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SHORT COMMUNICATION

Signature of selection on the rhodopsin gene in the marine radiation of American seven-spined gobies (Gobiidae, Gobiosomatini)

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Abstract

In comparison with terrestrial and freshwater ecosystems, information about speciation modes and the role of selection in marine environments is scarce. Recent studies have indicated that spectral adaptation could play an important role in the diversification of marine species flocks. Natural selection influences specific amino acids (AAs) that are involved in the spectral tuning mechanism of visual pigment genes. To study the wider occurrence and the characteristics of spectral adaptation in marine radiations, a reinterpretation of the rhodopsin (*RH1*) data of American seven-spined gobies (genus *Elacatinus*; Gobiidae; Teleostei) was carried out. Reanalysis revealed that some AAs, which are well known in the literature as spectral tuning sites, are variable in *Elacatinus*. Those crucial AA substitutions originated polyphyletically, indicating convergent evolution within the genus *Elacatinus*. Moreover, statistical tests based on the d_N/d_S ratio detected selection in several phylogenetic lineages and at specific AAs. Many of these AAs were previously shown to be under selection in other marine radiations. Therefore, the current phylogenetic approach provided an extended list of AAs that are probably involved in spectral tuning, and which should be validated by mutagenic experiments.

Introduction

The processes of speciation in the marine environment remain largely undocumented compared to the terrestrial and freshwater ecosystems. Classical models of divergence in allopatry are difficult to apply, due to the absence of clear geographic barriers and the seemingly high (albeit strongly species specific: Goetze, 2003) dispersal potential of many species (Rocha *et al.*, 2005; Taylor & Hellberg, 2005). The latter is true for planktonic organisms and many benthic species with a planktonic larval stage (Bierne *et al.*, 2003). However, species diversity can be high in geographically restricted areas such as the endemic radiations in the coral reefs, the Caribbean or the Indo-West Pacific (Briggs, 1999; Taylor & Hellberg, 2005), whereas incipient species may coexist in virtual sympatry over extensive contact zones (Bierne *et al.*, 2003). Hence, isolation mechanisms other than vicariant allopatry or limited dispersal capacity have to be inferred. Especially, the role of standing selection in speciation is currently under debate in marine science (Conover *et al.*, 2006). For marine species, visual pigments (VP) such as rhodopsin (RH1) are expected to be under particularly strong selection pressures in dim and spectrally restricted light conditions.

The water column of coastal habitats shows a range of optical characteristics, which puts special constraints on visual predators or animals with a visually based mating system. Moreover, aquatic environments differ in photic characteristics by differences in turbidity, and colour and brightness of the downwelling light (Bowmaker, 1995). Vertebrates will tune their VPs to deal with this diversity. VP molecules are bound in dense membrane stacks in retinal photoreceptors to mediate vision. The VP consists of a protein moiety, the opsin, bound to a light-absorbing

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chromophore. Each pigment shows a characteristic peak of maximal absorbance (λ_{max}), its precise value depending on the interactions between the opsin and the chromophore (Yokoyama, 2000). By changing specific amino acids (AAs) in the opsin, vertebrates have the possibility to modify their visual system to cope with the specific photic environment. Those AAs are called 'spectral tuning sites'. A clear correlation between the AA sequence, the λ_{max} of the VPs and the photic characteristics of the habitat has been observed in several marine vertebrates (Hunt *et al.*, 2001; Yokoyama & Takenaka, 2004). A recent study demonstrated the role of divergent selection on sensory genes in promoting speciation through sensory drive in cichlid fishes (Seehausen *et al.*, 2008). Natural selection acting on the visual system may contribute to reproductive barriers and the formation of new species because animals prefer to spend time in habitats in which they see best (Kirkpatrick & Price, 2008). Therefore, VP genes are promising models to study the molecular basis of evolutionary adaptations driven by marine environmental selection pressures.

Recently, strong signatures of evolutionary adaptation on the *RH1* gene were detected in marine species flocks (Larmuseau *et al.*, 2010a; Sivasundar & Palumbi, 2010). Adaptive radiations of fishes can provide crucial knowledge to understand how evolution operates; unfortunately, they are less studied in the ocean compared to freshwater systems (Ingram, 2011; Puebla, 2011). Genera of the goby family (Teleostei, Gobiidae), perhaps the most speciose fish family worldwide with currently around 1950 species described (Miller, 1986; Nelson, 2006), are often put forward as an example of adaptive radiation. To further elucidate the potential for visual adaptation in marine adaptive radiations, the radiation of Neotropical reef gobies (Teleostei, Gobiidae, Gobiosomatini) (Hoese & Larson, 1985) provides an excellent research goal. *Elacatinus* is the most species-rich fish genus on Neotropical coral reefs (Taylor & Hellberg, 2005). Its phylogeny is well known and encompasses three related sister clades: *Tigrigobius*, *Elacatinus* and *Risor* (Taylor & Hellberg, 2005). We use *sensu lato* [s.l.] and *sensu stricto* [s.s.] to distinguish between the 'genus' and subgenus *Elacatinus*, respectively. Some species, previously considered to belong to the genus *Elacatinus* Jordan, 1904, have meanwhile been assigned to *Tigrigobius* Fowler, 1931, and *Risor* Ginsburg, 1933, represents a different genus; we follow Eschmeyer (2010) for taxon and author names. The whole genus shows a high diversity in colour, ecology and behaviour (Rüber *et al.*, 2003) and reveals strong microhabitat preferences (Böhlke & Robins, 1968). Visual habitat characteristics will therefore differ between species, as has already been proven within *Elacatinus* (Lettieri *et al.*, 2009). Therefore, VP genes may have an important role in the adaptive speciation of *Elacatinus* species. Here, we assess directional selection in a phylogenetic framework to determine whether selection has played a significant role in the evolution of the *RH1* gene within the *Elacatinus* gobies and therefore in their radiation. This aspect was not covered by the study of Taylor & Hellberg (2005), where the *RH1* data were only used to construct the phylogenetic relationship between the *Elacatinus* gobies.

Materials and methods

Rhodopsin data of 28 valid species of *Elacatinus* [s.l.], including *Risor ruber* and *Ginsburgellus novemlineatus*, were reanalysed and reinterpreted from Taylor & Hellberg (2005) (Table 1). Since their publication, two new species have been described. First, the individuals identified as *E. oceanops* from Belize, unlike those from Florida, have been assigned to *E. lobeli* (Randall & Colin, 2009); the so-called *E. xanthiprora* individuals caught in Belize now belong to the new taxon of *E. colini* (Randall & Lobel, 2009). A summary of all specimens, sample locations, distribution and ecological/behavioural data per species is provided in the supplementary materials (Table S1). Taylor & Hellberg (2005) sequenced an 800-bp fragment of *RH1* for 64 samples from 28 species (GenBank accession no. AY846565–AY846628), which represents 76% of the full protein. All well-known 25 AAs involved in the spectral tuning of the VP are included in this gene fragment (Yokoyama *et al.*, 2007 and references herein). Based on the robust phylogeny of Taylor & Hellberg (2005) using 3230 bp from one mitochondrial and two nuclear – including *RH1* – gene regions (hereafter referred to as 'consensus phylogeny'), the AA sequence of the ancestral pigment of the three Neotropical reef goby clades was inferred using a likelihood-based Bayesian method (Yang, 1997) implemented in CODEML in PAML v.4.2 (Yang, 2007). The analysis was rerun based on the phylogeny of Taylor & Hellberg (2005) using only 1140 bp of the mtDNA cyt *b* (hereafter referred to as the 'mtDNA phylogeny') to study the impact of the rhodopsin data on the phylogeny. An analysis based on the phylogeny using only the *RH1* data was not performed because a tree based on a locus that is potentially under selection itself may not represent the actual phylogeny, and as a consequence, the signature of selection on specific AAs will not be observed (Larmuseau *et al.*, 2010a).

Two kinds of analyses were performed to determine whether positive selection was involved in the evolution of the *RH1* gene in *Elacatinus* [s.l.]. First, MEGA v.4 (Tamura *et al.*, 2007) was used to compare the relative abundance of synonymous and nonsynonymous substitutions between pairs of sequences using a *Z*-test. Second, the CODEML program of PAML was used to perform two tests (among lineages and among sites) using two types of models ('branch-specific' models and 'site-specific' models). The 'branch-specific' models allow the d_N/d_S ratio (hereafter referred to as ω ratio) to vary among branches in the phylogeny, and therefore, they are useful in detecting positive selection operating on a particular lineage. The level of selection also varies at

Table 1 Amino acid (AA) replacements at 23 variable sites in the *Elacatinus* genus. The AA sequence of the ancestral pigment (ancestral haplotype) was inferred using a likelihood-based Bayesian method (Yang, 1997). Dots represent the comparison of the AAs with those of the ancestral haplotypes. The asterisks indicate the AA replacements that either line the chromophore-binding pockets or are located in close proximity to the chromophore (Spady *et al.*, 2005; Bowmaker, 2008). Shaded columns indicate the AA replacements that were identified as sites significantly under positive selection by a likelihood-based Bayesian method (Yang *et al.*, 2000a).

AA number	50	54	56	83*	112	133*	137	162	165	173	189	205	209	210	214*	217*	255	279†	281	283	290	299*	305
Helix number	I	I	I	II	III	III	III	IV	IV	IV	–	V	V	V	V	V	VI	–	–	–	VII	VII	VII
Ancestor sequence	V	I	F	D	L	I	V	I	S	V	V	I	I	V	I	F	I	Q	S	F	L	S	I
Elacatinus lineage																							
Elacatinus atronasus (Böhlke & Robins, 1968)	.	L	.	N	.	.	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	A	.
E. evelynae (Böhlke & Robins, 1968)	.	L	.	N	.	.	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	A	.
E. figaro Sazima, Moura & Rosa, 1997	.	.	.	N	V	.	M	.	.	.	.	.	.	.	.	.	V	.	.	S	.	A	.
E. genie (Böhlke & Robins, 1968)	.	L	.	N	.	.	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	A	.
E. illecebrosus (Böhlke & Robins, 1968)	.	L	.	N	V	.	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	./A	.
E. oceanops Jordan, 1904	.	L	L	N	V	.	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	A	.
E. lobeli‡ Randall & Colin, 2009	.	L	L	N	.	./M	M	.	.	.	.	V	.	.	.	T	V	.	.	S	V	A	.
E. prochilos (Böhlke & Robins, 1968)	.	L	.	N	./V	.	M	.	.	.	.	V	./V	.	.	./T	V	.	.	S	V	A	.
E. randalli (Böhlke & Robins, 1968)	.	L	.	N	V	.	M	.	.	.	.	V	T	.	.	.	V	.	.	S	V	A	.
E. chancei (Beebe & Hollister, 1933)	.	L	L/.	N	.	V	M	.	.	.	.	V	./T	.	.	./T	V	H	./A	S	V	A	.
E. horsti (Metzelaar, 1922)	./A	L	.	N	.	./V	M	.	.	.	.	V	T	.	.	./T/S	V	./H	./A/I	S	V	A	.
E. lori Colin, 2002	./A	L	.	N	.	V	M	.	.	.	.	V	T	.	.	T	V	.	I	S	V	A	.
E. louisae (Böhlke & Robins, 1968)	.	L	.	N	.	V	M	.	C	.	.	V	T	.	.	T	V	./H	.	I	V	A	.
E. puncticulatus (Ginsburg, 1938)	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	V	.	.	S	.	.	.
E. colini§ Randall & Lobel, 2009	.	L	.	N	.	V	M	.	.	.	.	V	T	.	.	.	V	.	T	S	V	A	.
Tigrigobius lineage																							
Tigrigobius dilepis (Robins & Böhlke, 1964)	.	.	.	N	V	.	M	M	.	.	.	V	V	.	.	.	.	.	.	S	V	A	.
E. macrodon (Beebe & Tee-Van, 1928)	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	V	.	.
E. saucrus (Robins, 1960)	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	V	.	.	S	.	.	.
Risor lineage																							
E. digueti (Pellegrin, 1901)	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	V	.	.	S	.	.	.
T. gemmatus (Ginsburg, 1939)	.	.	.	.	.	.	.	.	C	F	I	.	T	C	.	V	.	.	T	S	V	.	.
E. inornatus¶	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	V	.	.	S	.	.	./M
E. janssi Bussing, 1981	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	V	.	.	S	.	.	.
E. limbaughi Hoese & Reader, 2001	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	V	.	.	S	.	.	.
E. multifasciatus (Steindachner, 1876)	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	./I	.	.	.	.	.	.	.
E. nesiotes Bussing, 1990	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	V	.	.	S	.	.	.
T. pallens (Ginsburg, 1939)	.	./V	./L	N/.	.	.	.	.	.	F	I	.	T	.	.	.	.	.	T	S	I	./A	.
Ginsburgellus novemlineatus (Fowler, 1950)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.
Risor ruber (Rosén, 1911)	.	./L	.	N	.	.	.	M	.	F	I	.	T	.	./T	.	.	.	I/T	S	I	A	.

†AA279 was only identified to be under selection based on the phylogeny using the mtDNA Cyt *b* data and not using the mtDNA, rag and *RH1* data.
‡*E. lobeli* is a newly described species of Randall & Colin (2009), namely the formerly known *E. oceanops* from Belize.
§*E. colini* is a newly described species of Randall & Lobel (2009), namely the formerly known *E. xanthiprora* from Belize/Honduras.
¶*E. inornatus* is currently a synonym of *E. digueti* (Pellegrin, 1901).

various AA positions along the protein sequences, and several 'site-specific' models have been developed that account for ω ratio variation between particular codon sites (Nielsen & Yang, 1998). For the test among lineages, the model M0, which assumes a single ω ratio for all nucleotide sites and branches of the phylogeny, was compared with a model that estimates two different ω ratios, one for the lineage of interest ('foreground lineage') and another one for all other lineages ('background lineages') (Yang, 1998). For the tests among sites, parameters were estimated under two different models, namely M7 (beta), which does not allow for positive selection on a specific gene, and M8 (beta and ω), which accounts for sites under positive selection on the gene under study (Yang *et al.*, 2000a). Although recombination on the nuclear rhodopsin gene may generate false positives in the detection of positive selection, these models are more robust in case of recombination compared with the other models implemented in CODEML (Anisimova *et al.*, 2003). Likelihood-ratio tests (LRTs) were used to evaluate the two-codon-based models of sequence evolution, as described by Yang (2000) and Yang *et al.* (2000b). Positively selected codons ($\omega > 1$ with $P > 95\%$) were identified through an empirical Bayesian approach (Yang *et al.*, 2005). All analyses were run based on the 'consensus phylogeny' but also on the 'mtDNA phylogeny', again to exclude the impact of rhodopsin data on the phylogeny.

Finally, the results of *Elacatinus* spp. were compared with the *RH1* data of *Pomatoschistus* spp. and *Sebastes* spp. derived from Larmuseau *et al.* (2010a) and Sivasundar & Palumbi (2010), respectively.

Results

In all available *RH1* sequences of the 28 *Elacatinus* taxa, 106 variable nucleotides were found (13.25% of the total fragment). The alignment in AAs showed 23 AA substitutions (8.6% of the total number of AAs), from which 19 are located in the transmembrane helices and four in the C-loops (Fig. 1). Six variable AA positions are close to the retinal-binding pocket, namely AA83, AA133, AA189, AA214, AA217 and AA299 (Fig. 1; Table 1). Intraspecific variation was found for 11 species at 14 different AA; based on the GenBank sequences, three individuals from *E. lobeli*, *E. horsti* and *E. prochilos* were even heterozygous at one AA. The assumed sequence of the ancestral pigment based on the 'consensus' and 'mtDNA phylogeny' of the genus *Elacatinus* was identical and is given in Table 1.

Several *Z*-tests between species revealed positive selection, especially between pairs of species within *Elacatinus* [s.l.] (Table S2). All *Z*-tests between pairs including *E. horsti*, *E. chancei* or *E. lori* showed a significant $\omega > 1$. With the branch-specific models, several branches showed $\omega > 1$, as well for the 'consensus' as for the 'mtDNA' phylogeny; however, only the branch with *E. horsti*, *E. chancei*, *E. lori* and *E. louisae* had a significant ω ratio > 1. The LRT of the maximum likelihood analysis demonstrates that M8, the model that accounts for sites under positive selection, showed a significantly better fit than the M7 model, which does not allow for positive selection (*P*-value < 0.01 as recommended by the software). Based on the 'consensus phylogeny,' Bayesian identification showed that sites AA54, AA112, AA165,

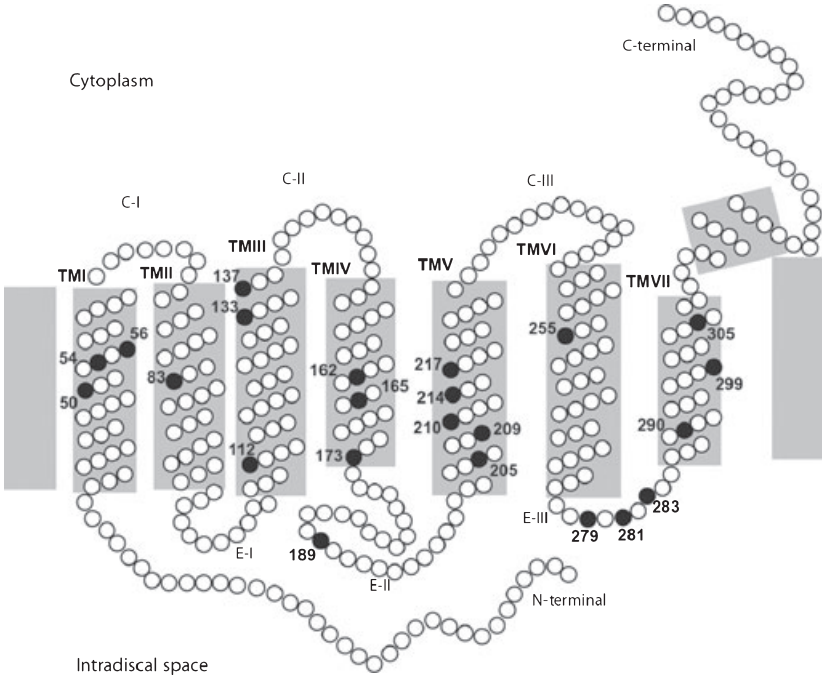

Fig. 1 Two-dimensional model of the seven transmembrane α-helices of the rhodopsin protein (RH1) (Palczewski *et al.*, 2000). The seven transmembrane helices (TM) are numbered, as well as the three loops at the cytoplasmic side (C) and the extracellular side (E) of the cell membrane. The 23 AA substitutions found in *Elacatinus* gobies are shown with closed circles and numbered.

AA209, AA217, AA281 and AA290 of the *RH1* gene are significantly under selection with a *P*-value < 0.01 and sites AA54 and AA165 with *P*-value < 0.05. The same result was found based on the 'mtDNA phylogeny,' with the addition of AA279, with *P*-value < 0.01. Those sites, and all others that have previously been identified in other marine radiations (the north-eastern Atlantic 'sand gobies' and *Sebastes* Cuvier, 1829) as being under selection (Larmuseau *et al.*, 2010a; Sivasundar & Palumbi, 2010), showed similar AA substitutions (Table 2).

Discussion

Mechanisms of allopatric speciation, many of which rely on geographic barriers or restricted dispersal abilities, often fail to explain biodiversity patterns in marine environments. Indeed, a range of isolation mechanisms other than vicariance or limited dispersal have been suggested to drive speciation in the ocean (Briggs, 1999). Examples include gamete interaction, differential spawning times, mate recognition, recent historical isolation (Palumbi, 1992, 1994), and larval retention (Palumbi & Warner, 2003; Taylor & Hellberg, 2005). Colour-assortative mating combined with disruptive selection was proposed by Puebla *et al.* (2007). Ecological differentiation followed by adaptation was also suggested on the basis of either trophic adaptation (Briggs, 1999) or habitat selection (Schroth *et al.*, 2002; Goetze, 2003; Rocha *et al.*, 2005). As visual perception may be vital to several of those phenomena (for instance for visual predation, mate choice and microhabitat selection), we assessed the occurrence of visual molecular adaptation in *Elacatinus* [s.l.] species. We assessed the possibility of selection on the *RH1* gene using a phylogenetic approach. The *Elacatinus* [s.l.] gobies provide an excellent study system. First, their diversity in coloration, ecology and behaviour (Rüber *et al.*, 2003; Colin, 2010) provides clear grounds for differential demands to their VPs. Second, they present a well-studied case of a marine adaptive radiation (Hoese & Larson, 1985), and as such might help to elucidate speciation in marine organisms (Rüber *et al.*, 2003; Taylor & Hellberg, 2005).

Table 2 Amino acids at specific sites of the RH1 opsin in *Elacatinus* [s.l.] spp, *Pomatoschistus* spp. and *Sebastes* spp. The amino acids that were indicated to be under selection within a genus are listed in bold and highlighted in grey.

AA position	*Elacatinus* [s.l.] spp.	*Pomatoschistus* spp.	*Sebastes* spp.
AA54	**I/L/V**	I	?*
AA112	**L/V**	**L/V/I**	L
AA116	F	F	**F/S**
AA119	L	L	**L/I/V**
AA133	V/I/M	**V/I**	V
AA158	A	A/G	**A/G**
AA165	**S/C/A**	**S/C/A/G**	**S/C**
AA205	I/V	I/L	**I/V**
AA209	**I/V/T**	I	V
AA213	C	S/L/V	**S/A/F/T**
AA217	**T/V/F/I/S**	**T/V/F/I**	**T/V/M**
AA274	Y	Y	**Y/F**
AA277	T	T	**S/C/L**
AA279	**Q/H**	Q	Q
AA281	**T/I/A/S**	**T/I/A**	S
AA290	**I/S/A/T**	I/V	I

*Only information from AA63 is known for *Sebastes* spp.

Functional variability at the *RH1* gene in *Elacatinus* [s.l.]

Variation at the *RH1* gene cannot be automatically considered to be neutral. According to the paradigm of efficient adaptive tuning of the VP by AA changes, some AA substitutions on the VPs may have a direct phenotypic effect. Based on the literature, several AAs that are variable in the *RH1* gene of *Elacatinus* [s.l.] are known spectral tuning sites, suggesting that the observed variability has a direct functional effect.

The actual effect of such substitutions has been documented in the literature for just two AAs. The first well-known tuning site is AA83, close to the retinal-binding site, where an aspartic acid to asparagine substitution causes a strong blue-shift of the λ_{max} values in retinal rods of many vertebrate families (Hunt *et al.*, 1996, 2001; Yokoyama & Takenaka, 2004; Sugawara *et al.*, 2005). The second well-known mutation is on AA299. This site is located towards the interior of the retinal-binding pocket in helix VII (Fig. 1) and close to the Schiff base linkage between the opsin and the chromophore (Hunt *et al.*, 2007). This suggests that it directly interacts with the chromophore (Fasick & Robinson, 1998). A blue-shift of the λ_{max} values of retinal rods caused by an alanine to serine or threonine substitution on AA299 has already been documented for many vertebrate families (Yokoyama *et al.*, 1995; Fasick & Robinson, 1998; Hunt *et al.*, 2001). Moreover, this AA was suggested to be under selection in, e.g. cichlids (Spady *et al.*, 2005) and *Pomatoschistus minutus* (Pallas, 1770) (Larmuseau *et al.*, 2009).

In *Elacatinus* [s.l.], we indeed found a clear link between the two aforementioned AAs, namely consistently either blue-shifted substitutions or red-shifted substitutions on AA83 as well as on AA299 for all species (Table 1). This already points to a link between the environment and the molecular/AA sequence. Moreover, the two red-shifted substitutions for AA83 and AA299 for *E. puncticulatus* in contrast to other species of the *Elacatinus* [s.s.] clade (Table 1) are remarkable. In comparison with the other studied species, *E. puncticulatus* occupies a different habitat. It only occurs in small caves and depressions, and at the lowest maximum depth of 6 m (Table S1). In shallow water, the best visual strategy is indeed to have a red-shifted λ_{max} in comparison with species living deeper in the water column

(Hunt *et al.*, 2001). Due to the lack of clear information about the behaviour and habitat range for each individual species, it is not possible to infer other links between AA sequence and microhabitat (Table S1). In view of the interesting ecological and behavioural range of the goby clade under study, a closer examination linking this diversity to molecular variability is recommended.

The effects of substitution of three remaining variable AAs that are closely located to the retinal-binding pocket of the opsin and also assumed to be spectral tuning sites (AA133, AA214 and AA217) have not yet been experimentally validated (Yokoyama, 2000; Bowmaker, 2008). Although the effect of variation at AA214 and AA217 on λ_{max} values has been tested by mutagenic experiments on red/green opsins of humans (Asenjo *et al.*, 1994), this effect is not yet known for rhodopsin. AA214 has been shown to be under selection within the sand goby *P. minutus* (Larmuseau *et al.*, 2009). Selection on AA217 has been detected in rockfishes *Sebastes* spp. (Sivasundar & Palumbi, 2010) and 'sand gobies' (Larmuseau *et al.*, 2010a). Finally, other AAs earlier indicated as being under selection in other marine radiations, and therefore maybe involved in spectral tuning mechanisms, were variable in *Elacatinus* [s.l.]. AA112, AA165 and AA281 were significantly under selection in the 'sand gobies' (Larmuseau *et al.*, 2010a), and AA162 and AA205 in *Sebastes* (Sivasundar & Palumbi, 2010).

Selection on the *RH1* gene in marine radiations

Different statistical tests indicated positive selection on the *RH1* fragment of the *Elacatinus* [s.l.] gobies. The *Z*-tests and 'branch-specific models' tests of neutrality are in general conservative because the substitution rates are averaged across all AA sites (Bamshad & Wooding, 2003). Nevertheless, several ω ratios revealed positive selection between different species and on specific branches within the *Elacatinus* [s.s.] subgenus. Furthermore, the Bayesian analysis in the 'site-specific model' identified eight positively selected sites at *RH1* of *Elacatinus* [s.l.] gobies, namely 54, 112, 165, 209, 217, 279, 281 and 290. However, AA279 was identified to be under selection based on the 'mtDNA phylogeny' but not based on the 'consensus phylogeny' of Taylor & Hellberg (2005). In the 'consensus phylogeny,' the *RH1* gene is included together with the rag gene and mtDNA Cyt *b* data and may therefore bias the phylogeny when *RH1* is strongly influenced by selection. It is, however, remarkable that there are no other differences between the 'consensus' and 'mtDNA' phylogeny, as previously observed by Taylor & Hellberg (2005). Finally, based on both phylogenies, independent similar changes at those AA positions reinforce the idea that they may in fact be under selection and functionally important, as their occurrence within *Elacatinus* [s.l.] seems to be polyphyletic rather than a result of the phylogenetic relationships.

There were four AAs that appeared to be under selection in this study (AA54, AA209, AA279 and AA290) that have not been detected in analogous studies on marine radiations. Remarkably, four other AAs (namely AA112, AA165, AA217 and AA281) were shared with those found to be under selection in another gobiid radiation, the north-eastern Atlantic 'sand gobies' (Larmuseau *et al.*, 2010a). In *Sebastes* spp., nine AAs were detected to be under selection, including AA165 and AA217 (Sivasundar & Palumbi, 2010). It should be noted that both of these AAs were shown to be under selection in the three studied marine radiations (Table 2), with AA217 assumed to be an important spectral tuning site (see earlier on in the discussion). The low number of shared AAs that were significantly under selection both in *Sebastes* and in the goby radiations could be the result of the ecological distance, i.e. in depth range, between gobies and rockfishes (Sivasundar & Palumbi, 2010). However, the comparison of the AA substitutions for those particular sites revealed that for most of them, the same substitutions occurred in all three radiations (Table 2). This apparent convergence confirms that these AAs are potential spectral tuning sites that merit further investigation. Mutagenic experiments or studies on other marine radiations are therefore recommended to study the effect of substitutions of those AAs.

Conclusion and perspectives

The study found clear indications for functional variability and positive selection on the rhodopsin gene in *Elacatinus* [s.l.] gobies, which had not been observed in the phylogenetic study based on *RH1* data by Taylor & Hellberg (2005). Therefore, the adaptive speciation in *Elacatinus* [s.l.] is presumed to be associated with photic divergence between local environments or microhabitats due to variation in depth, turbidity or light spectrum. It illustrates that the visual tuning system and the selection on light climate may play an important role in the speciation of marine taxa. A comparison with other marine radiations identified a clear set of AAs that are potential spectral tuning sites. They await validation by mutagenic studies. Nevertheless, the framework using VPs provided an excellent link between phylogeny, variable AAs associated with phenotypic changes and environmental variation.

This study suggests a close coupling between genotype, phenotype and environmental conditions for VP in *Elacatinus* [s.l.]. Nevertheless, future research has to focus on this link by measuring λ_{max} of the VPs and by a much better description of the behaviour and light climate of the microhabitat for each species in next sampling programs and species descriptions. It would also be interesting to analyse the link between high intraspecific variation and local adaptation as it was recently detected within the sand goby *Pomatoschistus minutus* (Larmuseau *et al.*, 2009, 2010b). Although only one to four individuals were

sequenced per species, intraspecific polymorphism was detected on AAs in *Elacatinus* [s.l.]. Understanding speciation in the marine realm associated with photic adaptation will benefit from the accumulation of more genetic data combined with a better knowledge on the ecology and behaviour of marine fishes.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Summary of specimens, sample locations, distribution and ecological/behavioural data based on a literature review.

Table S2 Results of a *Z*-test between pairs of *RH1* sequences in *Elacatinus* [l.l.].

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