PNAS INNER WORKINGS

Noncoding RNAs have a key role in butterfly speciation. What about other flora and fauna?

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Recent research suggests that the famous case study of peppered moth evolution has a new twist: their intriguing coloration changes are driven by RNA molecules that don't code for proteins. Just how common such cases are remains the subject of debate. Image credit: Shutterstock/IanRedding.

In 1848, naturalist R. S. Edleston stumbled upon an unusual-looking peppered moth (*Biston betularia*) in a Manchester, England, suburb. Rather than the usual speckled black-and-white, this one was an inky shade of black. By 1895, nearly 98% of Manchester's peppered moths had gone to the dark side.

At first blush, the story of the peppered moth seems straightforward. When England's thriving industries spewed soot and darkened tree bark, light-colored peppered moths became easy prey, while their dark-colored cousins thrived. It was a textbook example of evolution in action, an adaptation based on mutations in protein-coding genes.

But that conclusion drew controversy, including from proponents of intelligent design, a theory of evolution promoted by religious groups opposed to Darwinian evolution (1). Years later, multiple findings validated the mechanism of adaptation, notably in a 2016 study by a team at the University of Liverpool. The group identified the likely cause of this quick adaptation—insertion of a mutation in a protein-coding gene called *córtex*, which may have led to the moths' color switch (2).

But biology is rarely as simple as it seems—and, as it turns out, there's more to this intriguing case study of evolution. Recent research suggests that this coloration is driven by RNA molecules that don't code for proteins.

And this isn't the only example of the phenomenon. While many noncoding RNAs are known to have regulatory functions, a few striking studies have shown that noncoding RNAs can orchestrate moth and butterfly wing coloration (3–5). Examples also exist in the plant kingdom, governing dramatic shifts in floral pigmentation (6)

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Only in recent years have researchers discovered that noncoding RNAs have a big role in the evolution of the oft-studied plant model system, monkeyflowers. Image credit: Science Source/Stuart Wilson.

and regulating the timing of flowering (7). "The RNAs that don't even produce proteins are doing really cool stuff to create diversity in nature," says Shen Tian, a molecular biologist and postdoctoral fellow at Duke University, in Durham, North Carolina. "And that's just exciting."

But whether these RNAs influence evolution beyond a handful of examples is anything but clear-cut. Carrying out experiments to demonstrate that noncoding RNA is driving some facet of evolution in a complex organism is challenging because most of these RNAs aren't identical across species, and standardizing the tools to map and study them has been tricky. And, while some researchers suspect that noncoding RNAs may even be shaping human cognitive abilities (see Sidebar), others are not convinced, since these noncoding RNAs often appear in specific brain areas and aren't conserved across different species, which makes them hard to compare.

A Different Concept of RNA

Once viewed primarily as an intermediary between DNA and proteins, RNA's role started to appear much more nuanced since the late 20th century. Scientists began to realize that noncoding RNAs, first identified in the 1980s (8), were more than genome junk. The 2006 Nobel Prize in Physiology or Medicine was awarded for the 1998 identification of crucial noncoding small interfering RNA in *Caenorhabditis elegans*, silencing genes involved in muscle function (9). The finding brought a spotlight to noncoding RNAs and the other roles the molecules might have. Researchers have identified numerous microRNAs and small interfering RNAs, typically 20–22 nucleotides long, and long noncoding RNAs (lncRNAs) that are 200 nucleotides or longer.

Then, in 2008, researchers scanning for noncoding RNAs across species noticed an intriguing pattern: complex animals such as vertebrates contained higher numbers of families of microRNAs (10), compared with simple animals like sponges and jellyfish. They suspected that noncoding RNAs might be linked to body complexity among vertebrates.

Then, in 2023, a group of researchers from the University of Connecticut studying a common plant model system—monkeyflowers—noticed that noncoding RNAs played a role in their color evolution. monkeyflowers come in various shapes and colors, each attracting different pollinators. A genetic study revealed that around 5 million years ago, monkeyflowers lost their typical yellow pigmentation and developed pink petals (6). Later, the plants regained yellow pigments. But scientists were clueless as to how the plants made that color switch.

Back in 1999, botanists had identified a chunk of a chromosome that controlled yellow pigmentation in these species (11). Multiple cross-breeding changed not only the colors of the flowers, but also the type of pollinator. Altered pink Lewis' monkeyflowers—once almost exclusively pollinated by bumblebees—now attracted 68 times more hummingbirds. Meanwhile, altered scarlet monkeyflowers, which had only attracted hummingbirds, were drawing in nearly 74 times more bumblebees.

The botanists who did that work 25 years ago "didn't know what they were changing," says molecular biologist Yaowu Yuan at the University of Connecticut, who was part of the 2023 work but not the 1999 work. While the researchers may have assumed the change was the result of a protein-coding gene, they couldn't find the gene, as the monkeyflower genome hadn't been fully mapped at the time.

For decades, the lack of technological advances held researchers back from uncovering the molecular mechanisms behind this color switch. For the 2023 work, Yuan and his team used advanced small RNA sequencing to finally zoom in on this supposed color-coding segment in monkeyflower species, called the YUP segment (6). They learned that there were no protein-coding genes linked to the pigmentation. Instead, they found that the locus produced small interfering RNAs. In high amounts, these noncoding RNAs suppressed the production of carotenoids, which caused the color change and affected pollinator preferences.

> **"These findings are not that surprising because evolution tinkers with what is available."**
>
> **—Alexander Palazzo**

A Hidden Switch

Within a year of Yuan's monkeyflower discovery, three independent teams reported a role for noncoding RNAs in butterflies. The findings have implications for the infamous peppered moth. Tian learned that microRNAs near the *cortex* locus, the gene segment credited with peppered moth coloration, influenced wing color in *Bicyclus anynana*, a small African brown butterfly (3).

Scientists had found microRNAs in some moths and butterflies in the early 2010s (12). However, their function remained unknown. Tian extracted wing tissue from *B. anynana* at different developmental stages and sequenced all the microRNAs that he could find. Near the *cortex* locus, he identified two microRNAs, along with a long noncoding RNA.

Knocking out several protein-coding genes in the *cortex* locus didn't change the coloration of the butterfly's wing pattern. But when Tian knocked out the two microRNAs, he produced a light-colored butterfly from a dark-colored one.

Tian and his coworkers learned that in nonmutant butterflies, the microRNA degraded an enzyme that produces light-colored pigment precursors, leading to darker pigmentation. But in the mutant butterflies with microRNAs knocked out, the enzyme was overexpressed, resulting in the lighter wing coloration. The microRNAs are derived from a long noncoding RNA, which overlaps with the *cortex* locus. Two other research groups working at the same time as Tian further revealed this RNA's role in wing coloration (4, 5).

Working with the buckeye butterfly, *Junonia coenia*, biologist Richard Fandino, then a postdoctoral researcher at Cornell University in Ithaca, New York, and colleagues learned that it was this long noncoding RNA, not the *cortex* gene, that is responsible for giving out the insect's color patterns. When researchers deleted specific regulatory elements within the noncoding RNA, the deletion created new color variations, changing the appearance of eye-like markings on the wing of the butterfly (5).

Independently, developmental biologist Luca Livraghi of George Washington University in Washington, DC, and coworkers, using gene editing, deleted parts of the long noncoding RNA and learned that the molecule determined which wing scale would become dark or light (4). They confirmed its function in five different butterfly species. With all these new findings, it looks as if "we're seeing this kind of genetic 'dark matter' come to light," Fandino says.

For Antónia Monteiro, evolutionary biologist at the National University of Singapore and a co-author of Tian's microRNA study, these parallel discoveries involving noncoding RNAs near *cortex* finally explain the peppered moth's story. "What might have happened is that a mutation might have changed the expression of the long noncoding RNA, and thereby leading to the expression of microRNA," she explains. And that then affected the moths' wing color.

While a lot of noncoding RNAs are unique, some microRNAs are also "super-conserved" across species. In fact, Monteiro says humans have the exact same microRNA sequence as the one her team discovered in African brown butterflies. "It's possible that this microRNA is also quite important in regulating melanism in our skin, our hair, and anything that has melanic bits in our bodies," she adds. "But nobody has tested it yet."

Ilik Saccheri, a co-author of the study that attributed the moths' coloration changes to *cortex*, now agrees that noncoding RNAs are the likely orchestrators. The peppered moth has a one-year reproductive cycle, making it less than ideal for experiments, says Saccheri, who's an ecological geneticist at the University of Liverpool in the United Kingdom. "To be honest, one of my team members noticed those microRNAs a long time ago, but at the time, we didn't know about microRNAs or what they could do," he says. Saccheri says that the examples scientists have found are generalizable within the order of insects that includes butterflies and moths. "In other taxons, it's difficult to say," he adds. "Probably less so."

Yuan, the monkeyflower expert, says that it's challenging to link noncoding RNAs to specific evolutionary adaptations in complex organisms. That may be why insights took so long to emerge. Compared to protein-coding genes, their effects may be indirect. Still, the examples that do exist have added new nuances to the molecular understanding of evolution. "I've been teaching evolution for so long, I thought I knew evolution really well," he says. "But I am just constantly surprised by what we find."

However, Alexander Palazzo, molecular biologist at the University of Toronto in Canada, who has done extensive work on production and transportation of protein-coding RNAs within cells, says that scientists should be careful not to overgeneralize these findings. Not every genetic change leads to an effect on observable, phenotypic traits, and overinterpreting data can misrepresent how much of the genome matters. He suggests that while the work in monkeyflowers and butterflies is impressive, these are probably rare instances of noncoding RNA directly controlling a species' fitness; these findings may not carry over to other species.

"These findings are not that surprising because evolution tinkers with what is available," Palazzo says. He adds that

scientists know, based on research like the 2006 Nobel Prize-winning studies, that noncoding RNAs can play a crucial role in regulating gene expression. If a particular microRNA controls a subset of genes and those regulatory changes prove beneficial, it makes sense that the noncoding RNAs would become substrates for evolution.

John Rinn, a molecular biologist at the University of Colorado Boulder, agrees that overgeneralizations can be problematic. Yet, the monkeyflower and butterfly examples demonstrate that noncoding RNA plays a role in these species' evolution. "Nobody can argue with that," he says. Researchers may interpret the implications differently, but he hopes the examples inspire researchers and others to consider noncoding RNAs as one of several evolutionary forces. "We now know of some examples of RNA driving speciation—could there be more?"

Did noncoding RNA help spur the evolution of human cognition?

Could the role of noncoding RNAs and their adaptive influence extend beyond moths or monkeyflowers, perhaps even contributing to the evolution of human intelligence? Based on early work, some researchers suspect exactly that.

In 2006, University of California, Santa Cruz, computational biologist David Haussler was interested in building computer programs to determine which genomic regions in humans had changed the most from our last common ancestors with chimpanzees. His team discovered a region that had undergone the most change. They dubbed it Human Accelerated Region 1 (HAR1) (13). Haussler, whose team had previously assembled the first draft of the human genome, was surprised to find that noncoding RNA from this region was highly expressed in the human neocortex, a region associated with higher cognitive function.

Other researchers involved in the GENCODE project, a multi-institutional gene annotation project aiming to identify and classify human and mouse genetic elements (14), have found that human genomes code for thousands of long noncoding RNAs, with nearly 40% of them being expressed exclusively in the brain (14, 15).

Many long noncoding RNAs can change their sequences quickly, says molecular biologist John Rinn of the University of Colorado Boulder. He speculates that these molecules might have helped the brain adapt in ways that improved cognitive abilities, making humans different from other animals.

Rinn, who discovered a type of noncoding RNA called lincRNAs (long intervening noncoding RNAs), likes to use the analogy of a house—every house looks different, but they all need a framework. "I think long noncoding RNAs are the two-by-fours that are helping build the right structure for a species," Rinn adds. This type of RNA can be cut and shaped differently, allowing for unique biological designs. Protein-coding genes, meanwhile, are like nails; they don't change much. Rinn co-founded a biotech company called LincSwitch that aims to harness long noncoding RNA to treat diseases.

The problem with studying the functions of these noncoding RNAs in humans is obvious: "You can't experiment in humans very easily," says John Mattick, a molecular biologist at the University of New South Wales Sydney, in Australia. Mattick, known for his longtime work on designating functions for noncoding RNAs (16), notes that most of the research has to be done on postmortem human brain samples, which only provides evidence for a probable link. To date, conclusive findings in humans remain elusive.

Looking ahead, researchers should be examining the role of noncoding RNA in evolution, brain development, biodiversity, and more, Mattick says. "We need an army of studies around the world," he adds, noting the importance of piecing together a holistic picture of how these molecules shape life.

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