

Study tracks the changes in a vision protein as fish evolved

Genetic engineering, statistics, and spectroscopy are combined to address a fundamental question in biology.

How does Darwinian evolution work on the molecular scale? That is, how do genetic mutations, which occur in single molecules of DNA, lead to changes that help an organism of 10²³ or so molecules adapt to its environment?

The answer might seem straightforward. Because DNA encodes proteins, evolution presumably entails proteins becoming better at their jobs and, through improved performance, bestowing an advantage on a plant or animal as it struggles to survive.

Thanks to gene sequences and statistical algorithms, one can often infer the mutations a protein has undergone through the ages. And thanks to genetic engineering, one can coax certain cells into making a protein of one's choice, even a protein from a long-extinct species.

Measuring a protein's evolving job performance is harder. Proteins fulfill their roles by binding to other molecules. In most cases the consequences of that binding are hard to observe. But, as Emory University's Shozo Yokoyama realized, there is at least one class of protein, opsins, whose function has a clear, quantifiable signature.

Opsins help fish and other animals see in dim light. The molecule ultimately responsible for vision is retinal, a light-sensitive derivative of vitamin A. Isolated retinal absorbs in the UV, but when it's swaddled by a much larger opsin, retinal's peak absorption wavelength, $\lambda_{\max}$, shifts redward.

Depending on its amino acid sequence, an opsin can shift $\lambda_{\max}$ from the UV all the way through the colors of the rainbow to the IR. Fish exploit opsin tuning ability to evade predators during twilight, a particularly dangerous time of day.

In shallow, clear water, the twilight spectrum peaks broadly between 400 nm (violet) and 500 nm (green). In deep water, the spectrum narrows around a peak at 480 nm (blue). And in shallow, muddy

water, the spectrum shifts redward.

As you might expect, fish that swim today in those diverse habitats have opsins that engender an appropriate, survival-enhancing shift. Do those shifts constitute proof that Darwinian evolution works on the molecular scale? Not quite.

What's missing is evidence that opsins have repeatedly changed over the ages as fish spread into new environmental niches and diversified. To look for that evidence, Yokoyama and his collaborators undertook a seven-year project. They inferred the gene sequences of a family tree of fish opsins, re-created those opsins, and measured the $\lambda_{\max}$ values in the lab.¹

The results, which have just been

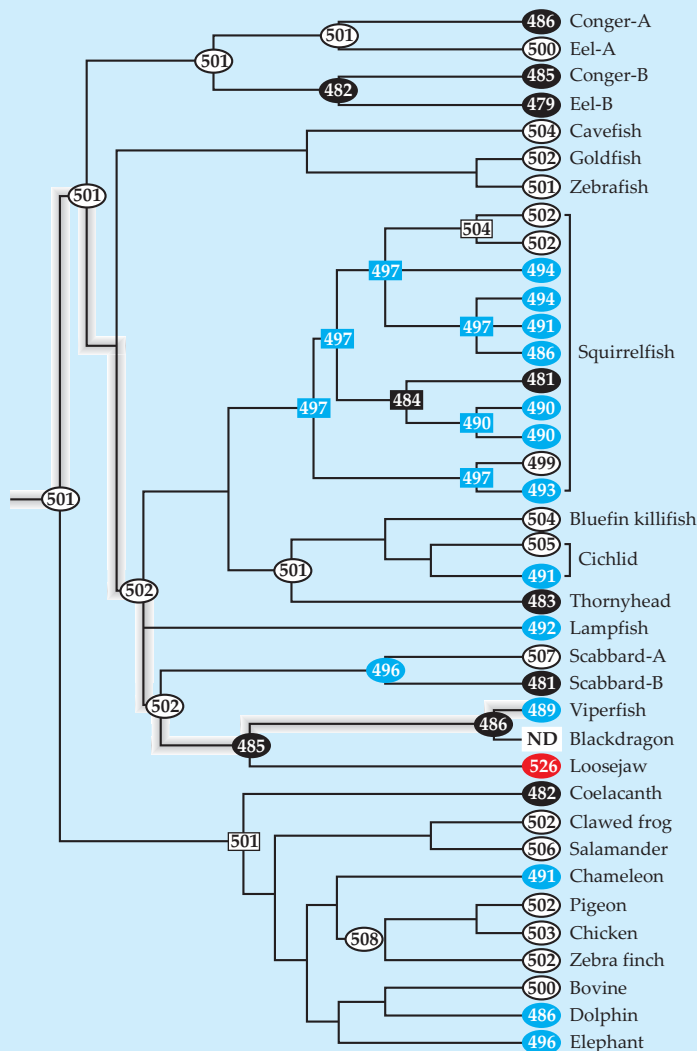
published, provide strong circumstantial evidence of molecular evolution. They also reveal the limitations of using statistical techniques alone to identify adaptive mutations in proteins.

Family ties

Yokoyama chose to focus on eight species: two eels that migrate from deep water to shallow water (Japanese eel, Japanese conger), five deep-sea fish (Pacific blackdragon, Northern lampfish, shining loosejaw, scabbardfish, viperfish), and one fish that lives in shallow freshwater (bluefin killifish).

The eight fish have opsins that suit their diverse habitats. Indeed, the Japanese eel has *two* opsins: one optimized for the shallow water where it

Figure 1. This family tree shows how 38 vertebrate opsins are descended from their common ancestor, an opsin from a prehistoric fish. Each node corresponds to an extant or extinct opsin, whose wavelength of peak absorption, $\lambda_{\max}$, is given in nanometers. For the fish, the colors indicate, based on $\lambda_{\max}$, the animal's known or presumed habitat: white for shallow water or land, blue for deep water, and black for intermediate depths. The shining loosejaw's opsin, indicated in red, is redshifted to sense IR. Having made and measured the opsins in the lab, Emory University's Shozo Yokoyama and his team could infer a fish's evolutionary path. The ancestors of the viperfish, for example, have occupied progressively deeper water. Ovals denote values measured directly in the lab; rectangles denote indirectly measured values. (Adapted from ref. 1.)



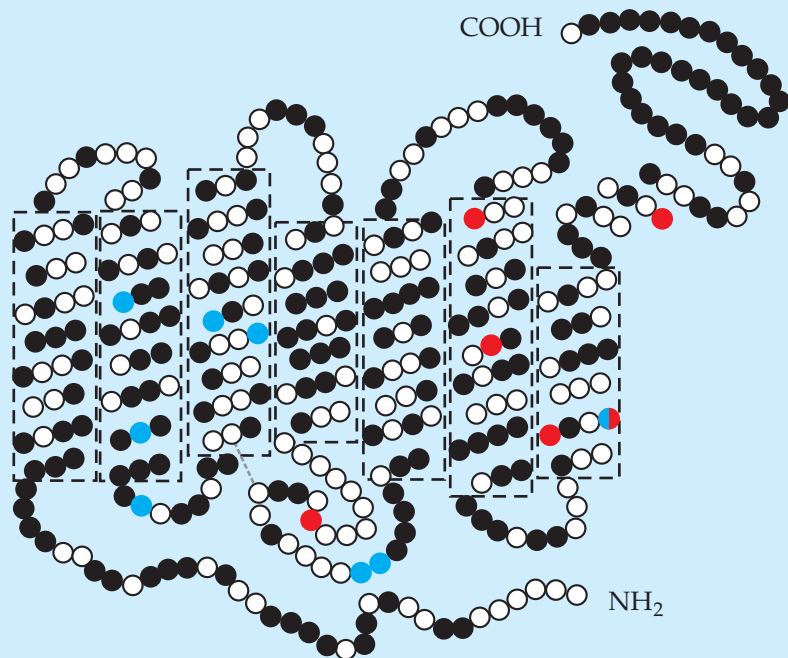


Figure 2. Structurally, an opsin consists of seven transmembrane helices, which wrap around a retinal molecule, and two floppy tails. Each circle represents one of the 354 amino acid sites in bovine opsin, a conventional benchmark. In their study of 38 opsins, Yokoyama and his team found that substitutions had occurred at a total of 203 sites. How those substitutions changed peak absorption is indicated by the colors: blue for decreasing $\lambda_{\max}$, red for increasing $\lambda_{\max}$, and black for keeping $\lambda_{\max}$ the same. No substitutions were found at the white sites. (Adapted from ref. 1.)

lives, another for deep water where it spawns. The shining loosejaw glows luminescently in the IR. Its opsin is tuned to the IR, presumably to see the prey it illuminates, find its fellow shining loosejaws, or both.

To trace how those opsins might have evolved, Yokoyama first applied a statistical technique called phylogenetic reconstruction. As input he used the genetic sequences of 38 opsins drawn from the eight species, supplemented by those of other fish and vertebrates.

The result is the family tree of opsins in figure 1. Its trunk springs from the ancestor of today's vertebrates, an animal that likely swam in fresh, shallow water more than 400 million years ago.

Each node of the tree, downstream of the trunk and upstream of the present-day vertebrates, represents the opsin of an extinct species. Applying the techniques of genetic engineering, the Emory team re-created those ancestral opsins in the lab and equipped them with retinal molecules. Then they measured $\lambda_{\max}$.

The combination of spectral meas-

urements and genetic sequences revealed which DNA mutations—that is, amino acid substitutions—were responsible for the shift in $\lambda_{\max}$.

Vertebrate opsins, extinct and extant, differ slightly in the total number of amino acids. Those differences in length appear at the protein's two tails. Mutations aside, the protein's functional core has remained more or less constant. It consists of seven helices that form a tube to hold the retinal.

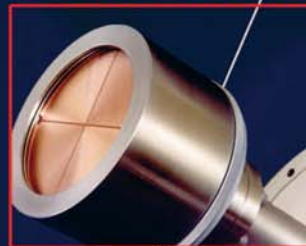
A flattened representation of bovine opsin appears in figure 2. Black, red, and blue dots indicate amino acid sites where the Emory team found substitutions in its opsin family tree. Substitutions at blue sites decreased $\lambda_{\max}$; substitutions at red sites increased $\lambda_{\max}$; substitutions at black sites left $\lambda_{\max}$ unchanged.

Bovine opsin has 354 amino acids. Substitutions at just 12 sites, 3%, could account for all the variation in $\lambda_{\max}$ that appeared in the opsin family tree.

Which substitutions changed $\lambda_{\max}$ proved surprising. In previous work, Yokoyama had found that substituting alanine with serine at the 292nd site

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(A292S) usually decreases $\lambda_{\max}$ by 10 nm. That substitution was acquired by one of the conger eel opsins from its immediate ancestor. Making the reverse substitution (S292A) in the conger eel opsin increased $\lambda_{\max}$ by 10 nm, as you'd expect.

Here's the surprise. When A292S was applied to the immediate ancestor, it had no effect on $\lambda_{\max}$! Evidently, two other substitutions found by the Emory team were responsible for the decrease, not A292S. Other cases occurred in which quite different substitutions could shift $\lambda_{\max}$ by more or less the same amount.

Opsins are important not only for vision; they are also candidates for light-switched, high-density data storage. When physicists and chemists calculate $\lambda_{\max}$, they typically focus on amino acids that lie within 4.5 Å of retinal. However, the substitutions that blueshifted the conger opsin's $\lambda_{\max}$ occurred at sites 20 Å away from retinal. Moreover, as figure 2 shows, some sites that shifted $\lambda_{\max}$ don't even belong to opsin's functional core.

A bigger surprise occurred when Yokoyama ran the opsins' genetic sequences through programs designed to identify naturally selected mutations. Three DNA bases suffice to encode a particular amino acid, but the coding has redundancies. For example, GCT, GCC,

GCA, and GCG all encode alanine. Changing the third base in any of those four codons would still yield alanine.

Changing the first base from guanine (G) to cytosine (C) would yield a different amino acid, proline. Usually, such "nonsynonymous" substitutions impair a protein's ability to fold or function. They aren't passed on to the next generation.

But some nonsynonymous substitutions turn out to be beneficial. As DNA mutates randomly from generation to generation and from species to species, more synonymous substitutions than nonsynonymous substitutions accumulate. Any nonsynonymous substitutions that get passed on are likely to be evolutionarily significant.

Statistical algorithms implement those expectations. They digest a family tree of genetic sequences and, by comparing rates of synonymous and nonsynonymous mutations, identify putative mutations that constitute evolutionary adaptations.

Yokoyama had determined which mutations really do change $\lambda_{\max}$. Could the statistical methods find them too? The answer turned out to be no. Why that should be the case isn't clear. Ziheng Yang of University College London has developed some of the statistical methods used by evolutionary

biologists. Those methods, he points out, may lack the sensitivity to detect adaptive mutations that become fixed within a short period of time, as may be the case in vertebrate opsins.

Gavin Naylor of Florida State University in Tallahassee points out another possible limitation: Statistical methods don't take into account the many-body nature of proteins. "Shozo shows quite compellingly that just because you change one site doesn't mean you're going to get a functional change," he says. "It depends, rather, on the context of the other amino acids."

Showing conclusively how the survival of the fittest plays out on the molecular level would require reconstructing not only the protein but also the whole organism. And you'd have to observe the organism and its descendants in their original, long-lost habitats.

Still, the Emory researchers did find a piece of reassuring evidence: The opsin of the vertebrates' piscine ancestor tuned retinal to absorb at 501 nm. That $\lambda_{\max}$ is consistent with the shallow-water habitat of its fossilized remains.

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Reference

1. S. Yokoyama, T. Tada, H. Zhang, L. Britt, *Proc. Natl. Acad. Sci. USA* **105**, 13480 (2008).

Experiment probes pattern formation during debonding of viscoelastic adhesives

In at least one respect, adhesives always behave as either viscous liquids or elastic solids, and the boundary between the two regimes is sharp.

Poke your finger into a jar of honey, and you feel a drag force that depends on how fast you're moving your finger. Remove it, and some honey clings to your finger and stretches into a long fibril that eventually breaks. Those properties are characteristic of a viscous liquid. When you push against a piece of soft rubber, on the other hand, the force you feel is proportional not to speed but to displacement, and when you pull away, the rubber remains in one piece; such are the properties of an elastic solid. Typical commercial adhesives have both viscous and elastic properties—that is, they are viscoelastic.

When a film of viscous, elastic, or viscoelastic material is sandwiched between two smooth, rigid surfaces that are then pulled apart, patterns form that include "fingers" of air penetrating the gap between the surfaces. Fingering patterns in the viscous and elastic cases have both been studied before. But pat-

tern formation in the viscoelastic regime, which is most relevant to adhesive failure and thus to adhesive performance, has remained unexplored. Now, Julia Nase, Anke Lindner, and Costantino Creton of the École Supérieure de Physique et de Chimie Industrielles in Paris have taken a first look at fingering patterns in adhesive materials with a range of properties, spanning the continuum between viscous liquid and elastic solid.¹

The researchers' first task was to find a good model material whose viscous and elastic properties they could tune reproducibly. They settled on a commercially available kit consisting of a polymer fluid and a curing agent that forms cross-links among the polymers. Typically, the fluid and curing agent are mixed in a 10:1 ratio to form a clear elastic solid that can be used for microfluidics devices. But the researchers found that smaller amounts of curing agent

produced materials with the desired range of viscoelastic properties. A mixture with 2–3% curing agent forms a softer solid, similar to the protective film that comes on a new computer monitor. A mixture with 1% curing agent forms a material at its gel point: the minimum density of cross-links needed to connect all the polymers in an infinite network. That material feels sticky to the touch and forms visible fibrils, like the adhesive on a piece of packing tape. (For more on polymers, viscoelasticity, and stickiness, see the PHYSICS TODAY articles by Tom McLeish, August 2008, page 40, and by Cyprien Gay and Ludwik Leibler, November 1999, page 48.)

To study the fingering patterns, the researchers coated a glass slide with a layer of the polymer adhesive between 50 μm and 500 μm thick. They brought a 6-mm-diameter steel probe in contact with the material from below and