Genome Sequencing and Gene Editing: Ancient DNA in a Living Species

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Known for their large size — sometimes growing up to six feet in length — dark fur and bone-cracking back teeth, dire wolves used to be the most prominent species in continental North America (1). The canines became extinct roughly 10,000 years ago, gradually dying out as their prey did. However, recent news claims that through gene sequencing and genetic engineering, the large predator has been resurrected.

Biotech company Colossal recently announced they had restored the long-extinct canine by extracting ancient DNA from fossilized remains of the animal. While scientists outside the company say the pups are not true dire wolves, they do represent a significant step in synthetic biology and gene editing.

The science behind it: how ancient DNA can create a modern species

The pups, named Romulus, Remus, and Khaleesi, come from learnings derived from DNA that was extracted from an ancient tooth and ear bone of dire wolves' fossilized remains. The DNA from these samples underwent genome sequencing, and scientists compared the genome of the dire wolf with that of the modern gray wolf to identify genetic differences and specific genetic variants unique to dire wolves.

The Colossal team published a paper (1) on *bioRxiv* on April 11, outlining their analysis of the ancient dire wolf DNA. The study found that dire wolves have a complex heritage, with roughly two-thirds of their lineage derived from a group related to gray wolves, coyotes, and dholes and the remaining one-third from a lineage near the base of Canini diversity. The researchers also identified genes evolving under diversifying selection in dire wolves, highlighting their unique evolutionary adaptations. Specifically, the study identified 80 genes evolving under diversifying selection in dire wolves.

The researchers began their analysis by downloading ancient DNA sequencing reads from previously sampled dire wolves and obtained permission to collect new samples from two dire wolves that previously yielded authentic dire wolf mitochondrial and nuclear DNA. The first dire wolf sample, referred to as DireSP, is from an isolated incisor estimated to be 13,000 years old. The second dire wolf sample, designated as DireGB, is from an isolated skull approximately 72,000 years old. After filtering, trimming, and merging overlapping reads from these genomes, the researchers retained 2.8 billion unique sequence reads for the incisor, and 33.3 million unique sequence reads for the skull (1). After mapping it against the reference Greenland gray wolf genome, it was found both individuals were male.

The analysis also identified several genes of the dire wolf that explain why the canines were so large. One key gene, called *NCAPG*, is linked to body size and shows strong signs of evolutionary pressure in dire wolves, suggesting it played a significant role in making them large. Interestingly, both dire wolves and modern gray wolves have very little change in this gene compared to other dog-like animals. Right next to *NCAPG* is another gene, *LCORL*, also linked to growth, which has an inverse relationship with *NCAPG* — when *NCAPG* is more active, animals grow more, and when *LCORL* is more active, they grow less.

The researchers also identified several genes in dire wolves that are linked to male reproduction and may indicate differences in how dire wolves reproduced compared to modern wolves and other canids. One gene, *ROS1*, plays a role in the development of the epididymis, a part of the male reproductive system that stores and matures sperm. Another gene, *PDGFA*, is important for the structure of that system. While interesting, the paper states that none of these genes were linked to baculum size, which can be an important reproductive trait in mammals and leaves aspects of the reproductive biology of dire wolves a mystery (1).

The reconstructed reference paleogenome provides a comprehensive overview of dire wolf genomics, evolution, and adaptations. It not only allowed Colossal to create wolf pups with dire-wolf traits, but also offers insights, and provides a resource for the broader research community and any future studies intending to leverage high-coverage ancient DNA.

Beyond DNA analysis, the many Colossal laboratories focus on several technologies to achieve their de-extinction goal, including stem cell technologies for non-model species, diseaseresistance gene editing, population genomics, and assisted reproductive technologies for critically endangered species.

Ultimately, Colossal scientists identified 20 genetic differences that were key to the extinct animal's appearance, including jaw muscle strength, coat color, size, and specialized bone structure. These specific single nucleotide polymorphisms (SNPs), which corresponded to 14 genes, were then edited into the genome of a gray wolf using the gene-editing tool CRISPR-Cas9, which cut the gray wolf DNA at precise locations and inserted the dire wolf genetic sequences. The modified cells were then used to create embryos, which were implanted into surrogate gray wolves and later born as pups with dire wolf-like traits.

The debate: dire wolf or genetically modified gray wolf?

Colossal declares itself a de-extinction company and states it "is closer to restoring the past, preserving the present and safeguarding the future than anyone before."

However, many scientists say that while the science behind the company's latest creation is impressive, the result is not an actual dire wolf but more akin to a designer dog or genetically modified gray wolf.

Jacquelyn Gill, a paleoecologist at the University of Maine, explained in a recent article that the pups don't have any traits that would provide a better insight into dire wolves and that understanding is more than just knowing what they looked like or ate but also knowing what they did in their ancient ecosystems. "Some of those things are coded genetically; some of those are cultural."

Additionally, the wolves are not being raised in a pack by other dire wolves or hunting in the wild, said David Gold, a professor of Paleobiology at UC Davis, meaning their behavior will likely be different from a real dire wolf.

However, Beth Shapiro, Colossal's chief science officer, explained that creating an animal via gene editing that is genetically identical to an ancient dire wolf is not possible, given the number of simultaneous edits required, "But it's also not the goal…we want to create functional versions of extinct species. We don't have to have something that is 100 percent genetically identical."

Colossal's co-founder and CEO Ben Lamm explained that only 0.3% of gray wolves' genes were changed to make the dire wolf, and the remaining 0.2% variation was unaltered. The reason the company didn't use all of the dire wolf's recovered genes is because the scientists worried those genes could cause deafness and blindness and felt it was ethically irresponsible to include them.

Could a woolly mouse be the first step in bringing back the woolly mammoth?

The dire-like wolves are not Colossal's first time dabbling with bringing back a long-extinct species. Previously, the company introduced woolly mammoth genes into mice, dubbing it the "woolly mouse."

Scientists identified 10 genetic variants in mammoths related to hair length, thickness, texture, color, and body fat to create the cute creature. Next, CRISPR was used to simultaneously edit seven genes in mouse embryos, including genes that affect hair growth cycles, like *FGF5*, and those influencing melanin production, like *MC1R* (2).

The process involved three cutting-edge editing techniques: RNP-mediated knockout to disable specific genes, multiplex precision genome editing to allow simultaneous editing of multiple genes, and homology directed repair (HDR), which facilitated the precise insertion of mammoth DNA sequences. The result was mice with mammoth traits like long, thick, woolly hair, a lighter coat color, and increased body fat.

"The researchers have succeeded in nudging the mouse genome in the direction of a mammoth genome, which is a first," said Louise Johnson, an evolutionary biologist at the University of Reading. "But of the 10 different mutations engineered into the mice, only a few actually make the mouse gene closer to a known mammoth gene."

While the woolly mouse gene may be just a nudge closer to the mammoth gene, the project is a prelude to Colossal's landmark "de-extinction" project: resurrecting the woolly mammoth. The company aims to have a mammoth-like calf born to an elephant by 2028.

"It will walk like a woolly mammoth, look like one, sound like one, but most importantly, it will be able to inhabit the same ecosystem previously abandoned by the mammoth's extinction," the company states.

Importantly, the project could help with the declining population of elephants as the company investigates the genome of both the woolly and modern elephants. Its research looks at ways to make elephants more resilient to climate change, and it is also working on a vaccine for the elephant herpes virus — a dangerous and fatal condition that wreaks havoc in young Asian elephants.

Altogether, Colossal's projects showcase the advanced capabilities of CRISPR in precise genome editing and the potential for de-extinction and trait engineering.

The cost of de-extinction: a boom or bust to biodiversity?

Some scientists have raised concerns about the ethics and conservation impact of de-extinction. While Colossal claims that reviving extinct species could aid in making modern-day habitats more resilient to climate change, others counter that it would be extremely challenging to place a once-extinct animal in an ecosystem that has dramatically changed since it went extinct.

Additionally, some researchers argue that de-extinction could detract from current conservation efforts, and the focus should not be on reviving a lost species, but on protecting existing animals and ecosystems.

In a paper published in Nature Ecology and Evolution, researchers used databases that methodically track the cost of conserving endangered species and determined that caring for a population of resurrected mammoths, for example, would be on par with the cost of caring for the endangered Asian elephant (3). The team also says that if the government assumed responsibility for the conservation of resurrected species, the money would take away limited funding from existing conservation projects, resulting in an overall loss of biodiversity. Specifically, the researchers state two species would go extinct for every species that could be revived (3). Alternatively, the researchers calculated that if the costs were taken on by private organizations or corporations, biodiversity would increase (3).

"However, such benefits would be outweighed by opportunity costs, assuming such discretionary money could directly fund conservation of extant species," the paper states. "Potential sacrifices in conservation of extant species should be a crucial consideration in deciding whether to invest in de-extinction or focus our efforts on extant species" (3).

However, Colossal explains that gene-editing techniques could help with currently endangered species. The red wolf saw a sharp drop in population numbers in the 20th century due to hunting and habitat loss, and in 1980, it was declared extinct in the wild before being reintroduced via a captive breeding program. A few years ago, Ben Lamm and Matt James, Colossal's Chief Animal Officer, learned about the red wolves near extinction and used the same technology they used to engineer dire wolves to create four red wolf clones. The company plans to make more red wolves and eventually re-wild them, which could save them from extinction and improve biodiversity.

While work is underway to collect genetic material from skin punches of various species so scientists can introduce more diversity into surviving populations, Shapiro says that Colossal's techniques of using routine blood draws produce cells that are easier to reprogram than those that come from the skin and could be a better way of diversifying the red wolf gene pool.

"It's actually using technology to prevent species from going extinct," said James.

A mammoth project: the future of "de-extinction"

Currently, Romulus, Remus, and Khaleesi live on a large preserve in an undisclosed location in the northern United States. While Colossal currently has no plans to breed the wolves, it does plan to create five more dire wolves so they can live in a pack. The company is also speaking with Indigenous communities about potentially re-wilding the dire wolves on their lands.

Genetically modifying animals to reintroduce traits of extinct species could benefit endangered species by addressing genetic bottlenecks and inbreeding issues, and help make them more resistant to climate change and pernicious diseases. However, conservation efforts for existing species must also be considered. While the pups are not true dire wolves, they represent a significant step in synthetic biology and gene editing and raise important questions about the ethical and ecological responsibilities of resurrecting and reintroducing species to the world.

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