

## Calibrating the Mitochondrial Clock

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*Mitochondrial DNA appears to mutate much faster than expected, prompting new DNA forensics procedures and raising troubling questions about the dating of evolutionary events.*

In 1991, Russians exhumed a Siberian grave containing nine skeletons thought to be the remains of the last Russian tsar, Nicholas II, and his family and retinue, who were shot by firing squad in 1918. But two bodies were missing, so no one could be absolutely certain of the identity of the remains. And DNA testing done in 1992--expected to settle the issue quickly--instead raised a new mystery.

Some of the DNA from the tsar's mitochondria--cellular organelles with their own DNA--didn't quite match that of his living relatives. Forensic experts thought that most people carry only one type of mitochondrial DNA(mtDNA), but the tsar had two: The same site sometimes contained a cytosine and sometimes a thymine. His relatives had only thymine, a mismatch that fueled controversy over the authenticity of the skeletons.

The question of the tsar's bones was finally put to rest after the remains of his brother, the Grand Duke of Russia Georgij Romanov, were exhumed; the results of the DNA analysis were published in *Nature Genetics* in 1996. Like the tsar, the duke had inherited two different sequences of mtDNA from their mother, a condition known as heteroplasmy. But solving the mystery of the Romanov's remains raised another puzzle that first troubled forensics experts and is now worrying evolutionists. "How often will this heteroplasmy pop up?" wondered Thomas J. Parsons, a molecular geneticist at the Armed Forces DNA Identification Laboratory in Rockville, Maryland, who helped identify the tsar's bones.

Several new studies suggest that heteroplasmy may in fact be a frequent event. They have found that it occurs in at least 10% and probably 20% of humans, says molecular biologist Mitchell Holland, director of the Armed Forces lab. And because heteroplasmy is caused by mutations, this unexpectedly high incidence suggests that mtDNA mutates much more often than previously estimated--as much as 20-fold faster, according to two studies that are causing a stir. Other studies have not found such rapid mutation rates, however.

Resolving the issue is vital. For forensic scientists like Parsons, who use mtDNA to identify soldiers' remains and to convict or exonerate suspects, a high mutation rate might cause them to miss a match in their samples. It could also complicate the lives of evolutionary scientists who use the mtDNA mutation rate as a clock to date such key events as when human ancestors spread around the globe.

Evolutionists have assumed that the clock is constant, ticking off mutations every 6000 to 12,000 years or so. But if the clock ticks faster or at different rates at different times, some of the spectacular results--such as dating our ancestors' first journeys into Europe at about 40,000 years ago--may be in question. "We've been treating this like a stopwatch, and I'm concerned that it's as precise as a sun dial," says Neil Howell, a geneticist at the University of Texas Medical Branch in Galveston. "I don't mean to be inflammatory, but I'm concerned that we're pushing this system more than we should."

## Counting mutations

The small circles of DNA in mitochondria have been the favored tool for evolutionary and forensic studies since their sequence was unraveled in 1981. Unlike the DNA in the nucleus of the cell, which comes from both egg and sperm, an organism's mtDNA comes only from the mother's egg. Thus mtDNA can be used to trace maternal ancestry without the complicating effects of the mixing of genes from both parents. And every cell in the body has hundreds of energy-producing mitochondria, so it's far easier to retrieve mtDNA than nuclear DNA.

It seemed like a relatively straightforward genetic system. Researchers could count the differences in the same sequence of mtDNA in different groups of people and, assuming a constant mutation rate, calculate how long ago the populations diverged. But the case of the tsar highlights how little is known about the way mtDNA is inherited. His mother must have carried or acquired a mutation, so there were hundreds of copies of each of two kinds of mtDNA in her egg cells. She then passed some of each kind to her sons. But just how often do such mutations occur?

The most widely used mutation rate for noncoding human mtDNA relies on estimates of the date when humans and chimpanzees shared a common ancestor, taken to be 5 million years ago. That date is based on counting the mtDNA and protein differences between all the great apes and timing their divergence using dates from fossils of one great ape's ancestor. In humans, this yields a rate of about **one mutation every 300 to 600 generations**, or one every 6000 to 12,000 years (assuming a generation is 20 years), says molecular anthropologist Mark Stoneking of Pennsylvania State University in University Park. Those estimates are also calibrated with other archaeological dates, but nonetheless yield wide margins of error in published dates. But a few studies have begun to suggest that the actual rates are much faster, prompting researchers to think twice about the mtDNA clock they depend upon.

For example, after working on the tsar's DNA, Parsons was surprised to find heteroplasmy popping up more frequently than expected in the families of missing soldiers. He and his colleagues in the United States and England began a systematic study of mtDNA from soldiers' families and Amish and British families. Like most such studies, this one compares so-called "noncoding" sequences of the control region of mtDNA, which do not code for gene products and therefore are thought to be free from natural selection.

The researchers sequenced 610 base pairs of the mtDNA control region in 357 individuals from 134 different families, representing 327 generational events, or times that mothers passed on mtDNA to their offspring. Evolutionary studies led them to expect about one mutation in 600 generations (one every 12,000 years). So they were "stunned" to find 10 base-pair changes, which gave them a rate of

one mutation every 40 generations, or one every 800 years. The data were published last year in *Nature Genetics*, and the rate has held up as the number of families has doubled, Parsons told scientists who gathered at a recent international workshop\* on the problem of mtDNA mutation rates.

Howell's team independently arrived at a similar conclusion after looking deep within the pedigree of one Australian family affected with Leber hereditary optic neuropathy, a disease caused by an mtDNA gene mutation. When the researchers analyzed mtDNA from 40 members of this family, they found that one individual carried two mutations in the control region (presumably unrelated to the disease, because it is noncoding mtDNA). That condition is known as triplasm, because including the nonmutated sequence, he had three different mtDNA sequences in his cells.

By tracing the mutations back through the family pedigree, Howell was able to estimate that both mutations probably arose in the same woman who was born in 1861, yielding an overall divergence rate of one mutation every 25 to 40 generations. "Both of our studies came to a remarkably similar conclusion," says Howell, whose study was published in late 1996 in the *American Journal of Human Genetics*. Both also warned that phylogenetic studies have "substantially underestimated the rate of mtDNA divergence."

Several teams of evolutionists promptly went back to their labs to count mtDNA mutations in families of known pedigree. So far, Stoneking's team has sequenced segments of the control region in closely related families on the Atlantic island of Tristan da Cunha, where pedigrees trace back to five female founders in the early 19th century. But neither that study nor one of 33 Swedish families has found a higher mutation rate. "After we read Howell's study, we looked in vain for mutations in our families," says geneticist Ulf Gyllensten of Uppsala University in Sweden, whose results are in press in *Nature Genetics*. More work is under way in Polynesia, Israel, and Europe.

Troubled by the discrepancy in their results, the scientists have pooled their data with a few other studies showing heteroplasmy, hoping to glean a more accurate estimate of the overall mutation rate. According to papers in press by Parsons, and Stoneking and Gyllensten, the combined mutation rate—one mutation per 1200 years—is still higher than the one mutation per 6000 to 12,000 years estimated by evolutionists, although not as fast as the rate observed by Parsons and Howell. "The fact that we see such relatively large differences among studies indicates that we have some unknown variable which is causing this," says Gyllensten.

Because few studies have been done, the discrepancy in rates could simply be a statistical artifact, in which case it should vanish as sample sizes grow larger, notes Eric Shoubridge, a molecular geneticist at the Montreal Neurological Institute. Another possibility is that the rate is higher in some sites of the DNA than others—so called "hot spots." Indeed, almost all the mutations detected in Parsons and Howell's studies occur at known hot spots, says University of Munich molecular geneticist Svante Pääbo.

Also, the time span of observation plays a role. For example, because hot spots mutate so frequently, over tens of thousands of years they can revert back to their original sequences, overwriting previous mutations at that site.

As a result, the long-term mutation rate would underestimate how often hot spots mutate--and the average long-term mutation rate for the entire control region would be slower than that from near-term studies of families.

"The easiest explanation is that these two rates are caused by hot spots," says Pääbo. If so, these short-term rates need not perturb long-term studies. "It may be that the faster rate works on the short time scale and that you use the phylogenetic rate for long term events," says Shoubridge.

But Parsons doubts that hot spots account for all the mutations he has observed. He says that some of the difference between the long-term and short-term rates could be explained if the noncoding DNA in the control region is not entirely immune to selection pressure. The control region, for example, promotes replication and transcription of mtDNA, so any mutation that interferes with the efficiency of these processes might be deleterious and therefore selected against, reducing the apparent mutation rate.

Regardless of the cause, evolutionists are most concerned about the effect of a faster mutation rate. For example, researchers have calculated that "mitochondrial Eve"--the woman whose mtDNA was ancestral to that in all living people--lived 100,000 to 200,000 years ago in Africa. Using the new clock, she would be a mere 6000 years old.

No one thinks that's the case, but at what point should models switch from one mtDNA time zone to the other? "I'm worried that people who are looking at very recent events, such as the peopling of Europe, are ignoring this problem," says Laurent Excoffier, a population geneticist at the University of Geneva. Indeed, the mysterious and sudden expansion of modern humans into Europe and other parts of the globe, which other genetic evidence puts at about 40,000 years ago, may actually have happened 10,000 to 20,000 years ago--around the time of agriculture, says Excoffier. And mtDNA studies now date the peopling of the Americas at 34,000 years ago, even though the oldest noncontroversial archaeological sites are 12,500 years old. Recalibrating the mtDNA clock would narrow the difference (*Science*, 28 February 1997, p. 1256).

But not everyone is ready to redate evolutionary history on the basis of a few studies of mutation rates in living people. "This is all a fuss about nothing," says Oxford University geneticist Martin Richards, who thinks the fast rate reaches back hundreds of years at most.

That, however, is squarely within the time frame of forensics cases. Heteroplasmy isn't always a complicating factor in such analyses. When it exists in more than one family member, the confidence in the identification gets stronger, as in the case of the tsar. But otherwise, it could let a criminal off the hook if his mtDNA differed by one nucleotide from a crime scene sample. Therefore, Parsons and Holland, in their work identifying 220 soldiers' remains from World War II to the present, now have new guidelines--adopted by the FBI as well--to account for a faster mutation rate. When a missing soldier's or criminal suspect's mtDNA comes up with a single difference from that of a relative or at a crime scene, the scientists no longer call it a "mismatch." Instead the results are considered "inconclusive." And, for now, so are some of the evolutionary results gained by using the mtDNA clock.