby includes a wide range of photic environments, varying in turbidity, colour and brightness. Therefore, adaptation to the local spectral environment may be crucial. Vertebrates have visual pigment (VP) molecules bound in dense membrane stacks in retinal photoreceptors to mediate vision. The VP protein moiety is opsin, which is a G protein-linked receptor, bound to a light-sensitive chromophore, 11-*cis* retinal (A1) or 11-*cis* 3, 4-dehydroretinal (A2) (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. The pigment that mediates vision in dim light and absorbs light with λ_{\max} of about 500 nm, is rhodopsin; It is located in rod cells.

Vertebrates have various possibilities to modify their visual system to cope with the photic environment. The spectral tuning of the VP proteins can be assessed on a physiologically time scale through exchange of the chromophore (A1 or A2), consistent with an anticipated change in photic environment (Bowmaker 1995). Tuning can also be achieved at the DNA level on an evolutionary time scale through amino acid (AA) substitutions in the protein part (the opsin) (Yokoyama 2000). The first possibility seems unlikely in sand gobies. Jokela *et al.* (2003) measured the absorbance spectra microspectrophotometrically in retinal rods of various sand goby populations. They found considerable variation in λ_{\max} values within and between populations. The shapes of the absorbance spectra-indicated polymorphism at the rhodopsin gene rather than admixture of A1 and A2 chromophores, suggesting that the variation in λ_{\max} values is genetic. Therefore, evolutionary adaptation, rather than physiological change, is presumed to be responsible for spectral tuning.

The tuning mechanism of VPs should be a suitable candidate to understand the opportunities and characteristics of local adaptation in the marine environment.

The aim of this study was to assess if sand gobies are evolutionary adapted to local photic environments on the rhodopsin gene (*RH1*). Our strategy to demonstrate local adaptation on *RH1* consists of three steps: first, we demonstrate differentiation in the functional variation of the *RH1* gene between sand goby populations. Next, we demonstrate that the population differentiation of *RH1* is the result of selection. Finally, we establish a link between the functional variation of *RH1* and selection regimes.

Materials and methods

Sampling and species identification

A total of 165 *Pomatoschistus minutus* individuals were caught at seven locations along the European coast between January 2006 and February 2007 (Table 1, Fig. 1). Samples were taken either by fyke, hand net or beam trawling. The 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 polymerase chain reaction (PCR)-restriction fragment length polymorphism species identification protocol developed by Larmuseau *et al.* (2008).

Gene amplification and sequencing

Genomic DNA was extracted from fin clips, stored in 100% ethanol, using the NucleoSpln Extraction Kit (Machery-Nagel GmbH). An 868 bp fragment of the *RH1* gene was amplified in PCR with the forward primer PminRh1F GCGCCTACATGTTCTTCCTT and the reverse primer Rh1039r TGCTTGTTTCATGCAGATGTAGA (Chen *et al.* 2003). The forward primer was designed using the Primer 3 program (Rozen & Skaletsky 1998) on conserved regions of the alignment of *RH1* gene sequences from *P. minutus* (Acc no. X62405), *Gobius niger* (Y18675), *Zeus faber* (Y14484), *Sargocentron diadema* (U57537) and *Sargocentron microstoma* (U57542). Developing new primers to amplify a larger fragment of the *RH1* gene was not successful because of co-amplification of other opsin genes. PCR reactions were carried out on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems) in a total volume of 25 μ L, containing 1 μ L of genomic DNA, 1X PCR buffer, 0.2 mM dNTPs, 0.8 μ M of each primer, 2.0 mM MgCl₂, 0.5 U of *Taq* DNA polymerase (Silverstar; Eurogentec) and mQ-H₂O. The PCR profile was: 4 min at 94 °C followed by 35 cycles of 30 s at 96 °C, 30 s at 54 °C and 1 min at 72 °C; with a final 10-min extension at 72 °C. To avoid contamination, different pipettes, aerosol

Table 1 Collection information for the seven populations of *Pomatoschistus minutus*, including code, marine system, country, site, date, longitude and latitude and sample sizes for the *RH1* and microsatellite analyses (N_r and N_s , respectively)

Code	Marine system	Country	Site	Date	Latitude	Longitude	N_r	N_s
TBS	(Northern) Baltic Sea	Finland	Tvärminne	Jul/2006	59°50'N	23°12'E	20	96
PBS	(Southern) Baltic Sea	Polen	Sopot, Bay of Gdańsk	Feb/2007	54°27'N	18°35'E	10	—
BNS	North Sea	Belgium	Oostduinkerke	Nov/2006	51°08'N	02°40'E	27	47
WIS	Irish Sea	UK (Wales)	Llanfairfechan	Nov/2006	53°20'N	03°59'W	21	45
GOA	Atlantic Ocean (Bay of Biscay)	France	Gironde estuary	Aug/2006	45°36'N	01°01'W	22	40
AAO	Atlantic Ocean (Iberian Peninsula)	Spain	Guadalquivir river estuary	Nov/2006	36°58'N	06°10'W	37	95
VMS	Western Mediterranean Sea	France	Vaccarès lagoon	Jan/2006	43°32'N	04°35'E	28	94

barrier tips and different sections of the laboratory were used for pre- and post-PCR work. In every other 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 gels with ethidium bromide. After purification with the 'GFX PCR DNA and Gel Band Purification kit' (GE Healthcare), 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 of 756 bp (252 AA) were checked and aligned to each other with SEQSPACE v. 2.1 (Applied Biosystems). The full rhodopsin sequence of *P. minutus* counts 1056 bp and thus 352 AAs (Archer *et al.* 1992). The 252 AA' fragment under study thus represents 72% of the protein. However, all known 25 AAs involved in the spectral tuning of the VPs are included in this gene fragment (Yokoyama *et al.* 2007 and references herein). Automated detection of point mutations was realized with the GAP4 subprogram embedded in the STADEN package (<http://sourceforge.net/projects/staden>) and checked manually by eye.

For several reasons, we are convinced that no other member of the opsin gene family than the *RH1* gene was co-amplified and analysed. First, when designing primers for rhodopsin, sites were selected that differ among paralogous genes. Second, other opsin genes have introns, unlike the rhodopsin genes of bony fishes (Bowmaker 1995). Third, the duplication event separating rhodopsin from other opsin genes occurred before the diversification of vertebrates (Yokoyama 2000). If we had sequenced by mistake, a paralogous opsin gene, the sequence alignment would have shown this extreme divergence. Finally, different PCR-products were cloned to control for a recent duplication event of the *RH1* gene in *P. minutus*. A total of 21 individuals with more than one heterozygote single nucleotide polymorphism (SNP) locus was cloned into bacterial vectors using the TOPO-TA cloning kit (Invitrogen). Five to ten clones

originating from two independent PCR reactions (for protocol see above) per specimen were sequenced. No more than two haplotypes were observed in each reaction, suggesting that only one gene was sequenced and analysed. All 38 rhodopsin haplotypes determined in this study were deposited in the GenBank database (Accession numbers: FJ410451–FJ410488; Table S1).

Microsatellite genotyping and analysis

Variation at eight high-quality microsatellite markers (Pmin03, Pmin04, Pmin09, Pmin16-2, Pmin29, Pmin31, Pmin35 and Pmin38) (Larmuseau *et al.* 2007) was assessed for 417 sand gobies of six populations (Table 1). Deviation from Hardy–Weinberg equilibrium and population differentiation quantified as F_{ST} were quantified with GENETIX v.4.05 (Belkhir *et al.* 2004).

Haplotype reconstruction and network analysis

Rhodopsin haplotypes of the 21 cloned individuals and 105 sequenced individuals with less than two heterozygous sites were available. The haplotypes of the 39 remaining individuals were inferred from the genotypes using the Bayesian statistical methods in the program PHASE v. 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). Using this program, haplotypes have been resolved based on the assumption that unsolved haplotypes tend to be more similar to previously sampled known haplotypes. Runs were conducted separately for each population, with known haplotype information (i.e. homozygous haplotypes and cloned haplotypes) 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 results and the goodness-of-fit values were very similar among runs, indicating that the run lengths were sufficient. Haplotypes of individuals with more than one heterozygous site for which the phase could not be determined with a probability of > 95% (averaged over the ten runs) were

excluded from the haplotype network (19 out of 165 analysed individuals). A haplotype network of the rhodopsin haplotypes was constructed using the statistical parsimony method implemented in the program TCS v. 1.21 (Clement *et al.* 2000). Interpopulation relationships were assessed by estimating pairwise F_{ST} -values based on the haplotype distributions with Arlequin v. 3.11 (Excoffier *et al.* 2005). These values were then used for a classical multidimensional scaling (CMDs) analysis in the VEGAN package in R (Oksanen *et al.* 2007) for detecting group structure. CMDs plots having a stress value <0.20 provide interpretable information concerning intersite relationships (Clarke 1993).

Genetic diversity and neutrality tests

The number of segregating sites (S), the mean number of pairwise differences (k) and estimates of nucleotide polymorphism (π , θ) were calculated using DnaSP v. 4.10.9 (Rozas *et al.* 2003).

Several analyses were performed to determine if positive selection was involved in the evolution of *RH1* in *P. minutus*. The number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N) were estimated using the Z-test implemented in MEGA v. 4.0 (Tamura *et al.* 2007) according to Nei & Gojobori (1986) with the correction of Jukes & Cantor (1969) for multiple substitutions. The variances of d_S and d_N were computed by bootstrap (10 000 replicates). With this information, the null hypothesis of neutral evolution ($H_0: d_N = d_S$) vs. the hypothesis of positive selection ($H_1: d_N > d_S$) was tested using a Z-test: $Z = (d_N - d_S) / \sqrt{\text{Var}(d_S) + \text{Var}(d_N)}$. The maximum-likelihood method (Yang *et al.* 2000) implemented in the program CODEML of the PAML 4.1 software package (Yang 2007) was used to test whether codon sites on the *RH1* gene were affected by positive selection (Yang *et al.* 2005). The models were M7 (beta) and M8 (beta and ω ; Yang *et al.* 2000). While recombination can potentially generate false-positives in the detection of positive selection, these models are more robust against the occurrence of recombination than the other models implemented in CODEML (Anisimova *et al.* 2003). The models M7 and M8 are compared pairwise using the likelihood-ratio test (LRT; Nielsen & Yang 1998). To provide phylogenetic information for the analysis, the best tree for *RH1* sequences was identified with the maximum likelihood method under the one-ratio model (M0) in CODEML. Positively selected codons ($\omega > 1$ with $P > 95\%$) were identified through an empirical Bayesian approach implemented in CODEML (Yang *et al.* 2005).

Another method used to test for the effects of differential selection among populations is to compare the

distribution of the variation on *RH1* and neutral nuclear markers. If SNPs of the *RH1* gene code for adaptive variation, the *RH1* gene is expected to reveal aberrant population structures in comparison to nuclear markers (such as microsatellite markers), which may be behaving neutral (Bamshad & Wooding 2003). To compare the degree of population differentiation between the *RH1* and the microsatellite markers, several methods were applied. First, correlations between pairwise F_{ST} -values of the two markers were calculated and tested using simple Mantel procedures (Mantel 1967) in the VEGAN package in R (Oksanen *et al.* 2007). Because the number of Mantel test permutations is limited for small sample sizes ($n = 6$) (Legendre 2000), complete enumeration of all possible $6! = 720$ permutations was carried out for all tests. Second, two-dimensional CMDs ordinations of pairwise F_{ST} -values of different marker types were compared by a Procrustes Analysis (PA) (Gower 1975) with the VEGAN package. PA searches for the best match between two configurations of points in a multivariate Euclidean space using rotation, translation, reflection and dilation of one configuration. The criterion used to assess the best fit is the minimization of the sum of squares between the differences for each observation (m^2). The significance of the result, an optimal superposition of one configuration on the other (reference) configuration, is obtained through a permutation test (PROTEST, Jackson 1995). PROTEST uses $R = \sqrt{1 - m^2}$ as a test statistic, which can be interpreted as a correlation. Finally, a selection detection workbench LOSITAN (Antao *et al.* 2008) based on the FDIST F_{ST} outlier methods of Beaumont & Nichols (1996) was used to evaluate the neutrality of the microsatellites and the presumed outlier status of *RH1*. Different runs were assessed: one run with only microsatellite data, 14 different runs with all microsatellites and a polymorphic SNP of the *RH1* gene, and a final run with all microsatellites and all polymorphic SNPs of the *RH1* gene. For all runs 30 000 simulations were generated with 'neutral mean F_{ST} ' and 'force mean F_{ST} ', to increase the reliability of the mean F_{ST} .

Environmental light measurements

To correlate the differences in the rhodopsin gene variation between *P. minutus* populations with the light transmittance of the respective waters, the spectral distribution of environmental ambient light was measured in the northeastern Atlantic Ocean, Mediterranean Sea and adjacent seas. Lindström (2000) introduced the concept of 'wavelength of maximally transmitted light' (WMTL) to characterize the spectral content and depth variation of the underwater light climate by a single parameter for comparison with the spectral sensitivity

of the eyes of marine animals. Whereas a combination of underwater light measurements and optical modelling is used by Audzijonyte *et al.* (2005) to estimate the WMTL at various locations, a new method is described in Appendix S1 to estimate this parameter from satellite remote sensing data. The method has the advantage of providing information at almost any location on earth without the need for *in situ* measurements or a priori knowledge, and of relying on a more uniform methodology. Water-leaving radiance data as measured by the MODIS-AQUA satellite sensor was downloaded on 19th November 2008 from the NASA 'Ocean Color' web site (<http://oceancolor.gsfc.nasa.gov/>) as the annual composite for 2007 (4-km standard map image file) for each of the available spectral bands (412, 443, 488, 531, 551 and 667 nm).

Results

Nucleotide diversity of the RH1 gene

Sequences matched the general properties of the *Pomatoschistus minutus* RH1 gene (X62405, Archer *et al.* 1992). In total, 19 segregating sites or SNPs were noticed across all genotypes (Table S2). Five SNPs (SNP 2, 3, 5, 8 and 18) were not polymorphic according to the 99% criterion; three polymorphic SNPs were part of the same codon and were merged (written further as SNP_9_10_11). The alignment in AA shows five nonsynonymous AA substitutions; four are located in the transmembrane helices and one in the C-II loop (Fig. 2). The sequencing and cloning reactions revealed 38 confidently resolved haplotypes (Table S1). Nucleotide diversity (π) of the RH1 gene fragment was estimated to be 0.0074 in total. The within-population RH1 nucleotide diversity values were highest in populations BNS, WIS and GOA (southern North Sea, Irish Sea and Bay of Biscay, respectively); the lowest value was found in PBS (southern Baltic Sea; Table 2).

Population differentiation on the RH1 gene

A maximum parsimony network of confidently resolved haplotypes revealed that three haplotypes are common in at least four populations (Fig. 3). Haplotype RhP7 is shared among all populations. Haplotypes clustered roughly into two groups, one containing the majority of haplotypes observed in the Iberian Peninsula, North Sea and Irish Sea, and another group containing haplotypes occurring mainly in the Mediterranean and Baltic Sea (Fig. 3). Accordingly, pairwise F_{ST} -values (Table 3) showed a clustering of Mediterranean and northern Baltic Sea (VMS and TBS) samples; the Atlantic samples (GOA, WIS and AAO)

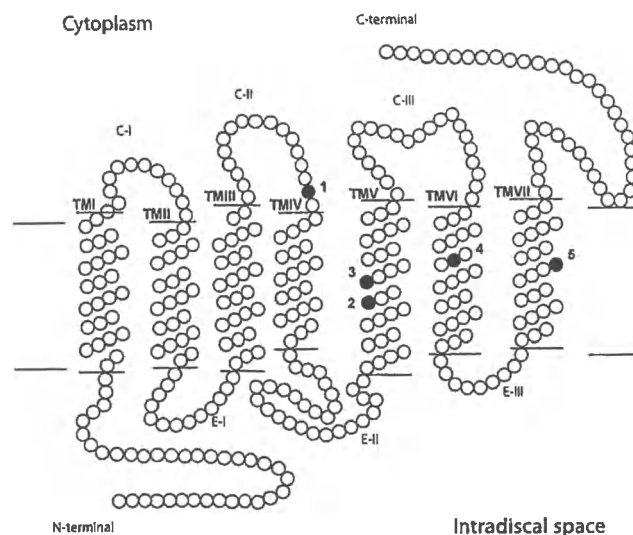


Fig. 2 Two-dimensional model of the seven transmembrane α -helices of the bovine rhodopsin (RH1) as in Hargrave & McDowell's (1992). The seven transmembrane helices (TM) are numbered, as well as the three loops at the cytoplasmic side (C) and the extracellular side (E) of the cell membrane. The different nonsynonymous mutations found in *Pomatoschistus minutus* are shown in filled circles. (1) AA151 (SNP4); (2) AA214 (SNP9_10_11); (3) AA217 (SNP12); (4) AA261 (SNP14); (5) AA299 (SNP19).

clustered together. Finally, the frequency of nonsynonymous SNPs and polymorphic AA differed between the populations (Table 4).

Neutrality tests

The null hypothesis of evolution according to the neutral model could not be rejected with a general Z-test for all samples combined as well as for the samples from the Baltic Sea, North Sea and Mediterranean Sea separately. The d_S -values were significantly larger than the d_N -values only for the Z-tests with the Atlantic samples WIS, GOA and AAO. However, the LRT of the maximum-likelihood analysis demonstrated that M8, the model that accounts for sites under positive selection, showed a significantly better fit than model M7, which does not allow for positive selection. The ω ratio is more than 1 (Table 5), indicating positive selection in the RH1 sequences of *P. minutus*. Bayes identification showed that sites AA151, 214 and 299 of the RH1 gene were significantly under positive selection.

Overall population differentiation was considerably higher for the full fragment of the RH1 gene ($F_{ST} = 0.4549$) than at the nuclear microsatellite markers ($F_{ST} = 0.0126$). Exact tests showed that no locus or sample exhibited consistent deviations from Hardy-Weinberg equilibrium with respect to microsatellites. The pairwise F_{ST} -values of the RH1 gene and the

Population	N	No. haplotypes		S_n	S_s	k	π	θ
		Total	Private					
TBS	20	8	2	5	3	1.763	0.0023 ± 0.0015	0.0028
PBS	10	4	1	4	2	0.895	0.0012 ± 0.0010	0.0012
BNS	27	14	8	5	8	4.197	0.0056 ± 0.0006	0.0038
WIS	21	7	1	5	7	3.987	0.0053 ± 0.0008	0.0037
GOA	22	8	4	4	6	3.755	0.0048 ± 0.0007	0.0030
AAO	37	9	5	6	8	1.287	0.0017 ± 0.0006	0.0038
VMS	28	12	6	4	4	1.449	0.0019 ± 0.0003	0.0023
Total	165	38	27	6	13	5.608	0.0074 ± 0.0001	0.0039

Table 2 Summary of diversity indices for the 19 polymorphic sites analysed for variation at the population level of the sand goby *RH1* gene

N , number of individuals surveyed; S_n , number of nonsynonymous segregating sites; S_s , number of synonymous segregating sites; k , mean number of pairwise differences; π , average number of nucleotide differences per site; θ , theta per site. For site codes, see Table 1.

microsatellite data (Table 3) did not correlate with each other (Procrustes $R = 0.218$, P -value = 0.910; Mantel $R = 0.307$, P -value = 0.100) (Table 6). Additionally, the simulation-based LOSITAN tests confirmed the neutrality of the microsatellites and the highly significant (> 0.99) outlier position of each SNP of the *RH1* gene in comparison with the microsatellites (data not shown).

Light measurements

Figure 1 plots the broad spatial variation of the WMTL in the northeastern Atlantic Ocean and Mediterranean Sea. The default value of 615 nm (coloured in orange) was substituted in case of saturated data at 531, 555 or 667 nm. This means that it is difficult to interpret the results along the coasts of Belgium, the Netherlands and the United Kingdom. The underwater light climate is mainly blue in the deep offshore oligotrophic waters of the Mediterranean and the Atlantic Ocean, west of the continental shelf break. Greener waters are found in the Southern North Sea, the southern Baltic Sea, near river mouths and in various shallow coastal waters in the North Sea, Mediterranean Sea and Black Sea. A few isolated areas with an underwater light climate shifted towards red light are found in nearshore regions of the northern Baltic Sea and for a few inland waters. The map therefore shows congruent, but more detailed results than previous maps of optical water types (Jerlov 1976).

Discussion

Functional polymorphism of the *RH1* gene in *Pomatoschistus minutus*

Jokela *et al.* (2003) found individual differences in the λ_{\max} values of the retinal rods of *Pomatoschistus minutus*. As the differences could not be explained by a chromo-

phore change, they suggested polymorphism of the opsin gene instead of physiological changes. Sequence analysis revealed substantial variation at the *RH1* gene with 19 SNPs, of which 14 were polymorphic, in seven sand goby populations (Table S2). This is the first observation of such a high level of intraspecific variation at a spectral opsin gene in vertebrates. There are five AA replacements, of which some are known to have a significant effect on the λ_{\max} values of retinal rods in aquatic vertebrates.

One of the AA substitutions present in sand goby is a phenylalanine to tyrosine substitution of AA261 (SNP14), known for causing a strong red-shift of the λ_{\max} values in retinal rods of many teleost families (Hunt *et al.* 1996, 2001; Yokoyama & Takenaka 2004). A comparative study on Salmonidae showed that the Phe261Tyr substitution causes a red-shift of ca. 10 nm in *Salmo salar* in comparison with *Oncorhynchus* sp. (Dann *et al.* 2004). A mutagenesis experiment of this mutant in *Astyanax fasciatus* confirmed a red-shift of 8 nm in λ_{\max} values (Yokoyama *et al.* 1995). The second well known mutation is on AA299 (SNP19). This site is localized towards the interior of the retinal binding pocket in helix VII (Fig. 2) and close to the Schiff base linkage between the opsin and the chromophore (Hunt *et al.* 2007). It suggests that this AA directly interacts with the chromophore (Fasick & Robinson 1998). A weak blue-shift of the λ_{\max} values of retinal rods caused by the Ala299Ser/Thr substitution has already been documented in many teleost families (Yokoyama *et al.* 1995; Hunt *et al.* 2001) and in the bottlenose dolphin (*Tursiops truncatus*) (Fasick & Robinson 1998).

Limited information is available for the three other AA-substitutions in the data set (AA151, AA214 and AA217). A comparative analysis of all available *RH1* genes of Teleostei on GenBank showed that those three AA sites are not conserved in Teleostei (results not

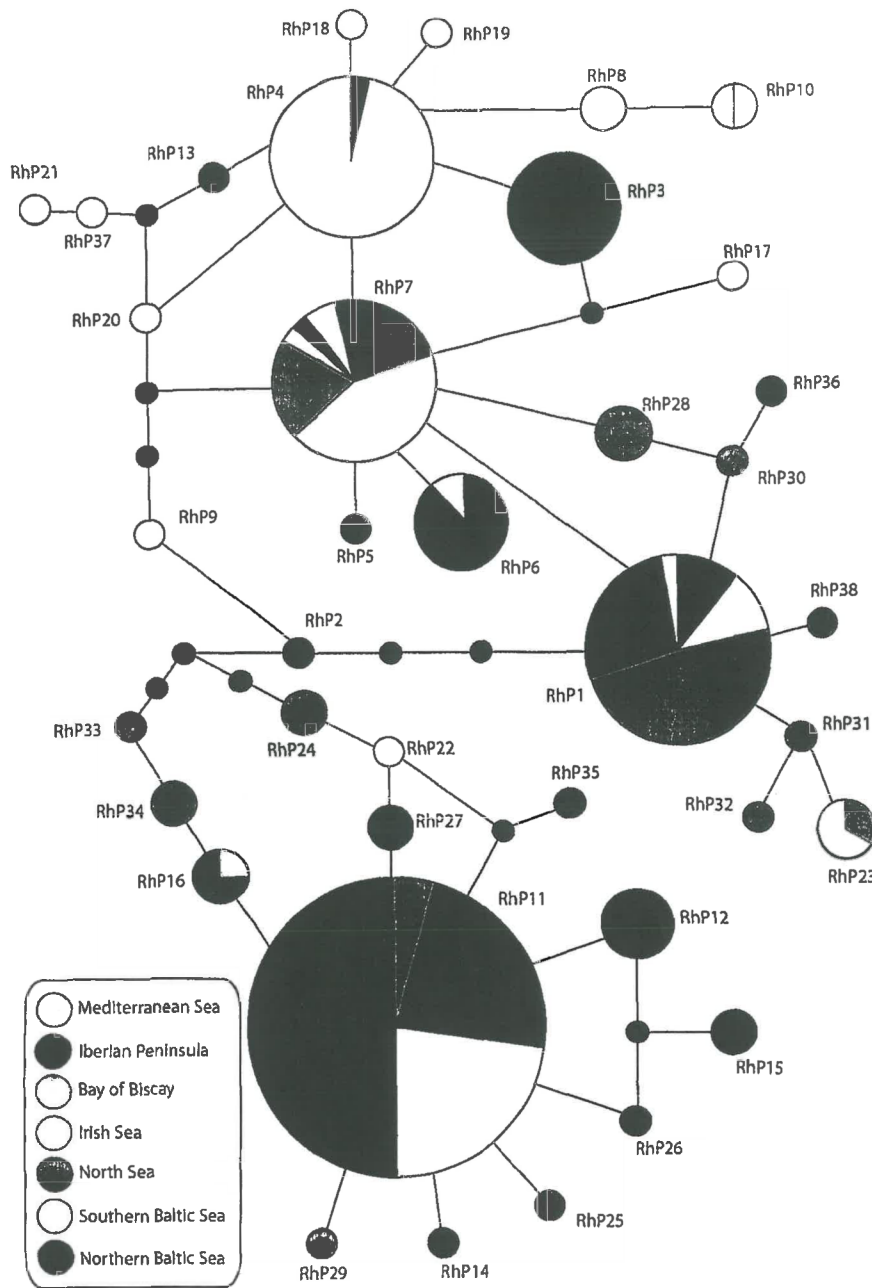


Fig. 3 Statistical parsimony network of the rhodopsin haplotypes of *Pomatoschistus minutus*. The size of the circles is proportional to the number of gobies sharing that haplotype. Haplotypes are indicated by numbers as given in Table S1. Black dots are undetected haplotypes.

	TBS	PBS	BNS	WIS	GOA	AAO	VMS
TBS	—	—	0.0095	0.0128	0.0132	0.0125	0.0251
PBS	0.1322	—	—	—	—	—	—
BNS	0.2976	0.2013	—	0.0038	0.0038	0.0122	0.0184
WIS	0.6551	0.6205	0.2893	—	0.0086	0.0097	0.0153
GOA	0.6647	0.6317	0.2961	-0.0213	—	0.0130	0.0145
AAO	0.8598	0.8732	0.6119	0.1653	0.1668	—	0.0166
VMS	0.1509	0.3108	0.3809	0.6942	0.7030	0.8688	—

Table 3 Pairwise F_{ST} -estimates based on the *RH1* gene (below diagonal) and based on the microsatellite loci (above diagonal) between sand goby populations (significant P -values after Bonferroni correction are in bold)

For site codes, see Table 1.

shown). The effect on λ_{max} values of substituted AA214 and AA217 (SNP9_10_11 and SNP12, respectively) has been tested by mutagenesis experiments on red/green

opsins (Yokoyama 2000). Only substitution Ile214Thr caused a substantial difference of <5 nm from red to green in red/green opsins (Asenjo *et al.* 1994). How-

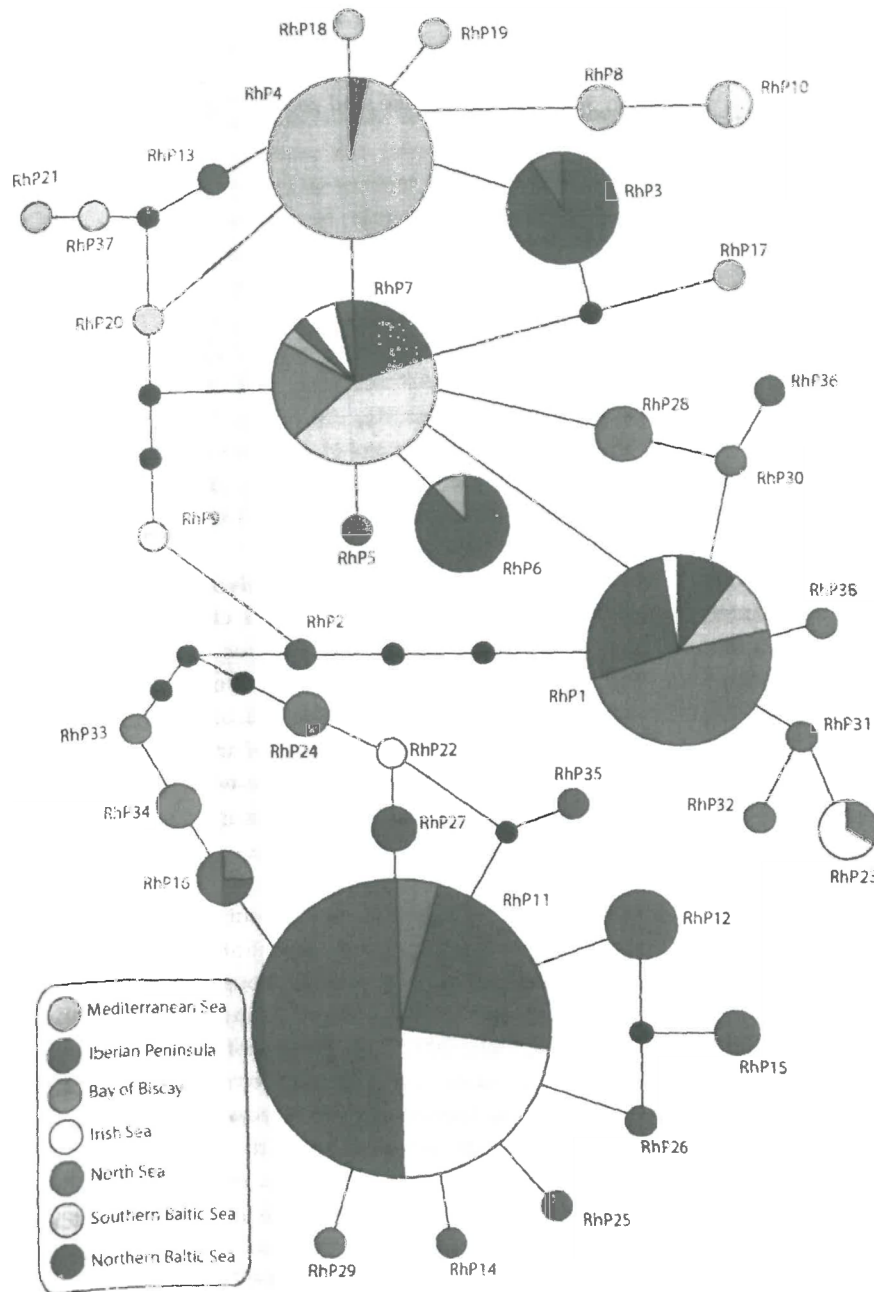


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	Population						
	TBS	PBS	BNS	WIS	GOA	AAO	VMS
AA151 (or SNP4)							
Asn	0.125	0.250	0.741	0.929	0.977	0.960	0.107
Thr	0.875	0.750	0.259	0.071	0.023	0.040	0.893
AA214 (or SNP9_10_11)							
Ala	0.975	0.950	0.704	0.286	0.273	0.040	0.893
Ile	0.025	0.050	0.296	0.714	0.727	0.960	0
Thr	0	0	0	0	0	0	0.107
AA217 (or SNP12)							
Ile	0.625	0.950	0.981	0.976	1	0.973	0.179
Thr	0.375	0.050	0.019	0.024	0	0.027	0.821
AA261 (or SNP14)							
Phe	0.450	1	1	1	1	0.973	0.857
Tyr	0.550	0	0	0	0	0.027	0.143
AA299 (or SNP19)							
Ala	1	1	0.667	0.238	0.250	0.054	1
Ser	0	0	0.333	0.762	0.750	0.946	0

Table 4 Frequency of the AA substitutions detected at the *RH1* gene in seven sand goby populations

The highest frequency in a population is given in bold for each AA. For site codes, see Table 1.

Table 5 Log-likelihood values and parameter estimates for the *RH1* gene sequences of *Pomatoschistus minutus*

Model	ln L	Estimates of parameters	Positively selected sites
M7 (beta)	-1361.766	$P = 0.02286$ $q = 0.14384$	
M8 (beta & ω)	-1337.268	$q_0 = 0.97818$ $(P_1 = 0.02182)$ $P = 0.00500$ $q = 0.27884$ $\omega = 6.02381$	151** 214** 261 299**

ln L is the log-likelihood value, ω is the selection parameter and P_n is the proportion of sites that fall into ω_n site class. Sites inferred to be under positive selection are given at the 99% (**) confidence interval level.

ever, the effect of a substitution on the AA214 in the rhodopsin gene remains unknown. AA151 (SNP4) is the only AA-substitution that is not located in the helix structure (Fig. 2), but may still affect λ_{\max} values (Yokoyama *et al.* 2007). Mutagenic experiments on the *RH1* gene are required to study the effect of these five non-synonymous mutations on the λ_{\max} values of retinal rods in *P. minutus*.

Network analysis of the significant *RH1* haplotypes (Fig. 3) and the highly significant F_{ST} -values (Table 3) revealed that variation at the *RH1* gene is not randomly distributed. Several populations are differentiated for several AA sites of *RH1*, including the two well known AA (AA261 and 299) that most probably influence the λ_{\max} values of retinal rods (Table 4). The differentiation

is consistent with the λ_{\max} values on the retinal rods as measured by Jokela *et al.* (2003). North Sea and Atlantic samples have blue-shifted AA-substitutions instead of the red-shifted substitution in the northern Baltic Sea sample (TBS) (Table 4), consistent with the larger λ_{\max} values in the northern Baltic Sea gobies compared with their Atlantic relatives.

Based on the wide rod λ_{\max} distributions within sand goby populations, Jokela *et al.* (2003) suggested the presence of within-population polymorphism on the *RH1* gene. AA variation on *RH1*, including on AA261 and AA299, is indeed polymorphic in various populations, demonstrating a genetic basis for within-population variation in spectral sensitivity (Table 4). The equal distribution of tyrosine and phenylalanine on AA261 (SNP14) in the northern Baltic individuals, can explain the particularly broad λ_{\max} distribution spanning 5.7 nm of the spectrum in the population (Jokela *et al.* 2003).

Population differentiation on *RH1* due to selection

Our results suggest that interpopulation allelic variation of the *RH1* gene is linked to selection as opposed to neutral processes like genetic drift. The d_N/d_S substitution rate ratios of the complete *RH1* fragment in *P. minutus* did not reveal selection. However, tests of neutrality are generally conservative because substitution rates are averaged across all amino-acid sites tested (Bamshad & Wooding 2003). Consequently, analyses of d_N/d_S ratios and likelihood ratio tests under site-specific models detected significant signal of positive Darwinian selection on the *RH1* gene. Bayesian analysis identified

Baseline	Marker	Mantel	P-value Mantel	Procrustes R	P-value Procrustes R
μ_{sats}	SNPsyn+non	0.307	0.100	0.218	0.910
	SNPsyn	0.202	0.167	0.143	0.972
	SNPnon	0.428	0.053	0.245	0.919
	SNP1 (s)	0.087	0.315	0.158	0.916
	SNP4 (n)	0.340	0.111	0.153	0.939
	SNP6 (s)	0.188	0.197	0.174	0.946
	SNP7 (s)	0.199	0.190	0.169	0.932
	SNP9 (n)	0.240	0.152	0.601	0.092
	SNP10 (n)	0.208	0.171	0.220	0.916
	SNP11 (s)	0.208	0.172	0.221	0.894
	SNP12 (n)	0.633	0.048	0.453	0.433
	SNP13 (s)	0.175	0.242	0.112	0.977
	SNP14 (n)	0.300	0.273	0.351	0.524
	SNP15 (s)	0.012	0.395	0.400	0.410
	SNP16 (s)	0.629	0.108	0.581	0.175
	SNP17 (s)	0.184	0.237	0.288	0.753
	SNP19 (n)	0.223	0.162	0.172	0.931

Significant *P*-values (<0.05) are given in bold. μ_{sats} , microsatellite markers; SNPsyn+non, all rhodopsin SNPs of the *RH1* gene; SNPsyn, all synonymous SNPs of the *RH1* gene; SNPnon, all nonsynonymous SNPs of the *RH1* gene; (s), a polymorphic synonymous SNP of the *RH1* gene; (n), a polymorphic nonsynonymous SNP of the *RH1* gene.

three individual positively selected sites in *RH1*, including AA299, which was verified as a true tuning site for rhodopsin (Table 5).

Moreover, selective forces most probably influence the rhodopsin gene of *P. minutus*, as the samples did not group according to geographical or historical proximity with regards to *RH1* variation (Hemmer-Hansen *et al.* 2007). Strong discrepancies were found between the distribution of the variation at *RH1* and the phylogeographic pattern of the sand goby based on the distribution of the variation at the mtDNA Cyt *b* gene (Larmuseau *et al.* 2009) and nuclear allozyme (Stefanni *et al.* 2003) and microsatellite markers (M. H. D. Larmuseau, unpublished data). Samples of the northern Baltic Sea and Mediterranean Sea carry a similar allelic profile of the *RH1* gene (Fig. 3; Tables 2 and 3), although historically the Mediterranean *P. minutus* individuals belong to a different phylogeographic mtDNA Clade (MS-Clade) than the Atlantic and Baltic sand gobies (AO-Clade) (Larmuseau *et al.* 2009). The *RH1* gene was also congruent between sand gobies from the Iberian Peninsula and the Irish Sea—Bay of Biscay. However, the Iberian sand gobies belong to a different historical unit (IB-Group) compared with the North Atlantic gobies (NA-Group), which includes all the populations from the Bay of Biscay to the northern Baltic. Additionally, distributions of the *RH1* and microsatellite variation were statistically significant different from each other (Table 6). No convincing evidence was found for non-neutrality of any of the microsatellites used to represent the neutral baseline. In contrast, each SNP of the

Table 6 Summary of the Mantel tests and Procrustes analyses correlating pairwise F_{ST} -values based on microsatellites markers vs. the *RH1* gene in *Pomatoschistus minutus*

RH1 gene was clearly identified as an outlier locus in comparison with the microsatellite markers. Therefore, random processes may be ruled out to explain the functional differentiation on the *RH1* gene between the different sand goby populations.

The link between functional variation on RH1 and environmental light climate

The significant discrepancies at various levels between the distribution of neutral markers and *RH1* gene data suggest that its variation is influenced by the optical environment instead of genetic drift. Differences in optical characteristics clustered the sampling locations into three groups: the Mediterranean Sea, Iberian Peninsula, the Bay of Biscay and Irish Sea (VMS, AAO, GOA and WIS) with a mainly blue light climate; the southern Baltic and North Sea (PBS and NBS) with greener water and the northern Baltic Sea (TBS) with water with the highest WMTL values (Fig. 1, Jerlov 1976). In general, these robust differences in environmental light transmittance correspond well with the differences in the absorbance spectra of the retinal rods (Jokela *et al.* 2003) and with functional variation at the *RH1* gene. The blue-shift of the λ_{max} values of the dim-light receptors and the highest frequency of the Ala299Ser substitution in the Bay of Biscay, Irish Sea and the Iberian Peninsula is characteristic for offshore blue water. The red-shift of the λ_{max} values of the rods and the highest frequency of Phe261Tyr substitution in the northern Baltic might be an adaptation to the red-shifted light condition in this region. One remark-

able observation is that the individuals of the Mediterranean Sea clustered with the northern Baltic samples based on the *RH1* variation. In the Mediterranean Sea, coastal lagoons play an important role in the life cycle of sand gobies as nurseries and feeding sites (Bouchereau & Guelorget 1998), whereas this is not the case for Atlantic sand gobies (Guelinckx *et al.* 2008). These lagoons, which are not included in Fig. 1, are characterized by a much higher turbidity than offshore (Poizat *et al.* 2004). Such conditions are thought to require spectral adaptations similar to those in the Northern Baltic.

Conclusion

The three conditions to demonstrate local adaptation at the rhodopsin gene of the sand goby are fulfilled. First, functional polymorphism was observed in the *RH1* gene. Then, it was demonstrated that population differentiation at the *RH1* gene was because of selection. Finally, a correlation was found between *RH1* variation and the specific spectral characteristics of the habitat environment of the sand goby. Therefore, there are good indicators for local adaptation of the rhodopsin gene in *Pomatoschistus minutus*. Further molecular research with a higher sampling resolution in space and time is required to disentangle the temporal variability of the *RH1* polymorphism and the small-scale differentiation on the *RH1* gene for *P. minutus* inside the various marine systems.

The hypothesis that sand gobies are evolutionary adapted to their optical environment implies that rapid changes in optical habitat characteristics may have negative consequences. For example, increased water turbidity by algal blooms in the highly polluted Baltic Sea negatively influenced sexual behaviour of fishes with a visual mating system. Cases have been documented for sand goby (Järvenpää & Lindström 2004) and three-spined stickleback (*Gasterosteus aculeatus*) (Engström-Öst & Candolin 2007; Candolin *et al.* 2008). Water quality of lagoonal and coastal waters can be influenced by anthropogenic changes in the nutrient load and by climatic factors. Therefore, if temperature continues to rise, spectral transmission of the water may shift with temperature (Archer *et al.* 2001). A marine monitoring program for water clarity and optical properties is therefore recommended, not only to consider their effect on primary productivity but also because of their direct influence on the visual capacity of the fish community (Aksnes 2007) and other organisms.

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Supporting information

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

Table S1 Rhodopsin haplotypes and their geographical distribution in *Pomatoschistus minutus*. The numbers of haplotypes reconstructed with a probability $\geq 95\%$ (estimated using PHASE 2.0.2) are listed in bold. The numbers of haplotypes that were nonsignificantly reconstructed are given in italic and between brackets. Dots indicate homology with haplotype RhP1. For site abbreviations, see Table 1

Table S2 Nucleotide polymorphisms at the *RH1* gene in seven sand goby populations. Dots indicate homology with the reference sequence (Accession no. X62405 or haplotype RhP1). AA numbers are listed for the nonsynonymous mutations, which are listed in bold. For site codes, see Table 1

Appendix S1 Estimates of wavelengths of maximally transmitted light for European seas: theory and description of method.

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