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Parallel amino acid replacements in the rhodopsins of the rockfishes (*Sebastes* spp.) associated with shifts in habitat depth

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Abstract

Among various groups of fishes, a shift in peak wavelength sensitivity has been correlated with changes in their photic environments. The genus *Sebastes* is a radiation of marine fish species that inhabit a wide range of depths from intertidal to over 600 m. We examined 32 species of *Sebastes* for evidence of adaptive amino acid substitution at the rhodopsin gene. Fourteen amino acid positions were variable among these species. Maximum likelihood analyses identify several of these to be targets of positive selection. None of these correspond to previously identified critical amino acid sites, yet they may in fact be functionally important. The occurrence of independent parallel changes at certain amino acid positions reinforces this idea. Reconstruction of habitat depths of ancestral nodes in the phylogeny suggests that shallow habitats have been colonized independently in different lineages. The evolution of rhodopsin appears to be associated with changes in depth, with accelerated evolution in lineages that have had large changes in depth.

Introduction

Marine species live in a range of photic environments determined by factors such as turbidity, depth, salinity, phytoplankton load and productivity (Thurman & Trujillo, 2004). Correspondingly, a correlation between photic environment and the visual sensitivity of animals has been noted (reviewed by Bowmaker, 1995). Changes in visual sensitivity are known to be because of shifts in the wavelength of maximal absorption (λ_{max}) of visual pigments. Such shifts, commonly referred to as spectral tuning, can occur through a number of mechanisms. Among the cichlids of the East African Rift Lakes, for example, visual sensitivity varies among species as a consequence of differential expression of the cone opsin genes, with only a subset of these genes being expressed in any one species (Carleton & Kocher, 2001; Parry *et al.*, 2005). A more common means of altering visual sensitivity in opsins is by amino acid replacement. In opsins, the seven transmembrane domains form a binding pocket for the photosensitive chromophore, usually 11-*cis*-retinal, which is attached via a protonated Schiff's base (Yokoyama, 1995; Sakmar, 2002; Yokoyama & Takenaka, 2004). Changes in the amino acid sequence within these transmembrane domains alter the spectral sensitivity of the chromophore by changing the distance between the protonated Schiff's base and its counterion, changing the orientation of the chromophore or by otherwise altering its immediate environment (Takahashi & Ebrey, 2003). Individual amino acid substitutions can shift the λ_{max} of the pigment in question by as much as 75 nm in some cases, whereas in other cases there may be no shift associated with a particular replacement (Yokoyama, 2002; Takahashi & Ebrey, 2003; Spady *et al.*, 2005). Spectral shifts as a result of amino acid replacement have been well documented in a number of species. Among the cottoid species flock of Lake Baikal, there is a stepwise blue shift in λ_{max} among the deeper-dwelling species compared to the nearshore shallow-water species. The overall shift in λ_{max} is from about 516 nm in the littoral species to 484 nm in the species from the deepest abyssal habitats (Hunt *et al.*, 1996). Likewise, among the Holocentridae (squirrelfishes and soldierfishes), there is a range of variation in λ_{max} that is associated with depth of the preferred habitats of the various species (Yokoyama & Takenaka, 2004). These studies have identified particular amino acid replacements that are important in causing

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specific shifts in λ_{max}. It thus appears clear that some amino acid substitutions, e.g. E122M, F261Y and A292S (Yokoyama & Takenaka, 2004) are responsible for blue shifts in λ_{max} and appear to be adaptations to deep-water environments.

Rockfishes, belonging to the genus *Sebastes*, comprise a rare example of a marine species flock (Johns & Avise, 1998). There are over a hundred species in the genus, with about 65 of them occurring along the Pacific coast of the United States. While individuals of any given species can be found in a fairly wide range of depths, species have preferred depth ranges that are much narrower than their recorded depth ranges (Love, 1996; Love *et al.*, 2002). Here, we examine 32 species of *Sebastes* from the north-east Pacific, inhabiting a wide range of depths, and ask whether previously identified, critical sites show evidence of adaptation to habitat depth via amino acid substitution. The species vary from those that prefer very shallow environments, including the intertidal, such as *S. chrysomelas* to those commonly found as deep as 600 m such as *Sebastes melanostomus*. We test the hypothesis that shallow-water environments have been colonized independently in different lineages of *Sebastes* by reconstructing ancestral habitat depths, and ask whether these parallel colonizations are accompanied by parallel evolution in the rhodopsin gene.

Materials and methods

Sequencing of the rhodopsin gene

Genomic DNA was extracted from fin clips by boiling a small piece of tissue in 10% Chelex (Bio-Rad Laboratories, Hercules, CA, USA). The portion of the rhodopsin gene spanning codons 63-302 was amplified by PCR. As the rhodopsin gene in teleost fishes lack introns (Fitzgibbon *et al.*, 1995), a single reaction was sufficient to amplify the portion of interest. PCR products were sequenced in both directions using BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) using the forward PCR primer and additional internal primers on an ABI-3100 sequencer (Applied Biosystems). PCR protocols and all primer sequences can be obtained from the authors. Sequences were assembled and aligned using Sequencher 4.6 (Gene Codes Corporation, Ann Arbour, Michigan, USA). The sequences have been deposited in GenBank (accession numbers EF212407–EF212438). Sites along amino acid sequences are referenced by positions of the corresponding sites in the bovine rhodopsin amino acid sequence (GenBank accession NM_001014890).

Phylogenetic analyses

The nonsynonymous and synonymous substitution rates, d_N and d_S, respectively, as well as their ratio, ω, were estimated by maximum likelihood using the software package paml version 3.15 (Yang, 1997). The estimates were made using a codon model which accounts for the transition to transversion ratio, κ, and the base frequencies at the three codon positions. The maximum likelihood tree for a portion of the *cytochrome b* gene, constructed using PHYLIP (Felsenstein, 2005), was used for the PAML analyses, as it appears to be a closer representative of the species phylogeny than is the tree for rhodopsin (see later). *Cytochrome b* sequences were available from GenBank for all species from a recent phylogenetic study of the genus (Hyde & Vetter, 2007). In addition, the ratio of nonsynonymous to synonymous substitution at the level of the entire gene among all pairs of taxa was estimated for both the rhodopsin and the *cytochrome b* sequences using the program dnasp (Rozas & Rozas, 1999).

To identify amino acid sites under positive selection ($\omega > 1$), we used models of variable ω among sites. The following pairs of models were used in likelihood ratio tests (LRTs) to infer the presence of such sites and to identify them: model M0 (one ratio for all sites) vs. model M3 (discrete distribution of site classes), model M1 (neutral; ω = 0 or 1) vs. model M2 (selection; model M1 with an additional class of sites where ω can exceed 1) and model M7 [beta; ω values drawn from a beta distribution over (0,1)] vs. model M8 (beta and ω; model M7 with an additional class of sites where ω can exceed 1). The LRT statistic, $2\Delta l = 2(l_1 - l_0)$, where l_0 is the log-likelihood under the null model with p_0 parameters and l_1 is the likelihood under the alternative model with p_1 parameters, has a χ^2 distribution with $p_1 - p_0$ degrees of freedom.

Reconstruction of ancestral states

To test the possibility that some species of *Sebastes* colonized shallow habitats secondarily, we reconstructed the habitat states of the various ancestral nodes in the phylogeny. As closely related species might share habitat preferences simply because of their relatedness, it is necessary to account for the phylogeny when attempting such a reconstruction. One method that has widely been employed to account for the confounding effect of phylogeny on trait evolution is that of phylogenetically independent contrasts (Felsenstein, 1985; Garland *et al.*, 1992). For continuous traits, such as depth, a Brownian process model is employed, which makes the assumption that the evolutionary process does not include any trends (Schluter *et al.*, 1997; Garland *et al.*, 1999). Using the average depth at which each species occurs most commonly (Love, 1996; Love *et al.*, 2002), and a phylogenetic tree including branch length estimates, we can reconstruct the value of the trait at various ancestral nodes using the maximum likelihood framework of Schluter *et al.* (1997). Based on the *cytochrome b* phylogeny and known depth preferences of extant species, ancestral states were reconstructed using the software program ancml (Schluter *et al.*, 1997).

Results

Rhodopsin nucleotide variation and phylogenetic relationships

Of the 720 bp of rhodopsin nucleotide sequence under consideration, 27 sites were found to be variable among the 32 species of *Sebastes* studied. Of these, substitutions at 14 codon positions resulted in amino acid replacements, the rest being synonymous changes. The phylogenetic tree for *Sebastes* rhodopsins has several topological incongruencies with the *cytochrome b* tree (Fig. 1). This is particularly striking in some subgeneric groups within the genus. The monophyly of these subgenera is strongly supported in this and other phylogenetic studies of the genus (Rocha-Olivares *et al.*, 1999a; Rocha-Olivares *et al.*, 1999b; Hyde & Vetter, 2007). However, none of these groupings are supported by the rhodopsin sequences.

A nontree based analysis of the ratio of nonsynonymous to synonymous substitutions (K_a/K_s) in rhodopsin at the level of the entire gene shows a large proportion of pairwise comparisons to be greater than 1, suggesting that positive selection is a force directing the evolution of this gene in at least some lineages. For *cytochrome b* on the other hand, the same set of pairwise comparisons has a very narrow range of K_a/K_s, values, never exceeding 0.05 (Fig. 2) suggesting strong negative, or purifying selection at the *cytochrome b* locus. This, together with the fact that almost all substitutions in *cytochrome b* are silent changes, suggests that the phylogenetic signal present in the *cytochrome b* data is attributed to neutral variation and thus a better indicator of species relationships. The large values of K_a/K_s for rhodopsin result from nonsynonymous substitutions at specific codon positions across multiple pairwise comparisons. Apart from these, most changes are synonymous ($K_a/K_s < 1$). Thus it appears that for the most part the gene is under purifying selection but a subset of codons is under positive, or diversifying, selection.

Parsimony analyses of the rhodopsin data resulted in 25 equally parsimonious trees of length 64. Enforcing the topology of the *cytochrome b* tree on the rhodopsin

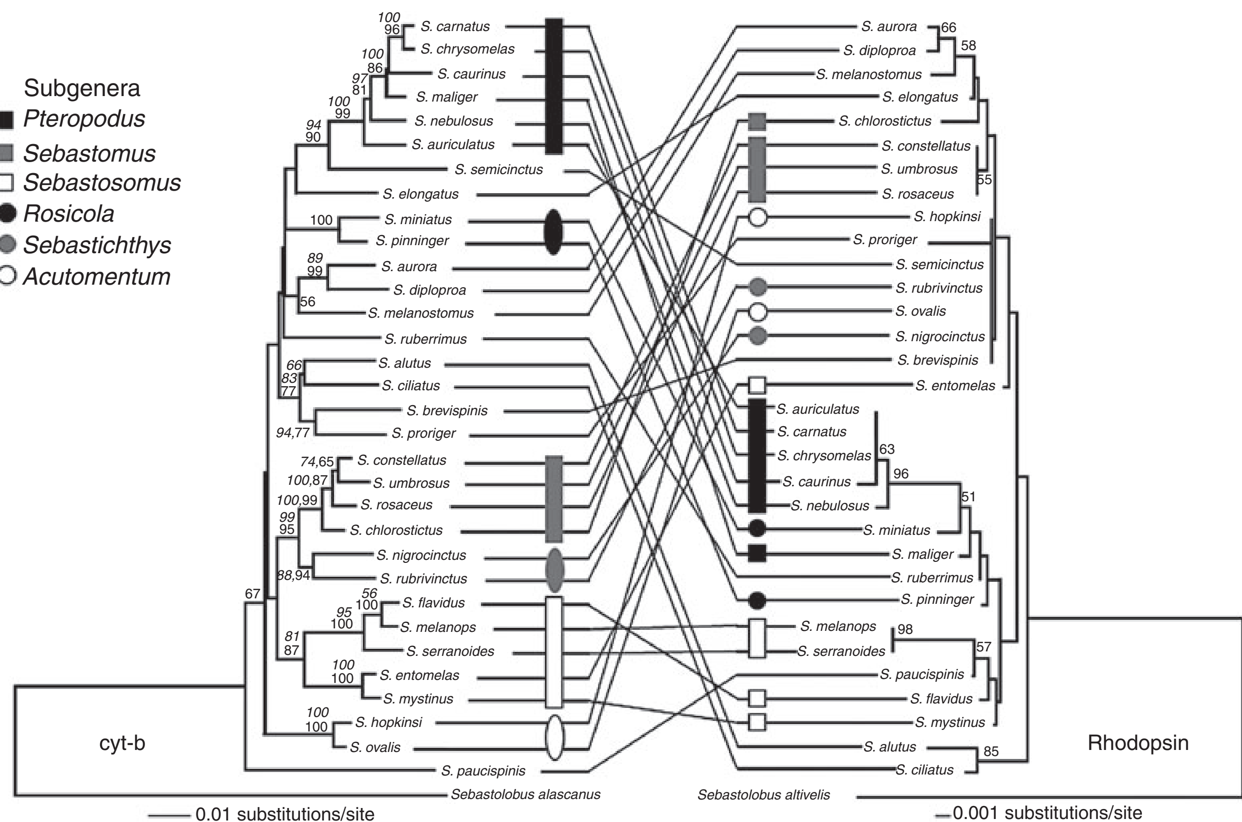

Fig. 1 Neighbour-joining tree showing the phylogenetic relationships among *Sebastes* species based on the cytochrome b (left) and rhodopsin sequences (right). Cytochrome b tree: numbers in regular typeface above branches are bootstrap support values for those branches based on 10000 pseudoreplicates of a neighbour-joining bootstrap analysis. Numbers in italics indicate maximum posterior probability in the consensus tree generated by Bayesian analysis. Three subgeneric groupings within *Sebastes* are shown on the trees. Rhodopsin tree: distances were calculated using the HKY85 model of substitution (Hasegawa *et al.*, 1985). Numbers next to branches are bootstrap support values based on 10 000 pseudoreplicates of a neighbour-joining bootstrap analysis.

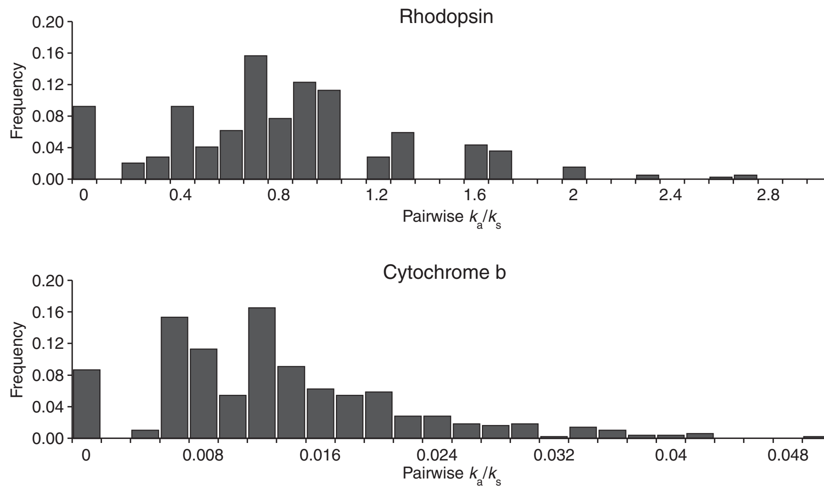

Fig. 2 Distribution of pairwise K_a/K_s ratios for rhodopsin (top panel) and *cytochrome b* (bottom panel). Note the different scales on the *x*-axis in the two plots.

sequence data increased the length of the most parsimonious tree by about 34% to 86. Methods of assessing topological incongruence described by Nye *et al.* (2006) and by Page (1994) strongly indicate incongruence between these two gene trees. The topology of the rhodopsin tree thus appears to reflect parallel evolution rather than true species relationships.

Tests for positive selection

All three likelihood models tested which allow for the presence of positively selected sites fit the data significantly better than their corresponding null models (Table 1). Moreover, there is considerable variation in selective pressure among amino acid. The 'Bayes empirical Bayes' (BEB) method, an improvement over the previous 'naïve empirical Bayes' method (NEB) in PAML, was used to identify amino acid sites under selection (Yang, 1997) except under model 3 where the BEB method is not implemented (and NEB was used). Five amino acid positions (119, 158, 205, 213, 217) were identified as being under selection in all models allowing selection with probability > 99%. Four other positions (116, 165, 274, 277) were identified as being under selection at either the 99% or the 95% level of probability depending on the model (Table 2). Figure 3 shows the distribution of variable amino acid residues among the species of *Sebastes* included here.

Table 1 Results of likelihood ratio test of selection for *Sebastes* rhodopsins. The test statistic $2\Delta l$ follows a χ^2 distribution with degrees of freedom shown in the column labelled d.f. The last column shows the critical value of the χ^2 distribution at the $\alpha = 0.001$ level, for the relevant degrees of freedom.

Likelihood ratio test	$2\Delta l$	d.f.	χ^2 0.001
M0 (one ratio) vs. M3 (discrete)	124.9	4	18.5
M1 (neutral) vs. M2 (selection)	69.3	2	13.8
M7 (beta) vs. M8 (beta and $\omega > 1$)	85.9	2	13.8

The PAML analyses described earlier were conducted using the maximum likelihood *cytochrome b* tree. It is possible that the outcome of these analyses is a result of the particular *cytochrome b* tree chosen for the analyses. To evaluate the effect of the tree used on the results of the PAML analyses, the analyses were repeated twenty times using different, randomly chosen most parsimonious trees of equal length. Five of the sites, where selection was detected at the probability > 99% level in the original analyses (119, 158, 205, 213, 217), were also identified as targets of selection at the 99% level in all subsequent runs. Two other sites (165 and 277) which were only supported at the 95% level in the original analyses were supported at the 99% level in all the subsequent runs. Position 274, which was supported at either the 95% level or the 99% level in the original analysis, was supported at the 95% level in only 75% of the repeat runs, with no support in the remaining runs. One other site, position 116, which appeared to be under selection in the original analysis at the 95% level, was never detected in any of the twenty subsequent reruns. The identification of this site as a target of selection thus seems likely an artefact of the tree used in the original analysis. Another amino acid position, 218, was detected as significant in 60% of the reruns, but not in the original analysis. The results of these comparisons are summarized in Table 3. All these results were obtained within the first ten repeated runs of PAML; ten further runs did not reveal any additional patterns. Hence, the PAML re-analyses were terminated after twenty runs.

Phylogenetic analysis of habitat shifts

Given that many close relatives of the genus, such as *Sebastolobus* spp., as well as the majority of *Sebastes* species commonly inhabit deep waters, it is possible that members of the group colonized shallower waters secondarily. Reconstruction of the ancestral depths (Fig. 4) suggests that colonization of shallower habitats

Table 2 Parameter estimates from PAML analyses, and amino acid sites under positive selection in the *Sebastes* rhodopsin gene.

Model	d.f.	Parameter estimates	d_N/d_S	ln*L*	Positively selected amino acids†
M0	1	$\omega = 0.8306$	$= \omega$	−1290.14	None
M1	1	$p_0 = 0.8591$, $\omega_0 = 0$	0.1409	−1262.34	NA
		$p_1 = 0.1409$, $\omega_0 = 1$			
M2	3	$p_0 = 0.9462$, $\omega_0 = 0.0365$	1.0241	−1227.69	116, **119**, **158**, 165, **205**, **213**, **217**, **277**
		$p_1 = 0.0001$, $\omega_1 = 1.0000$			
		$p_2 = 0.0537$, $\omega_2 = 18.4239$			
M3	5	$p_0 = 0.7515$, $\omega_0 = 0.0365$	1.0244	−1227.69	**116**, **119**, **158**, **165**, **205**, **213**, **217**, **274**, **277**
		$p_1 = 0.1947$, $\omega_1 = 0.0365$			
		$p_2 = 0.0537$, $\omega_2 = 18.4238$			
M7	2	$p = 0.005$, $q = 0.0118$	0.3000	−1270.64	NA
M8	4	$p_0 = 0.9463$	1.0244	−1227.69	116, **119**, **158**, 165, **205**, **213**, **217**, 274, 277
		$p = 3.7912$, $q = 99.000$			
		$p_1 = 0.0537$, $\omega = 18.4253$			

†Bold: $P < 0.01$; non-bold: $P < 0.05$.

indeed occurred secondarily and independently in several lineages.

The *cytochrome b* tree, rather than that for rhodopsin, was used in these analyses for two reasons. First, it is likely to be a better representative of the species tree than is the rhodopsin tree based on various lines of evidence including morphological data (Cramer, 1895; Phillips, 1957; Rocha-Olivares *et al.*, 1999a; Hyde & Vetter, 2007). Second, because the underlying objective of this exercise is to examine possible associations between rhodopsin evolution and evolution of depth preference, using the rhodopsin tree represents a possible confound (Felsenstein, 1985).

A number of substitutions are associated with shifts to shallower habitats. The clade defined by branch 1 in Fig. 4 represents an inferred habitat shift of 44 m by a deeper water ancestor to shallower water. This clade contains members of the subgenus *Pteropodus* (*Sebastes chrysomelas*, *S. caurinus*, *S. carnatus*, *S. maliger*, *S. nebulosus* and *S. auriculatus*). Five amino acid replacements are observed along this branch. Glycine at position 158 is replaced by alanine (G158A). In one species within this

Species	Common name	116	119	158	162	165	205	213	217	218	255	270	274	277	299
		F	**L**	**G**	**V**	**C**	**V**	**S**	**T**	**V**	**V**	**G**	**Y**	**S**	**A**
S. chrysomelas	Black and yellow	.	.	A	.	S	I	A	M	.	.	.	.	.	S
S. carnatus	Gopher	.	.	A	.	S	I	A	M	.	.	.	.	.	S
S. melanops	Black	S	.	.	.	.	.	F	V	.	.	.	F	.	S
S. mystinus	Blue	.	.	.	.	.	.	.	M	.	.	.	.	.	S
S. maliger	Quillback	.	.	.	.	.	I	.	M	.	.	.	.	.	S
S. caurinus	Copper	.	.	A	.	S	I	A	M	.	.	.	.	.	S
S. umbrosus	Honeycomb	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. nebulosus	China	.	.	A	.	S	I	T	M	.	.	.	.	.	S
S. auriculatus	Brown	.	.	A	.	S	I	A	M	.	.	.	.	.	S
S. serranoides	Olive	S	.	.	.	.	.	F	V	.	.	.	F	.	S
S. hopkinsi	Squarespot	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. rosaceus	Rosy	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. miniatus	Vermilion	.	.	A	.	.	I	.	M	.	.	.	.	.	S
S. constellatus	Starry	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. ovalis	Speckled	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. semicinctus	Halfbanded	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. pinniger	Canary	.	.	.	.	.	I	.	M	.	.	.	.	.	-
S. rubrivinctus	Flag	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. ruberrimus	Yelloweye	.	.	.	.	.	I	.	M	.	.	.	.	.	S
S. flavidus	Yellowtail	.	.	.	.	.	.	.	M	.	.	.	F	.	S
S. paucispinis	Bocaccio	.	.	.	.	.	.	F	M	.	.	.	.	.	S
S. nigrocinctus	Tiger	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. chlorosticus	Greenspotted	.	I	.	.	.	.	.	.	.	.	.	.	C	.
S. elongatus	Greenstriped	.	I	.	.	.	.	.	.	.	.	.	.	.	.
S. entomelas	Widow	.	.	.	.	.	.	.	.	.	.	.	.	.	S
S. brevispinis	Silvergray	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. ciliatus	Dusky	.	.	.	.	.	I	.	.	.	.	.	F	L	S
S. proriger	Redstripe	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. diploproa	Splitnose	.	V	.	.	.	.	.	.	I	.	.	.	.	.
S. alutus	Pacific Ocean Perch	.	.	.	.	.	I	.	.	.	I	S	F	L	.
S. aurora	Aurora	.	I	.	.	.	.	.	.	I	.	.	.	.	.
S. melanostomus	Blackgill	.	I	.	L	.	.	.	.	I	.	.	.	.	.

Fig. 3 Amino acid substitutions in *Sebastes* rhodopsin sequences. The panel to the right of the common name shows amino acid substitutions observed for each species, with the top line being the consensus sequence. Dots indicate identity with the consensus, whereas a dash indicates missing data. Amino acid position numbering follows that of the bovine rhodopsin (GenBank accession NM_001014890). Position numbers shaded grey fall within transmembrane regions of the protein. The second panel shows the approximate range of depths at which each species is most commonly found (Love, 1996; Love *et al.*, 2002).

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Table 3 Effect of the tree used in the PAML analyses on its identification of sites under positive selection.

Amino acid position	Significance in original analysis under model 8	Percentage of reruns in which significant		
		At 99% level	At 95% level	Not significant
116	> 95%	0	0	100
119	> 99%	100	0	0
158	> 99%	100	0	0
165	> 95%	100	0	0
205	> 99%	100	0	0
213	> 99%	100	0	0
217	> 99%	100	0	0
218	ns	0	60	40
274	> 95%	0	75	25
277	> 95%	100	0	0

group, *S. maliger*, however, there is a reversion to the ancestral state that is not associated with any inferred habitat shift. *Sebastes miniatus*, associated with a shift into habitats 33 m shallower, also shows this same replacement in parallel.

Position 205 is another example of parallel substitutions associated with shifts into shallower habitats. The change V205I occurs independently along branch 1, along the branch leading to the (*S. miniatus*, *Sebastes pinniger*) group as well as along that leading to *Sebastes ruberrimus*. In these cases, the inferred shift in habitat is between 44 and 64 m into shallower water. In one case, however, namely branch 3, this same substitution is associated with a small shift of 6 m into deeper water.

The amino acid at position 213 appears to be of some importance in shallow-water species, with S213A occurring along branch 1. Again, in *S. maliger* there is a reversion to serine at this position. In *S. nebulosus* there is a further replacement, A213T. Further evidence that this site might play an important functional role comes from its parallel replacement, to phenylalanine, in the two closely related shallow-water species *S. melanops* and *S. serranoides*, associated with depth shifts of 52 and 23 m, respectively, into shallower habitat. The closely related *S. flavidus*, which prefers deeper habitats, retains the ancestral state. The independent, parallel substitution of serine at this site, albeit by different residues in different shallow-water species or clades, suggests the possible functional importance of this site.

A similar situation occurs at amino acid position 217, with T217M occurring along branch 1. This same amino acid substitution occurs in four other cases. *Sebastes mystinus*, *S. ruberrimus*, *S. paucispinis* and the (*S. miniatus*, *S. pinniger*) pair display this replacement, all associated with shallow shifts of between 18 and 78 m. Along branch 4, associated with a 44-m shallow shift, there is a T217V replacement. Furthermore, within this clade, the only species associated with recolonization of deeper habitats, *S. flavidus*, carries an additional V217M replacement.

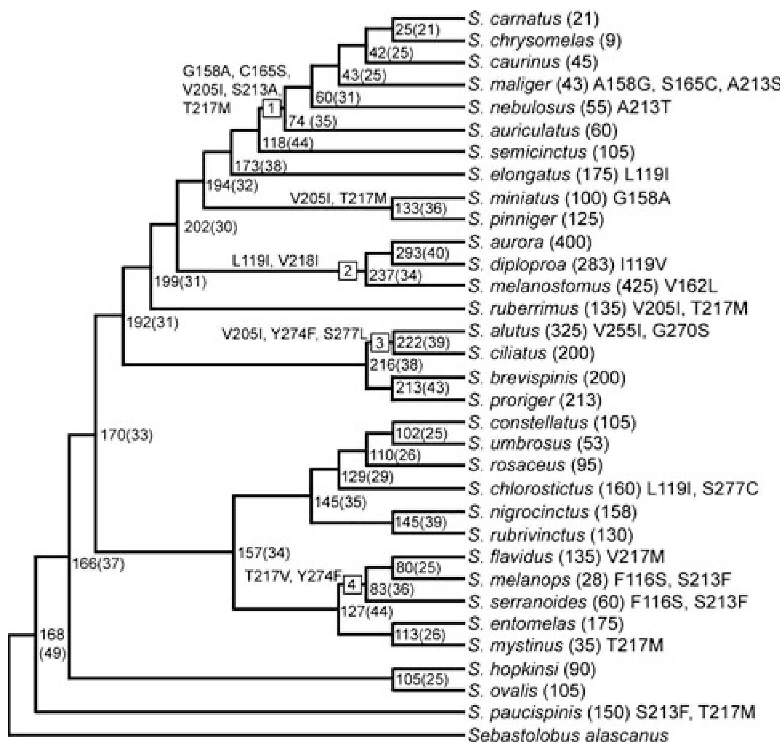

Fig. 4 Estimated average habitat depths of ancestral *Sebastes* species. Maximum likelihood estimates of ancestral depths are indicated next to internal nodes, with standard errors of estimates in parentheses. Average depth of extant species is in parentheses next to the species names. Numbers next to internal nodes are the maximum likelihood estimates of depths. Also shown, in Roman font, are amino acid substitutions along branches. Inferred replacements along terminal branches are shown next to species names.

Particular amino acid substitutions are likewise associated with shifts into deeper habitats. The replacement L119I is seen in parallel in *Sebastes chlorostictus* and along branch 2 of the tree, associated with inferred depth changes of 31 and 35 m, respectively, into deeper water. Within the clade defined by branch 2, there is one case of a species shifting back into shallower habitat. This species, *Sebastes diploproa*, has a further substitution resulting in a valine at this position instead. *Sebastes elongatus* also carries the L119I replacement, although the inferred shift in habitat depth is small in this case (2 m deeper). Position 277 carries the replacement S277L in *S. chlorostictus*, associated with a depth shift of 31 m. The same position has the replacement S277C along branch 3, also associated with a deep shift, although small in magnitude (6 m). Other amino acid substitutions (positions 162, 218, 255, 270) only occur once each and probably reflect neutral variation at this gene; PAML analyses never detected evidence for selection at these sites in all analyses. The same is true of position 299, which has no particular association with depth. However, this position has been postulated as a possible spectral tuning site in the rhodopsins of cichlids, where this site is similarly variable (Spady *et al.*, 2005). Hence, the possible functional importance of replacements at this position cannot be completely ruled out. Amino acid substitutions and their associated inferred shifts in habitat depths are summarized in Table 4.

Table 4 Amino acid substitutions in rhodopsin and associated inferred shifts in habitat depth. Inferred shifts into shallower habitats are denoted by negative numbers, and those into deeper habitats by positive numbers.

Position	From	To	Species or branch	Depth change (m)
116	F	S	*Sebastes melanops*	−52
	F	S	*Sebastes serranoides*	−23
119	L	I	*Sebastes chlorostictus*	31
	L	I	*Sebastes elongatus*	2
	L	I	Branch 2	35
	I	V	*Sebastes diploproa*	−10
158	G	A	Branch 1	−44
	A	G	*Sebastes maliger*	0
158	G	A	*Sebastes miniatus*	−33
162	V	L	*Sebastes melanostomus*	188
165	C	S	Branch 1	−44
	S	C	*S. maliger*	0
205	V	I	Branch 1	−44
	V	I	(*S. miniatus*, *Sebastes pinniger*)	−61
	V	I	*Sebastes ruberrimus*	−64
	V	I	Branch 3	6
213	S	A	Branch 1	−44
	A	S	*S. maliger*	0
	A	T	*Sebastes nebulosus*	−5
	S	F	*S. melanops*	−52
	S	F	*S. serranoides*	−23
	S	F	*Sebastes paucispinis*	−18
217	T	M	Branch 1	−44
	T	V	Branch 4	−44
	V	M	*Sebastes flavidus*	55
	T	M	*Sebastes mystinus*	−78
	T	M	(*S. miniatus*, *S. pinniger*)	−61
	T	M	*S. ruberrimus*	−64
	T	M	*S. paucispinis*	−18
218	V	I	Branch 2	35
255	V	I	*Sebastes alutus*	103
270	G	S	*S. alutus*	103
274	Y	F	Branch 4	−44
	Y	F	Branch 3	6
277	S	L	Branch 3	6
	S	C	*S. chlorostictus*	31

Patterns of habitat preference and rates of rhodopsin evolution

The rate of evolution of rhodopsin relative to *cytochrome b* was measured by estimating the branch lengths for rhodopsin given the *cytochrome b* topology. Rates of change of habitat preference, which can be thought of as branch lengths for this character, were also measured along this tree. This gave us three sets of branch lengths for this topology, namely *cytochrome b*, rhodopsin and depth. Branch lengths for rhodopsin are significantly positively correlated with absolute values of inferred branch lengths for depth (Fig. 5) when we consider amino acid replacement changes ($r = 0.238$; $P = 0.03$) but not when we consider all substitutions ($r = 0.185$; $P = 0.07$). Branch lengths for *cytochrome b* are not correlated with inferred changes in depths along the tree ($r = 0.157$; $P = 0.11$).

Some branches of the rhodopsin tree indicate accelerated rate of evolution in this gene relative to *cytochrome b*. In particular, for branch 1 of Fig. 4, the branch length is the highest among all branches of the tree for rhodopsin but not for *cytochrome b*. This is consistent with the notion of accelerated evolution of rhodopsin relative to the *cytochrome b* gene in this shallow-water lineage. The same is true for the branch leading to the Pacific Ocean Perch, *Sebastes alutus*, which inhabits the other end of the depth range spectrum. Thus it appears that lineages that have a history of change in habitat depth have had a corresponding acceleration in the rate of rhodopsin evolution.

Discussion

Sebastes species inhabit habitats ranging from surface waters to over 600 m. Even though juveniles of species in this group generally recruit in shallow-water and then move to the adult habitat, the adults spend most of their life localized over home sites with little, if any, movement (e.g. Larson, 1980; Gascon & Miller, 1981; Matthews & Barker, 1983; Hartmann, 1987; Stanley *et al.*, 1994). These are long-lived species, with individuals over 200 years old reported in some species (Love *et al.*, 2002). Thus, given the length of time these fish spend in their specific habitats, it is likely they have

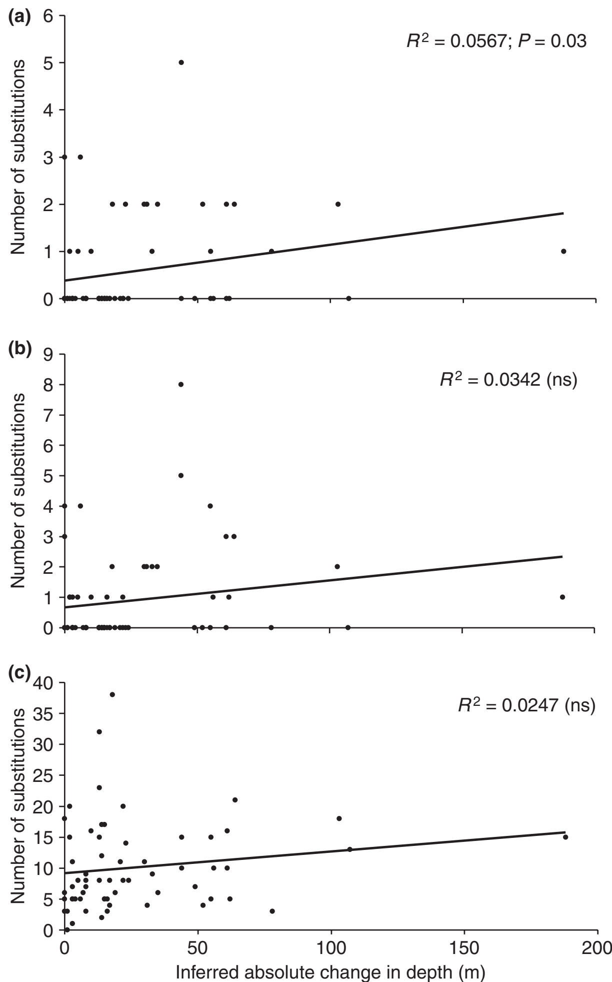

Fig. 5 Relationship of branch lengths for habitat depth with branch length for rhodopsin measured as (a) amino acid replacements in rhodopsin, (b) all nucleotide substitutions in rhodopsin and (c) substitutions in *cytochrome b* (bottom panel) measured along the tree in Fig. 4.

diverged in their adaptations to their habitats and visual systems are likely an important aspect of adaptation to different depths.

The gene encoding the visual pigment rhodopsin in rockfishes appears to be evolving adaptively in relation to the habitat preference of these species. Visual systems are important to many animals for prey acquisition, predator avoidance and mate choice. The cichlid fishes of the African Rift Valley lakes have provided us an excellent system to study the evolution of these systems, because they contain large assemblages of closely related and recently diverged species that have colonized a wide range of photic environments (Carleton & Kocher, 2001; Terai *et al.*, 2002; Carleton & Kocher, 2003; Parry *et al.*, 2005; Spady *et al.*, 2005; Terai *et al.*, 2006; Seehausen *et al.*, 2008). The rockfishes of the Pacific Ocean offer us a marine equivalent of the cichlid example (Johns & Avise, 1998; Alesandrini & Bernardi, 1999) and present us with a unique system in which to study the evolution of traits that contribute to the species divergence and the colonization of novel habitats.

Fourteen variable amino acid positions were observed in the 240 amino acid region of the rhodopsin gene studied in 32 species of *Sebastes*. All but two of these sites occur within transmembrane domains of the protein. This far exceeds random expectations ($P < 0.01$), given the relative proportions of amino acid sites that fall within (109 sites) and outside (131 sites) these domains. Most known spectral tuning sites in rhodopsin (and for that matter in all the opsins) fall within transmembrane domain regions (Yokoyama, 1995; Hunt *et al.*, 1996; Yokoyama, 2002; Takahashi & Ebrey, 2003; Yokoyama & Takenaka, 2004). However, only one of these known sites was variable among the species we studied. Nonetheless, the nonrandom occurrence of these changes in functionally important regions of the protein as well as the fact many of the variable amino acid sites were identified as being targets of positive selection (Tables 2 and 3) together suggest a possible functional role for these amino acid replacements in *Sebastes* rhodopsins.

The structure of the rhodopsin protein (Fig. 6) shows that three sites (119, 165 and 213) are adjacent to known critical sites, and it is possible that replacements at these sites might impact the structure, and consequently function, of the protein. Four of the same amino acid positions (158, 165, 213 and 217) were also predicted by PAML analyses to be selection targets in cichlids (Spady *et al.*, 2005). While *in-vitro* experiments have not been performed to verify the role of these positions in spectral tuning, the strength of evidence presented in this study as well as their identification in an unrelated group strongly argues for a role in adaptation.

Two lines of empirical evidence are needed to explicitly test the thesis that amino acid replacements in rhodopsin are associated with habitat preference. First, measurements of λ_{max} have to be made in species from different depth categories, to verify if, in fact, there are differences in λ_{max} between species that inhabit different depths. Secondly, it would be instructive to conduct site-directed mutagenesis and microspectrophotometry experiments examining the effects of these particular amino acid substitutions on λ_{max}. Ideally, such experiments would use a *Sebastes* rhodopsin system, or at the very least a teleost rhodopsin model, rather than the bovine rhodopsin as has been the norm thus far.

The differences in topology between the phylogenetic trees for rhodopsin and *cytochrome b* are striking (Fig. 1). Different and nonmutually exclusive factors, such as incomplete lineage sorting, gene flow and natural selection, could lead to the discrepancies between the two trees. Another possible cause for the incongruence

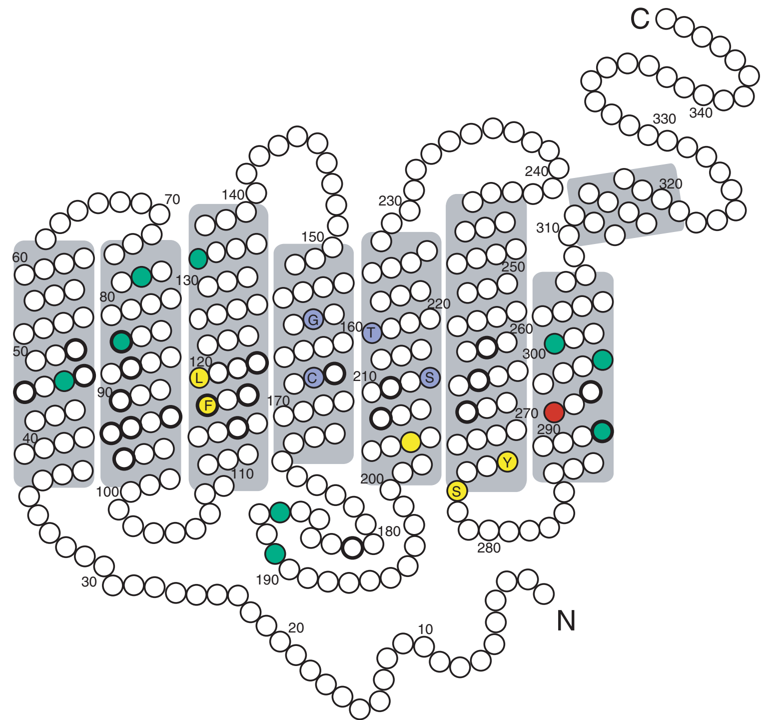

Fig. 6 Two-dimensional representation of the structure of rhodopsin based on the crystal structure of rhodopsin (Palczewski *et al.*, 2000) and redrawn from that article. Positions outlined in bold are sites identified as functionally critical or important in spectral tuning in other studies (Yokoyama & Takenaka, 2004; Sugawara *et al.*, 2005). Sites coloured yellow are sites identified in this study as being targets of selection, and those coloured blue are sites predicted to be under selection in both this study as well as in cichlids (Spady *et al.*, 2005). Sites shaded green are those that either line, or are in close proximity to the chromophore (Sugawara *et al.*, 2005). Position 296, coloured red, is the binding site of the retinal. The seven transmembrane helix domain is shown as shaded grey vertical cylinders, and the eighth amphiphilic helix domain is shown as a horizontal grey cylinder.

between the two trees is that the rhodopsin gene has not achieved reciprocal monophyly among species because of a lower rate of genetic drift relative to the mitochondrial locus (Sunnucks, 2000; Simon *et al.*, 2006). Then, by sampling a single individual from each species, as we have performed in this study, we might have sampled from a pool of shared ancestral polymorphism, and species share rhodopsin genotypes simply because of chance. To investigate this possibility, for seven of the species (*S. auriculatus*, *S. mystinus*, *S. melanops, S. serranoides, S. miniatus, S. constellatus* and *S. flavidus*), we sequenced rhodopsin from a number of different individuals (between 8 and 14). Four of the species showed no intraspecific variation whatsoever, whereas for *S. flavidus* there is a single synonymous polymorphism. The remaining two species, *S. mystinus* and *S. miniatus*, each have one amino acid replacement polymorphism, which in both cases is a singleton change. This low level of intraspecific variation is not consistent with the possibility that there is widespread shared ancestral polymorphism. Based on these arguments, it is likely that the *cytochrome b* tree may be a better representative of the species phylogeny than is the rhodopsin tree. The *cytochrome b* tree is also more consistent with systematic treatments of the group based on morphological characters (Cramer, 1895; Phillips, 1957; Rocha-Olivares *et al.*, 1999a; Hyde & Vetter, 2007).

Another possible reason for the incongruence between the two trees is that there is ongoing gene flow between species at this nuclear locus. Introgression has been documented between some species of *Sebastes* (Seeb, 1998), and it is possible that introgressed alleles could be selected for in species that shift depth preferences. For example, *S. melanops* and *S. serranoides* share depth preference and have identical rhodopsin genotypes, even sharing synonymous substitutions. Yet, *S. melanops* groups with *S. flavidus*, and not *S. serranoides* on the *cytochrome b* tree, a relationship strongly supported in this and other analyses (Johns & Avise, 1998; Rocha-Olivares *et al.*, 1999a; Kai *et al.*, 2003; Hyde & Vetter, 2007). This phylogenetic discrepancy could arise as a result of parallel substitutions at rhodopsin in *S. melanops* and *S. serranoides* as adaptations to shallow environments. However, given that four independent amino acid substitutions as well as shared synonymous substitutions have to be invoked, this pattern could be the result of introgression and subsequent fixation under selection of a shallow-adapted rhodopsin genotype from *S. serranoides* to *S. melanops*.

In other cases, it might be more likely that selective pressure has resulted in convergent amino acid replacements in the rhodopsin gene as an adaptation to habitat depth in various lineages. The rhodopsin of *S. mystinus* differs from its close relative *S. entomelas* by a single base change. This amino acid replacement change is shared with *S. flavidus*, *S. melanops* and *S. serranoides*, resulting in the grouping seen in the rhodopsin tree. No synonymous changes, however, are shared by these species. *Sebastes elongatus* likewise shares a replacement substitution but

no synonymous changes with *S. aurora* and *S. melanostomus*, resulting in its placement with that group in the rhodopsin tree. Similarly, *S. maliger*, which is part of the clade defined by branch 1 in the *cytochrome b* tree, falls outside that group because of the two nonsynonymous changes that it shares with *S. miniatus* and *S. ruberrimus*, but it does not share any synonymous changes with those species. This predominance of shared amino acid replacements that are not accompanied by shared silent variation argues for convergent.

Given that none of these possible factors are mutually exclusive, it may be that some combination of these processes is responsible for the patterns of replacement in rhodopsin as well as the incongruencies between the two gene trees. The most definitive evidence for adaptation in this gene would come from *in-vitro* demonstration of spectral tuning resulting from these replacements, but such data are presently unavailable. Regardless, multiple lines of evidence suggest that the rhodopsin gene in *Sebastes* might be undergoing adaptive evolution, and that replacement at this locus is associated with habitat depth. If so, this study has uncovered a group of functionally important amino acid sites in the rhodopsin molecule that have not been previously characterized as spectral tuning sites. These sites may be important in adaptations to various photic environments in marine ecosystems.

A recent study (Yokoyama *et al.*, 2008) employed a similar maximum likelihood approach to the one we have used to predict sites under selection in squirrelfishes. Several amino acid positions predicted in their analyses coincide with ones we have proposed in this study. However, functional analyses of these replacements did not result in a spectral shift *in vitro*. Regardless of this finding, we still propose a functional role for these sites because of multiple parallel substitutions at these positions within *Sebastes* and the overlap in predicted critical sites in three divergent groups of fishes, namely *Sebastes*, squirrelfishes (Yokoyama *et al.*, 2008) and cichlids (Spady *et al.*, 2005).

Genes other than rhodopsin also contribute to adaptation to photic environments. Prominent among these are the cone opsins which have been shown to undergo adaptive evolution by amino acid replacements and by differential gene expression (Carleton & Kocher, 2001; Terai *et al.*, 2002; Parry *et al.*, 2005; Spady *et al.*, 2005). As with rhodopsin, spectral tuning sites have been identified in several of these genes. The findings of this study raise predictions for cone opsin genes in *Sebastes*. Species inhabiting deeper habitats would be expected to show amino acid replacements that lead to blue shifts in λ_{max}, while shallow-water species would be expected to have red-shifted λ_{max} values. Some of the deep-dwelling species might even be predicted to have lost functionality of some or all cone opsins, given their decreasing importance in darker environments (Yokoyama, 2000).

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