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Molecular evolution

Out of the blue: adaptive visual pigment evolution accompanies Amazon invasion

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Incursions of marine water into South America during the Miocene prompted colonization of freshwater habitats by ancestrally marine species and present a unique opportunity to study the molecular evolution of adaptations to varying environments. Freshwater and marine environments are distinct in both spectra and average intensities of available light. Here, we investigate the molecular evolution of rhodopsin, the photosensitive pigment in the eye that activates in response to light, in a clade of South American freshwater anchovies derived from a marine ancestral lineage. Using likelihood-based comparative sequence analyses, we found evidence for positive selection in the rhodopsin of freshwater anchovy lineages at sites known to be important for aspects of rhodopsin function such as spectral tuning. No evidence was found for positive selection in marine lineages, nor in three other genes not involved in vision. Our results suggest that an increased rate of rhodopsin evolution was driven by diversification into freshwater habitats, thereby constituting a rare example of molecular evolution mirroring large-scale palaeogeographic events.

1. Introduction

Evolutionary transitions of species colonizing new ecological niches provide excellent systems for studying molecular adaptation. Visual pigments, light-sensitive molecules mediating the initial steps in the visual transduction cascade, are amenable to these studies, because they represent a direct interface between an organism and its environment. Mutations in the opsin protein component of visual pigments can shift peak sensitivity towards the wavelengths of light most prevalent in the environment [1]. Rhodopsin is the visual pigment predominantly expressed in rod photoreceptors and functions in dim-light vision. Rhodopsin is particularly important in aquatic environments, where light attenuation is much greater than in air, and has been extensively studied in deep-sea fishes, where its peak spectral sensitivity has been shifted to match the predominately blue environment [2]. In contrast to oceans, many large rivers are most transparent to red light owing to the selective scattering of short wavelengths by suspended particulate matter. This causes freshwater systems to appear dimmer and red-shifted when compared with marine systems of the same depth [3].

During the Miocene in South America, profound palaeogeographic and climatic changes caused massive incursions of seawater into formerly freshwater continental habitats [4,5]. These incursions resulted in complex habitat mosaics with varying salinity levels that facilitated evolutionary transitions between marine and freshwater in several lineages of fishes, including anchovies [6]. The New World clade of anchovies (subfamily Engraulinae) includes marine

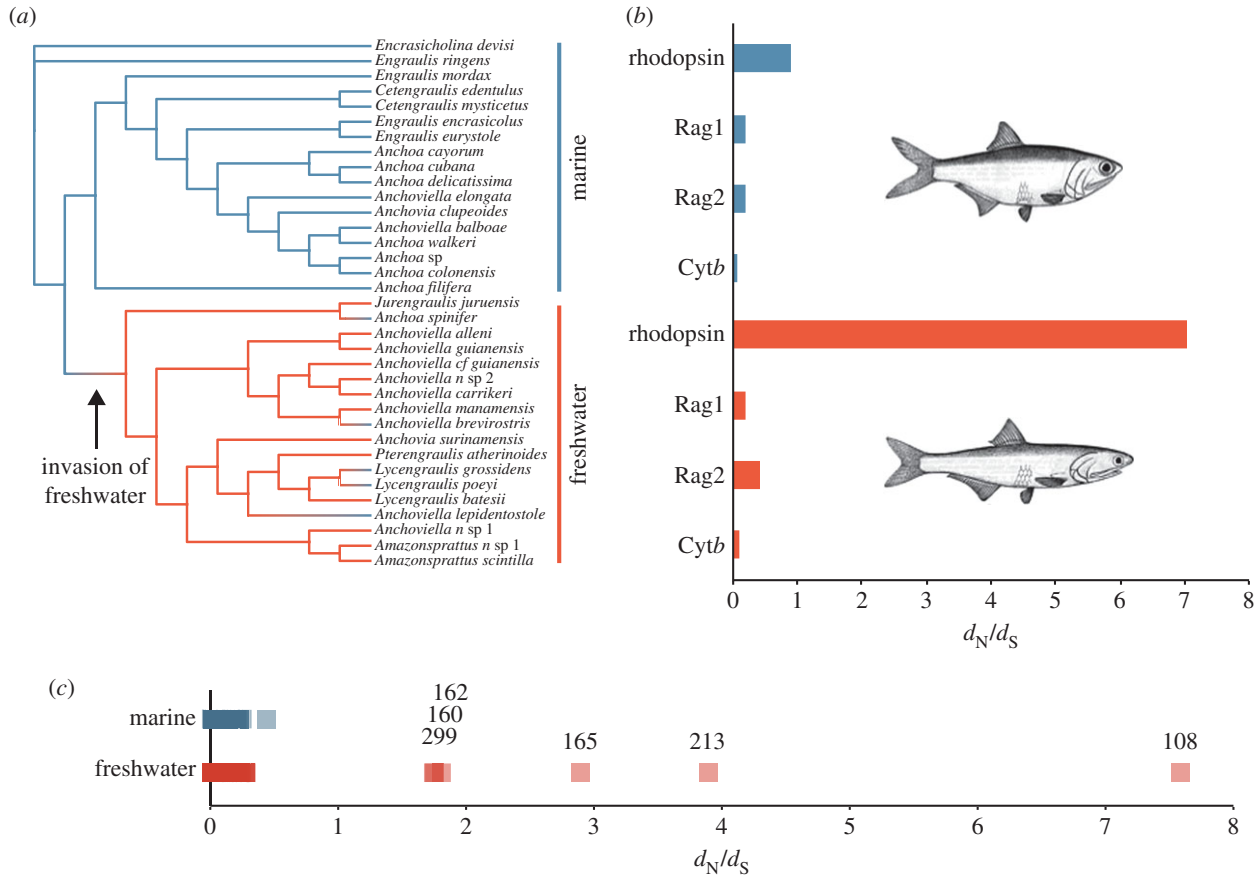

Figure 1. Phylogeny and molecular evolution in New World anchovies. (*a*) Phylogenetic relationships among marine (blue) and freshwater (red) species of the subfamily Engraulinae [5]. Colours on branches show optimization of habitat type; partitions used for clade model analyses indicated by vertical bars. (*b*) d_N/d_S estimates for the divergent site class in marine and freshwater partitions from clade model analyses implemented in PAML. (*c*) Sites with a greater than 0.75 posterior probability for inclusion in the positively selected site class as inferred by FUBAR. Fish images after [13]. (Online version in colour.)

species distributed along the coasts of North, Central and South America, as well as freshwater species in the Amazon, Orinoco and other large Neotropical rivers. Recent phylogenetic analyses revealed that the South America river anchovies are the product of a single freshwater invasion by a marine ancestor [7,8]. Subsequent radiation throughout the basins of South America produced a profusion of morphologically and ecologically distinct species not seen in marine habitats, such as the miniaturized *Amazonsprattus scintilla* and the piscivorous *Lycengraulis batesii*. A few freshwater anchovy lineages even reinvaded marine habitats [7].

In this study, we use the striking marine to freshwater habitat transition as a unique natural experiment to study the effects of different light environments on rhodopsin gene evolution. At the shallow depths occupied by the majority of marine anchovies, the spectral attenuation of light is negligible [3]. By contrast, at similar depths in South American rivers, the amount of available light is substantially decreased and richer in longer wavelengths. In addition, the degree of spectral attenuation can vary among South American rivers, broadly classified as white water, black water or clear water based on their optical qualities [9]. Given the contrast in spectra of downwelling light between marine and freshwater habitats, as well as the diversity of visual environments in South American rivers, we predicted that the freshwater-invading anchovy lineage would show evidence of positive Darwinian selection in the rhodopsin gene. To test this hypothesis, we sequenced rhodopsin from New World anchovies and used codon-based models of molecular evolution to compare the strength of selection acting on freshwater invaders versus their marine relatives.

2. Material and methods

The rhodopsin gene was amplified and sequenced from genomic DNA extracted from 35 species spanning the taxonomic diversity of freshwater and marine New World anchovies. We used codon-based models of molecular evolution to estimate the ratio of non-synonymous to synonymous substitutions (d_N/d_S) [10]. We used several methods of likelihood-based comparative sequence analysis to estimate d_N/d_S rates across sites and lineages, including models implemented in PAML and HyPhy [11,12]. The non-vision-related recombination-activating gene 1 and 2 (Rag1, Rag2), and cytochrome *b* (Cyt*b*) were analysed for the same set of species for comparison. Additional details are provided in the electronic supplementary material.

3. Results

A d_N/d_S value greater than one (positive selection) is indicative of evolutionarily advantageous amino acid substitutions and is often observed in proteins undergoing adaptive functional change [10]. We first implemented models in PAML that allow variation in d_N/d_S across sites but assume a uniform distribution across the entire phylogeny. These analyses identified a subset of sites under positive selection in the rhodopsin gene. We then defined 'marine' and 'freshwater' partitions using the phylogeny in figure 1*a* (note that

Table 1. Clade model C (PAML) analyses of rhodopsin and non-vision-related genes. (lnL, ln likelihood; ω, d_N/d_S for site class and percentage of sites in site class; m/f, marine partition (background)/freshwater partition including marine reinvading species (foreground); asterisk denotes significantly different marine versus freshwater d_N/d_S values (*$p < 0.01$); d.f., degrees of freedom.)

		parameters				
model	lnL	ω_0	ω_1	ω_2	null	p (d.f.)
rhodopsin						
M2a_REL	−4099.1	0.03 (91.6%)	1.00 (7.4%)	2.82 (1.0%)		
constrained CmC (m/f)	−4099.7	0.03 (91.7%)	1.00 (6.9%)	3.38/1.00 (1.4%)		
CmC (m/f)	−4095.6	0.03 (91.6%)	1.00 (7.7%)	0.89/7.04* (0.7%)	M2a_REL	0.008 (1)
					constrained CmC	0.004 (1)
Rag1						
M2a_REL	−6444.0	0.01 (85.2%)	1.00 (0.6%)	0.19 (14.2%)		
CmC (m/f)	−6444.0	0.01 (85.2%)	1.00 (0.6%)	0.19/0.19 (14.2%)	M2a_REL	0.972 (1)
Rag2						
M2a_REL	−3505.2	0.02 (89.2%)	1.00 (0.5%)	0.31 (10.3%)		
CmC (m/f)	−3504.0	0.02 (89.9%)	1.00 (0.7%)	0.21/0.41 (9.4%)	M2a_REL	0.112 (1)
Cyt*b*						
M2a_REL	−11291.8	0.00 (94.4%)	1.00 (0.0%)	0.06 (5.4%)		
CmC (m/f)	−11290.0	0.00 (94.4%)	1.00 (0.0%)	0.07/0.11 (5.6%)	M2a_REL	0.065 (1)

Table 2. Random sites (PAML) analyses of rhodopsin. (lnL, ln likelihood; p and q, parameters of beta distribution of site classes in models M8a and M8; ω, d_N/d_S for site class and percentage of sites in site class; asterisk denotes significantly different marine versus freshwater d_N/d_S values (*$p < 0.01$); d.f., degrees of freedom.)

		parameters				
model	lnL	p	q	ω	null	p (d.f.)
freshwater						
M8a	−2682.2	0.07	0.64	1.00 (3.9%)		
M8	−2677.0	0.09	1.07	5.23* (0.8%)	M8a	0.001 (1)
marine						
M8a	−2329.9	0.21	9.80	1.00 (7.3%)		
M8	−2329.3	0.10	2.79	1.42 (5.3%)	M8a	0.258 (1)

the freshwater partition includes five secondarily marine species), and implemented clade models that allow for a class of sites with d_N/d_S values that differ between defined partitions [14]. Incorporation of model parameters that allow d_N/d_S to differ between marine and freshwater partitions provided a statistically better fit to the data than simpler models with uniform d_N/d_S values across the tree (table 1). These clade models indicate significant positive selection ($d_N/d_S = 7.0$) in the freshwater partition but provide no evidence for positive selection in the marine partition. Transferring the five secondarily marine species from the freshwater to the marine partition results in an even higher freshwater value ($d_N/d_S = 9.5$) and improves support for the model allowing variation between partitions (electronic supplementary material, table S1). By contrast, analyses of non-vision-related genes provided no evidence for positive selection, nor any significant differences in d_N/d_S between freshwater and marine partitions (figure 1*b* and table 1; electronic supplementary material, table S1).

To confirm our clade model results, we also examined selection pressures acting on the rhodopsin gene by using PAML to implement random sites models to estimate d_N/d_S [11]. For these analyses, marine and freshwater partitions were considered as separate datasets. As with the clade model analyses, we found clear evidence for positive selection occurring in the freshwater anchovy dataset (table 2), with positively selected sites identified at positions in the rhodopsin gene previously implicated in tuning spectral sensitivity to longer wavelength light (figure 1*c* and electronic supplementary material, tables S3 and S4) [2]. By contrast, random sites analysis did not provide evidence for positive selection in the marine partition (table 2), nor for positive selection in either the freshwater or marine partitions for any of the non-vision-related genes (electronic

supplementary material, table S2). To ensure that these results were not owing to artefacts in d_S estimation, we also conducted analyses using HyPhy that allow for independent estimation of d_N and d_S [12] (figure 1*c* and electronic supplementary material, tables S3 and S4).

4. Discussion

Positive selection has occurred in the rhodopsin gene of freshwater anchovies, but not their marine relatives. This result is consistent with our hypothesis that the invasion of the much dimmer and red-shifted freshwater rivers of South America is accompanied by visual adaptation in the dim-light sensitive visual pigment rhodopsin. The relatively low d_N/d_S observed in marine anchovies may be owing to the negligible attenuation of light at depths inhabited by the majority of anchovy lineages [15], or because the peak absorbance of rhodopsin is already tuned to the ideal wavelength of light for marine environments. The lack of evidence for positive selection in the three non-vision-related genes indicates that the increased d_N/d_S found for rhodopsin in freshwater is not owing to differences in population structure or genome-wide shifts in evolutionary rates.

South American rivers are also more spectrally diverse than marine ecosystems [9]. Lineages distributed across highly stratified or disparate freshwater light environments may have increased d_N/d_S values owing to divergent selection pressures acting on the visual systems of species occupying spectrally distinct habitats [16]. In freshwater anchovies, different adaptations may be required for optimal visual performance in the highly turbid Amazon, the clear waters of the Rio Tapajos or the tannin stained black waters of the Rio Negro. Also, the ecological diversification of freshwater anchovies (for example, into actively hunting piscivores) has probably altered visual demands and correspondingly shifted patterns of natural selection in rhodopsin [7].

Amino acid substitutions within the chromophore binding pocket of rhodopsin can change the wavelength of its maximal absorbance [1]. Of the positively selected amino acid sites identified in this study, sites 124 and 299 are spectral tuning sites in rhodopsin [2], and site 108 is a spectral tuning site in the homologous cone opsin SWS2 [17]. Sites 165 and 213 have been identified as positively selected in recent studies of fishes inhabiting environments of varying turbidity [16,18]. Aspects of rhodopsin function other than spectral tuning have not been as well investigated but are also likely to be adaptive under different light conditions. For example, the thermal stability of both the dark- and light-activated state of rhodopsin may be important for visual perception in dim-light environments, where the signal-to-noise ratio is very low [19]. In addition, potential site reversions to marine states in secondarily marine lineages (for example, site 96 in *Anchoa spinifer*) are attractive candidates for mutagenesis studies.

Several other groups of marine fishes (including stingrays, pufferfishes, needlefishes and flatfishes) have independently invaded the optically diverse freshwaters of South America. Our results suggest that these taxa represent a superb natural experiment for future studies of habitat-driven molecular adaptation.

Ethics. All work on fishes was approved by the Research Oversight and Compliance Office at the University of Toronto.

Data accessibility. DNA sequences: Genbank accession nos. KT201093-KT201146.

Authors' contributions. A.V.N. collected the rhodopsin sequence dataset, analysed data, participated in study design and drafted the manuscript; D.B. collected field data and participated in manuscript preparation; B.S.W.C. and N.R.L. conceived of the study, coordinated the study and helped write the manuscript.

Competing interests. We have no competing interests.

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References

1. Bowmaker J, Hunt D. 2006 Evolution of vertebrate visual pigments. *Curr. Biol.* **16**, R484–R489. (doi:10.1016/j.cub.2006.06.016)
2. Hunt DM, Dulai KS, Partridge JC, Cottrill P, Bowmaker JK. 2001 The molecular basis for spectral tuning of rod visual pigments in deep-sea fish. *J. Exp. Biol.* **204**, 3333–3344.
3. Lythgoe J. 1979 *The ecology of vision*. Oxford, UK: Clarendon Press.
4. Lovejoy NR, Bermingham E, Martin AP. 1998 Marine incursion into South America. *Nature* **396**, 421–422. (doi:10.1038/24757)
5. Lovejoy NR, Albert J, Crampton W. 2006 Miocene marine incursions and marine/freshwater transitions: evidence from Neotropical fishes. *J. S. Am. Earth Sci.* **21**, 5–13. (doi:10.1016/j.jsames.2005.07.009)
6. Bloom DD, Lovejoy NR. 2011 The biogeography of marine incursions in South America. In *Historical biogeography of neotropical freshwater fishes* (eds JS Albert, RE Reis), pp. 137–144. Berkeley, CA: University of California Press.
7. Bloom DD, Lovejoy NR. 2012 Molecular phylogenetics reveals a pattern of biome conservatism in New World anchovies (family Engraulidae). *J. Evol. Biol.* **25**, 701–715. (doi:10.1111/j.1420-9101.2012.02464.x)
8. Bloom DD, Lovejoy NR. 2014 The evolutionary origins of diadromy inferred from a time-calibrated phylogeny for Clupeiformes (herring and allies). *Proc. R. Soc. B* **281**, 20132081. (doi:10.1098/rspb.2013.2081)
9. Costa M, Novo E, Telmer K. 2012 Spatial and temporal variability of light attenuation in large rivers of the Amazon. *Hydrobiologia* **702**, 171–190. (doi:10.1007/s10750-012-1319-2)
10. Anisimova M, Kosiol C. 2008 Investigating protein-coding sequence evolution with probabilistic codon substitution models. *Mol. Biol. Evol.* **26**, 255–271. (doi:10.1093/molbev/msn232)
11. Yang Z. 2007 PAML 4: Phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**, 1586–1591. (doi:10.1093/molbev/msm088)
12. Murrell B, Moola S, Mabona A, Weighill T, Sheward D, Kosakovsky Pond SL, Scheffler K. 2013 FUBAR: a fast, unconstrained Bayesian approximation for inferring selection. *Mol. Biol. Evol.* **30**, 1196–1205. (doi:10.1093/molbev/mst030)
13. Whitehead PJP, Nelson GJ, Wongratana T. 1988 *FAO species catalogue, vol. 7. Clupeoid fishes of the World*. Rome: FAO.
14. Bielawski J, Yang Z. 2004 A maximum likelihood method for detecting functional divergence at individual codon sites, with application to gene family evolution. *Mol. Biol. Evol.* **59**, 121–132. (doi:10.1007/s00239-004-2597-8)

15. Warrant E, Johnsen S. 2013 Vision and the light environment. *Curr. Biol.* **23**, R990–R994. (doi:10.1016/j.cub.2013.10.019)
16. Schott R, Refvik S, Hauser F, López-Fernández H, Chang BSW. 2014 Divergent positive selection in rhodopsin from lake and riverine cichlid fishes. *Mol. Biol. Evol.* **31**, 1149–1165. (doi:10.1093/molbev/msu064)
17. Chinen A, Matsumoto Y, Kawamura S. 2004 Spectral differentiation of blue opsins between phylogenetically close but ecologically distant goldfish and zebrafish. *J. Biol. Chem.* **280**, 9460–9466. (doi:10.1074/jbc.m413001200)
18. Larmuseau M, Vanhove M, Huyse T, Volckaert FA, Decorte R. 2011 Signature of selection on the rhodopsin gene in the marine radiation of American seven-spined gobies (Gobiidae, Gobiosomatini). *J. Evol. Biol.* **24**, 1618–1625. (doi:10.1111/j.1420-9101.2011.02290.x)
19. Liu J, Liu M, Nguyen J, Bhagat A, Mooney V, Yan E. 2011 Thermal properties of rhodopsin: insight into molecular mechanism of dim-light vision. *J. Biol. Chem.* **286**, 27 622–27 629. (doi:10.1074/jbc.M111.233312)