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Time of the Deepest Root for Polymorphism in Human Mitochondrial DNA

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Summary. A molecular clock analysis was carried out on the nucleotide sequences of parts of the major noncoding region of mitochondrial DNA (mtDNA) from the major geographic populations of humans. Dates of branchings in the mtDNA tree among humans were estimated with an improved maximum likelihood method. Two species of chimpanzees were used as an outgroup, and the mtDNA clock was calibrated by assuming that the chimpanzee/human split occurred 4 million years ago, following our earlier works. A model of homogeneous evolution among sites does not fit well with the data even within hypervariable segments, and hence an additional parameter that represents a proportion of variable sites was introduced. Taking account of this heterogeneity among sites, the date for the deepest root of the mtDNA tree among humans was estimated to be 280,000 ± 50,000 years old (±1 SE), although there remains uncertainty about the constancy of the evolutionary rate among lineages. The evolutionary rate of the most rapidly evolving sites in mtDNA was estimated to be more than 100 times greater than that of a nuclear pseudogene.

Key words: Nucleotide sequences — Major noncoding region — Evolutionary rates — Molecular clock — Rate heterogeneity among sites — Effective proportion of variable sites — Maximum likelihood

Introduction

From restriction enzyme analysis of mitochondrial DNA (mtDNA) from major human races, several authors suggested that the time of the deepest root of the mtDNA tree of humans was about 200,000 years ago (Brown 1980; Johnson et al. 1983; Horai et al. 1986; Cann et al. 1987). The restriction enzyme analysis, however, is subject to several ambiguities in estimating genetic distances (Kocher et al. 1989).

Recently, Vigilant et al. (1989) challenged the problem by directly sequencing 740 nucleotides in the major noncoding region (which they called the control region) of mtDNAs of 83 individuals from the major geographic populations. They estimated that the deepest root of the mtDNA tree of humans was about 238,000 years ago, consistent with the previous estimates from restriction enzyme analysis. The human/chimpanzee divergence was taken as a reference to calibrate the clock, which suggested that a large number of multiple substitutions had taken place between these two species. How Vigilant et al. (1989) corrected for multiple substitutions, however, is approximate and it seems to be worthwhile to reanalyze their data by a more rigorous statistical method.

Furthermore, Horai and Hayasaka (1990) sequenced at least 482 nucleotides in the major noncoding region that partially overlaps with the region sequenced by Vigilant et al. (1989) from 95 individuals of major races. In the present work, by using a generalized least-squares method developed by Hasegawa et al. (1985) and by Kishino and Hasegawa (1990), we have analyzed the sequence data from these two papers in addition to those of Anderson et al. (1981) who sequenced the complete mtDNA genome from a European, to those of Greenberg et al. (1983) who sequenced most of the

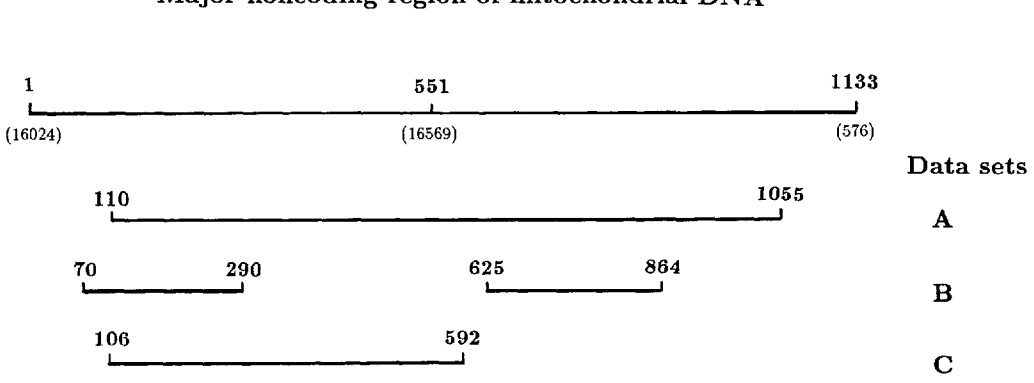

Fig. 1. Parts of the major noncoding region used in this work. The numbering of nucleotides follows Foran et al. (1988), and Anderson et al.'s (1981) numbering is given in parentheses.

Table 1. Three data sets used in the analysis

Individuals sequenced	Geographic origin	Data set A	B	C
Common chimpanzee[a]	Africa	+	+	+
Pygmy chimpanzee[a]	Africa	+	+	+
ANDER[b]	Europe	+	+	+
DCK1[c]	Africa	+	+	+
CDK1[c]	Africa	+		
CJK5[c]	Africa	+		
!Kung5[d]	Africa		+	
!Kung11[d]	Africa		+	
!Kung12[d]	Africa		+	
SB17[e]	Africa			+
MS10[e]	Japan			+
MS14[e]	Japan			+
MS24[e]	Japan			+
Number of nucleotide sites[f]		921	449	475

References for sequence data are as follows: [a] Foran et al. (1988), [b] Anderson et al. (1981), [c] Greenberg et al. (1983), [d] Vigilant et al. (1989), [e] Horai and Hayasaka (1990)
[f] Sites that experienced deletion or insertion are excluded

major noncoding region of five individuals from Europe and Africa, and to those from the common chimpanzee and pygmy chimpanzee sequenced by Foran et al. (1988), and we have estimated the time of the deepest root of the mtDNA tree of humans.

Materials and Methods

Sequence Data. The data used in this study are nucleotide sequences of the major noncoding region of mtDNA. Data from the common chimpanzee and pygmy chimpanzee sequenced by Foran et al. (1988) are used as the outgroup. The regions covered by the three data sets used in this work are shown in Fig. 1. Designations of individual mtDNAs sampled in the three data sets are given in Table 1.

Methods. A statistical method used in this paper was developed by Hasegawa et al. (1985) and by Kishino and Hasegawa (1990). To calibrate the mtDNA molecular clock, the branching between humans and chimpanzees is taken as a reference. Under the assumption that the orangutan diverged from the African apes–human clade 13 million years (Myr) ago, the mtDNA data of 896 nucleotides sequenced by Brown et al. (1982) gave a date of 3.9 ± 0.7 Myr (± refers to 1 SE) for this branching (Hasegawa et al. 1990), and the small rRNA genes of mtDNA sequenced by Hixson and Brown (1986) gave a date of 4.2 ± 1.3 Myr (Hasegawa and Kishino 1990). In this work, therefore, the human/chimpanzee branching is assumed to be 4 Myr ago.

As was discussed by Greenberg et al. (1983) and by Horai and Hayasaka (1990), it is apparent that not all the sites in the major noncoding region are equally variable. Actually there must be several different stages of variability among nucleotide sites. But we simplify the problem by assuming that fraction f of nucleotide sites is equally variable and the remaining sites are invariable in the time scale under consideration. Furthermore we assume that each variable site evolves independently from other sites according to a Markov process in which a nucleotide x (T, C, A, or G) is replaced by another nucleotide y in an infinitesimally short time interval, dt, with a probability of

$$P_{xy}(dt) = \begin{cases} \alpha\pi_y dt & \text{(for transition)} \\ \beta\pi_y dt & \text{(for transversion)} \end{cases} \quad (1)$$

where π_y is the frequency of nucleotide y, and α and β are parameters that determine transition rate and transversion rate, respectively (Hasegawa et al. 1985). These parameters are assumed to be equal among different lineages. Another Markov model approach has been proposed by Lanave et al. (1984).

We consider n homologous nucleotide sequences that consist of r nucleotides. Between every possible pair among n sequences [$n(n-1)/2$ pairs], we count numbers of transversion differences, V_{ij} ($i = 1, \ldots, n-1; j = i+1, \ldots, n$), and those of transition differences, S_{ij}, and construct a vector $\mathbf{D} = [V_{12}, \ldots, V_{1n}, V_{23}, \ldots, V_{2n}, \ldots, V_{(n-1)n}, S_{12}, \ldots, S_{1n}, S_{23}, \ldots, S_{2n}, \ldots, S_{(n-1)n}]$. The distribution of this vector can be approximated by a multivariate normal distribution represented as follows,

$$\mathbf{D} \sim N(\bar{\mathbf{D}}, \Omega)$$

where $\bar{\mathbf{D}} = [\overline{V_{12}}, \ldots, \overline{V_{1n}}, \overline{V_{23}}, \ldots, \overline{V_{2n}}, \ldots, \overline{V_{(n-1)n}}, \overline{S_{12}}, \ldots, \overline{S_{1n}}, \overline{S_{23}}, \ldots, \overline{S_{2n}}, \ldots, \overline{S_{(n-1)n}}]$ and Ω is the variance–covariance matrix (see Hasegawa et al. 1985). We can determine the maximum likelihood estimates of the parameters, that is, f, α, β, and t_k's (date of the kth splitting; $k \neq c$, where the cth splitting is a reference point for the calibration of the clock) by minimizing (generalized least-squares method)

$$R(\mathbf{D}, \Theta) = [\mathbf{D} - \overline{\mathbf{D}(\Theta)}]^T \Omega^{-1} [\mathbf{D} - \overline{\mathbf{D}(\Theta)}], \quad (2)$$

where $\Theta = [t_k\text{'s}\,(k \neq c), f, \alpha, \beta]^T$ (superscript T denotes a transposed vector) and Ω is given by Kishino and Hasegawa's (1990) pro-

cedure. The variance of the estimates of the parameter Θ is given by Eq. (17) in Hasegawa et al. (1985).

Compared to the conventional model of homogeneity among different sites, the inhomogeneity model that is specified by introducing an additional parameter f should always improve, or at least not make worse, the apparent fit of the model to the data, because the latter model has a greater degree of freedom. However, we cannot accept a model with an unnecessarily large number of parameters as an appropriate model. A penalty should be imposed for increasing the number of parameters. Based on theoretical information, Akaike (1974) showed that, in comparing the goodness of the approximation of models with different numbers of parameters, AIC (*A*kaike *I*nformation *C*riterion) defined by

$$\text{AIC} = -2(\text{maximum log-likelihood}) + 2\,(\text{number of parameters}) \quad (3)$$

provides a good criterion of model selection. The better the fit of the model to the data, the lower is the first term. On the other hand, the more complex the model, the higher is the second term. A model that minimizes AIC is considered to be the most appropriate model (Akaike 1974; Hasegawa et al. 1990; Kishino and Hasegawa 1990). By using AIC, we can determine whether or not we should introduce an additional parameter f.

Results and Discussion

Branching Order of Individual Human mtDNAs

Branching orders are inferred at first by the maximum likelihood method of Felsenstein (1981) (DNAML in Felsenstein's program package PHYLIP ver. 3.1). When there are alternative trees whose likelihoods are nearly the same as that of the maximum likelihood tree, as is mostly the case for these data, AIC under the constant rate model given by Kishino and Hasegawa (1990) is used in selecting a tree among the alternatives. When branching order seems to be ambiguous, trifurcation or quadrifurcation models are compared with bifurcation ones by using AIC. The trees inferred by these procedures are shown in Fig. 2.

Vigilant et al. (1989) rooted the mtDNA tree of humans by using the parsimony method and by using the common chimpanzee as an outgroup. The parsimony method as well as the maximum likelihood method of Felsenstein (1981) cannot determine placement of the root unless a proper outgroup is provided. From the data for a short stretch of sequences, chimpanzees might be too remotely related to humans to be used as an outgroup for rooting the human tree (the human/chimpanzee divergence is more than 10 times the divergence within humans). In this situation, the branching order such as ((((A, B), C), D), O) (O: outgroup) can be roughly equivalent to ((((D, C), B), A), O) by these methods, even though these two trees differ drastically from the biological point of view. Therefore confidence limits for the inferred tree must be evaluated. When

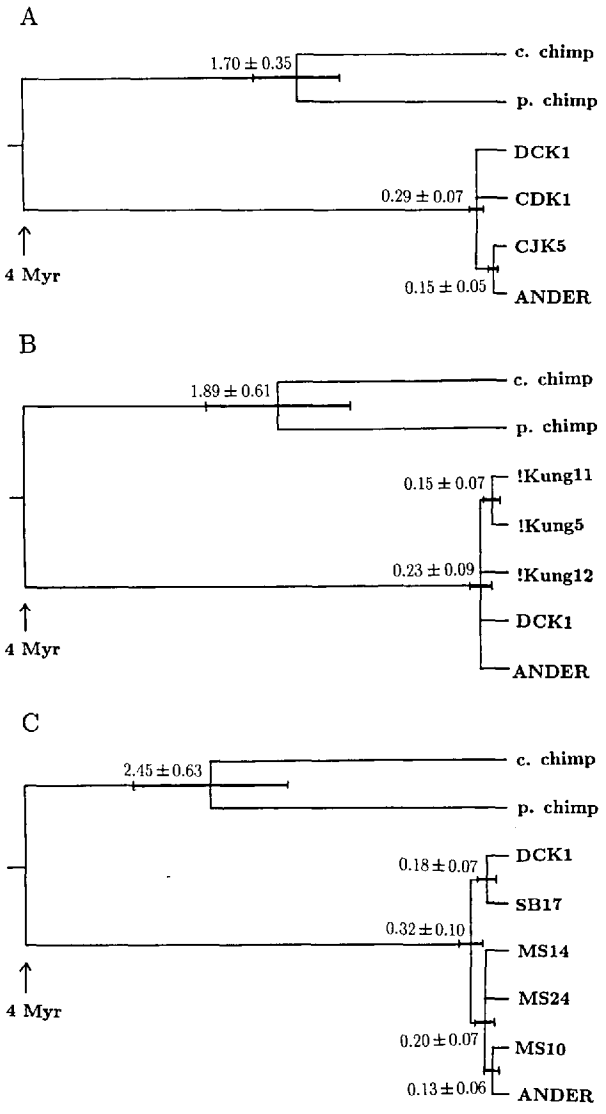

Fig. 2. Three mtDNA trees inferred from data sets A, B, and C. Branching dates were estimated from molecular clocks calibrated by assuming human/chimpanzee splitting to be 4 Myr ago (± refers to 1 SE). The 68% confidence intervals of the estimates are shown by |——|. Abbreviations: c. chimp, common chimpanzee; p. chimp, pygmy chimpanzee.

the significance level discriminating between these trees is low, it seems justifiable to select a tree by using AIC based on the constant rate model presented in the Methods section.

Proportion of Variable Sites

Generalized least-squares fittings are shown in Fig. 3 for data set A. Theoretical curves of the best fitting f as well as those for $f = 1$ (homogeneous evolution among different sites) are shown. It is apparent that for $f = 1$ the difference points (V_{ij}/r, S_{ij}/r) between humans and chimpanzees locate on the lower-right side of the theoretical curve (expected from the model) and those among humans locate on the upper-left side, that is, the numbers of transversion

Table 2. Divergence times and rates of change in parts of the major noncoding region

Parameters	Estimates based on three data sets					
	A		B		C	
f	0.27	1	0.31	1	0.27	1
AIC	86.05	91.12	63.91	71.79	60.09	62.20
t_1 (Myr)	1.70 ± 0.35	2.61 ± 0.27	1.89 ± 0.61	3.18 ± 0.42	2.45 ± 0.63	3.15 ± 0.40
t_2 (Myr)	0.29 ± 0.07	0.60 ± 0.12	0.23 ± 0.09	0.74 ± 0.17	0.32 ± 0.10	0.67 ± 0.17
$\hat{v}_S$ (Myr^{-1})	0.157 ± 0.036	0.016 ± 0.001	0.265 ± 0.116	0.020 ± 0.002	0.158 ± 0.052	0.016 ± 0.002
$\hat{v}_V$ (Myr^{-1})	0.019 ± 0.003	0.004 ± 0.001	0.021 ± 0.005	0.003 ± 0.001	0.024 ± 0.005	0.005 ± 0.001
$\hat{v}$ (Myr^{-1})	0.177 ± 0.036	0.020 ± 0.002	0.286 ± 0.119	0.023 ± 0.003	0.182 ± 0.055	0.020 ± 0.002
$\hat{\alpha}/\hat{\beta}$	17.1 ± 3.9	9.1 ± 1.8	27.0 ± 10.6	12.3 ± 3.9	14.5 ± 4.6	7.3 ± 1.8

These and other estimates are given for the models with variable f (best fit f) and with fixed $f = 1$. For all three data sets, a model with variable f was selected from AIC. t_1 is the branching date between common and pygmy chimpanzees, and t_2 is the date of the deepest node within humans in the respective trees of Fig. 2. v_S, v_V, and v are transition rate, transversion rate, and total substitution rate per variable site given by

$$v_S = 2(\pi_T\pi_C + \pi_A\pi_G)\alpha,$$
$$v_V = 2(\pi_T + \pi_C)(\pi_A + \pi_G)\beta,$$
$$v = v_S + v_V$$

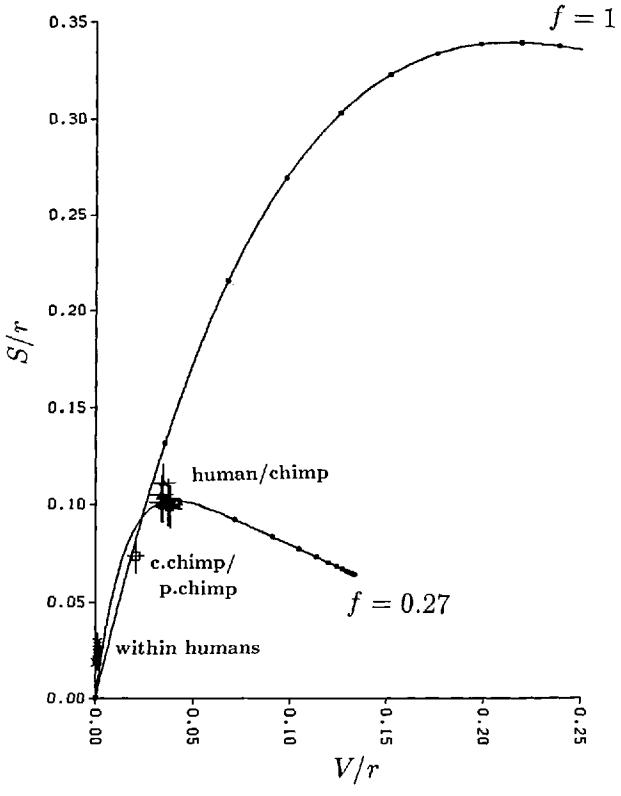

Fig. 3. Relationship between S/r and V/r for data set A. Theoretical curves of the best fit f(=0.27) and of $f = 1$ are shown. Vertical and horizontal lines indicate 1 SE of S_{ij}/r and V_{ij}/r, respectively. The interval between neighboring small circles along the curves is 5 Myr.

differences among humans are smaller than expected. This systematic deviation is apparent also for data sets B and C (data not shown) and seems to indicate that not all the sites are equally variable and that the effect of multiple substitutions is not fully taken into account in the model of $f = 1$. When f is taken as a variable and the generalized least-squares fit is performed on this variable, the deviation is reduced, and the estimated value of f turns out to be 0.27, 0.31, and 0.27, respectively, for data sets A, B, and C. The AIC of the model with variable f is smaller than that with fixed $f = 1$ consistently for all the data sets (Table 2), and therefore an additional rationale for introducing the parameter f is provided.

The importance of estimating the proportion of variable sites when estimating genetic distances between DNA sequences has been stressed by Hasegawa et al. (1985), by Fitch (1986), and by Hasegawa and Kishino (1989). Our analysis indicates that even the regions that Vigilant et al. (1989) called hypervariable parts (data set B) do not evolve homogeneously among sites and contain relatively conservative sites. From the neutral theory (Kimura 1983), this suggests that some selective constraint is operating even in these hypervariable regions. Although transitions in the third codon positions of protein-coding genes are always synonymous in mammalian mitochondria, the transition rate parameter α in the third positions of codons (0.241 ± 0.051 Myr^{-1} estimated by Hasegawa et al. 1990) is lower than that in the variable sites of the major noncoding region (0.666 ± 0.153, 1.158 ± 0.509, and 0.690 ± 0.229 Myr^{-1} for data sets A, B, and C, where ± refers to 1 SE). This suggests that the selective constraint, although weak, is operating on synonymous change in the third codon positions. It might be reasonable to assume that transition and transversion rates shown in Table 2 represent the upper limits of mtDNA evolutionary rates and therefore the mutational rates.

Rate of transition plus transversion, v, is estimated to be 0.177 ± 0.036, 0.286 ± 0.119, and

0.182 ± 0.055 Myr^{-1} per variable site, respectively, for data sets A, B, and C. Kishino and Hasegawa (1990) estimated that this rate for the η-globin pseudogene in nuclear DNA is $(0.781 \pm 0.189) \times 10^{-3}$ for humans and $(1.212 \pm 0.104) \times 10^{-3}$ Myr^{-1} for the great apes, by assuming the human/orangutan divergence to be 13 Myr ago. Because the pseudogene is considered to be free from selective constraints, the analysis was made under the assumption that all sites are equally variable. The rate for variable sites in the major noncoding region of mtDNA is thus larger by more than 100 times than that of the pseudogene. Therefore the mutation rate of mtDNA is likely to be more than 100 times that of nuclear DNA, if the evolutionary rate of the η-globin pseudogene represents the average mutation rate of nuclear DNA. The ratio α/β is estimated to be 17.1 ± 3.9, 27.0 ± 10.6, and 14.5 ± 4.6 for data sets A, B, and C. The ratio was estimated to be about 5 for the η-globin and for the intergenic spacer (Hasegawa et al. 1989). Although both transition and transversion rates are much higher in mtDNA than in nuclear DNA, the amount of elevation of transition rate in mtDNA is larger than that of the transversion rate.

The introduction of the parameter f suggests a large amount of multiple nucleotide substitutions in a site even between closely related species such as humans and chimpanzees. For example, the data set A, the expected number of substitutions between the two species is 2×4 Myr $\times$ 0.177 Myr^{-1} = 1.42 per variable site (Table 2) rather than $2 \times 4 \times 0.020 = 0.16$ per site, which is suggested by the homogeneous model, or 0.14, which is suggested by a simple difference. Because of its high evolutionary rate, mtDNA has been widely used in solving phylogenetic problems of mammalian evolution, particularly regarding human evolution. Our present work indicates that evaluation of the amount of multiple substitutions based on a proper model of evolution is very important in solving these problems.

Branching Dates

The estimated dates of the branchings are also shown in Fig. 2. The date of the branching between the common chimpanzee and pygmy chimpanzee is estimated to be 1.70 ± 0.35, 1.89 ± 0.61, and 2.45 ± 0.63 Myr ago, respectively, from data sets A, B, and C. These estimates from the three data sets are mutually consistent if the standard errors are taken into account, and they are consistent with an estimate based on restriction analysis of mtDNA (Wilson et al. 1985). The dates of the deepest branchings among humans in the respective mtDNA trees are 0.29 ± 0.07, 0.23 ± 0.09, and 0.32 ± 0.10 Myr ago, respectively, for data sets A, B, and C. The weighted mean of these estimates, where a weight is inversely proportional to the variance of the estimate, is 0.28 ± 0.05 Myr ago. The deepest root for mtDNA polymorphisms in the human population is estimated to be some 280,000 years ago, although a large amount of error is still attached to this estimate.

Based on restriction enzyme analysis of mtDNA, Horai and Matsunaga (1986) suggested that the Japanese population consists of two clusters (groups I and II). In data set C in this work, MS14 and MS24 are members of group I, whereas MS10 is a member of group II. Our estimate of the date for this separation is $200{,}000 \pm 70{,}000$ years.

Root for Polymorphism in the Human Population

The uncertainty in the branching order of individual human mtDNA exists and we cannot determine which of the individual mtDNAs diverged first from others, yet the available estimates indicate that the date of the root for the human mtDNA tree is some 280,000 years ago, which is roughly consistent with previous estimates from restriction analysis and with the data of Vigilant et al. (1989) if the uncertainties of this estimate mentioned below are taken into account.

It should be noted that the estimates of evolutionary rates and of branching dates depend strongly on f as shown in Table 2, but that the error in estimating f was not taken into account in evaluating the standard errors of these estimates given in this paper. In order to obtain a more reliable estimate of f, distantly related pairs of sequence data, in which the number of transition differences decreases as t increases, are needed (Hasegawa et al. 1985). In our case, orangutan and gibbon sequences can be useful for this purpose.

Recently, S.H. and coworkers sequenced 4.9 kbp of mtDNA from chimpanzee, gorilla, and orangutan. A preliminary analysis of the data suggests a slower rate of transition in the chimpanzee than in humans. If this is the case in the major noncoding region also, then branching dates among humans given in this paper are overestimates (biased toward old). In Fig. 3 it is evident that the common/pygmy chimpanzee point locates on the lower-right side of the best fit curve, which assumes an equal rate between humans and chimpanzees, and that the points among humans still locate on the upper-left side. This is also the case for data sets B and C (data not shown). These deviations are consistent with the suggestion that the transition rate is slower in chimpanzees than in humans. Additional sequence data are still highly desirable for evaluating the extent of

rate variation quantitatively. We assumed that the split between human and chimpanzee mtDNAs took place 4 Myr ago. This time could be greater. If it was 5 Myr, our estimates of branching dates should increase by a factor of 5/4, but the time is unlikely to be much greater than this (Hasegawa and Kishino 1990). It is also possible that human individuals with mtDNA more divergent from others than known to date may be found in the future. However, we think that, because of the extensive collection of the data for individuals from diverse geographic and racial backgrounds, most of the divergence of mtDNA in the present human populations is accounted for in this work.

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