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Opsin Evolution in Damselfish: Convergence, Reversal, and Parallel Evolution Across Tuning Sites

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Abstract The visual system plays a role in nearly every aspect of an organism's life history, and there is a direct link between visual pigment phenotypes and opsin genotypes. In previous studies of African cichlid fishes, we found evidence for positive selection among some opsins, with sequence variation greatest for opsins producing the shortest and longest wavelength visual pigments. In this study, we examined opsin evolution in the closely related damselfish family (Pomacentridae), a group of reef fishes that are distributed widely and have a documented fossil record of at least 50 million years (MY). We found increased functional variation in the protein sequences of opsins at the short- and long-wavelength ends of the visual spectrum, in agreement with the African cichlids, despite an order of magnitude difference in the ages of the two radiations. We also reconstructed amino acid substitutions across opsin tuning sites. These reconstructions indicated multiple instances of parallel evolution, at least one definitive case of convergent evolution, and one evolutionary reversal. Our findings show that the amino acids at spectral tuning sites are labile evolutionarily, and that the same codons evolve repeatedly. These findings emphasize that the aquatic light environment can shape opsin sequence evolution. They further show that phylogenetic approaches can provide important insights into the mechanisms by which natural selection "tinkers" with phenotypes.

Keywords Opsin · Ancestral state reconstruction · Parallel evolution · Pomacentridae

> It is at the molecular level that the tinkering aspect of natural selection is perhaps most apparent. Jacob 1977

Introduction

Organisms use their visual systems to detect food, avoid predators, find mates, navigate over short and long distances, and communicate information. These factors result in strong selective pressure to optimize visual sensitivities for specific tasks in specific environments. Visual ecologists have documented convergent changes in visual sensitivity in response to changes in the light environment repeatedly (Loew and Lythgoe 1978; Lythgoe 1979; Partridge et al. 1989; Goldsmith 1990; Bowmaker 1995; Hart 2001), across many animal taxa. Because there is a relatively direct link between genotype and visual phenotype (Bowmaker 1995; Yokoyama and Yokoyama 1996), the molecular mechanisms underlying many of these changes have been elucidated (Yokoyama 2000; Hart 2001; Hunt et al. 2001), and the visual system has become a model for understanding functional adaptation.

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The fundamental unit of the visual system is the visual pigment. Visual pigments are composed of an opsin protein that is covalently bound to a light-sensitive chromophore (Wald 1968). Interactions between the opsin protein and the chromophore determine the peak spectral sensitivity of the visual pigment (Yokoyama 2008). This spectral sensitivity can by altered by changing amino acids at key tuning sites, typically sites that form the chromophore binding pocket or are in close proximity to it (Bowmaker 1995; Chang et al. 1995; Yokoyama 2000, 2002; Hart 2001; Hunt et al. 2001, 2007; Hart and Hunt 2007). Visual pigments are the primary components of rod and cone photoreceptors, which confer maximal sensitivity under scotopic and photopic conditions (dim and bright light), respectively (Ebrey and Koutalos 2001). With few exceptions, color vision in vertebrates utilizes cone opsins, and phylogenetic studies suggest that the common ancestor of all vertebrates had four classes of cone opsin genes: short-wavelength sensitive 1 (SWS1), short-wavelength sensitive 2 (SWS2), rhodopsin-like (RH2), and long-wavelength sensitive (LWS) (Collin et al. 2003). Two of these opsin genes, SWS2 and RH2, were duplicated early in the acanthopterygian lineage of fishes (approximately 200 million years ago), suggesting that the genomes of many fishes contain at least one rod and six cone opsins (Hofmann and Carleton 2009).

Opsin evolution is one of the prime examples of molecular adaptation, where peak spectral absorption is tuned to match the light environment (Yokoyama and Yokoyama 1996). Convergent evolution of key tuning sites, among taxa that share common habitats or life histories, has been used repeatedly as an example of functional adaptation (e.g., Sugawara et al. 2002, 2005; Terai et al. 2002; Shen et al. 2010; Nagai et al. 2011; but see Yokoyama et al. 2008). Numerous studies have examined opsin evolution in a phylogenetic manner, and important insights about the molecular evolution of opsins have been gained from them (e.g., Chang et al. 1995; Briscoe 2001; Carvalho et al. 2007; Ward et al. 2008; Yokoyama et al. 2008; Owens et al. 2009; Shen et al. 2010). Studies that have focused at the population level or on rapid radiations can provide important insights about selection and speciation (e.g., Terai et al. 2002; Spady et al. 2005; Seehausen et al. 2008; Larmuseau et al. 2010). In one recent study, Nagai et al. (2011) found evidence for evolutionary reversals in the RH1 opsin. Further studies to elucidate the evolutionary pathways that specific amino acids have taken will provide a better understanding of the mechanisms through which selection shapes adaptive phenotypes.

Most of these previous studies have focused on the role of spectral tuning through sequence evolution of opsin genes. However, in cichlid fishes, we have found that sequence evolution is complemented by spectral tuning through changes in opsin gene expression. These studies focused on cichlids from the clear lakes of Malawi (Hofmann et al. 2009) and Tanganyika (O'Quin et al. 2010). For these species, sequence tuning works primarily on genes at the edges of the spectrum, the SWS1 and LWS genes. Making these genes shorter or longer wavelength sensitive can only happen by altering their sequence, as there are no other genes to use for gene expression tuning. We wanted to determine whether this was a common phenomena in other fish species and turned to the damsel fish radiation for further investigation.

Damsels and cichlids have many parallels. Both inhabit clear, relatively shallow aquatic habitats close to the substrate. Damsels live in the clear marine habitat near coral reefs while cichlids live in fresh water lakes. Both groups are quite diverse with a range of colorful patterns as well as foraging styles which might impact the visual tasks that they perform (Marshall 2000a, b; Konings 2007). The two groups are phylogenetically closely related. We can estimate their divergence time from the date of the common ancestor between cichlids and medaka (104 MY; www.timetree.org) and the fact that the mtDNA sequence divergence from damsels to cichlids is only 75 % of that from cichlids to medaka. This suggests there was a common ancestor for damsels and cichlids in the last 75 MY. Damselfish have a fossil record back to 50 MY (Bellwood 1996) with a well-resolved phylogeny (Cooper and Westneat 2009; Jang-Liaw et al. 2002; Quenouille et al. 2004, Tang 2001). By contrast, the lacustrine African cichlids, such as those from Lake Malawi, are 1–2 MY in age and it is difficult to get a well-resolved phylogeny for the closest related species (Meyer et al. 1990; Kocher 2004; Genner et al. 2007). We therefore examine a diverse set of damselfish taxa to assess whether the patterns we see in cichlid opsin sequence variation extends deeper in phylogenetic time.

The damselfish family (Pomacentridae) is a diverse, monophyletic family of coral reef fishes that includes 368 currently described species from 18 genera (valid names listed at http://www.fishbase.org, accessed August 2011; Allen 1991; Quenouille et al. 2004; Cooper et al. 2009). Damselfish are distributed in many of the world's oceans, typically in association with tropical coral or temperate rocky reefs and vary considerably in their ecology (Allen 1991). This includes areas in both the Atlantic and Pacific including the Great Barrier Reef, the Hawaiian Islands, and the Caribbean. Pomacentrids have several other factors that make them an ideal system for studying opsin evolution. Previous research using microspectrophotometry (MSP) suggests that damsel fish have three to four cone pigments and a rod pigment. Species vary in the peak absorbance of their short- (UV/violet), middle- (blue-green/green), and long-wavelength (yellow/red) sensitive cones, as well as their rods (Fig. 1) (McFarland and Loew 1994; Losey et al. 2003; Hawryshyn et al. 2003). This spectral variation suggests opsin sequences will also likely vary. Variation in damselfish ocular media absorbance has also been reported (Siebeck and Marshall 2001, 2007; Losey et al. 2003); a

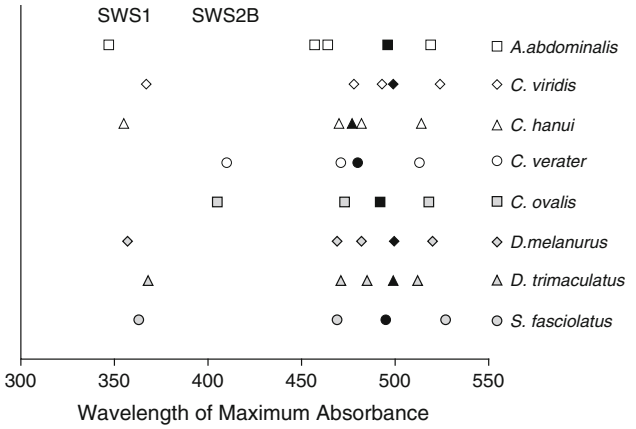

Fig. 1 Visual pigment absorbances from eight species of damselfish that were measured previously using MSP (Losey et al. 2003; Hawryshyn et al. 2003). These measurements suggest that most species express either their SWS1 or SWS2B opsin genes and that there is variation in the spectral sensitivity of the resulting visual pigments. Rod sensitivities are shown in *black*

trait that is correlated with variation in UV sensitivity in some fishes (Hofmann et al. 2010). In addition, damselfish have considerable variation in color, ranging from short-wavelength UV and blue colors to longer wavelength greens, yellows, and reds (Marshall et al. 2003a; for illustrations, see Randall et al. 1996). Though the relationship between color and vision remains elusive, color has been demonstrated to play an important role in behavioral interactions (Siebeck et al. 2010).

We wanted to know how opsin evolution proceeds in damselfish. We hypothesized first that damselfish would have multiple opsin genes. The duplication of the SWS2 and RH2 opsin genes occurred approximately 200 MY ago (Spady et al. 2006, Rennison et al. 2012). Since damsels and cichlids diverged only 75 MY ago, and cichlids have duplicates of both genes, we might expect that damsels would have six opsin genes, including SWS1, SWS2A, SWS2B, RH2A, RH2B, and LWS. Next, we hypothesized that because of the similar light environment and the similar absorption properties of the water near coral reefs and in Lake Malawi (Marshall et al. 2003b, Hofmann et al. 2009), and the diversity of opsin genes, damselfish opsins might evolve in a similar way as cichlid opsins, with the most variation for the shortest and longest wavelength sensitive opsin genes where the light environment is the most variable with depth.

Materials and Methods

Sample Collection

Fish were collected off the coast of North Stradbroke Island, Queensland, Australia by a professional collector and kept in aquaria at the Moreton Bay Research station for up to 1 week. Fish were anaesthetized and euthanized using clove oil (Reilly 2001) and their eyes were enucleated. The lens and cornea were removed for transmission measurements, retina were removed and stored in RNA-later (Ambion), and fin clips were collected and stored in ethanol. At the University of Maryland, RNA was extracted and reverse transcribed using protocols optimized previously for cichlids (Hofmann et al. 2009). All animals were collected and exported according to state or national laws and all animal care was performed in accordance with the relevant institutional guidelines.

We focused on 10 species in this study: *Parma oligolepis, Plectroglyphidodon dickii*, *Stegastes gascoynei*, *Chromis viridis*, *Dascyllus trimaculatus*, *Abudefduf sexfasciatus*, *Chrysiptera rex*, *Amphiprion akindynos*, *Neopomacentrus bankieri*, and *Pomacentrus amboinensis*. These taxa represent four of the five major damselfish clades identified in a previous phylogenetic study by Cooper et al. (2009), and represent a diversity of ecologies. For example, *Amph. akindynos* is a species of anemonefish, *S. gascoynei* and *Parma oligolepis* typically inhabit rocky, temperate reefs and feed on algae, and *Chromis viridis* is a zooplanktivore that feeds in the water column above coral heads (Coughlin and Strickler 1990; Manica 2004; Cooper and Westneat 2009; Frederich et al. 2009).

Opsin Sequencing

We used opsin sequences from *Lucania goodei*, *Oryzias latipes*, *Takifugu rubripes*, *Tetraodon nigroviridis*, and several species of Cichlidae to design degenerate PCR primers for the rod (RH1) and six (SWS1, SWS2B, SWS2A, RH2B, RH2A, and LWS) cone opsin genes (Table S1–2). These degenerate primers were used to amplify partial coding sequence from each species. We blasted these partial coding sequences against the Genbank database and generated gene trees using the neighbor joining algorithm in the program MEGA 4 (Tamura et al. 2007) to confirm that we had in fact amplified the target opsin genes. These partial sequences were then used to develop 5′ and 3′ RACE primers (Table S2). Once complete, or near complete, coding sequence had been obtained for each species, a final set of damsel specific primers were developed that amplified each opsin in two overlapping segments (Table S2–3). Because of the phylogenetic diversity of the damselfish, the designed opsin primers successfully amplified anywhere from one to ten species, depending on the opsin gene (Table S3).

Degenerate primers were developed using the program GeneFisher2 (Giegerich et al. 1996). All PCR amplifications used Dynazyme (New England Biolabs) or GoTaq Polymerase (Promega). PCR products were run on a 1 %

Table 1 Focal amino acid sites that were reconstructed in this study

Bovine rhodopsin	SWS1	SWS2B	Rh2B	LWS	Rh1
46[a]	39	52			
49[a]	42				
97[a]	90				
109[a]	102				
114[a]	107				
118[a]	111				
164[a]				177	
207[a]			208		
299[b]					299

The positions in bovine rhodopsin and the corresponding cone opsin are represented

[a] Yokoyama (2008)

[b] Fasick and Robinson (1998), Hunt et al. (2001)

agarose gel, bands were excised and purified using Qiagen columns, then sequenced using Applied Biosystem's Big Dye chemistry. RACE and damsel specific primers were designed using the program Primer 3 (Rozen and Skaletsky 2000). RACE was performed using a Smart RACE kit (Clonetech).

Opsin Sequence Analysis

Sequences were aligned and edited using Sequencher (Genecodes Corp.). Forward and reverse sequences were used to generate a consensus sequence from each species, which was trimmed and exported to MEGA 4 (Tamura et al. 2007). We applied methods that have been used previously in cichlids to identify amino substitutions that were potentially functional (Hofmann et al. 2009). In brief, we focused on substitutions between amino acids with different physical properties (polar, non-polar, acidic, or basic) that occurred in the transmembrane or retinal binding pocket region of the opsin protein (based on the alignments of Carleton et al. 2005). We also examined known tuning sites that were described in a recent review by Yokoyama (2008), plus one additional tuning site, Rh1 site 299 (Fasick and Robinson 1998; Hunt et al. 2001), that also varied in cichlid rods (Hofmann et al. 2009). Table 1 shows the gene specific location of each tuning site, as well as well as their location relative to bovine rhodopsin. To avoid confusion due to differences in opsin gene length, we refer to each site by its location relative to bovine rhodopsin.

We also used MEGA to calculate the number of synonymous substitutions per synonymous site (d_s) and non-synonymous substitutions per non-synonymous site (d_n), nucleotide diversity (π), and to generate gene trees for each opsin. Neighbor joining trees were generated using MEGA's maximum composite likelihood nucleotide model with gamma distributed rates among sites. Modeltest (Posada and Crandall 1998) was used to estimate the gamma parameter. 1000 bootstrap replicates were performed for each tree, and values >50 % are included.

Phylogenetic Sequencing

We amplified the mitochondrial DNA *12s* sequence as well as the nuclear gene, *rag1*, to confirm the phylogenetic relationship of these 10 species. We performed this work in the context of previous phylogenies and used the same primers as previous work (Cooper et al. 2009; Quenouille et al. 2004) (Table S4). The *12s* gene was amplified with Dphe22F or D12s53F and D12s991R primers. The *rag1* gene was amplified with Drag1F1a and Drag1Q8R. PCR products were amplified using Dynazyme EXT polymerase, run on a 1 % agarose gel, excised, and cleaned on Qiaquick columns. The product was then sequenced using the amplification primers and additional internal primers using ABI Big Dye chemistry on an ABI 3130 or 3730xl sequencer.

The *12s* and *rag1* gene sequences (accession #s in Table S5) were concatenated and aligned with sequences from both Cooper et al. (2009) and Quenouille et al. (2004) using MAFFT (Katoh et al. 2002, 2005). They were then used to make a phylogenetic tree using either distance methods in PAUP 4.0b (Swofford 2003) or maximum likelihood in a parallelized online version of Garli (http://www.molecularevolution.org/; Zwickl 2006; Bazinet and Cummings 2008, 2011). Since the damselfish phylogeny requires numerous genes to resolve the families, our goal was not to generate a new phylogeny based on only two genes, but to use these two genes to confirm the placement of several congeners that were not included in the original phylogenetic studies.

Tests for Positive Selection

We used the codeml program from the PAML package v4.5 (Yang 2007) to look for evidence of positive selection in order to identify sites that might have functional importance in the six opsin genes LWS, RH2A, RH2B, SWS2B, SWS1, and RH1. This program calculates the ratio of non-synonymous substitution (d_N) to synonymous substitution (d_S) rates using a codon-based model of sequence evolution (Goldman and Yang 1994). Site-specific models M1a vs M2 and M8 vs M8a were used that allow d_N/d_S ratios to vary among codon sites of the proteins. M1a and M8a are null models that do not allow for positive selection; rather a proportion of sites are under purifying selection and another proportion of sites are neutral ($0 < d_N/d_S < 1$ and $d_N/d_S = 1$; Yang 2007). These models were compared to models M2a and M8 which add

Table 2 Summary of damselfish opsin variation

	SWS1	SWS2B	Rh2B	Rh2A	LWS	Rh1
Total number of nucleotides	1017	1059	1038	1059	1074	1059
Total number of amino acids	338	352	345	352	357	352
Number of missing aa sites	15	14	0	0	0	0
Percent coverage	95.6	96.0	100.0	100.0	100.0	100.0
Variable nt sites	228	315	154	134	211	130
Indels	0	2	0	0	0	1
Variable amino acid sites	65	109	41	29	45	24
Functionally variable amino acid sites	33	52	17	8	20	7
Variable transmembrane sites	52	59	26	18	27	22
Functionally variable transmembrane sites	27	25	11	6	13	8
Variable retinal binding pocket sites	8	12	3	0	3	2
Functionally variable retinal binding pocket sites	7	5	0	0	4	1
Number of substitutions at known tuning sites	6	1	1	0	1	1
Number of substitutions at sites known to tune other opsins	2	3	3	0	3	0
Nucleotide diversity (π)	0.083	0.106	0.049	0.045	0.067	0.038
Synonymous substitutions (d_s)	0.204	0.219	0.133	0.127	0.218	0.120
Nonsynonymous substitutions (d_n)	0.040	0.070	0.023	0.019	0.021	0.013
d_n/d_s	0.198	0.318	0.174	0.151	0.096	0.104

an additional category which allows for sites to be under positive selection. Likelihood ratio tests (LRT) for positive selection were performed by comparing twice the difference in the log-likelihoods ($2\Delta\ell$) between models M1a and M2a and models M8a and M8. These comparisons were made against a χ^2_2 with critical values of 5.99 ($p > 0.95$) and 9.21 ($p > 0.99$) for M1a vs M2a and a χ^2_1 with critical values of 2.71 ($p > 0.95$) and 5.41 ($p > 0.99$) for M8 vs M8a (Yang 2007). The null models were rejected and positive selection was inferred if $2\Delta\ell$ fell above the critical values and d_N/d_S values were >1. If LRT were significant, sites inferred to be under positive selection were identified using the Bayes Empirical Bayes (BEB) method which is employed by the codeml program under models M2 and M8 (Yang et al. 2005).

Ancestral State Reconstruction

We used two different phylogenies to perform ancestral state reconstruction using Mesquite (Maddison and Maddison 2008). We began with the phylogeny from Cooper et al. (2009) which included six of the species that we examined (*Plect. dickii*, *Chromis viridis*, *D. trimaculatus*, *Abu. sexfasciatus*, *Chrys. rex*, and *Pom. amboinensis*). Placement of the remaining four species (*N. bankieri*, *Amph. akindynos*, *S. gascoynei*, and *Parma oligolepis*) was determined based on the location of other congeners. This placement agreed with the results of our phylogenetic tree, which always grouped congeners together. Second, we used one of the alternative phylogenetic hypothesis from Quenouille et al. (2004), based on mtDNA genes and rag1, to test the robustness of our results. These phylogenies vary in the relative placement of *Abu. sexfasciatus* as well as the relationships among *Chrys. rex*, *Amph. akindynos*, *N. bankieri*, and *Pom. amboinensis*. The cichlid, *Oreochromis niloticus,* and the guppy, *Poecilia reticulata* were included as outgroups for phylogenetic hypotheses.

We used these phylogenetic hypotheses to examine the number and direction of changes in known amino acid tuning sites, and to identify cases of parallel and convergent evolution. We used a strictly phylogenetic definition of these two terms, where we defined parallel evolution as two or more changes from the same ancestral character state to the same derived character state, and convergent evolution as two or more changes from different character states to the same derived character state (Zhang and Kumar 1997).

We used two different methods to perform ancestral state reconstructions. These included parsimony in Mesquite (Maddison and Maddison 2008) and the codeml program in PAML (Yang 2007) using an empirical Bayes procedure (Yang et al. 1995).

Results and Discussion

We were able to identify and sequence six opsin genes from 10 species of damselfish, obtaining full length coding sequence for the RH2B, RH2A, LWS, and RH1 opsins, and slightly more than 95 % of the coding sequence for SWS1 and SWS2B (Table 2, Genbank accession #s in Table S5).

We were not successful in sequencing the SWS2A opsin. Our initial set of degenerate primers amplified SWS2B. An additional 40 primers in various combinations failed to amplify SWS2A from either cDNA or genomic DNA. Because the SWS2B opsin typically sits downstream of SWS2A, we tried two additional approaches. First, we tried long PCR, using a degenerate forward primer designed from a region upstream of SWS2A that is conserved in cichlids and medaka, and a reverse primer located in damselfish SWS2B. However, the PCR product did not contain any SWS2A sequence. We also attempted to genome walk upstream from SWS2B which did not produce long enough sequences to reach the SWS2A gene, assuming it was 5 kb away. The conclusion from these attempts suggests the SWS2A gene may not be present in the damselfish genome. However, we did not find evidence for a pseudogene or a transposon insertion to prove this.

Based on these results, it appears that damsels have five cone opsin genes and one rod opsin. The RH2 gene duplicates, RH2A and RH2B, do occur in these fish and both appear to be functional. However, for the class of SWS2 genes, only the SWS2B gene was found with no evidence for the SWS2A gene. It is possible that the SWS2A gene has been lost.

Opsin Gene Sequence Diversity

There was considerable sequence diversity in all the opsins. The SWS2B opsin (violet) had the greatest variation (Table 2, Fig. 2); in this gene 315 of the 1059 nucleotides and 108 of the 352 amino acid sites varied across species. Nucleotide diversity for SWS2B opsin (0.106) was more than that of the SWS1 (UV) opsin (0.083), which was the second most diverse opsin, and more than three times as diverse as the RH1 (rod) opsin (0.038), which was the least diverse (Table 2).

Complete amino acid alignments are given in Fig. S1. These show the retinal binding pocket sites (Carleton et al. 2005) as well as sites known to cause spectral tuning (Yokoyama 2008). These also show sites that we identified as likely spectral tuning sites (i.e., amino acids with different physical properties that were located in the retinal binding pocket or at known tuning sites of the protein, labeled in the supplementary figures as class 3, 4, or 5). The number of likely spectral tuning sites varied with 13 sites for SWS1, 7 for SWS2B, 4 for RH2B, none for RH2A, 5 for LWS and 1 for RH1. SWS1 and SWS2B had comparable levels of variation for nucleotide variation and also likely tuning sites, although SWS1 had slightly more variation in the retinal binding pocket (Fig. 2; Fig. S1). Many of these likely tuning sites overlap with sites known to cause spectral tuning of the same opsin gene. These known tuning sites, along with the specific damsel fish amino acid changes given in bovine rhodopsin site numbers are as follows: SWS1 (F46I or F46A, F49I or F49C, S97A, M109L, S114A, S118A); SWS2B (F46V, S118T); RH2B (M207L*); RH2A (none); LWS (S164A*); and RH1 (S298A). Both the RH2B and LWS sites are the exact same amino acid changes which are known to cause spectral tuning. Comparisons of these sites to known mutational studies are shown in Table S6. This offers support that several of these changes are likely functional.

The PAML method has been developed to identify potentially functional sequence changes. The idea is that those sites under positive selection are changing adaptively to alter protein function. We ran PAML to identify genes and then sites under positive selection (Table S7). Using a comparison of codeml models 1a and 2, genes SWS2B ($p = 0.0088$), RH2B ($p = 0.026$), RH2A ($p = 5.7e{-}5$), and RH1 ($p = 4e{-}8$) were under positive selection. Similar results were found for the model 8/8a comparison, which identified the same genes with more significant p values (SWS2B: 4.4e−4; RH2B: 3.5e−3; RH2A: 5.6e−6; RH1: 5e−9). It was a bit surprising that neither of the model comparisons identified the SWS1 gene as being under positive selection, as it varies at six sites known to tune SWS1. LWS was also not identified as under positive selection, though the damselfish sequences contain a mutation known to cause a 2–6 nm change in LWS opsin.

The PAML model 8/8a comparison identified a few sites to be under positive selection at $p > 0.95$ (Table S7 and shown in Supplementary Fig. 1a–f). These include two sites for SWS2B, four sites for RH2B, five sites for RH2A, two sites for LWS, and two sites for RH1. These tend to be the amino acids that show the greatest variation involving several different amino acids and several different changes across the tree. Interestingly, none of these sites are known tuning sites for the corresponding gene and only one of the LWS positively selected sites was a known tuning site for a different gene (site 116 known to tune SWS1 and 2). Based on homology with the bovine rhodopsin crystal structure, we find many of these positively selected sites to be in the transmembrane regions. However, they are unlikely to be directed into the retinal binding pocket and so unlikely to tune peak visual pigment absorption. However, these mutations require future site directed mutagenesis studies to test their effect.

Based on the analyses of retinal binding pocket sites with polarity changes, we identified the greatest variation within the SWS1, SWS2B, and LWS opsin amino acid sequences (Fig. 2). For LWS, this amino acid diversity occurred in spite of LWS having the lowest dN/dS ratio of all of the opsin genes. Thus, the opsins that have the greatest potential functional diversity are those that produce visual pigments sensitive to the short- and long-

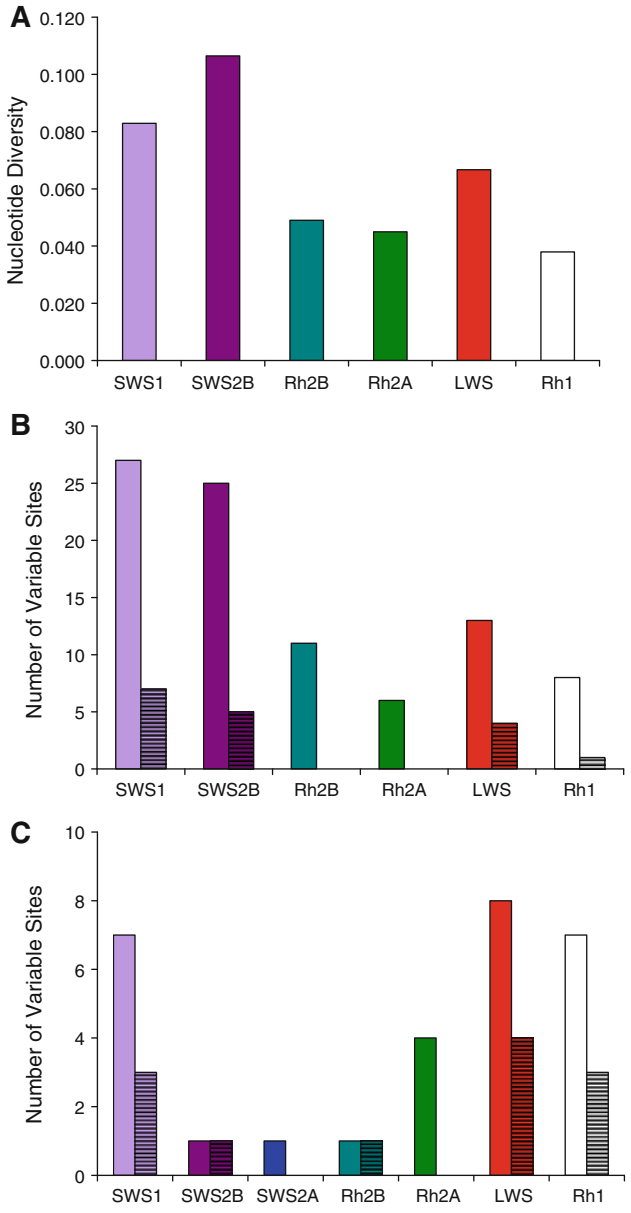

Fig. 2 **a** Nucleotide diversity (π) across species for each opsin. **b** Potentially functional amino acid sequence variation in the transmembrane (*solid*) and retinal binding pocket (*hashed*) regions of each damselfish opsin. Only substitutions between amino acids with different physical properties were counted. **c** The same variation in cichlids (adapted from Hofmann et al. 2009)

wavelength ends of the spectral range. This pattern of variation is similar to the one that we observed across cichlids from Lakes Malawi and Victoria (Hofmann et al. 2009; Fig. 2c). Further studies have shown that across these two lakes, the sequence diversity of SWS1 and LWS cone opsins corresponds to functional diversity (Smith and Carleton 2010; Carleton et al. 2005; Seehausen et al. 2008). Thus, the damselfish and cichlid radiations show similar patterns of sequence variation, even though their timescales differ by an order of magnitude (Meyer et al. 1990; Bellwood 1996; Kocher 2004; Genner et al. 2007). The explanation for this pattern of variation in cichlids was that differential gene expression provided an evolutionarily labile mechanism for tuning visual pigment sensitivities across the spectrum, but that only sequence changes could extend the sensitivities of the short- and long-wavelength visual pigments. Whether gene expression varies across species in damselfish remains an intriguing avenue for future research.

Although there are similarities, there also appear to be some differences between the pattern of variation we observed in damselfish and that of cichlids. Interestingly, damselfish appear to have considerable variation in their SWS2B opsins, such that SWS1 and SWS2B have comparable amino acid sequence variation (Fig. 2), and this variation is greater than that observed in LWS. Also, the RH1 amino acid sequences have considerably less variation (relative to SWS1 and LWS) in damselfish. Together, these findings suggest there is more potential functional variation at the short-wavelength end of the spectrum in damselfish. Differences in the light environment may be one explanation for these observations. Previous work examined the effects of photic environment in cichlids from Lakes Malawi and Victoria. The cichlids that colonized the clear waters of Lake Malawi did so within the past 2 million years from more turbid riverine environments (Kocher 2004), and Lake Victoria is considerably turbid (Hofmann et al. 2009). These turbid environments, which are dominated by long-wavelength light, may have resulted in stronger divergent selection on the LWS opsins of cichlids than damselfish. Damselfish live in relatively clear light environments with broad spectral irradiance, and all of the damselfish species that we sampled inhabit relatively shallow depths, which may not provide strong enough light gradients to influence LWS.

Matching Genes with Visual Pigment Absorbances

Physiological measurements of photoreceptor absorption have been taken using MSP from several species of damselfish. These include data for *Chromis viridis* and *Dascylus trimaculatus* included in this study (Hawryshyn et al. 2003). Previous studies have shown that cone opsin genes of a particular class produce visual pigments within a particular spectral sensitivity (SWS1 350–420 nm; SWS2 405–475 nm; RH2 467–516 nm; LWS 508–565 nm; Yokoyama 2008). We can use these MSP data to match damselfish photoreceptor absorbances with the likely opsin genes that are expressed in these photoreceptors. Many of the damselfish utilize three or four cone pigments and one rod pigment. All have a long wavelength pigment, several medium wavelength pigments, and a short wavelength pigment. These visual pigments are best explained by

expression of the LWS, RH2A, and RH2B opsin genes and then one of the SWS genes. Most of the damselfish short wavelength photoreceptors have ultraviolet sensitivity and would therefore contain the SWS1 opsin protein (Fig. 1). A few species appear to have violet sensitive cones, which would contain the SWS2B opsin protein (Fig. 1). The SWS gene not expressed in the adults could be expressed at a different life stage. MSP measurements of juveniles and adults from *Chromis punctipinnis* suggest there is a switch in gene expression from SWS2B to SWS1 over the course of development (McFarland and Loew 1994). All the species we examined have SWS1 and SWS2B suggesting this could be a common expression strategy. Interestingly, this change toward shorter wavelengths is the reverse of what we have observed in cichlids such as *O. niloticus* (Carleton et al. 2008) and *A. burtoni* (O'Quin et al. 2011).

Based on the MSP data, we could not definitively confirm or rule out the presence of SWS2A-based visual pigments. The medium wavelength pigments in the 470–480 nm range are typically in the RH2B opsin range, but SWS2A and RH2B visual pigment sensitivities may overlap (Hofmann and Carleton 2009). In previous damselfish MSP studies, the medium wavelength pigment (460–480 nm) was found in double cones 93 % of the time, suggesting it is an RH2 pigment (Losey et al. 2003). Therefore, this could be consistent with the loss of SWSA from the damselfish lineage.

Phylogenetic Relationships

The *12s* mtDNA gene and *rag1* nuclear gene were used to infer the relationships of the 10 focal damselfish species within the larger context of damselfish species studied previously by Cooper et al. (2009) and Quenouille et al. (2004). In all cases, the species grouped with their conspecifics or their congeners from the previous studies (Fig. S2). To account for the fact that relationships of the most closely related genera (*Chrys rex*, *N bankieri*, *Pom amboinensis*, and *Amph akindynos*) as well as the placement of *Abu sexfasciatus* varied between Cooper et al. (2009) and Quenouille et al. (2004), we considered both of these topologies in the ancestral state reconstructions. These alternate topologies did not change the conclusions of this study.

Ancestral State Reconstruction

Understanding the evolutionary pathways that are taken at the amino acid level can provide interesting insights into the molecular mechanisms through which selection shapes phenotypes. We reconstructed the evolutionary changes that occurred at the nine known tuning sites, six for SWS1 (bovine rhodopsin sites 46, 49, 97, 109, 114, 118) and one each for SWS2B (46), RH2B (207), and LWS (164) (Yokoyama 2008). One additional tuning site that varies in cichlid rods (bovine rhodopsin site 299) was also included. We did not identify any potentially functional variation in the RH2A protein at these sites.

We were able to infer the number and direction of changes at most of the known tuning sites that varied (Fig. S3). Of the 110 nodes that were internal to our 10 focal taxa, only 10 were ambiguous based on the parsimony method. Using the codon model in PAML, most of these could be assigned to one amino acid. Based on these reconstructions, we identified parallel evolution occurring for SWS1 (114, 118), RH2B (207), and LWS (164). For example, SWS (114) has parallel changes from alanine to serine for several taxa including *N. bankieri*, *Amp. akindynos*, and the common ancestor of *D. trimaculatus* and *Chromis viridis* (Fig. 3; Fig. S3). A clear reversion can be seen at SWS1 site 118 where a change from alanine to serine occurred in the common ancestor of the clade containing *Chrys. rex*, *Amph. akindynos*, *N. bankieri*, and *Pom. amboinensis* and a reversion back to alanine occurred in *Pom. amboinensis* (Fig. 3).

In most of the cases of parallel evolution that we observed, the same codon was used, suggesting a potential codon bias. However, one notable exception occurred for RH2B site 207. Four independent changes from methionine to leucine, using different codons, occurred in *S. gascoynei*, *D. trimaculatus*, *Abu. sexfasciatus*, and *Amph. akindynos* (Fig. 3). In *Amph. akindynos* and *D. trimaculatus* there was a change from ATG (M) to TTG (L) while in *Abu. sexfasciatus* and *S. gascoynei* there was a change from ATG (M) to CTG (L). This site is a known RH2 tuning sites and the fact that the same amino acid phenotype has arisen through different codons provides strong evidence of selection. Interestingly, PAML does not identify this as a positively selected site though at $p = 0.88$ it is close to the 0.95 threshold.

Finally, we consider one additional LWS site (116). This site is not a known tuning site in LWS but it known to tune SWS1 and SWS2. This site shows evidence of convergent evolution. At this site, a change from threonine to isoleucine occurred in *Plect. dickii, D. trimaculatus*, and *Abu. sexfasciatus* (Fig. 3). A change from threonine to valine occurred in the common ancestor of *Amph. akindynos*, *N. bankieri*, and *Pom. amboinensis*. Finally, a change from valine to isoleucine occurred in *Pom. amboinensis*. The PAML reconstructions follow a slightly different path, since it takes account of the fact that a T to V change requires two nucleotide changes (Fig. S3). This suggests that this site might be worth further investigation as a tuning site in LWS.

One additional point of interest is that changes at several of these sites appear to be correlated both within and across opsin genes. If we treat these as bivariate sites with states 1 and 0, we can calculate an r^2 value. Sites 114 and 118 of the SWS1 have nearly identical transitions across the

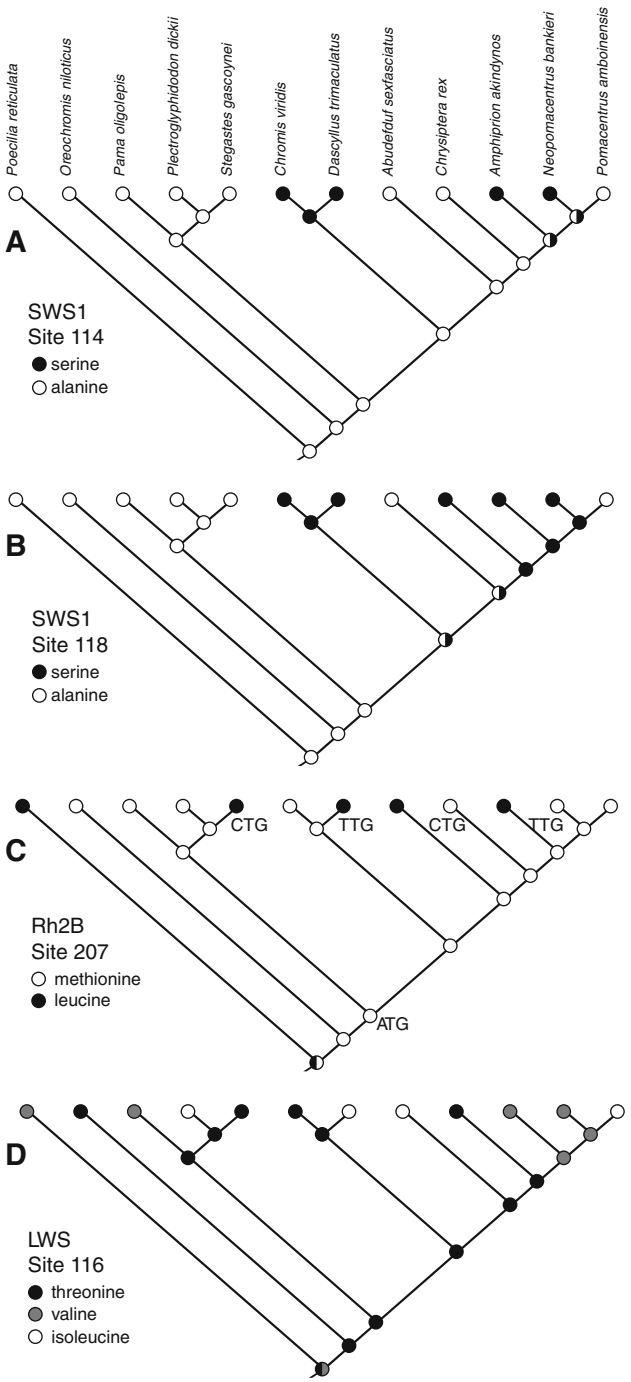

Fig. 3 Reconstruction of amino acid changes that demonstrate parallel evolution **a**, convergent evolution **b**, differential codon use **c**, and reversion **d**. All reconstructions were performed using maximum parsimony in Mesquite (Maddison and Maddison 2008).

phylogeny and have an r^2 of 0.67 ($p = 0.004$). In addition, SWS1 site 118 is correlated with LWS site 164 with an r^2 of 0.67 ($p = 0.004$). Because the light spectrum typically narrows with depth in the clear waters above a coral reef, it is possible that these long and short wavelength genes will be subject to selection in a correlated fashion.

Using alternative phylogenetic hypotheses did not change our overall observation of repeated parallel evolution, convergence and reversal. Although the character states at a few internal nodes became ambiguous, other nodes that were previously ambiguous could be resolved (see Fig. S4 for results using the tree from Quenouille et al. 2004). Thus, the net result was little change in the overall number of instances of reversal, convergence, or parallel evolution. This robustness is likely because many of the examples of parallel evolution occurred across major clades of the damselfish phylogeny (Fig. 3; Fig. S3).

Gene Trees

We generated neighbor joining trees for each opsin gene (Fig. 4). The relationships among *Amph. akindynos*, *Chrys. rex*, *N. bankieri*, and *Pom. amboinensis* varied across the gene trees, although all four always formed a single clade. This could be due to the relatively rapid radiation of this clade into the Indo-Pacific. In contrast, *D. trimaculatus* and *Chromis viridis* always formed a single clade, as did *Plect. dickii* and *S. gascoynei*. Thus, the deeper topology of the species tree is maintained in the gene trees, despite the fact that parallel evolutionary changes occurred across all of these lineages (Fig. 3; Fig. S3). However, given that the damsel radiation is >50 MY it is not surprising that parallel evolution at one or a few tuning sites does not provide enough phylogenetic signal to alter the overall topology of the gene trees.

We also observed some interesting differences in tree topology across the opsin genes. RH1, RH2A, and RH2A have short internal nodes, while the internal nodes of SWS2B, LWS, and SWS1 (to a lesser extent) tend to be longer. Both groups had long terminal branches. These differences in topology suggest SWS2B, LWS, and SWS1 were diversifying as these lineages were radiating, while much of the variation in RH1, RH2A, and RH2B occurred after they had radiated. This topology is consistent with SWS2B, LWS, and SWS1 opsins playing a role in the early radiation of the damselfish. Although circumstantial, this finding suggests future studies with denser taxon sampling might provide interesting insights into the role that vision played in the damselfish radiation. It also complements previous work suggesting that jaw morphology, especially adaptations for planktivory, have facilitated the radiation of damselfish. Ultraviolet vision has also been associated with planktivory, both behaviorally, and phylogenetically (Jordan et al. 2004; Hofmann et al. 2009).

Areas for Future Research

Our findings highlight several interesting directions for future research. The parallel evolution that we observed at known tuning sites provides strong evidence that selection was

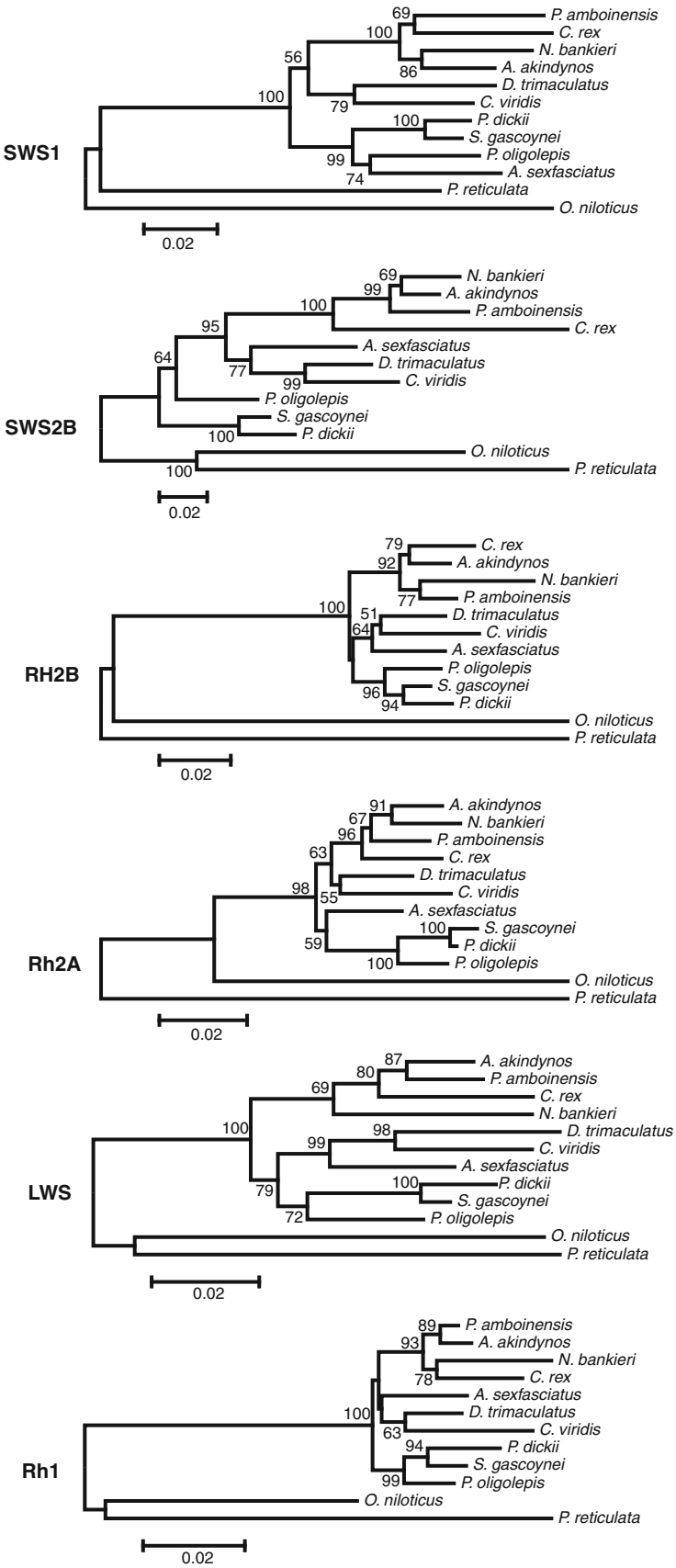

Fig. 4 Neighbor joining trees for each opsin gene for the 10 damsel fish species: *Parma oligolepis*, *Plectroglyphidodon dickii*, *Stegastes gascoynei*, *Chromis viridis*, *Dascyllus trimaculatus*, *Abudefduf sexfasciatus*, *Chrysiptera rex*, *Amphiprion akindynos*, *Neopomacentrus bankieri*, and *Pomacentrus amboinensis*. Trees were generated using nucleotide sequence data in Mega (Tamura et al. 2007) and bootstrap values >50 are shown. Sequences from *Oreochromis niloticus* and *Poecilia reticulata* opsins were included as outgroups. The general topology of these trees is conserved across species, although the relative relationships between *Chrys. rex*, *Amph. akindynos*, *N. bankieri*, and *Pom. amboinensis* vary. Note that SWS2B, LWS, and SWS1 gene trees have longer internal branch lengths than those for RH2A, RH2B, and RH1

acting on damselfish visual pigments during the course of the damselfish radiation. This is not surprising since visual cues play an important role in damselfish behavior (Siebeck et al. 2008, 2010), and damselfish vary considerably in their ecology and life history (Randall et al. 1996). However, whether selection remains strong among extant taxa requires a different sampling regime. Addressing this question will require sampling from multiple groups of closely related species, rather than the phylogenetically diverse species examined in this study.

The tools we have developed also provide the groundwork for future studies examining changes in opsin gene expression across species, which can produce dramatic shifts in photoreceptor sensitivity (Hofmann et al. 2009). Changes in gene expression can also be examined throughout the course of development, for example, between pre- and post-settlement larvae (Siebeck and Marshall 2007). Previous studies have found these larvae undergo changes in lens transmission Siebeck and Marshall 2007), and lens transmission is correlated with SWS1 opsin gene expression (Hofmann et al. 2010).

Conclusions

This study provides an overview of opsin evolution in a radiation with a well-resolved molecular phylogeny. Although this radiation is an order of magnitude older than the cichlid radiations of Lakes Malawi and Victoria, we observed similar patterns of variation across the opsins, with most of the variation occurring in the opsins at the extremes of the spectral range, and some notable differences between radiations in the SWS2B (violet) and RH1 (rod opsins). We used the damselfish phylogeny to demonstrate multiple examples of parallel evolution at known and potential tuning sites. We also demonstrated that character states at individual tuning sites can be labile, and that these amino acids undergo parallel evolution and reversal, often utilizing the same codon, though sometimes different codons. These findings suggest damselfish may be a useful model system for understanding the evolution of the visual system, especially since damselfish are also being used to study the evolution of jaw morphology. They also emphasize that phylogenetic approaches can provide important insights into the process of molecular adaptation.

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