EEB 122: Introduction to Ecological and Evolutionary Biology
Professor Steve Stearns

Recombination in Mitochondrial DNA: Nonzero but Rare

by Christina Hueschen

In the study and reconstruction of the history of man and of the evolutionary events that created us, mitochondrial DNA has been our most essential molecular tool. Using mtDNA and our assumption of its clonal, purely maternal inheritance in humans, scientists have created a phylogenetic tree following and dating the migration of modern humans from Africa. They have even traced all human mtDNA to a single maternal ancestor, "mitochondrial Eve," who lived approximately 171,000 years ago (Slate and Gemmell, 2004). However, in the past decade, researchers have proposed and begun to seriously consider the existence of mitochondrial recombination: the crossing-over of parts of mitochondrial DNA during replication.

If mitochondrial DNA does undergo recombination, the use of mtDNA in population genetics may not be so simple. Under the assumption of clonal inheritance, scientists successfully estimated a relatively constant rate of mutation for mtDNA, which we have used as a "molecular clock" to measure time as a function of accumulated mutations (Stearns and Hoekstra, 2005). The molecular clock we have relied on for decades to date events on the human genetic tree is based on this assumption of clonal inheritance, with all genetic variation in mtDNA arising from mutations that occur at a roughly constant rate (Slate and Gemmell, 2004). The dating of "mitochondrial Eve" or

of events like the migration of humans from Africa to Europe did not factor in the added variation in mtDNA that recombination would cause.

At the same time that the possibility of recombination is opening up new questions and doubts about our knowledge of human evolutionary history, the same possibility presents a possible solution to an unanswered and very important question: how supposedly asexual mitochondria have escaped extinction by Muller's ratchet. Without recombination to clear out deleterious mutations, Muller's ratchet predicts that these mutations should accumulate irreversibly in mtDNA (Stearns and Hoekstra, 2005). Instead, mitochondrial genomes have remained functional – an indication, some scientists contend, of the existence of mitochondrial recombination (Ladoukakis and Eyre-Walker, 2004; Barr et al., 2005).

Over the past decade, the body of evidence supporting recombination has become substantial enough that we cannot ignore it. However, proponents of pure maternal mtDNA inheritance have accused much of the key research supporting recombination of data misinterpretation, exaggeration, and sloppy procedures (Jorde and Bamshad, 2000; Piganeau and Eyre-Walker, 2004; Bandelt et al., 2005). They continue to argue for clonal inheritance of mtDNA, also pointing out the lack of population genetic evidence for recombination (Bandelt et al., 2005). The debate over mitochondrial DNA recombination continues today, but research on both sides is consistent with a very low level of mitochondrial DNA recombination.

Much of the earliest research that claimed to prove the existence of mitochondrial recombination, such as that of Awadella et al. in 1999, looked for evidence of recombination in linkage disequilibrium, the non-random tendency of alleles to be

inherited together more frequently than would be expected given their distance. Without recombination, these associations of alleles occur because an entire chromosome is inherited clonally. Recombination would give alleles the opportunity to form new combinations and therefore break down linkage disequilibrium. Awadella and his colleagues analyzed the relationship between linkage disequilibrium and the distance between allele sites in human and chimpanzee mtDNA. They wanted to investigate the hypothesis that if recombination occurred, it would be more likely to disrupt the linkage disequilibrium of two alleles that are far apart than two alleles that are close together, since the far-apart alleles would be likely to be separated during crossing-over and end up on different segments of the DNA. Because their statistical analysis showed that linkage disequilibrium declined with distance between alleles in both humans and chimpanzees, Awadella et al. concluded that recombination must exist.

Awadella et al.'s methods contained some important controls, such as restricting their analysis to synonymous variation – genetic variation with no effect on fitness. By only examining alleles whose corresponding phenotypes had equal reproductive success, they ensured that natural selection could not be a force creating linkage disequilibrium. They could therefore attribute variation in linkage disequilibrium to recombination, not selection, because the mitochondrion gained no phenotypic advantage by inheriting two particular alleles together. In addition, when measuring linkage disequilibrium, they used a reliable and established formula, the Hill and Robertson measure, to calculate it.

However, when Jorde and Bamshad re-analyzed Awadella et al.'s data in 2000, they first used the same Hill and Robertson measure for linkage disequilibrium. Jorde and Bamshad found a significant correlation with distance in only two of the five human

data sets, not in four as Awadella et al. claimed. In addition, when Jorde and Bamshad then used an alternative formula, *D'*, to calculate linkage disequilibrium, they did not find a significant correlation with distance for any of the data sets. Awadella et al.'s results, therefore, do not represent unquestionable proof of mitochondrial DNA recombination. They provide evidence for the possibility of recombination, but because another team of scientists did not reproduce or confirm their findings when using an alternative method of analysis, Awadella and his collegues' evidence is disputable.

Although Awadella et al. explored a clever way of detecting recombination in mitochondrial DNA, others have questioned their interpretation of their data (Jorde and Bamshad, 2000; Piganeau and Eyre-Walker, 2004). Awadella et al. overstated their conclusions when they claimed that their results "can be attributed to one mechanism only: recombination." Awadella et al.'s research did not provide direct detection of mitochondrial recombination; it implied recombination through of the decline of linkage disequilibrium in alleles farther apart from each other on the mitochondrial genome. Therefore, indisputable statistical correlation of the relationship between linkage disequilibrium and distance is necessary to back such a strong conclusion.

In the past five years, scientists have further questioned and discredited Awadella et al.'s evidence of recombination, as well as the evidence published in the following several years by scientists who copied their technique of analyzing the relationship between linkage disequilibrium and distance. In 2004, Piganeau and Eyre-Walker re-analyzed mtDNA data from Awadella et al. and three other published data sets that, according to their authors, supported the hypothesis of mitochondrial recombination. They used both the Hill and Robertson and the alternative *D'* measure of linkage

disequilibrium and determined the statistical significance of the negative relationship between the linkage disequilibrium of two alleles and the distance between them. Of analyses with the Hill and Robertson measure, 104 of 140 did show a negative correlation, but most were not statistically significant. Furthermore, of the analyses with *D'*, half showed a negative correlation and half a positive one. These results do not definitively demonstrate the existence of mitochondrial recombination; in fact, they reveal a lack of solid evidence in any of the published research using linkage disequilibrium. The work of both Jorde and Bamshad and of Piganeau and Eyre-Walker shows that current techniques, at least, have not conclusively detected recombination using the method of linkage disequilibrium analysis on sequence data. This method, used by a substantial fraction of the published research supporting recombination, has not proved capable of definitively demonstrating recombination's existence because the linkage disequilibrium-distance relationship is "hardly detectable" with available methods (Piganeau and Eyre-Walker, 2004).

At first glance, the failure of linkage disequilibrium analysis to show real evidence for recombination could seem like proof of pure, maternal mitochondrial DNA inheritance. To make that claim, however, would be to force an unsupported conclusion just as scientists like Bamshad, Jorde, Piganeau, Eyre-Walker, and Bandelt accuse the supporters of the recombination hypothesis of doing. It is very possible that recombination may occur, but occur at such low levels and so infrequently that it cannot be detected by linkage disequilibrium analysis to a significant degree.

Another argument for the existence of recombination is high levels of homoplasies in phylogenetic trees, where the same mutation (or its reverse) occurs at the

same genetic site in two different parts of the tree (Piganeau and Eyre-Walker, 2004). Recombination could cause this if a mutant mitochondrial genome underwent crossing-over with a mitochondrial genome of a different branch of the phylogenetic tree, passing the mutation into that branch. However, homoplasies can be created either by recombination or simply by multiple mutations, which may be selected for because of their phenotypic advantage (Piganeau and Eyre-Walker, 2004). Scientists arguing for clonal, maternal inheritance use the concept of multiple mutations, especially at hypervariable sites – genetic sites where mutation is unusually common – to invalidate this argument for recombination (Piganeau and Eyre-Walker, 2004; Slate and Gemmell, 2004). However, the existence of multiple mutations and hypervariable sites are not evidence of non-recombinant inheritance. Some occurrences of homoplasy could very possibly be the result of recombination; we cannot know for certain either way without further research.

Proponents of purely maternal inheritance also cite the lack of population genetic evidence for recombination as a strong argument against its existence. As Bandelt et al. stated in 2005, "the worldwide mtDNA phylogeny faithfully mirrors the non-recombinant nature of this genome." Bandelt and his colleagues argue that recombination would have been reflected in mtDNA phylogeny over the thousands of years of human history. According to Bandelt et al., every population has phylogenetically distant mtDNA haplotypes, and there exists therefore approximately a 50% probability that these genetically different, or "foreign," mtDNAs will be involved in an instance of recombination. If these genetically different mtDNAs recombined with the mtDNAs more common in the population, the resulting heterogeneous mtDNAs would be

noticeable generations later when studying that population's genomes. Bandelt et al.'s logic is compelling, but some of their statements beg skepticism. Have all populations in human history really contained phylogenetically distant, or "rare," mtDNA? Where did the authors get their statistic of a 50% chance of recombination of those unusual mtDNAs – a statistic whose basis they do not explain or prove at all? Perhaps because paternal leakage – the "accidental" transfer of mtDNA into a gamete from the father as well as the mother, a pre-cursor for recombination – occurs so rarely, the frequency of recombination is so miniscule that it escapes detection in population genetic data. Even thousands of years of phylogenetic history may not visibly reflect such an extremely low frequency of recombination events.

In 2004, a paper by Kraytsberg et al. presented the first truly solid evidence for recombination. Kraytsberg and her colleagues looked for mitochondrial DNA recombinants in the muscle tissue of a unique individual – a 28 year old man who, unlike almost all humans, had mtDNA from both his mother and father in his muscle tissue mitochondria (Ladoukakis and Eyre-Walker, 2004). They sequenced the extracted mitochondrial DNA and found 33 recombinants with alternating maternal and paternal segments. Recombinant mtDNA made up approximately 0.7% of total mtDNA. This corresponds to 7% of maternal mtDNA molecules, since the make-up of the individual's muscle tissue mtDNA was 90% paternal and 10% maternal.

Kraytsberg et al. provided direct evidence of recombinant mitochondrial DNA, instead of implying it through methods such as linkage disequilibrium analysis or homoplasy. Their findings demonstrate that recombination is possible; it occurs given paternal leakage. The subject of their research, however, was an extraordinary

individual, whose mitochondria had undergone paternal leakage and therefore contained heterogeneous mtDNA. How often mitochondrial DNA recombine, therefore, depends on the frequency of paternal leakage – and this frequency is probably very low.

Some scientists on the clonal inheritance side of the debate have questioned Kraytsberg et al.'s methods, pointing out that the single molecule PCR method they used can frequently be subject to contamination (Bandelt et al., 2005). Mixing of samples during PCR would have caused artificial recombination, leading the researchers to false conclusions. However, in addition to repeating their experiment many times with the same result, Kraytsberg and her colleagues conducted an important control experiment: mixing purely maternal and purely paternal mtDNA from the subject's mother and father and sequencing that mix. In this case, they observed no evidence of recombination at all. This control demonstrates that the recombinants found after PCR of the subject's mtDNA were not artificial, but actually created *in vivo*, in the mitochondria of the subject's muscle tissue.

Human mitochondrial DNA recombination would make sense in the context of what we know about plant and animal mtDNA. Current scientific consensus is that occasional recombination occurs in plant and fungal mitochondrial genomes, and researchers have reported recombination in several animal species (Barr et al., 2005). All animal mtDNA contains the "machinery" needed for recombination (Barr et al., 2005); even mammalian mtDNA possesses the necessary enzymes (Ladoukakis, Eyre-Walker, 2005). Besides showing directly that recombination between maternal and paternal mtDNA is possible, Kraytsberg et al.'s results reveal that human mitochondria have an

active pathway for recombination – not just the necessary "machinery" (Ladoukakis and Eyre-Walker, 2004).

Further direct evidence of mitochondrial DNA recombination between two human cytoplasmic hybrid cell lines supports the idea that recombination is possible in human mtDNA given paternal leakage (D'Aurelio et al., 2004). D'Aurelio and his colleagues used two cell lines whose mitochondrial DNA contained mutations preventing oxidative phosphorylation. The mtDNAs of the two cell lines had two different mutations, but with this same phenotypic result. Both types of mitochondria could not survive in a medium lacking either uridine or pyruvate because of their dysfunctional respiratory chains. However, after fusing the two cell lines, D'Aurelio et al. found that hybrids grew in media that did not contain uridine or pyruvate. The coexistence of the two mutant mtDNA cell species resulted in mitochondrial functional complementation – mitochondria with mtDNA from both species had repaired mitochondrial respiration. After using this uridine and pyruvate-lacking medium to select for hybrids, D'Aurelio et al. sequenced their mtDNA. They found that some of the nucleoids of these fused cells contained not just several mtDNA molecules of each type, but also recombinant mtDNA molecules made of pieces from both types. Because the region of the molecules in which the sequence transition from one species' mtDNA to the other's differed for different mtDNA molecules, they concluded that these recombinant mtDNA molecules resulted from independent recombination events. Then, using Southern blot, D'Aurelio and his colleagues found recombinant bands that did not correspond to any of the bands from the original species' mtDNA. These recombinant bands made up approximately 10% of the total mtDNA.

Because D'Aurelio and his colleagues produced direct evidence of mtDNA recombinants by two independent methods – sequencing and Southern blot – their conclusion that human mtDNA recombination can occur within heteroplasmic cells is difficult to refute. However, the heteroplasmic cells they created are an ideal condition for recombination – one that hardly ever occurs naturally in humans.

Research by Zsurka et al. in 2005 provided less definitive evidence of recombination than D'Aurelio et al.'s, but their analysis used mtDNA from 24 human individuals. Therefore, it is more relevant to determining whether human mtDNA recombination occurs in practice. However, these individuals do not represent the general population either; they had multiple mtDNA heteroplasmy, which means that their mtDNA contained a section of mutated mtDNA in more than one place.

Zsurka et al. analyzed the skeletal muscle mtDNA of these individuals with more than one mutant mtDNA sequence, looking for evidence of recombination. In particular, they wanted to determine if these individuals' mtDNA was characterized by triplasmy or by tetraplasmy, which is a hallmark of recombination. Triplasmic mtDNA has a mixture of three different genomes: one wild type, with no mutations, the second with mutation 1, and the third with mutation 2. Tetraplasmic mtDNA, on the other hand, has a mixture of four different types of genomes: wild type (W_1W_2), genomes with mutation 1 or with mutation 2 (W_1M_2) and (M_1W_2), and genomes with both mutations (M_1M_2). This last option, which separates tetraplasmy from triplasmy, can only occur if the two separate mutations that produced multiple mtDNA heteroplasmy undergo recombination, allowing the creation of mtDNA molecules containing both mutations.

In ten individuals (those with both a tRNA point mutation or a large deletion and a D-loop mutation, where the two mutations were far apart) Zsurka et al. observed tetrasplasmy. In twelve of the fourteen individuals whose two mutations were close together and therefore should have been unaffected by recombination, they observed triplasmy. These two results support the existence of human mtDNA recombination and even imply that it is common in human skeletal muscle.

Further evidence for mitochondrial recombination lies in the high rate of evolution of human mtDNA relative to the nuclear genome. Traditionally, scientists have cited mtDNA's lack of repair enzymes as the cause of unusually frequent mutations and unusually fast evolution (Ladoukakis and Eyre-Walker, 2004). But the existence of recombination and the new genetic variation it produces would provide another (and in the opinion of Barr et al., 2005, a better) explanation for mtDNA's high evolutionary rate.

In addition to the existence of new genetic variation, recombination provides a possible solution to the mystery of how mitochondria escape Muller's ratchet. By producing recombinant molecules, some of which are mutation-free, recombination would allow mitochondrial DNAs to rid themselves of deleterious mutations through selection of these higher-fitness recombinants. As a result, mtDNA would not lose their fitness irreversibly because of the accumulation of harmful mutations, as Muller's ratchet predicts. Recombination is definitely a candidate for the answer to the puzzle of how mitochondria have preserved themselves and their function for so long.

It is possible that because mitochondria are so essential, haploid (mutations cannot be hidden), and subject to intragenomic conflict with other mitochondria, almost all mutant mitochondria with reduced fitness are not reproductively successful.

Mitochondria undergo very strong natural selection within each human individual, so it is unlikely that mutant mtDNA with compromised function will be passed on by that individual to her (or his?) children. However, these unusually strong selective forces operating on mitochondrial DNA may slow down the effect of Muller's ratchet, but they cannot prevent it entirely. At some point, a deleterious mutation will occur – perhaps during the developmental stage of an individual, which would cause it to be clonally reproduced throughout the body and even in the gametes – and be passed on, and over enough time the accumulation of these mutations will result in extinction unless combated by another biological or evolutionary process. Even rare recombination events, on the other hand, can facilitate adaptive evolution and prevent mutation accumulation and Muller's ratchet (Barr et al., 2005). Mitochondrial DNA recombination – even a very low level of it – is a plausible explanation for the incredible preservation of mtDNA fitness.

Very infrequent recombination in the human population would not be detectable by methods such as the analysis of linkage disequilibrium and distance between alleles, because of the low statistical power of these analyses (Ladoukakis and Eyre-Walker, 2004). Critics of research like that of Awadella and colleagues point out that Awadella et al.'s data did not definitively demonstrate recombination. Kraytsberg et al. (2004), D'Aurelio et al. (2004), and Zsurka et al (2005) present less questionable conclusions in their papers, but they draw easy criticism for focusing on special mtDNA that seems irrelevant to the general human population. Kraytsberg et al. studied mtDNA from muscle tissue in an extraordinary individual with paternal and maternal mtDNA inheritance - a specific type of cell from an extremely unusual individual. D'Aurelio et

al. studied hybrid mtDNA fused from two specially created mitochondrial species – a situation created in the laboratory to produce ideal conditions for recombination. Zsurka et al. demonstrated the existence of mitochondrial recombinants in ten individuals, but specifically in their skeletal muscle. Unfortunately, when dismissing mtDNA recombination research on the basis of alternative data interpretation in some experiments or a lack of unequivocally direct relevance in others, critics who support pure maternal inheritance tend to dismiss the body of research as a whole. In doing so, they ignore the strong argument for mtDNA recombination that these researchers have built out of small, sound pieces. In fact, a low level of mitochondrial DNA recombination is consistent with the results of all of these experiments.

Kraytsberg et al. provided direct evidence that mtDNA recombination does occur when heterologous mtDNAs are mixed in one individual's cells, and D'Aurelio et al. provided additional evidence of recombination between two different mtDNAs within one cell. Zsurka et al. showed that recombination also takes place in mitochondrial genomes containing a mixture of mutated and wild-type sequences at multiple locations. The research by these three groups of authors directly supports the hypothesis that a pathway for recombination exists in human mitochondrial DNA, and that recombination does occur; however, it has no impact and is not consequential (or detectable) unless the mtDNA undergoing recombination is heterologous. Recombination between two homologous mtDNA molecules results in new mtDNA molecules identical to the old. Therefore, the new question we must ask is not whether recombination occurs, but how frequently (or rather, infrequently) heterologous mtDNA molecules come to exist

together within human cells. Both paternal leakage and multiple mtDNA heteroplasmy due to mutations can cause this to occur, and both occur very rarely.

In all mammals, paternal mtDNA does enter the egg when the sperm fertilizes it, but efficient mechanisms target and destroy the paternal mtDNA immediately (Ladoukakis and Eyre-Walker, 2004). Except rare cases like that of the individual studied by Kraytsberg et al., when paternal leakage does occur, different mtDNAs from the two parents would never have the opportunity to recombine. If a mutation arises within one of the mtDNA molecules in a human cell, that mtDNA molecule can recombine with other mtDNA inside the same cell, but unless the other mtDNA has another, different mutation, the resulting recombinants will not be anything new – they will either have the one mutation, or not. Even if two mtDNAs with two different mutations do recombine, in order to pass on to the next human generation and have any phylogenetic impact, the recombinant mitochondria must be inside an egg cell that is fertilized and grows into a child. The bottleneck of vertical transmission of mtDNA from human generation to generation – so few mtDNA pass on – makes the likelihood of inheritance of this recombination of multiple mtDNA heteroplasmy almost non-existent. When considering the frequency of mitochondrial DNA recombination, the most important factor is therefore the frequency of paternal leakage.

No precise estimate of the rate of paternal leakage yet exists, but Ladoukakis and Eyre-Walker (2004) estimate it at much less than 1 in 1,000. This very low rate of paternal leakage, and therefore of recombination, is consistent with the failure of linkage equilibrium analyses to show significant evidence of recombination because of their low statistical power. It also helps to explain why mtDNA phylogenies and work in

population genetics have not detected or confirmed the existence of recombination. Because of the very low rate of recombination needed to combat the accumulation of mutations (Barr et al., 2005), however, mtDNA recombination allows – or at least helps to allow – mitochondria to escape Muller's ratchet. Mitochondria may have additional, undiscovered mechanisms that have allowed them to retain their fitness and function, but the most important is probably recombination, the traditional opponent of mutation accumulation. Although we do not yet know how frequently mtDNA recombination occurs in humans, scientific research has demonstrated its existence. This existence makes sense in an evolutionary context. Occasional paternal leakage and the resulting mtDNA recombination would help to clear out deleterious mutations without conceding the control over selfish elements allowed by uniparental inheritance (Barr et al., 2005). Because mitochondrial DNA is usually inherited clonally from the mother, little variation in mtDNA exists within each individual, and therefore selfish elements have fewer opportunities to assert an advantage over other mitochondria and reproduce themselves continuously to the disadvantage of the host (in this case, the human) (Barr et al., 2005). If an allele promoting a high level of paternal leakage were to enter the population as a mutation, it would be cleared out of the population by selection on the human level for minimized variation between mitochondria. By retaining a norm of purely maternal inheritance, except for the occasional paternal leakage and recombination, mitochondrial DNA successfully controls the spread of selfish elements and clears out any deleterious mutations that slowly accumulate.

With the new consideration of rare, but nonzero human mitochondrial DNA recombination, our knowledge of the evolution and history of man must be re-evaluated.

Because of its supposed pure maternal inheritance, scientists have used mtDNA to trace the spread of humans across the globe, to date events in human evolutionary history, and to calculate the age and geographical location of "mitochondrial Eve."

Because of the rarity of mtDNA recombination and because very little gene flow occurs between distant populations, the mtDNA recombination factor will have little effect on the study of human migration across the world and the shape of the human phylogenetic tree (Ladoukakis and Eyre-Walker, 2004). An mtDNA mutation characteristic of a population that moved to a certain area would not be affected by recombination with a different population's mtDNA because of the lack of gene flow between the two.

The existence of mtDNA recombination will, however, affect our current dating of events in human evolution. Ignoring recombination results in an overestimate of the rate of mutation in mtDNA, since recombination, not mutation, caused some of the observed genetic variation (Ladoukakis and Eyre-Walker, 2004). Therefore, the mtDNA molecular clock ticks slower than scientists previously believed, and many important events in human evolutionary history probably occurred longer ago than was thought. Using a modified molecular clock that factors in mtDNA recombination, the new estimate of the time of migration of humans from Africa is closer to estimates based on nuclear genes (Slate and Gemmell, 2004).

The age of "mitochondrial Eve," too, must be modified when factoring in the existence, however infrequent and rare, of mitochondrial DNA recombination. Two different studies considering recombination place Eve's age at two times and at four times its current value of approximately 171,000 years (Slate and Gemmell, 2004).

However, Eve's existence as the current human population's most recent common female ancestor and her geographical location in Africa remain unchanged.

Although recombination does occur in human mitochondrial DNA, usually as a result of paternal leakage, leakage and recombination occur so rarely that their existence does not call for an overhaul of our knowledge about human evolutionary history, but rather for a fine-tuning of it. Recombination occurs so infrequently, in fact, that population genetic data contains no noticeable evidence for it. However, in combating a similarly slow process – the accumulation of mitochondrial mutations over time – recombination is effective, with the current preserved function of mitochondria as evidence of its success.

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