Counterbalancing the time-dependence effect on the Human Mitochondrial DNA Molecular Clock

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Background: The molecular clock is the most important genetic tool to estimate evolutionary timescales. However, the detection of a time dependency effect on the mutation rate estimates is complicating its application. It has been suggested that demographic processes could be the main cause of this confounding effect. In the present study I propose a new algorithm to estimate the coalescent age of phylogenetically related sequences, taking into account the observed time dependency effect on the molecular rate detected by others.

Results: Applying this method to real human mitochondrial DNA trees, with shallow and deep topologies, I have obtained significantly older molecular ages for the main events of human evolution than in previous estimates. These ages are in close agreement with the most recent archaeological and paleontological records that are in favor of an emergence of early anatomically modern humans in Africa at 315 ± 34
thousand years ago and the presence of recent modern humans out of Africa as early as 174 ± 48 thousand years ago. Furthermore, in the implementation process, we demonstrated that in a population with fluctuating sizes, the probability of fixation of a new neutral mutant depends on the effective population size which is more in accordance with the fact that, under the neutral theory of molecular evolution, the fate of a molecular mutation is mainly determined by random drift.

**Conclusions:** I suggest that the demographic history of populations has a more decisive effect than purifying selection and/or mutational saturation on the time dependence effect observed for the substitution rate.

**Introduction**

During the last three decades, the application of mitochondrial DNA (mtDNA) variation to studies of human evolution has dominated the scientific scenario. Recently, this small molecule is being substituted by the analysis of whole genomes. However, before turning the page, it would be convenient to solve the patent contradictions between the mtDNA molecular clock time estimations and those lately proposed by paleontological and archaeological data. A key achievement of early mtDNA analyses was the dating and origin of the most recent common female ancestor of all living women around 200 kya in Africa [1]. However, recently, hominin fossils and associated Middle Stone Age artifacts from Jebel Irhoud in Morocco have been aged at 315 ± 34 kya [2]. These older dates were genetically confirmed in a study of ancient African genomes that estimated modern human divergence at 350 to 260 kya [3]. Another controversial milestone of the mtDNA molecular clock was the dating, based on the coalescence age of macrohaplogroup L3, of the
dispersal of modern humans out of Africa 50 to 70 kya [4]. This is against the presence of early modern human remains in the Levant at the Skhul and Qafzeh caves dated at about 80-120 kya [5], the presence of middle stone age industries in the Arabian peninsula with similar dates around 80-130 kya [6–8], the recent discovery in southern China of unequivocally modern human teeth dated to 80-120 kya [9], or the lately reported detection of an ancient gene flow from early modern humans into the ancestors of eastern Neanderthals more than 100 kya [10]. Furthermore, the most recent finding of a *Homo sapiens* maxilla found at Misliya Cave, Israel, dated to 177-194 kya [11], could significantly anticipate the exit of *Homo sapiens* from Africa. Curiously, these dates within the MIS-7 last interstadial, are in agreement with the age proposed for an ancient African hominin introgression into the European Neanderthals [12]. In addition, OSL dating of stratigraphic undisturbed basal stone tool assemblages in Madjedbebe [13] placed the human colonization of Australia around 65 kya with minor age uncertainties of only ± 3-4 kyr. The above date is significantly older than the 43-47 kya coalescence age estimated recently from Australian aboriginal mitogenomes [14] Evidently, under the actual molecular rate estimates given for different sequences of the human genome, and the accepted constancy of the molecular clock along the time, all these older archaeological and fossil dates have to represent failed dispersals that, therefore, did not contribute to the present-day genetic pool of modern humans. In this paper I try to demonstrate that these molecular constrains could be released giving a significantly wider molecular age window.

**Results and Discussion**

**The Human Mitochondrial mutation rate.** The efficiency of the molecular clock [15] is based on the reliability of several implicit assumptions as a) the correctness of the mutation rate ($\mu$) of the gene under
study; b) constancy with time of the rate of molecular evolution (Θ) and c) rate homogeneity among the different lineages involved in the phylogeny. To accomplish the first point, in this study I have used the full-length mtDNA germ-line mutation rate of \(1.3 \times 10^{-8}\) mutations per site per year (assuming a generation time of 20 years) and its derived rate scalar of one mutation every 4651 years estimated by others [16]. This mtDNA mutation rate is about ten times lower than estimates in most pedigree studies which the authors explain because, at analyzing two tissues, they could discard somatic heteroplasmies. In this respect, it has to be mentioned that second-generation massive sequencing has made possible the direct calculation of the germ-line human genomic mutation rate which resulted in half of the phylogenetic mutation rate thus, doubling the estimated divergence dates of Africans and proposing that crucial events in human evolution have occurred earlier than suggested previously [17].

**Time-Dependence of the Rate of Molecular Evolution.** It is well known that rates of molecular evolution are not constant neither at interspecific nor intraspecific levels [18]. In general, they decline with increasing divergence time, but the rate of decay differs among taxa. This time-dependent pattern has also been observed for human mtDNA in coding as well as non-coding regions [19, 20]. Purifying selection on deleterious mutations and mutation saturation has been suggested as the main forces responsible for this time rate decay [20]. However, the unrealistic large effective population sizes required to explain the long-term persistence of significantly deleterious mutations cast doubts on whether purifying selection alone can explain the observed rate acceleration [21]. It has also been found unlikely that the apparent decline in rates over time is due to mutational saturation [20]. Congruently, correcting for the effects of purifying selection and saturation has only slightly modified the mtDNA evolutionary mutation rate,
providing molecular times still in apparent contradiction with archaeological and paleontological ages [20, 22]. Demographic processes such as serial bottlenecks and expansions have also been proposed to explain the differences in rate estimation over time [19]. It seems evident that some adjustment should be implemented to correct the time dependency of the molecular clock [23]. In this paper, I propose a practical approach to counteract the time dependency effect on the molecular rate estimates.

**Lack of Mutation Rate homogeneity between Lineages.** Since early molecular analyses, it was observed that rates of homologous nuclear DNA sequence evolution differ between taxonomic groups [24] which was also extensible to mtDNA [25]. Later, significant differences in rates of molecular evolution between mtDNA human lineages were also detected at haplogroup level [26–30]. Different relaxed molecular-clock methods have been implemented to incorporate rate variation among lineages [31, 32]. However, the application of these methods to the human mtDNA has yield age estimates for the main milestones of human evolution in agreement with previous molecular estimates [33, 34]. In this paper, when distributing mutations of lineages with significant rate differences within coalescent periods, I have used a simple proportionality criterion. I allowed a window from 0 to 5 mutations between sequences within coalescent periods, as it has been demonstrated that, under a Poisson distribution, even in an extended period of 10,000 years, there could be lineages still carrying the same mutations that their common ancestor, and lineages that have accumulated five new mutations with probabilities higher than 0.05 percent [35]. Another technical problem is the mutation distribution into coalescent periods of those isolated sequences that directly radiate from ancestral nodes. To resolve this issue I have used a weighted distribution, consisting
in multiplying the number of mutations in the isolates by the number of
mutations between internodes in each coalescent period, and then to divide
the result by the total number of mutations in all the internodes.

**Fluctuating Population Size effect on Mutation Substitution Rate.**

In a population, the fixation time of a mutation (forward) or the
coalescence time to the most recent common ancestor (backward), is
usually calculated by the estimator $\Theta = 4N_e\mu$ (being $N_e$ the effective
population size). For the haploid mtDNA genome, $\Theta$ equals to $N_{ef}\mu$ (being
$N_{ef}$ the female effective population size). Long time ago Kimura
demonstrated that under strict neutral theory parameters, the rate of
substitution is equated to the mutation rate [36]. However, the same author
warned us that a clear distinction exists between mutation rate ($\mu$) and
mutation substitution ($\Theta$). The former refers to the change of genetic
material at the individual level, and the latter refers to that at the population
level [37]. Thus, only when $N_e$ is constant across generations, keeping
small or large sizes, $\Theta$ equals to $\mu$. This holds because with large constant
sizes the number of new mutations incorporated into the population ($N_e\mu$)
increases but, on the same path, the probability of fixation ($1/N_e$)
decreases. Contrarily, with small constant sizes, the number of new
mutations decreases but the probability of fixation of any of them increases
at a similar level. It is widely admitted that $N$ fluctuated largely during the
human history and that global exponential population growth is happening
since recent times [23]. In this paper, I have taken into account the changes
in population size that occurred backward in time and its influence on the
rate of gene substitution. When the population size fluctuates across
generations, the probability of fixation of a mtDNA neutral variant ($q$) is no
longer the $1/N$ constant. It will depend on the difference in population size
of the next generation ($N_1$) with respect to the initial size ($N_0$):
\[ q = \frac{N_1}{N_0} \times \frac{1}{N_0} \]

For example, if \( N_1 \) is twice the size of \( N_0 \), \( q \) equals \( 2/N_0 \) and, on the contrary, if \( N_1 \) is half the size of \( N_0 \), \( q \) equals \( 1/2N_0 \). As a consequence, the rate of substitution for neutral mutations in a population with fluctuating size depends on the change in size between generations:

\[ \Theta = \frac{N_1}{N_0} \times \mu \]

Using a different approach, the dependence on population size of the substitution rate at neutral genes was already demonstrated for populations with fluctuating sizes and overlapping generations [38].

As human populations have been growing exponentially for several centuries, we should counterbalance this effect from the present-day generation (\( N_n \)) going backward in time by inverting the fraction between consecutive generations (\( N_{n-1}/N_n \)). Notice that this dependence might explain the differences in rate estimation over time observed empirically (Henn et al. 2009). I will take into consideration this important relationship for the calculation of \( \Theta \).

**A new Rho Statistic to estimate Coalescent Ages.** Several statistics, based on DNA polymorphism, exist to estimate the parameter \( \Theta \). One, \( S \), the number of segregating sites per nucleotide in a sample of sequences [39] is strongly dependent on the sample size. A second, \( \pi \), is defined as the average number of nucleotide differences per site in a sample of sequences [40]. These two estimators were used to implement a statistical method for testing the neutral mutation hypothesis [41]. A third statistic, rho (\( \rho \)), is referred to as the mean number of nucleotide differences of a sample of sequences compared to their common ancestral type [42]. This last statistic is calculated from a rooted phylogenetic tree relating the sampled
sequences. The accuracy of molecular dating with the rho statistic has been questioned by ones, because it shows downward biased data estimations, large asymmetric variances and strong dependency of demographic factors [43], but defended by others [44]. Anyway, it is still the most used method to measure intraspecific mtDNA divergence events in humans. Although the distribution of pairwise nucleotide site differences between individuals have been used to detected episodes of population growth and decline [45], none of the above mentioned statistics considers the past demography of the sample in their age estimations. In this paper, I propose the use of a modified rho ($\rho_m$) that, taking into account the coalescent genealogical structure, significantly improves the molecular date estimation of key events in the human history based on mtDNA genome data. In order to make explicit our modifications to the classical rho, I have depicted, in figure 1, a real genealogy constructed from five lineages (a), and an idealized star-like phylogeny of the same five lineages (b) supposing a population exponential growth short after a severe bottleneck [46]. The number of lineages sampled is represented by $n_i$; $t_i$ are the time periods defined by progressive coalescent events from the tips to the MRCA root; $i$ represents the number of independent lineages left after successive coalescences, $\Upsilon_i$ is the number of mutations accumulated during each coalescent period, and $m_i$ the number of mutations accumulated along each lineage. Mutations in the star-like phylogeny are distributed into periods following the pattern found in the real phylogeny. As the accumulation of mutations along each lineage is an individual process driven by the mutation rate, $\mu$, and distributed as independent Poisson processes, $\rho$, the average number of mutations per lineage, has the same value irrespective of the topology. However, as a consequence of the fact that the lineages in the real tree are not independent, because of their shared genealogy, the variance decay is much slower ($1/\log n$) than in the independent star-like
As a consequence of this, for the rho calculation, mutations within lineages in the star-like phylogeny are counted only once. On the contrary, in the real phylogeny, only mutations occurring at the tips are counted only once while mutations in subsequent coalescent periods going backward to the MRCA node are counted as many times as the number of the period to which they belong. For this reason, mutations in the older periods are overrepresented in the rho calculation. Because of lineage independence, star-like phylogenies are statistically optimal to calculate \( \rho \) and \( \pi \) estimators. Under this topology, as mutations along lineages are counted from the root to the tips in \( \rho \) and from tip to tip in \( \pi \) pairwise comparisons, the value of \( \pi \) doubles that of \( \rho \). Thus, to correct for dependence in the real tree, I propose a modified rho statistic (\( \rho_m \)) that is the summation of classical rho statistics calculated for each coalescent period in the tree:

\[
\rho_m = \sum_{i=2}^{\tau} \rho^i
\]

This compound Poisson distribution is also Poisson distributed, therefore mean and variance are equal and, the standard deviation is the square root of this variance. Another important difference between the real and star-like genealogies is that in the first the number of lineages decreases as one stepwise function across coalescent periods while in the second, the number of lineages is constant until the root is reached. Equating the number of lineages in the sample as an approximation to the effective population size in the population, we should take into account this backward real decrease in \( N_e \) to improve the estimation of the MRCA age. In an ideal coalescent model we should have i-1 population decreasing sizes, but in real phylogenies, in addition to bifurcations, there are also multifurcations and lineages with long internal segments without any branching event. Even so, I applied to each rho in consecutive periods
going backward the reverse proportion used to counteract the
dependence effect on the evolutionary rate. That is, multiplying in each i-1
period the mutation rate \( \mu \) by \((i-1)/i\) and leaving the \( \mu \) rate as calculated
from germ-line estimations for the most recent period, comprising the tips
of all the lineages sampled. With this method, I have obtained a time-
dependent scaled mutation rate, \( \Theta \), which gave human mtDNA intraspecific
ages congruent with the archaeological and paleontological calibrated
nodes representing key events in the human history.

\[
\Theta = \frac{1}{\mu} \left( \rho_n + \sum_{i=2}^{n-1} \frac{i+1}{i} \rho_i \right)
\]

Performing calculations on the empirical genealogy (Figure 1a), I have
obtained an age of 22 277 ± 4 720 years using the standard \( \rho \) and age of 30
396 ± 5 513 years, 1.36 times greater, when using the time-dependent \( \Theta \)
estimator proposed here (Table 1).

**Application of the new Rho Statistic to the main Human Evolution**

**Events.** To apply this statistic to real mtDNA human data we need a rooted
tree relating the sampled sequences. Using coalescent methodology we
could obtain a probabilistic tree. However, in the case of human mtDNA,
we have a very much contrasted phylogenetic tree [48], constructed using
the Network program [49]. In it, mutations are placed hierarchically from
the tips to the root and multiple hits, identified by network reticulations,
have been resolved attending to the relative mutation rate of the positions
involved [22]. Thus, following this standard, I constructed an African
mtDNA genome-based phylogenetic tree, using 86 previously published
complete mtDNA sequences, in which all the main African haplogroups are
represented (Figure S1). Likewise, using 142 already published complete
mtDNA sequences, I constructed a second phylogenetic tree for the
Australasian specific haplogroup P (Figure S2). Finally, to test a more
recent human colonization, I constructed a third tree including 48 already published complete mtDNA sequences belonging to the Americas specific haplogroup B2 (Figure S3). Using these trees, I applied the proposed time-dependent based estimator to calculate coalescence ages of several essential nodes of the human history (Tables S1 to S11). I found a TMRCA for all the extant human African mtDNAs of 315 801 ± 17 827 years (Table 2 and S2) which is highly compatible with the recent archaeological and paleontological estimations of modern human origin around 315 000 ya [2, 50]. It has been proposed an early out-of-Africa of modern humans recently, carrying haplogroup L3 precursor lineages, in a favorable time window around 125 000 ya [51], that is in harmony with the age calculated here for the L3′4 split in Africa of 165 610 ± 12 869 ya as a lower bound (Tables 2 and S3). Furthermore, this age frame is compatible with the presence of modern humans in the Levant [5] and in China [9] around 100 000 ya. In the same paper, a return to Africa of basal L3 lineages over 75kya was also suggested. Again, the age for the L3 African expansion calculated with the method reported here of 112 829 ± 10 622 ya makes this suggestion feasible (Tables 2 and S4). Furthermore, under this new temporal window the great morphological variability of the Skhul/Qazfeh remains and their corresponding wide range of ages (120-80 kya), could easily fit into the whole molecular period proposed elsewhere [51], beginning with the out-of-Africa of early modern humans and finishing with their early return to the same Continent, carrying basic L3 lineages (125-75 kya). However, notice that a return to Africa from the Arabian Peninsula would also be supported by the dates estimated from the archaeological record of the region. On the other hand, our TMRCA for the Australasian haplogroup P (103 267 ± 10 332 ya) is also in agreement with an early presence of modern humans in Asia (Tables 2 and S6, S7, S8, S9). Thus, it represents a lower bound for the colonization of Philippines [52],
Sumatra [53] and Australia [13] around 65,000 to 73,000 ya. It has to be
mentioned that from the genome sequencing of an Aboriginal Australian
[54], it was deduced that Aboriginal Australians are descendants of human
dispersal into Eastern Asia that occurred about 62-75 kya. Finally, the age
of human expansion into the American Continent, deduced from the
haplogroup B2 phylogeny, is around 37,000 ya (Table 2, and table S11).
This age is in support of a pre-Clovis occupation of the New World, well
before the last glacial maximum. As the calculated ages have wide
statistical confidence intervals (Table 2), different models could be
adjusted into their frames. For example, we might suppose an earlier out of
Africa matching the Misliya maxilla dated to 177-194 kya, then, the Skhul
and Qafzeh remains, dated around 80-130 kya might signal the return to
Africa of the carriers of the mtDNA basal haplogroup L3 lineages instead
of the out of Africa of early anatomically modern humans as proposed here.
In the same way, the controversial presence of *H. sapiens* in Java [55] and
Sulawesi [56] as early as 120 kya would fit into the window age of the
Australasian mtDNA haplogroup P (Table 2). Sure, future archaeological
discoveries and more precise fossil dating will outline the most appropriate
model.

**Conclusions**

Taking into account the time-dependence of the mtDNA evolutive rate in
humans as proposed here, and choosing a conservative mtDNA germ-line
mutation rate as experimentally obtained by others [16], has significantly
delayed the mtDNA molecular clock in humans, in such a way that all the
main events of the human history, dated by paleontological and
archaeological methods, fit in this new mtDNA temporal scale without the
necessity of external node calibrations.

**Ethics approval and consent to participate:** Not applicable

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Consent for publication: Not appliable

Availability of data and materials: All data analyzed during this study are included in this article and its Supplementary Information files.

Competing interests: The author declare he has no competing interests.

Funding: Not applicable.

Author’s contributions: VMC is the sole author of this article.

Acknowledgements: Not applicable.

Author’s information: The author, VMC, is actually retired.

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Figure legend

Figure 1 legend: a) Empirical coalescent tree of five lineages (a, to e), with four coalescent periods (t) and mutations along branches (numbers). b) Ideal star-like tree for the same five lineages.

Tables

**Table 1: Coalescence age for Figure 1 Tree using the compound rho**

| Period | Lineages | Mutation | Rho  | i/i + 1 | μ    | 1/μ  | Years |
|--------|----------|----------|------|---------|------|------|-------|
| 2      | 2        | 3        | 1.50 | 0.67    | 1.44 | 6944 | 10 416 |
|        |          |          |      |         | 10-4 |      |       |
| 3      | 3        | 6        | 2.00 | 0.75    | 1.61 | 6211 | 12 422 |
|        |          |          |      |         | 10-4 |      |       |
| 4      | 4        | 2        | 0.50 | 0.80    | 1.72 | 5814 | 2 907  |
|        |          |          |      |         | 10-4 |      |       |
| 5      | 5        | 5        | 1.00 | 1.00    | 2.15 | 4651 | 4 651  |

Coalescence age for Figure 1 tree: 30 396 ± 5 513
| Haplogroup split | Evolutive event | Mean age in years | 95% Coefficient interval |
|------------------|----------------|-------------------|-------------------------|
| L0/L1’2’5’6’4’3  | Most recent African common ancestor | 317 814 ya | (352 755-282 873 ya) |
| L3’4             | Out of Africa | 165 610 ya | (190 833-140 387 ya) |
| L3               | Return to Africa of L3 | 112 829 ya | (133 648-92 010 ya) |
| P                | Reaching the Pacific | 106 752 ya | (127 003-86 501 ya) |
| P                | Reaching Australia | 108 034 ya | (128 406-87 662 ya) |
| P                | Reaching Philippines | 111 545 ya | (132 244-90 846 ya) |
| P                | Reaching New Guinea | 112 070 ya | (132 818-91 322 ya) |
| B2               | Expansion Americas | 37 701 ya | (49 735-25 667 ya) |