The Journal of Experimental Biology 212, 3415-3421
Published by The Company of Biologists 2009
doi:10.1242/jeb.031344

Individual variation in rod absorbance spectra correlated with opsin gene polymorphism in sand goby (*Pomatoschistus minutus*)

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Accepted 28 July 2009

SUMMARY

Rod absorbance spectra, characterized by the wavelength of peak absorbance (λ_{max}) were related to the rod opsin sequences of individual sand gobies (*Pomatoschistus minutus*) from four allopatric populations [Adriatic Sea (A), English Channel (E), Swedish West Coast (S) and Baltic Sea (B)]. Rod λ_{max} differed between populations in a manner correlated with differences in the spectral light transmission of the respective water bodies [λ_{max}: (A)≈503 nm; (E and S)≈505–506 nm; (B)≈508 nm]. A distinguishing feature of B was the wide within-population variation of λ_{max} (505.6–511.3 nm). The rod opsin gene was sequenced in marked individuals whose rod absorbance spectra had been accurately measured. Substitutions were identified using EMBL/GenBank X62405 English sand goby sequence as reference and interpreted using two related rod pigments, the spectrally similar one of the Adriatic *P. marmoratus* (λ_{max}≈507 nm) and the relatively red-shifted Baltic *P. microps* (λ_{max}≈515 nm) as outgroups. The opsin sequence of all E individuals was identical to that of the reference, whereas the S and B fish all had the substitution N151N/T or N151T. The B fish showed systematic within-population polymorphism, the sequence of individuals with λ_{max} at 505.6–507.5 nm were identical to S, but those with λ_{max} at 509–511.3 nm additionally had F261F/Y. The substitution F261Y is known to red-shift the rod pigment and was found in all *P. microps*. We propose that ambiguous selection pressures in the Baltic Sea and/or gene flow from the North Sea preserves polymorphism and is phenotypically evident as a wide variation in λ_{max}.

Key words: opsin sequence, visual pigment, evolutionary adaptation

INTRODUCTION

Pure water transmits light maximally in the blue range of the spectrum, but in natural coastal and fresh waters, blue light is strongly absorbed and scattered by dissolved organic or inorganic matter. When going from marine to coastal, brackish or fresh water basins, the ambient light environments tends to shift from blue to green, yellow or even reddish brown, challenging the visual systems of the animals and creating a laboratory for studies on evolutionary adaptation of visual pigments (e.g. Hunt et al., 1996; Hunt et al., 2001; Hope et al., 1997; Yokoyama and Tada, 2000). The performance of rod vision is expected to depend in a straightforward manner on the degree of 'match' between the absorbance spectrum of the visual pigment and the illumination spectrum, because the visual information obtained at low light levels is critically dependent on photon catch, and rods alone convey information at these light levels. Thus there is a direct link between the structure of the receptor protein, opsin, and a critical functional property of the organism, i.e. absolute visual sensitivity. The rod opsin gene is extraordinarily directly 'targeted' by natural selection acting on phenotypes.

In many fishes and amphibians, large red-shifts can be achieved on a physiological time scale by changing the prosthetic group, the chromophore, from retinal (A1) to 3,4-didehydroretinal (A2). The use of A2, however, is associated with a strong increase in the spontaneous thermal activations of the pigment, which will constitute a dark noise, detrimental to absolute sensitivity (Donner et al., 1990; Ala-Laurila et al., 2007). Excepting chromophore exchange, shifts in the absorbance spectrum of a functional visual pigment are necessarily based on amino acid substitutions in the opsin. In our model species, the sand goby (*Pomatoschistus minutus* Pallas 1770), no A2 chromophore has ever been detected.

The sand goby is one of the most abundant marine fish species in Europe, occurring all the way along the coast from Norway and the Baltic Sea to the Mediterranean and the Black Sea (Miller, 1986). In the off-breeding season (winter), the fish migrate to depths of tens of metres, even 100 m, spending at least part of their life cycle at low light levels with a narrow spectral distribution that strongly depends on the transmission properties of the water. Large-scale dispersal (during the larval stage) is determined by oceanic and coastal currents (Miller, 1986), which in general leads to a low degree of population differentiation (Ward et al., 1994) and less resolved phylogeographical patterns than in freshwater and terrestrial species. However, a number of studies on the phylogeography and genetics of sand goby populations, especially on Venetian/Adriatic *P. minutus* (Stefanni et al., 2003; Gysels et al., 2004; Huyse et al., 2004), indicate that Adriatic and Baltic populations are genetically distinct, the latter grouping with other Atlantic Ocean *P. minutus* populations. The Adriatic and Baltic Seas are enclosed waters with barriers for dispersal and different spectral light transmissions, the former peaking around 450–475 nm and the latter around 550–575 nm. These factors will in principle favour adaptive divergence of the opsin gene.

The purpose of the present work was to investigate evolution of rod spectral sensitivity in a single species with populations isolated in environments with different spectral illumination for time scales even as short as 10^4 years. By combining microspectrophotometry and opsin sequencing in single individuals we obtain a correlated picture of phenotypic and genetic variation within as well as between populations.

MATERIALS AND METHODS

Animals

The sand gobies (*Pomatoschistus minutus*) were obtained by netting in the Baltic Sea (SGB; Tvärminne Zoological Station, Finland), the west coast of Sweden (SGS; Kristineberg Marine Research Station), the English Channel (SGE; the Marine Biological Association, Plymouth), and the Adriatic Sea (SGA; Chioggia Marine Biological Station, Italy). The other goby species included in this study were common goby (*P. microps*) from the Baltic Sea (CGB; Tvärminne Zoological Station, Finland) and marbled goby (*P. marmoratus*) from the Adriatic Sea (MGA; Chioggia Marine Biological Station, Italy; Fig. 1). After capture, fish were transferred in special water bags or tanks to the animal care facilities at the University of Helsinki. They were kept in aquaria with the salinity of their respective habitat (from 0.6% in the Baltic Sea to 3.5% in the Adriatic Sea).

Microspectrophotometry

For the microspectrophotometry (MSP), living fish were pithed and decapitated and both retinae isolated in cooled fish Ringer under dim red light. The Adriatic sand gobies had died soon after netting, however, and they were frozen (–18°C) and stored frozen in darkness until MSP and sequencing. We found no significant difference in λ_{max} of cells from retinas that had been frozen and then thawed compared with fresh retinas.

The methods for MSP have been described in detail by Jokela et al. (Jokela et al., 2003). For each individual, an average of 35 rods were recorded (range 15–65). After averaging, base-line correction, and removal of high-frequency noise by Fourier filtering (retaining 25–35 harmonics), the spectrum from each individual was fitted with Govardovskii et al. (Govardovskii et al., 2000) templates. In all individuals, the best fit was obtained with pure A1 template. Fits with any sum of A1 and A2 templates were always perceptibly poorer. Thus there was never any indication of the presence of A2, which, given the accuracy of our methods, means that any possible A2 fraction must have been <5%. In the spectra measured for the present work, differences of several nanometres in λ_{max} (e.g. within the B population) could under no circumstances be explained by varying proportions of chromophores A1 and A2 (Jokela et al., 2003).

Govardovskii et al. (Govardovskii et al., 2000) templates give standard curves with λ_{max} as the sole variable. Thus we use λ_{max} alone to characterize the entire spectra and the statistics of spectral variation within and between the different populations.

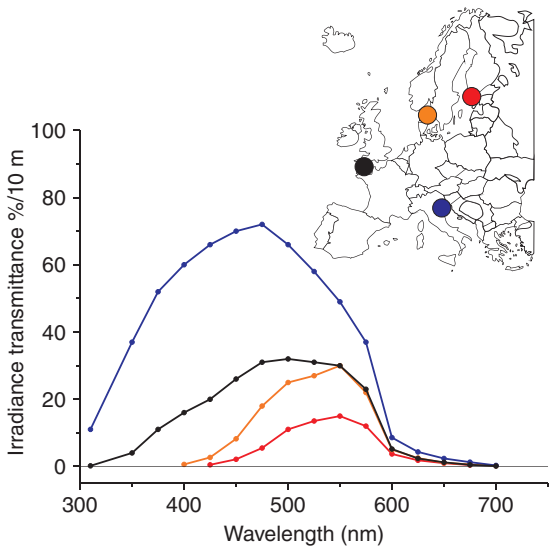

Fig. 1. Geographical locations and spectral light environments of the four sand goby populations studied (red, Baltic Sea; orange, Swedish West Coast; black, English Channel; and blue, Adriatic Sea). Individuals of two related species studied for comparison (*P. marmoratus* and *P. microps*) were caught in the same habitats as two of the sand goby populations (Adriatic Sea and Baltic Sea, respectively). Spectral light environments are from Jerlov's classification of types of natural water (Jerlov, 1976).

Sequencing of the rod opsin gene

After MSP recording of rod absorbance spectra, each individual was marked and frozen separately in carefully identified plastic bags for later DNA extractions and opsin gene sequencing. Identified individuals were thawed for about 30 min at room temperature and approximately 25 mg of the trunk muscles was dissected. The total genomic DNA from the trunk muscle was extracted and purified by the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The DNA was eluted in 30–60 µl of buffer EA and stored at –20°C before use. The first set of primers (SG1-SG2) for the polymerase chain reaction (PCR) was designed according to the sand goby rhodopsin sequence published by Archer et al. (Archer et al., 1992) (EMBL/GenBank X62405; Table 1). The primer sets were designed to split the gene in two pieces in order to increase the sequencing success rate. The exact nucleotide positions (sand goby rhodopsin) to generate shorter nucleotide chains are shown in Table 1. Additional primers were generated according to the opsin sequences obtained (Table 1).

The PCR conditions with different primers were as follows (50 µl reaction): from 3 to 8 µl of genomic DNA corresponding to 100–300 ng of genomic DNA, 1 µl (20 pmol l^{-1}) of primers (two per reaction), 0.5 µl (25 mmol l^{-1}) dNTPmix, 5 µl of 10× buffer, 1.25 U of enzyme (Biotools *Pfu* DNA Polymerase, B & M Labs, Madrid, Spain) and sterile water to 50 µl. Amplification was performed in 29 cycles with the following profile: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 5 min. The success of the PCR reaction was checked by analysing a 10 µl sample of PCR product on a 1% agarose gel. For the Adriatic gobies the following PCR conditions were used: 2–10 µl (150–400 ng) of genomic DNA, 1 µl (20 pmol l^{-1}) of primers (two per reaction), 0.5 µl (25 mmol l^{-1}) dNTPmix, 2 µl DMSO, 10 µl Buffer HF (5× Phusion™ HF Buffer, Finnzymes Oy, Finland) and 1 U enzyme (Phusion™ High-Fidelity DNA Polymerase, Finnzymes Oy, Finland). The amplifications were performed in 30 cycles with the following profile: denaturation at 98°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s.

Before sequencing, the PCR product was purified using a Millipore PCR-purification plate (Millipore, Watford, UK) and dissolved in 40 µl of distilled water. Sequencing was done using BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) and 1–5 µl PCR template on an ABI3100 capillary sequencer system (Applied Biosystems, Foster City, CA, USA). The obtained sequences were assembled and edited using the GAP4 program from the Staden Package (Bonfield et al., 1995). The obtained sequences were submitted to the EMBL. The accession numbers are given in Table 2.

RESULTS

Microspectrophotometry

The main MSP results have been described in Jokela et al. (Jokela et al., 2003). Briefly, the distributions of rod λ_{max} values among individuals representing four allopatric sand goby populations

Table 1. List of the oligonucleotide PCR and sequencing primers used and their nucleotide positions

Primer	Nucleotide sequence (5′→3′)	Direction +/–	Acc. no. X62405 nucleotide position (sand goby rhodopsin numbering)
SG1*	TGA ATT CAC CGC CAA CAA AC	+	19–38
SG2*	ATG ATC CAG GAG AAA GCC AC	–	514–533
SG3†	CAC CGC CAA CAA ACC GCA AC	+	25–44
SG4†	AGA ATT TAG TGG TTT CCC AG	–	1147–1166
SG5‡	ATG AAC GGC ACG GAG GGA CC	+	46–65
SG6‡	ATG AAC GGT ACG GAG GGA CC	+	46–65
SG7C‡	AGA GGC GGC ACA GCA CAG GT	–	541–560
SG8§	CTG CAA ACC CAT CAG CAA CT	+	462–481
SG9§	GTG AGT GTC GGT CTT GTT TTA	–	1102–1122
SG10**	TCG ACT CCA CAT GAG CAC TG	–	595–615

*Original PCR- and sequencing primer pair.
†Primer pair for most of the PCR and sequencing reactions, whole gene.
‡Primer pair used for the Adriatic sand gobies for the PCR and sequencing reactions, first part.
§Primer pair used for the Adriatic sand gobies for the PCR and sequencing reactions, last part.
**Additional primer for the sequencing reactions.
Altogether, three different PCR primer pairs were used (†,‡,§).
Original nucleotide numbering system follows that of Archer et al., 1992. The open reading frame begins from base position 46 and ends at position 1102.

(Baltic, Swedish, English and Adriatic), common goby (*P. microps*) from the Baltic Sea, and marbled goby (*P. marmoratus*) from the Adriatic Sea were determined by MSP recording from outer segments of single rods, averaging 15–65 of single-rod spectra in each individual. Small but statistically significant differences were found between the sand goby populations. Mean (± s.d.) wavelengths of peak absorbance (λ_{max}) were 508.3±1.9 nm (B, *N*=17), 505.4±0.5 nm (S, *N*=10), 506.2±1.0 nm (E, *N*=9) and 503.0±1.3 nm (A, *N*=18). *P. microps* is basically a freshwater species for which λ_{max}=515.7±0.4 (*N*=11). Both of the *P. marmoratus* individuals studied had λ_{max}=506.7 nm. All individuals had pure A1 pigments as judged by Govardovskii et al. (Govardovskii et al., 2000) templates fitted to the spectra. This indicates that there must be amino acid substitutions in the protein part of the pigment, the opsin. Our objective was to correlate differences in λ_{max} between identified individuals from all populations with differences in the amino acid sequences of their rod opsin.

Table 2. The wavelength of peak absorbance and the EMBL accession numbers of opsin sequences for the sand goby, the common goby and the marbled goby

Species/fish	Origin	λ_{max} (nm)	EMBL accession numbers
Sand goby			
$SGB1_I$	Baltic	507.5	FN430589
$SGB2_I$		506.5	FN430593
$SGB3_I$		507.9	FN430590
$SGB4_I$		505.6	FN430594
$SGB5_{II}$		511.0	FN430595
$SGB6_{II}$		510.4	FN430591
$SGB7_{II}$		511.3	FN430596
$SGB8_{II}$		509.0	FN430597
SGS9	Swedish	505.8	FN430598
SGE10	English	505.6	FN430599
SGE11		506.8	FN430600
SGE12		507.8	FN430601
SGA13	Adriatic	503.8	FN430592
SGA14		503.0	FN430602
SGA15		504.1	FN430603
SGA16		502.4	FN430604
SGA17		500.8	FN430605
Common goby			
CGB1	Baltic	514.2	FN430606
CGB2		516.7	FN430607
Marbled goby			
MGA1	Adriatic	506.7	FN430608
MGA2		506.7	FN430609

λ_{max}, wavelength of peak absorbance.

Sequencing of the rod opsin gene

The rod absorbance spectra of each fish used for sequencing had been carefully determined and the individual marked for reliable identification. With the exception of Baltic sand gobies (see below), individuals from each population were chosen essentially randomly for sequencing. Statistical analysis (SPSS, independent samples *t*-test) was applied to ascertain that the animals selected were fair representatives of the average λ_{max} of each population.

The Baltic sand gobies were divided into two groups on the basis of their rod λ_{max}, one for 'short-wavelength' (BI, λ_{max}=506–508 nm) and the other for 'long-wavelength' (BII, λ_{max}=509–511 nm) fish, and four individuals were drawn from each group (SGB1–4, and SGB5–8, respectively). Altogether we sequenced and analyzed five sand gobies from the Adriatic Sea, three from the English Channel, one from the west coast of Sweden and eight from the Baltic Sea. Two common gobies and two marbled gobies were included in the study for comparison (Table 3).

The first sets of primers (SG1–SG2: Table 1) were tested with two Baltic sand gobies to generate a gene fragment (size 500 pb) of the first part of the opsin gene. For the other fish we generated additional primers (SG3–SG10) to improve PCR success. The size of the whole genomic opsin fragments was 1 kb. No introns were found in the coding region, as is typical of fish rod opsin (Fitzgibbon et al., 1995). The highest PCR success rate was achieved with primer pair SG3–SG4 (Table 1), and most of the sequences were obtained with this pair. Additional primers used for the sequencing reaction were SG2, SG7 and SG8. For the Adriatic sand gobies, PCR and sequencing were performed in two pieces; the first part with SG5 or SG6 and SG7 and the second part with SG8–SG9.

We also studied 'silent' nucleotide polymorphisms, i.e. nucleotide differences that do not alter the amino acid sequence of the expressed opsin. Several such sites were detected between the

Table 3. The nucleotide and amino acid changes in the opsin sequence of sand goby populations, common goby and marbled goby

		Nucleotide/amino acid change														
Species/fish	λ_{max} (nm)	V14 GTG	I19 ATC	G41 GGC	A71 GCT	L74 TTA	V112 GTG	V133 GTT	T149 ACT	N151 AAC	I205 ATC	A214 GCT	I217 ATC	F261 TTT	T263 ACA	A299 GCT
Sand goby																
$SGB1_I$	507.5									→ T ACC						
$SGB2_I$	506.5									→N/T AA/CC						
$SGB3_I$	507.9									→ N/T AA/CC						
$SGB4_I$	505.6									→T ACC						
$SGB5_{II}$	511.0									→ N/T AA/CC				→F/Y TT/AC		
$SGB6_{II}$	510.4	→M/V A/GTG								→T ACC				→F/Y TT/AC		
$SGB7_{II}$	511.3									→T ACC				→F/Y TT/AC		
$SGB8_{II}$	509.0	→M/V A/GTG								→T ACC			→I/T AT/CC	→F/Y TT/AT		
SGS9	505.8									→N/T AA/CC						
SGE10	505.6															
SGE11	506.8															
SGE12	507.8															
SGA13	503.8	→M ATG										→T ACT	→ I/T AT/CC			
SGA14	503.0	→M ATG										→T ACT	→T ACC			
SGA15	504.1											→T ACT	→I/T AT/CC			
SGA16	502.4	→M ATG										→T ACT	→T ACC			
SGA17	500.8	→M ATG										→T ACT	→T ACC			
Common goby																
CGA1	514.2		→V GTG	→G/S A/GGC	→P CCT	→Y TAT	→L TTG		→S AGT		→V GTC	→I ATT	→V GTC	→Y TAC	→V GTA	→S TCT
CGA2	516.7		→V GTG		→P CCT	→Y TAT	→L TTG		→S AGT		→V GTC	→I ATT	→V GTC	→Y TAC	→V GTA	→S TCT
Marbled goby																
MGA1	506.7		→V GTG		→ P CCT	→Y TAT		→I ATT			→V GTC	→I ATT	→V GTC		→V GTA	→S TCT
MGA2	506.7		→V GTG		→P CCT	→Y TAT		→I ATT			→V GTC	→I ATT	→V GTC		→V GTA	→S TCT

λ_{max}, wavelength of peak absorbance.

species and between the sand goby populations. These polymorphisms also helped us to be sure that sequences were obtained from both alleles of the opsin gene.

Differences in amino acid sequences

Differences between species

Compared with the opsin sequence derived from the reference sand goby cDNA sequence in the EMBL database (X62405), we detected 11 amino acid substitutions shared by the two common gobies plus one additional substitution in one of them (Table 3, Fig. 2A,B) The two marbled gobies had nine shared substitutions, eight of them found in the common gobies as well (I19V, A71P, L74Y, I205V, A214I, I217V, T263V and A299S) and only one of them (V133I) was characteristic of the marbled goby. L74Y is located in the second transmembrane (TM) helix; I205V, A214I and I217V in the fifth; T263V in the sixth and A299S in the seventh TM helix. Of these, A299 forms hydrogen bond with side chains of N55 in TMI and D83 in TMII to stabilize the structure of the protein (Palczewski et al., 2000). Substitution A299S is also known to slightly red-shift (+2 nm) the absorbance spectrum (Fasick and Robinson, 1998). Note that the rhodopsins of the English sand gobies and the marbled gobies were spectrally indistinguishable in spite of these nine substitutions (mean $\lambda_{max}\approx$506 and 507 nm, respectively). By contrast, the four additional substitutions that were specific to the common goby red-shifted its absorbance spectrum by 9 nm. Of these substitutions, we would like to draw particular attention to F261Y, which is known to red-shift human cone pigments (Merbs and Nathans, 1993) and was also found in the 'red-shifted' individuals of the Baltic sand goby population (cf. below and Discussion).

Differences between the sand goby populations

All the English sand gobies (N=3) were congruent with the reference cDNA sequence in the EMBL database (X62405) (Archer et al., 1992). Compared with this reference, the Swedish individual (λ_{max}=506 nm) as well as all Baltic individuals, had the single substitution N151N/T or N151T. The Swedish (S) and a Baltic

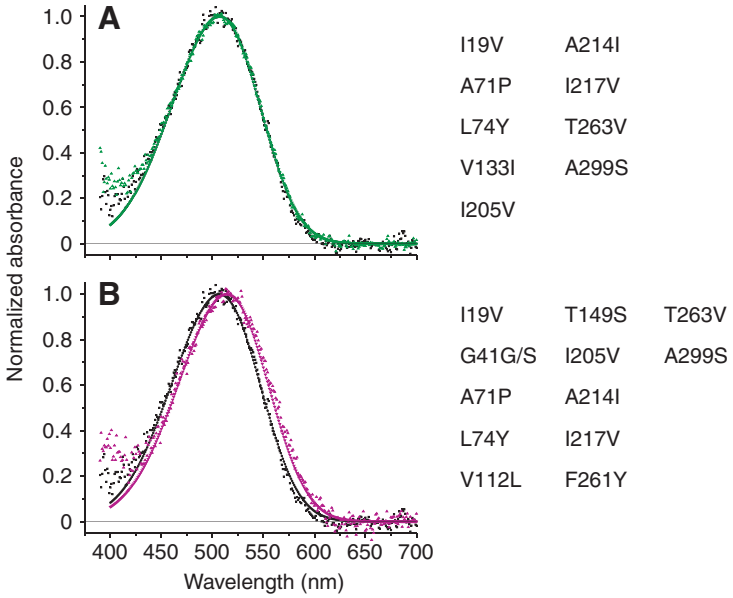

Fig. 2. Comparison of the rod absorbance spectrum of an E sand goby (black) with that of a marbled goby (green; A) and common goby (violet, B). Each spectrum is from a single individual, obtained as an average of recordings from 64, 42 and 16 single rods, respectively. The amino acid substitutions found in the rod opsin of that individual compared with the E individual [the sequence of which was identical to the reference of Archer et al. (Archer et al., 1992)] are shown beside the spectra. Eight of the substitutions are shared by the marbled and common goby. Only V133L is unique to the marbled goby, whereas G41G/S, V112L, T149S and F261Y are unique to the common goby.

individual representing the group with absorbance spectra that were virtually identical with those of the English are compared in Fig. 3A.

The Adriatic sand gobies had unique mutations. Four individuals out of five had mutation V14M and all had two mutations in the fourth TM segment: A214T and I217T (three of five) or I217I/T (two of five) (Table 3). The overall spectral result was a 3–4 nm blue-shift (Fig. 3B). Additionally, Adriatic sand gobies had silent nucleotide polymorphisms at seven sites.

Polymorphisms within the Baltic sand goby population

Compared with the English reference, the Baltic population fell into two groups. For sequencing, we selected eight individuals such that four had λ_{max} at 505.6–507.5 nm (mean ± s.d.: 506.9±0.5 nm) and four had λ_{max} at 509.0–511.3 nm (mean ± s.d.: 510.4±0.5 nm). All had the mutation N151T or N151N/T as already mentioned. In addition, all individuals in the 'red-shifted' group (BII) had mutation F261F/Y, located in the sixth helix and shared with the common gobies. In the red-shifted group, two further individuals had mutation

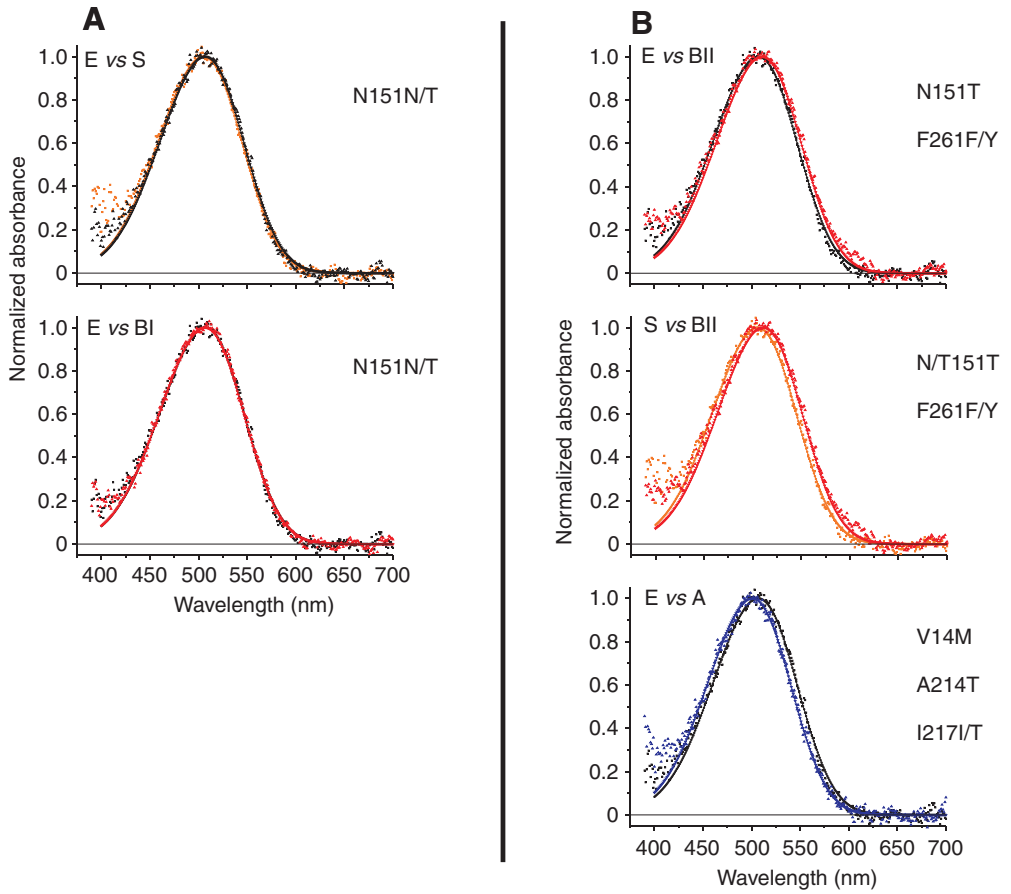

Fig. 3. Comparison of the rod absorbance spectrum of (A) the same E sand goby (black) with those of S and BI sand gobies (orange, top, and red, bottom, respectively) and (B) BII and A sand gobies (red, top, and blue, bottom, respectively). The middle part of panel B compares an S individual (orange) with the same BII individual as in the top part (red). Each spectrum is from a single individual obtained as an average of recordings from 64 (E), 17 (S), 49 (BI), 53 (BII) and, 23 (A) single rods. The amino acid substitutions found in the rod opsin of each individual compared with the E individual (or, for the middle part of panel B, the S individual) are shown beside the spectra.

V14V/M and one individual I217I/T. Comparisons of representative individuals that were spectrally different from the English reference are illustrated in Fig. 3B: 'red-shifted' Baltic BII and Adriatic A.

In Fig. 4, all 15 amino acid substitutions we found between the reference E sand goby sequence and the B, S and A sand goby populations, as well as the common goby, are marked in red in the two-dimensional rhodopsin model of Hargrave et al. (Hargrave et al., 1983). Also marked are K296, to which the chromophore is bound by a protonated Schiff base, and its counterion E113. Note that 10 of the sites were common to at least two of the populations/species studied, underscoring how certain regions of the opsin are better able to accommodate substitutions than others. In the opsin gene, there might be unusually little tolerance to changes that might compromise either the spectral, structural or enzymatic functionality of the protein.

DISCUSSION

Differences in mean λ_{max} between populations

Evolutionary adaptation of rods to the ambient light is particularly necessary and striking in aquatic environments (Lythgoe, 1972; Bowmaker, 1994; Yokoyama and Tada, 2000). In the dim and spectrally restricted illumination encountered even at moderate depths, maximizing quantum catch is imperative and sensitivity will depend even on small shifts of the visual pigment absorbance spectrum. Many studies have revealed spectral adaptations between different fish species (Hunt et al., 1996; Hunt et al., 2001; Hope et al., 1997; Carleton and Kocher, 2001; Parry et al., 2005; Carleton et al., 2005; Carleton et al., 2008; Seehausen et al., 2008). However, there has been little evidence on incipient evolutionary adaptation of rod spectral sensitivity between separated populations inhabiting spectrally different waters. In this paper we demonstrate correlated differences in spectral sensitivity and opsin sequence within a single species on the population level as well as on the individual level, within populations.

Each pair-wise comparison among the four sand goby populations studied revealed a statistically significant difference in λ_{max}, except for the comparison of E with S (post-ANOVA Scheffe's test) (see Jokela et al., 2003). Although the differences are small, they are qualitatively correlated with the differences in the ambient spectral light environments in the sense that each population catches more quanta in its own habitat than would the other populations (again excepting the comparison E *vs* S). For example, the quantum catch of an average Baltic sand goby in a Baltic light environment would be approximately 20% higher than that of an average Adriatic sand goby. Thus it seems justified to regard the differences in absorbance spectra between populations as adaptive, presumably based on selection-driven changes in the opsin sequences.

The λ_{max} values within the Baltic population spread over a 7 nm interval from 505.6 to 511.3 nm (mean value 508.3 nm). By contrast, the dispersion of λ_{max} in the English and Swedish populations was no wider than 3 nm. This suggested functional polymorphism within the Baltic population. The B fish that we sampled for sequencing (N=8) had λ_{max} values varying from 505.6 nm to 511.3 nm. Among these, we found that the 'short-wavelength-sensitive' individuals (BI; λ_{max} ranging from 505.6 to 507.9 nm, N=4), which were spectrally similar to the E and S populations, were congruent with these also with respect to locus F261. By contrast, the 'red-shifted' B individuals (BII; λ_{max} ranging from 509.0 nm to 511.3 nm, N=4) had a mixture of F261F/Y. The substitution F261Y was also found in both of the common gobies sequenced, the spectra of which were red-shifted by 8–10 nm compared with the BI sand gobies (CGB: λ_{max}=515.7±1.3 nm; mean ± s.d.). This substitution is known to red-shift human cone pigments, too (Merbs and Nathans, 1993), whereas the alternative substitution Y261F causes an 8 nm blue shift in cavefish spectra (Yokoyama et al., 1995). Position 261 is close to the β-ionone ring in the rhodopsin (Palczewski et al., 2000) and the substitution F261Y alters interhelical interactions. Since the mean λ_{max} difference between BI and BII sand gobies was as small as 4 nm, we assume that F261 and Y261 are co-expressed in BII.

In addition to this substitution, which can be given a robust functional interpretation, all Baltic gobies had one additional

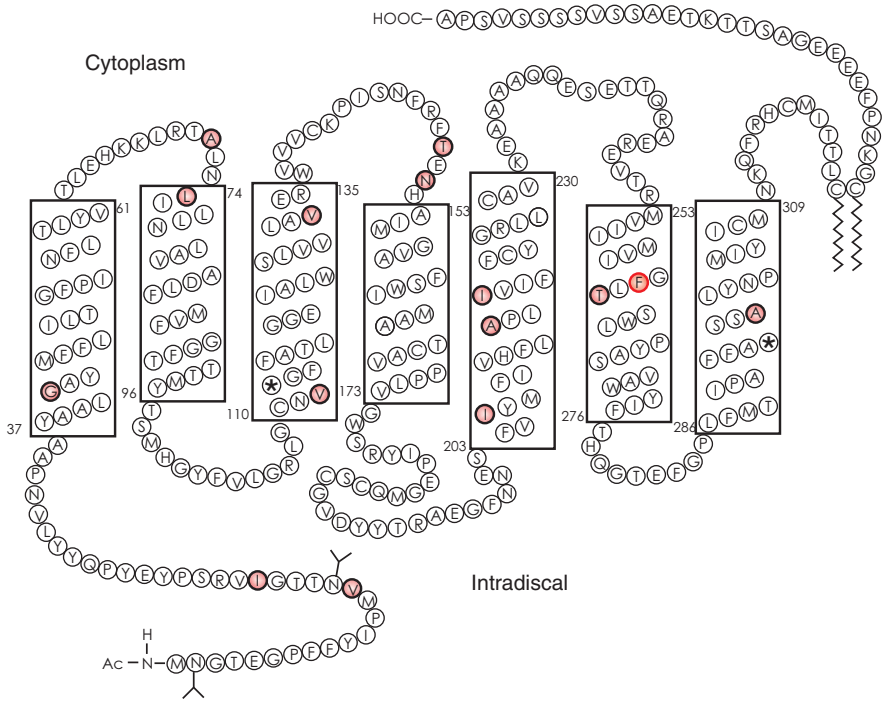

Fig. 4. Summary of all amino acid substitutions found between the E sand goby reference sequence and those of all the other sand goby populations, as well as that of the common goby. The 15 substitutions identified in the two-dimensional rhodopsin model of Hargrave et al. (Hargrave et al., 1983) have been marked in red; F261Y is highlighted by a red outline. Asterisks mark K296, to which the chromophore is bound by a protonated Schiff base, and its counterion E113.

substitution in the fourth helix, two had a mutation V14M/V and one had I217I/T compared with the E reference. We suggest that the polymorphism of the Baltic sand goby population may be due to ambiguous selection pressures in the Baltic Sea, as the organic matter content varies significantly with season and habitat. Considering that the immigration and isolation of *P. minutus* in the Baltic Sea occurred recently on an evolutionary time scale (less than 10,000 years ago), the Baltic population might not have attained equilibrium. Another possibility is that there is some gene flow from the North Sea through the Danish Straits.

The Adriatic population had three amino acid substitutions compared with the E reference. The correlation between the sequence and spectral absorbance in this case needs further analysis, since these substitutions have not previously been found to blue-shift spectra. Changes in sites 214 and 217 were found also in Baikal cottoid fishes by Hunt et al. (Hunt et al., 1996), but the authors did not suggest any functional significance. Two of the substitutions were in the TM segment IV. In addition, there were neutral nucleotide polymorphisms. The presence of a larger number of amino acid substitutions and nucleotide polymorphisms is consistent with the findings of Stefanni et al. (Stefanni et al., 2003) and Gysels et al. (Gysels et al., 2004), based on neutral markers, that the Adriatic sand gobies are genetically more distant from other sand goby populations.

We would like to thank Mrs Päivi Laamanen and Mrs Paula Collin-Olkkonen, Institute of Biotechnology, University of Helsinki, for their assistance with the DNA sequencing. This work was supported by The Academy of Finland (grants 206221 and 125666) and the Oskar Öflund Foundation.

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