High Mitochondrial Mutation Rates Estimated From Deep-Rooting Costa Rican Pedigrees

Lorena Madrigal,[1] Loredana Castrì (Posthumously),[2] Mauricio Melendez-Obando,[3] Ramon Villegas-Palma,[3] Ramiro Barrantes,[4] Henrieta Raventos,[4] Reynaldo Pereira,[4] Donata Luiselli,[2] Davide Pettener,[2] and Guido Barbujani[5]*

[1]*Department of Anthropology, University of South Florida, Tampa, FL 3360*
[2]*Dipartimento di Biologia evoluzionistica sperimentale. Area di Antropologia, Università di Bologna, Via Selmi 3, 40126 Bologna, Italy*
[3]*Academia Costarricense de Ciencias Genealógicas, 1771-1002, Paseo de los Estudiantes, San José, Costa Rica*
[4]*Escuela de Biología, Universidad de Costa Rica, Ciudad Universitaria Rodrigo Facio, San Pedro de Montes de Oca, 2050, Costa Rica Universidad de Costa Rica*
[5]*Dipartimento di Biologia ed Evoluzione, Università di Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy*

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ABSTRACT Estimates of mutation rates for the noncoding hypervariable Region I (HVR-I) of mitochondrial DNA vary widely, depending on whether they are inferred from phylogenies (assuming that molecular evolution is clock-like) or directly from pedigrees. All pedigree-based studies so far were conducted on populations of European origin. In this article, we analyzed 19 deep-rooting pedigrees in a population of mixed origin in Costa Rica. We calculated two estimates of the HVR-I mutation rate, one considering all apparent mutations, and one disregarding changes at sites known to be mutational hot spots and eliminating genealogy branches which might be suspected to include errors, or unrecognized adoptions along the female lines. At the end of this procedure, we still observed a mutation rate equal to 1.24×10^{-6}, per site per year, i.e., at least threefold as high as estimates derived from phylogenies. Our results confirm that mutation rates observed in pedigrees are much higher than estimated assuming a neutral model of long-term HVRI evolution. We argue that until the cause of these discrepancies will be fully understood, both lower estimates (i.e., those derived from phylogenetic comparisons) and higher, direct estimates such as those obtained in this study, should be considered when modeling evolutionary and demographic processes. Am J Phys Anthropol 148:327–333, 2012. © 2012 Wiley Periodicals, Inc.

Placing demographic events in the appropriate time frame is crucial in the study of human evolution. Dates of demographic changes, such as population expansions or splits, are often estimated from DNA variation, under the assumption that mutations accumulate at an approximately steady pace through time. This is often referred to as the molecular clock hypothesis, and considering how many studies on the origin and microevolution of the human species rely on mitochondrial DNA (mtDNA) molecular clocks (Forster et al., 1996; Bonne-Tamir et al., 2003; Gonzalez et al., 2007; Chaubey et al., 2008; Zhu et al., 2009; Brucato et al., 2010; Crubezy et al., 2010; Batini et al., 2011), one would imagine that the rate of change in mtDNA is well understood and precisely measured. However, that is not really the case. Indeed, evidence has been accumulating since the 1990s that estimates of the mtDNA mutation rate vary widely not only according to the estimation method used but also between populations studied using the same method—compare, e.g., Swedes and Icelanders, respectively in (Cavelier et al., 2000; Siguroardottir et al., 2000).

The two main classes of methods differ in that they infer the mutation rate either directly, counting the mutations observed in pedigrees, or indirectly, from phylogenetic comparisons. Under the latter approach, the nucleotide substitutions between different sequences are counted; corrections may be introduced for different rates of transition and transversion, and for the possibility of repeated mutations at the same nucleotide site; finally, the figures obtained are calibrated, i.e., divided by an independent estimate of the time since the common ancestor of the subjects studied (Ward et al., 1991; Lundstrom et al., 1992; Endicott and Ho, 2008; Nabholz et al., 2008). Studies of this kind, sometimes labeled either as "model-based" or "model-free" depending on the weight of the underlying assumptions, yield rates ranging from 0.12 to 0.38 per base pair per million years for the human first mitochondrial hypervariable region, or hypervariable Region I (HVR-I; Henn et al., 2009; Ho et al., 2011). Forster et al. (1996) used a more complex model incorporating the effects of changes in

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*Correspondence to: Guido Barbujani, Department of Biology and Evolution, University of Ferrara, Via L. Borsari 46, 44121 Ferrara, Italy. E-mail: g.barbujani@unife.it

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population size; they obtained an estimate of one transition every 20,180 years which, over the 375 base pairs considered, translates into 0.13 transitions per site per million years. That figure was widely used in successive studies. By contrast, much higher estimates were obtained by directly counting the mutations in pedigrees, up to 2.50 mutations per base pair per million years (Howell et al., 1996; Parsons et al., 1997; Siguroardottir et al., 2000; Heyer et al., 2001; Howell et al., 2003; Santos et al., 2005; Ho et al., 2007; Santos et al., 2008; Henn et al., 2009; Ho et al., 2011).

The higher pedigree-derived mutation rates have several potential causes. It might be that mutational events observed in recently recorded meiotic events are "erased" by events such as back mutations, when recorded at a macroevolutionary time frame. Other explanations for this disparity include random genetic drift, unaccounted heteroplasmy (Zaragoza et al., 2010) and a different tendency of different mtDNA sites to be damaged (Howell et al., 2003; Soares et al., 2009; Pereira et al., 2011). Indeed some sites within the mtDNA HVR-I (mutational hot spots or "speedy sites") seem to undergo substitution four to five times as fast as the average sites (Wakeley, 1993; Excoffier and Yang, 1999; Bandelt et al., 2000). Finally, even mutations in noncoding regions may evolve in a non-neutral way, because selection affects the transmission of mtDNA (Pereira et al., 2011), these mutations are in linkage disequilibrium with mutations in coding regions, and recombination is little (Awadalla et al., 1999) or nil. This implies that the haplotype on which a new HVR-I mutation occurs, i.e., the combination of alleles in the mitochondrial genome, affects the mutation's probability to be transmitted through generations. Haplotypes sharing the same mutations in coding regions will tend to have the same apparent mutation rate in HVR-I, because they will undergo the same selective pressure; by contrast, the same HVR-I site may appear to mutate at different rates in haplotypes having different fitnesses due to mutations in the coding region. As a consequence, one should not take for granted that mutation rates estimated in different populations should necessarily coincide, because the genetic background on which the mutation occurs may contribute to determining successive changes in its frequency.

In short, knowing exactly the mitochondrial mutation rate is at the same time complicated, and of crucial importance for accurately reconstructing population history. Because all available direct estimates refer to populations of European ancestry, an additional question is whether populations with different genetic backgrounds will show similar patterns, and comparable rates, of mutation. In this article, we took advantage of the availability of deep-rooting pedigrees collected in Costa Rica, and going back to the 1500s. These pedigrees come from a mixed population and so offered us the opportunity to estimate the HVR-I mutation rate in people in which such studies have never been carried out. We used a pedigree-based approach to compute the observed mutation rate, as well as a number of related statistics. We obtained very high initial estimates; by stepwise eliminating pedigrees which, one way or another, may not be considered reliable, and by disregarding the effects of DNA sites that may mutate at a higher-than-average rate, we showed that even under the most conservative assumptions our empirical estimate of the HVR-I mutation rate remains high.

MATERIALS AND METHODS

The data consist of maternal genealogies started from 152 living subjects, all of whom lived in Atenas, Costa Rica. The genealogies were reconstructed with records obtained from the Church Chancery and the National Document Register, and each included at least seven generations, sometimes extending all the way back to the 1,500s. Only adults able to give informed consent were recruited. The project was approved by the committee on bioethics of the University of South Florida and the Universidad de Costa Rica. For this article, we considered 19 pedigrees for which we could determine if mutational events had taken place because they included more than one living subject from whom we extracted mtDNA. Fieldwork and data collection are described elsewhere (Madrigal and Melendez-Obando, 2008). mtDNA was extracted from 152 blood samples with standard procedures. The HVR-I of the mtDNA control region was amplified by PCR between nps 16,024–16,383 using primers H16401 and L15997. Both strands of the HVR-I were sequenced. In addition, seven binary PCR-restriction fragment length polymorphisms were typed, known to be specific to maternal lineages within America, Europe, and Africa, to help define the haplogroups, namely 1663HaeIII, 13592HpaI, 110397AluI,110871MnlI, 211251 Tsp509I, 113262 AluI, 214766 MseI, and the COII/tRNAlys 9-bp deletion (Castri et al., 2009).

An initial mutation frequency was calculated by counting the number of HVR-I mutations and dividing this number by the total number of meiotic events recorded in all the pedigrees. The mutation rate per site per generation was then computed by dividing the mutation frequency by 360, the number of base pairs sequenced. The mutation rate per site per year was computed by dividing the former by the generation time. Although several authors considered a standard generation time of 20 years (Parsons et al., 1997; Cavelier et al., 2000; Siguroardottir et al., 2000; Howell et al., 2003) for this study, we had full information about the age at reproduction of the women in our sample. In the 289 meioses considered, it was 28.3 years (ranging from 15 to 50 years of age), and hence we decided to use that figure. Lastly, the divergence rate is twice the mutation rate.

For computing the age at motherhood, we considered all pedigrees, namely, those which did not have more than one descendant from a single common ancestor and those analyzed in this article. Each datum consisted of the age at motherhood for each woman in each of our pedigrees. In a few cases, the exact age was missing, and in this case, this data point was entered as missing. If a woman was the mother of more than one branch in a pedigree (as shown in the figures), her age at all of the pregnancies was considered, and a mean age at motherhood was computed for her. The mean age at motherhood used in this article is the mean considered using all data points obtained as described here.

Counting mutations was not always straightforward; sometimes the descendants of a common ancestor differed by more than one mutation, suggesting that an adoption might have taken place. In two pedigrees, the hypothesis of adoption appeared clearly the most parsimonious, but in one case, it was difficult to judge whether there had been repeated mutations in the same pedigree (#3), or several adoptions, or errors in genealogical reporting. In addition, there is no consensus on whether nucleotide changes at sites known to mutate at

TABLE 1. HVR I motifs and mutations observed in 19 Costa Rican pedigrees

Pedigree	*N* meioses in the pedigree	*N* subjects typed	Common HVR I motif in the typed subjects[a]	*N* mutations observed in HVRI	Birth year of MRCA
1	22	3	111 136 223 290 319 324 362	0	1720
2	11	2	182 183 189 217; 111 223 290 319 362	(9)[b]	1814
3	43	6	183 189 217; 182 183 189 217; 183 189 217 362; 183 189 217 311 362	3 (182, 311, 362)[c]	1600
4	45	6	111 223 290 319 362	0	1660
5	18	4	182 183 189 217 298	1 (131)[d]	1834
6	10	2	111 187 189 223 290 319 352 362	0	1834
7	31	4	111 187 189 223 290 319 352 362	0	1700
8	14	2	111 223 290 319 360 362 381	0	1750
9	17	3	CRS	0	1757
10	9	3	183 189 217	1 (335)	1846
11	10	2	183 189 217	1 (182)	1802
12	7	2	183 189 217	0	1868
13	15	2	183 189 223 325 362	0	1758
14	7	2	183 189 217	0	1845
15	8	3	111 187 189 223 290 319 352 362	0	1848
16	12	2	183 189 217	1 (344)	1794
17	5	3	183 189 217	0[e]	1848
18	11	2	111 223 290 319 362	0	1795
19	5	2	111 223 290 292 319 356 362	0	1876
TOTAL	289	53		7	

[a] Nucleotide position minus 16,000.
[b] Probable adoption; these mutations were not considered in any calculation.
[c] Three mutations or three adoptions needed to account for the presence of these sequences.
[d] This mutation occurs in a stretch of C-nucleotides, often leading to sequencing errors.
[e] Different SNPs outside HVR I; probable adoption.

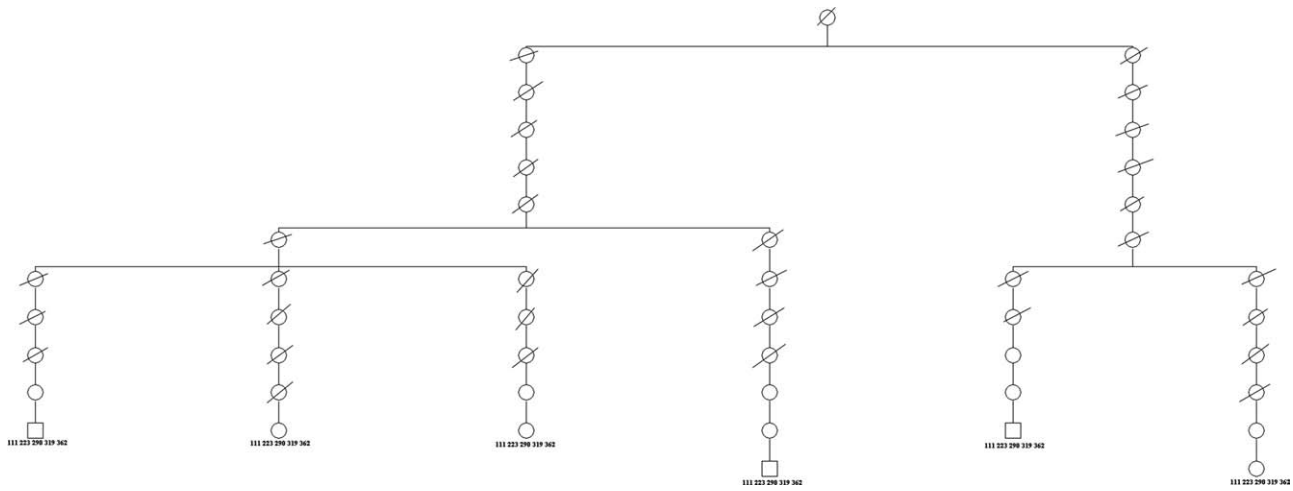

Fig. 1. Pedigree #4. This pedigree shows that no mutations occurred in any of the descendants of the last common ancestor.

a particularly high rate (the so-called "mutational hotspots" or "speedy" sites (Wakeley, 1993; Excoffier and Yang, 1999; Bandelt et al., 2000) should or should not be considered in the calculation of the mutation rate. As a consequence, we chose to compute both a maximum and a minimum estimate of mutation rates and related statistics, respectively with and without the ambiguous pedigree #3 and in the latter case also disregarding mutations occurring at one hotspot.

Point estimates are, of course, only mildly informative. Therefore, we resorted to a Bayesian procedure to estimate an exact confidence interval about them, assuming no prior knowledge, a binomial distribution of the parameter and a beta distribution of its posterior probabilities (Jaynes, 1976).

RESULTS

A total of 19 pedigrees met the requirement that more than one living subject was typed for mtDNA (Table 1). Table 1 also shows the age at birth of the most recent common ancestor. Sequence variations in each individual were scored relative to the revised Cambridge Reference Sequence (Andrews et al., 1999). Typically Amerindian sequences of haplogroups A, B, C, and D represented 89% of the total; typically European sequences of the haplogroups H and J account for a further 10% of the total; one individual sequence belongs to haplogroup L4b2, generally observed in subjects of African ancestry. Figure 1 shows one of the pedigrees (#4) in which no mutations were detected, while Figure 2 shows a

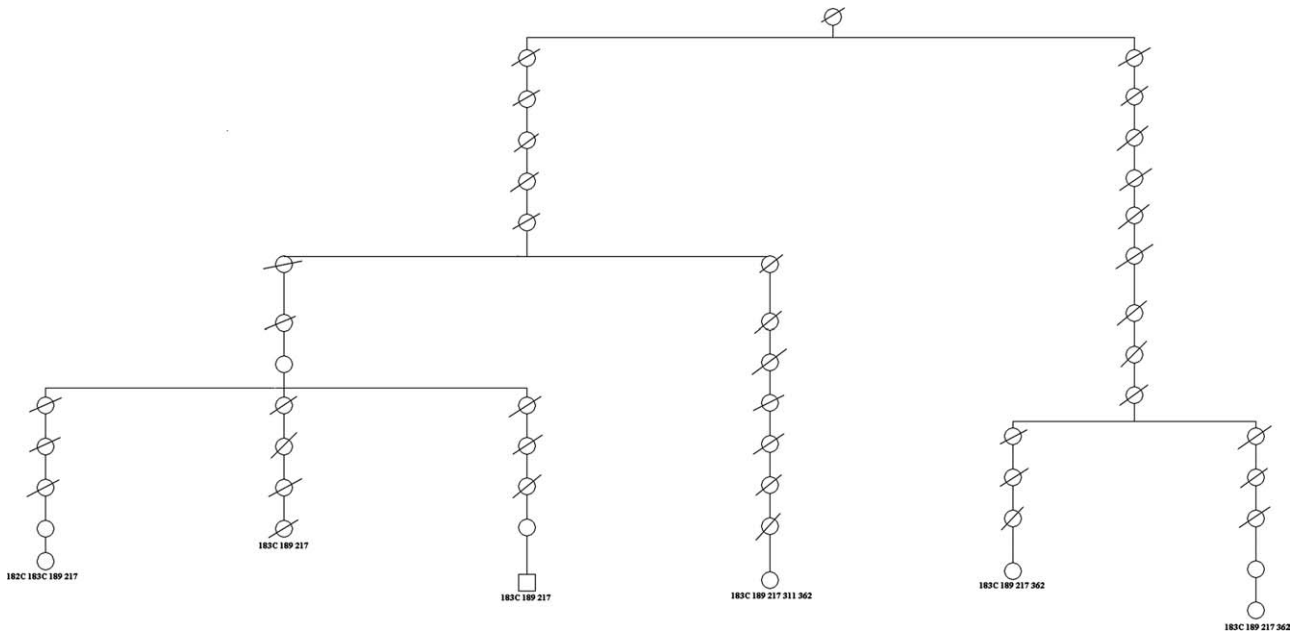

Fig. 2. Pedigree #3. There are three mutations, adoptions or genealogical errors in this pedigree.

pedigree (#3) in which there were either repeated mutations, several adoptions or errors in genealogical reporting. All other pedigrees are shown in Supporting Information Figures S1–S17. The pedigrees with the number of mitosis and mutations involved are listed in Table 1.

In pedigree #2, the two living subjects apparently differ by nine mutations. Clearly, the simplest explanation for that finding is that one of their female ancestors was actually adopted, and hence, we discarded this pedigree. Similarly, in pedigree #17, the three living subjects had the same HVR-I sequence, but one of them differed from the others by mutations at two sites in the coding region (573 nC and 10806Hpal). Therefore, we excluded from the analysis the relevant section of this pedigree, which, accordingly, contributes to further calculations no substitutions and five instead of 10 meiotic events.

After this, we observed a total of seven mutational events (plus one mutation in the coding region) over a total of 299 meioses, occurring at the following sites: 16,131; 16,182 (twice); 16,311; 16,335; 16,344; 16,362. In this and all following cases, the empirical HVR-I mutation rate was estimated by dividing the number of mutations by the number of meiotic divisions, namely $\frac{7}{273} = 0.0256$ per generation. Over the 360 sites of HVR-I, this means 71.1×10^{-6} substitutions per site per generation, and assuming a (female) average generation interval of 28.3 years, 2.51×10^{-6} substitutions per site per year. Our divergence rate is then $= 5.02 \times 10^{-6}$ substitutions per site per year. These values are much higher than those estimated by Howell et al. (2003), and 13–42 times as high as most phylogenetic estimates for HVR-I, all in the range $0.12–0.38 \times 10^{-6}$ substitutions per site per year (Forster et al., 1996; Henn et al., 2009; Ho et al., 2011). These are what we refer to as our maximum estimates.

Three apparent mutational events were observed in the same pedigree, pedigree #3 (shown in Fig. 2), where four different HVR-I motifs were observed, each differing from its nearest neighbor by one substitution. To be sure that no error had occurred in sequencing, all these sequences were independently replicated and confirmed. Therefore, one cannot easily rule out the possibility that what we observed is really the consequence of repeated mutations occurring in the same pedigree. On the other hand, it is also conceivable that this variation might result from adoption of several girls, or from errors in genealogical reporting, or from a combination of all these factors. We had no way to directly test whether that was actually the case, and hence we redid the estimation procedure, eliminating that pedigree from the analysis, thus losing three mutations and 43 meioses. We also considered that nucleotide position 16,182 (pedigree #11) occurs in a stretch rich in C-nucleotides, known to sometimes cause apparent length polymorphism and subsequent typing errors (Bandelt and Kivisild, 2006; Soares et al., 2009). Thus, we excluded this pedigree as well, thus losing one mutation and 10 informative meioses. Finally, of the sites where mutations were observed, Bandelt et al. (2000) report as mutational hot spots (or "speedy" sites) 16,311, 16,335, and 16,362. After elimination of pedigrees #3 and 17, only one such mutation remained (site 16,335) and is likely to inflate the apparent mutation rates. For the estimation of a minimum value of the mutation rate, it seemed logical to treat this mutation as not informative, without disregarding the information represented by the nine meioses in pedigree #10. Therefore, our minimum estimate of the observed mutation rate is $2/220 = 0.0091$, or 25.3×10^{-6} substitutions per site per generation, and 0.89×10^{-6} substitutions per year. Accordingly, the divergence rate became $= 1.78 \times 10^{-6}$ per year.

Table 2 shows the confidence intervals about our two estimates. Based on the pedigrees of this study, and under the most conservative assumptions (i.e., considering the minimum estimate obtained), we can conclude that the HVR-I mutation rate in the Costa Rican population of Atenas has 95% probability to fall between 0.27 and 3.17×10^{-6} substitutions per site per year. In other words, the lower limit of our estimate is approximately twice as high as the most commonly accepted phylogenetic estimate, namely 0.13×10^{-6}.

TABLE 2. Bayesian estimators of the confidence intervals about mutation rates

	Point estimate	0.995 Lower	0.95 Lower	0.95 Upper	0.995 Upper
Max mutation frequency (7/273)[a]	0.0256	0.0084	0.0127	0.0519	0.0652
Min mutation frequency (2/220)[b]	0.0091	0.0012	0.0028	0.0323	0.0450
Max mutation rate[c]	2.51×10^{-6}	0.71×10^{-6}	1.08×10^{-6}	4.40×10^{-6}	5.52×10^{-6}
Min mutation rate[c]	0.89×10^{-6}	0.12×10^{-6}	0.27×10^{-6}	3.17×10^{-6}	4.42×10^{-6}

[a] Excluding pedigrees 2 and 17. Total meioses 289 – 11 – 5 = 273.
[b] Excluding pedigrees 2, 3, 11, and 17, and the dubious mutation at site 16,335. Total meioses 289 – 11 – 43 – 10 – 5 = 220.
[c] Generation interval = 28.3 years.

TABLE 3. Comparison with other pedigree studies

References	Mutations/ generations	Generation interval (years)	Mut. Rate per site per million years	Population studied
Parsons et al., 1997	10/327	20	2.50	European-origin samples
Sigurdarðóttir et al., 2000	5/705	20	0.32	Icelanders
Cavelier et al., 2000	0/292	20	0.00	Swedish
Heyer et al., 2001	4/508	30	0.35	French-Quebecois
Howell et al., 2003[a]	3/263	20	0.51	Unspecified European
Santos et al., 2005[b]	6/321	25	0.24	Azores Islands
This study (minimum estimate)	2/212	28.3	0.92	Costa Rican

[a] There were several estimates in this paper. We report the one inferred from the pooled dataset, referred to as "University of Texas Medical Branch pooled."
[b] Both males and females were considered in the analysis, and so initial estimates of mutation rate were corrected taking into account the impossibility for males to transmit the mutations to the next generation.

TABLE 4. Assumed generation times and assumed HVRI mutation rates in studies of human evolution

References	Generation time	HVRI Mutation rate per site per million years	Subject of the study and time scale
Ray et al., 2003	25	0.13	Pleistocene spatial expansions >110,000 years BP
Destro-Bisol et al., 2004	25	0.042–0.18	Divergence between Pygmies and Bantu, >18,000 years BP
Tishkoff et al., 2007	25	0.10	Demographic history of African click-speakers, >35,000 years BP
Chaix et al., 2008	29	0.35	East-West population expansions in Eurasia, >25,000 years BP
Guimaraes et al., 2009	25	0.05–0.50	Genealogical relationships between Etruscans and Tuscans, >3,000 years BP
Zlojutro et al., 2009	25	0.05–0.50	Upper Paleolithic origin of the Yakuts, >30,000 years BP
de Filippo et al., 2010	25	0.10	African population divergence, >62,500 years BP
Ghirotto et al., 2011	25	0.05–0.50	Genealogical relationships between Neandertals and modern Europeans, >200,000 years BP

DISCUSSION

In this article, we showed that high empirical HVR-I mutation rates are inferred from the analysis of pedigrees of a mixed Central American population. We also found that neither assumptions on the interval between generations nor presence in the pedigree of adopted individuals are possible explanations for the high values observed. Indeed, the generation length for what we consider the most plausible estimate was not assumed, but calculated from the data, and is among the most conservative values used so far (Table 3). Similarly, we eliminated from the sample all instances of possible adoptions, and still the estimates (and their lower confidence limits) remained much higher than those resulting from phylogenetic analyses, and of the same order of magnitude as those inferred from studies of European pedigrees.

In the last decade, the controversy surrounding the rate of mtDNA evolutionary change has increased to include discussions of the rate of change in coding regions (Elson et al., 2004; Kivisild et al., 2006), the role of purifying selection (Soares et al., 2009), the relation between mtDNA phylogeny and pathogenicity of mitochondrial variants (Elson et al., 2004; Mishmar and Zhidkov, 2010), the possible role of climate as a selective force on mtDNA diversity and the relation between time scale and molecular rate estimates (Macaulay et al., 1997; Emerson, 2007; Ho et al., 2007; Endicott et al., 2009). Bandelt (2008) stressed that every individual is likely heteroplasmic, i.e., harbors several related variants; this within-individual variation creates uncertainty about the estimates obtained comparing mother and child. As a matter of fact, the process through which a mutation initially occurring in a single mtDNA molecule reaches fixation in an individual's cells is far from being understood. However, this way heteroplasmy introduces a large bias in the estimates only if one or a few meioses are considered; hence, it can hardly account for the repeatedly observed difference between the estimates coming from phylogenetic and pedigree studies. With one exception (Cavelier et al., 2000) the latter are much higher; our maximum estimate, 2.51×10^{-6} is very close to the highest value ever reported (Parsons et al., 1997),

whereas our minimum estimate is a still high 0.92 per site per million years.

Whether or not, and to what extent, it is legitimate to extrapolate rates of change estimated over a few generations across different evolutionary timescales is open to discussion. However, our results clearly support previous proposals suggesting that the apparent rate of change at the microevolutionary level is faster than that seen at a macroevolutionary level. Two open questions, then, are why different approaches lead to such different results, and which values represent a plausible approximation for dating events, or for simulation studies, when mutation rates cannot be estimated from the data. Our pedigrees do not allow us to speculate on the reasons for the disparity of estimates based on the two methods, but, as for the second question, it seems reasonable that the time-depth of the study might dictate the choice of the appropriate mutation rate, or at least that a range of values should be considered.

That does not seem to be currently the case. Table 4 shows the values chosen by several authors in studies at different time-scales, from 3,000 to several hundred thousand years. In most such studies, mitochondrial gene genealogies, either reconstructed from data on current variation or generated by computer simulation, were interpreted under the assumption that mutation rates are low or very low. Only in a subset of studies was a range of possible values explored (Destro-Bisol et al., 2004; Guimaraes et al., 2009; Zlojutro et al., 2009). However, in one such case (Destro-Bisol et al., 2004), the maximum mutation rate considered was well below the values estimated in almost all pedigree-based studies.

Until the causes of the discrepancy between mutation rates estimates will be fully understood, our study and similar pedigree-based analyses suggest at least that HVR-I mutation rates around 0.10 per site per million years should be taken with a grain of salt. By making rigid assumptions based on that figure, one radically dismisses the empirical evidence available in favor of a faster-ticking evolutionary clock. This way, the risk is to erroneously locate in a remote past relatively recent events, thus distorting our perception of our evolutionary trajectory. At present, a prudent choice seems then to consider in the analyses both a slow- and a fast-ticking mitochondrial clock, evaluating the evolutionary consequences of the models under a broad range of assumptions.

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