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Dating of the Human-Ape Splitting by a Molecular Clock of Mitochondrial DNA

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Summary. A new statistical method for estimating divergence dates of species from DNA sequence data by a molecular clock approach is developed. This method takes into account effectively the information contained in a set of DNA sequence data. The molecular clock of mitochondrial DNA (mtDNA) was calibrated by setting the date of divergence between primates and ungulates at the Cretaceous–Tertiary boundary (65 million years ago), when the extinction of dinosaurs occurred. A generalized least-squares method was applied in fitting a model to mtDNA sequence data, and the clock gave dates of 92.3 ± 11.7, 13.3 ± 1.5, 10.9 ± 1.2, 3.7 ± 0.6, and 2.7 ± 0.6 million years ago (where the second of each pair of numbers is the standard deviation) for the separation of mouse, gibbon, orangutan, gorilla, and chimpanzee, respectively, from the line leading to humans. Although there is some uncertainty in the clock, this dating may pose a problem for the widely believed hypothesis that the bipedal creature *Australopithecus afarensis,* which lived some 3.7 million years ago at Laetoli in Tanzania and at Hadar in Ethiopia, was ancestral to man and evolved after the human–ape splitting. Another likelier possibility is that mtDNA was transferred through hybridization between a proto-human and a proto-chimpanzee after the former had developed bipedalism.

Key words: Evolution of hominoids — Phylogenetic position of *Australopithecus afarensis* — Interspecies transfer of mitochondrial DNA

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Introduction

When humans and apes separated during evolution is still a matter of controversy. The fossil record, of course, can provide relevant data, but it does not provide conclusive evidence, because the data can be interpreted in several ways. The molecular record can provide additional powerful material to solve this problem.

Because of the approximate constancy of the rate of change in informational macromolecules, it has been suggested that they can serve as an evolutionary clock allowing us to date the divergence times of extant organisms (Zuckerkandl and Pauling 1962, 1965; Dickerson 1971; Wilson et al. 1977). This constancy is consistent with the neutral theory of molecular evolution (Kimura 1968, 1983; Kimura and Ohta 1974). Since the pioneering work of Sarich and Wilson (1967), many researchers have estimated the divergence time between humans and the African apes using molecular clock approaches (Sarich and Cronin 1976, 1977; Andrews and Cronin 1982; Sibley and Ahlquist 1984). In spite of the diverse materials and methods used, their results uniformly show an apparently recent divergence of less than 8 million years (Myr) ago between humans and the African apes, which indicates that *Ramapithecus,* which lived some 8–14 Myr ago, cannot have been an ancestor of humans that evolved after the human–ape separation (Andrews 1982; Pilbeam 1982, 1984; Ciochon and Corruccini 1983). It is now apparent that the molecular record can tell us much about the dates of branching during hominoid evolution.

The previous molecular clock studies were based on immunological distances (Sarich and Wilson

1967; Sarich and Cronin 1976, 1977), DNA hybridization (Sarich and Cronin 1977; Sibley and Ahlquist 1984), restriction endonuclease mapping of mitochondrial DNA (Brown et al. 1979), protein electrophoresis (Sarich and Cronin 1976; Nozawa et al. 1982), and amino acid sequencing (Goodman et al. 1983). Although these methods were powerful enough to exclude the possibility that *Ramapithecus* was ancestral to humans and evolved after the human–ape splitting, they provided only a rough estimate of the date of the separation.

These methods estimated genetic distances indirectly, and not on the basis of statistical models. They therefore contained some uncertainty, and the amount of error inherent in the estimates could not be evaluated in a proper way. Furthermore, since most of these previous methods did not take account of the effect of multiple changes in a site, their estimates of the divergence date are biased in favor of one more ancient than the actual one when a more distant splitting is taken as a reference. Therefore, in a preliminary report, we developed a statistical method that gives genetic distances by direct comparison between mitochondrial DNA (mtDNA) sequences, and obtained a more reliable estimate of the timing of the divergence events during the evolution of the Hominoidea (Hasegawa et al. 1984a).

Our estimate was heavily dependent on the assumption that the divergence between bovines and primates occurred 90 Myr ago (Dickerson 1971; Sarich and Cronin 1976; Simons 1976; Wilson et al. 1977; Goodman et al. 1983). However, we now know that no convincing fossils of the living orders of placental mammals have been found from the Cretaceous period (Novacek 1982; Savage and Russell 1983). Also, the presumed holocaust that occurred at the end of the Cretaceous (Alvarez et al. 1980, 1984; Raup and Sepkoski 1984), some 65 Myr ago, may have been responsible for starting a new radiation of placental mammals (Allan C. Wilson, personal communication). Therefore, it seems likely that the divergence between bovines and primates occurred as recently as 65 Myr ago. In this paper, we present full details of our method, and give estimates of divergence times among the Hominoidea obtained using a recalibrated molecular clock based on the revised reference time.

Mitochondrial DNA Sequence Data

The mtDNAs of human (Anderson et al. 1981), bovine (Anderson et al. 1982), and mouse (Bibb et al. 1981), each of which is about 16,500 nucleotides in length, have been completely sequenced. Brown and his coworkers sequenced a stretch of 896 nucleotides in mtDNAs from human, chimpanzee, gorilla, orangutan, and gibbon (Brown et al. 1982). This segment contains the genes for three tRNAs and parts of two proteins. The data set used in the present study is composed of the 896-nucleotide sequences from the above-mentioned five species of Hominoidea and the sequences of the corresponding regions from bovine and mouse (L-strand of mtDNA). These data provide us with the opportunity to date the divergence events during the evolution of the Hominoidea by a more reliable method than has been used before.

The rate of synonymous substitutions in DNA coding for proteins is much higher than both that of amino acid-altering substitutions (Kimura 1977; Brown et al. 1982; Miyata et al., 1982) and that of substitutions in tRNA genes. The rates of amino acid-altering substitutions and of tRNA substitutions have been approximately the same during the evolution of animal mtDNA (Brown et al. 1982). This is in sharp contrast with the situation for nuclear DNA, in which tRNA genes are much more conservative than are most of the genes for proteins (Hasegawa et al. 1984b). Since synonymous substitutions are confined mostly to the third codon positions of protein genes, we divide the nucleotide sites into two classes: Class 1 sites are third codon positions, and class 2 sites are first and second codon positions and sites in tRNA genes. These two classes of sites are treated separately in the statistical model presented in this paper.

Phylogenetic Relationships Among the Hominoidea

In analyzing the data, it must be taken into account that transition (A ↔ G, T ↔ C) has greatly predominated over transversion (A,G ↔ T,C) in the evolution of animal mtDNA (Brown and Simpson 1982; Brown et al. 1982). We therefore counted the numbers of transition- and transversion-type differences between species in class 1 and class 2 sites separately, as shown in Table 1 (Hasegawa et al. 1984a). It is remarkable that the number of transition-type differences in class 1 sites between human and chimpanzee is nearly the same as that between human and mouse. This means that a considerable number of multiple transitions have accumulated at these sites, even when we compare any pair of closest relatives in the present data set. Transition at the third codon position (class 1 site) is always synonymous in the genetic code of mammalian mitochondria (Barrell et al. 1979; Anderson et al. 1981, 1982; Bibb et al. 1981).

The transversion-type differences in Table 1 and other evidence indicate that of the living hominoids, gibbons separated first and orangutans second from

Table 1. Numbers of transition- (upper right half) and transversion- (lower left half) type nucleotide differences among mammalian mtDNAs

i	1 Mouse	2 Bovine	3 Gibbon	4 Orang.	5 Gorilla	6 Chimp.	Human	$S^{(i)}/r_i$
1 Mouse		68 (39)	81 (53)	81 (48)	87 (46)	79 (50)	79 (51)	0.119 (0.206)
2 Bovine	91 (82)		80 (42)	81 (44)	93 (52)	85 (61)	86 (57)	0.128 (0.221)
3 Gibbon	83 (83)	69 (71)		57 (59)	65 (59)	61 (64)	59 (58)	0.091 (0.259)
4 Orang.	90 (85)	65 (65)	18 (34)		64 (52)	59 (60)	55 (53)	0.089 (0.237)
5 Gorilla	85 (77)	72 (67)	19 (26)	15 (18)		28 (58)	32 (52)	0.045 (0.237)
6 Chimp.	86 (79)	71 (67)	18 (26)	16 (18)	5 (4)		24 (50)	0.036 (0.216)
Human	89 (77)	70 (67)	19 (26)	15 (20)	4 (4)	3 (2)		
$V^{(i)}/r_i$	0.131 (0.347)	0.104 (0.291)	0.028 (0.121)	0.023 (0.080)	0.007 (0.017)	0.005 (0.009)		

Number in parentheses is for the third codon positions of protein-coding regions (class 1 sites; 232 nucleotides) and the number preceding it is for the rest of the sites (class 2 sites; 667 nucleotides). From Hasegawa et al. (1984a). See text for explanation of $S^{(i)}/r_i$ and $V^{(i)}/r_i$

the line leading to humans (Goodman 1962, 1963; Zihlman et al. 1978; Ferris et al. 1981a; Andrews and Cronin 1982; Brown et al. 1982; Sibley and Ahlquist 1984), and that primates are related more closely to bovines than to the mouse (McKenna 1975; Eisenberg 1981). However, the branching order among human, chimpanzee, and gorilla is controversial. Templeton (1983) has developed an algorithm for a nonparametric test for comparing alternative phylogenies obtained from restriction endonuclease cleavage site data, and has applied it to the mtDNA data from hominoids. His conclusion was that the chimpanzee and gorilla separated after the divergence of humans. Because his analysis involved many synonymous transitions, a considerable number of which represent multiple transitions, his conclusion may not be correct. In fact, nine of the variations in the data used by him are in the protein-coding region in our data set. Seven of them involve transitions at third codon positions, one of the remaining two involves transitions at a first position, and the other involves transitions at a second position.

To clarify the phylogenetic relationships among the Hominoidea, we applied the maximum likelihood method developed by Felsenstein (1981) to our data set (Hasegawa and Yano 1984). The method originally assumed that transitions and transversions occur at the same rate. This assumption is invalid in animal mtDNA. Therefore, we separated transversion from transition, and examined only the former in calculating the maximum likelihood estimate. The topology of the maximum likelihood tree (Fig. 1) shows the chimpanzee as the unique closest relative of humans among extant apes. Although the branching order among humans and the African apes is confident only at 4.4% risk level by this analysis, the human–chimpanzee grouping has been suggested also by a single-copy nuclear DNA–DNA hybridization (Sibley and Ahlquist 1984), by hemoglobin sequences (Goodman et al. 1983), and by extensive comparison of high-resolution banding patterns of the chromosomes (Yunis and Prakash 1982). We tentatively adopt this tree topology in estimating divergence times in the Hominoidea.

A Statistical Model

Let us consider s homologous nucleotide sequences that consist of r nucleotide sites of a homogeneous class (either class 1 or class 2). For the data set analyzed in this work, $s = 7$, $r_1 = 232$ (class 1 sites), and $r_2 = 667$ (class 2 sites); sites that experienced deletion or insertion are included, but deletion–insertion events are not taken into account in our analysis. A basic assumption is that each site changes homogeneously and independently of others; that is, the probability of nucleotide substitution has an independently identical distribution (i.i.d.). A random variable is represented by $(x_1, \ldots, x_s)$, in which each component is T, C, A, or G, and the number of possible states is 4^s. Our purpose is to parametrize

$$P(x_1 = i_1, \ldots, x_s = i_s) = q_{i_1 \ldots i_s}$$
$$(i_1, \ldots, i_s = T, C, A, G)$$

based on a statistical model and to estimate divergence times among the extant hominoids.

We denote by $n_{i_1 \ldots i_s}$ the number of sites that have a value of $(i_1, \ldots, i_s)$. This follows the multinomial (4^s-nomial) distribution

$$\mathrm{Pol}(n; q_{i_1 \ldots i_s}, i_1, \ldots, i_s = T, C, A, G)$$

and represents the most detailed information about the data under the basic assumption of an i.i.d. The average and the covariance of these statistics are given by the following formulae:

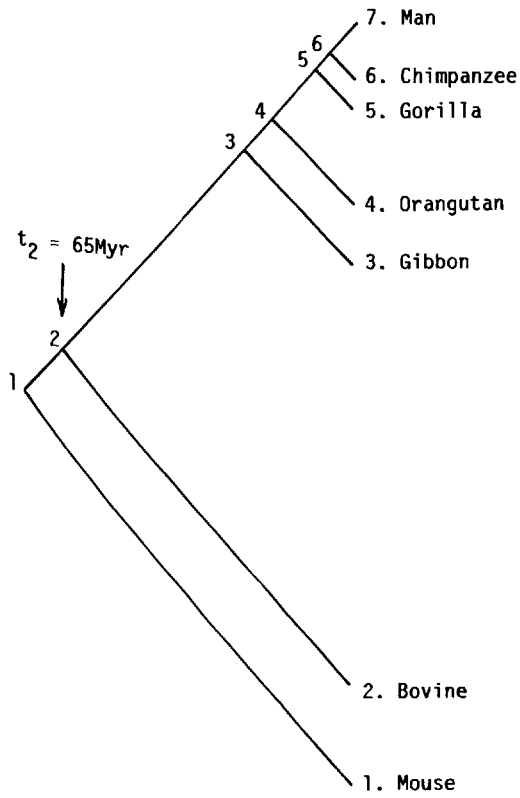

Fig. 1. Phylogeny inferred from the mtDNA sequences by a maximum likelihood method developed by Felsenstein (1981). In calibrating our molecular clock, the date of divergence between the primates and bovines (node 2) was taken to be 65 Myr ago

$$E\{n_{i_1 \ldots i_s}\} = rq_{i_1 \ldots i_s} \tag{1a}$$

$$\mathrm{Cov}\{n_{i_1 \ldots i_s}, n_{i_1' \ldots i_s'}\} = r(\delta_{i_1 \ldots i_s;\, i_1' \ldots i_s'} q_{i_1 \ldots i_s} - q_{i_1 \ldots i_s} q_{i_1' \ldots i_s'}) \tag{1b}$$

where $\delta_{i_1 \ldots i_s;\, i_1' \ldots i_s'}$ equals 1 when $i_1 = i_1', \ldots, i_s = i_s'$, and 0 otherwise.

We cannot handle Eqs. (1a) and (1b) as they are, because the number of states increases explosively as s increases. Therefore, we reduce the data to differences, and compare the differences with a probability distribution to which they conform.

A Stationary Markov Model

The probability that a given site is variable is denoted by f, which means that the probability of its being nonvariable is 1 − f. Each variable site evolves according to a Markov process in which a base i (T, C, A, or G) is replaced by another base j in an infinitesimally short interval of time, dt, with a probability of $P_{ij}(t)$, as follows:

$$P_{ij}(dt) = \Pr(x(t + dt) = j \mid x(t) = i) = \begin{cases} \alpha\pi_j dt & \text{(for transition)} \\ \beta\pi_j dt & \text{(for transversion)} \end{cases} \tag{2}$$

where π_j is the stationary composition of base j. In our data set, $\pi_T^{(1)} = 0.169$, $\pi_C^{(1)} = 0.429$, $\pi_A^{(1)} = 0.364$, and $\pi_G^{(1)} = 0.038$ for class 1 sites; and $\pi_T^{(2)} = 0.297$, $\pi_C^{(2)} = 0.267$, $\pi_A^{(2)} = 0.310$, and $\pi_G^{(2)} = 0.126$ for class 2 sites. This model is justified because the base composition of animal mtDNA is highly biased (particularly, G is scarce in the L-strand), and because the asymmetry of the substitution frequencies is in accord with the bias in base composition (the A → G frequency is much lower than the G → A) (Aquadro and Greenberg 1983). Our model is a generalization of the models of Kimura (1980) and of Felsenstein (1981). Kimura's model corresponds to the case of $\pi_T = \pi_C = \pi_A = \pi_G = 1/4$ in Eq. (2), and Felsenstein's model corresponds to the case of $\alpha = \beta$. Since transition predominates over transversion, Felsenstein's model is apparently inadequate for animal mtDNA. Furthermore, because the base composition of animal mtDNA is highly biased, Kimura's model does not fit the data. This will be further shown for the class 1 sites later in this paper.

The substitution probability matrix for an infinitesimally short interval of time can be written as

$$P(dt) = \begin{array}{c} \\ T \\ C \\ A \\ G \end{array} \begin{array}{c} \begin{array}{cccc} T & C & A & G \end{array} \\ \begin{bmatrix} 1 - (\alpha\pi_C + \beta\pi_A + \beta\pi_G)dt & \alpha\pi_C dt & \beta\pi_A dt & \beta\pi_G dt \\ \alpha\pi_T dt & 1 - (\alpha\pi_T + \beta\pi_A + \beta\pi_G)dt & \beta\pi_A dt & \beta\pi_G dt \\ \beta\pi_T dt & \beta\pi_C dt & 1 - (\alpha\pi_G + \beta\pi_T + \beta\pi_C)dt & \alpha\pi_G dt \\ \beta\pi_T dt & \beta\pi_C dt & \alpha\pi_A dt & 1 - (\alpha\pi_A + \beta\pi_T + \beta\pi_C)dt \end{bmatrix} \end{array}$$

$$\equiv I + Adt \tag{3}$$

For an arbitrary time interval t, the function P(t) satisfies the Chapman–Kolmogorov equation

$$P(t + dt) = P(t)P(dt) = P(t)(I + Adt)$$

This equation is a mathematical manifestation of the Markovian nature of the process. Therefore, we get

$$\frac{dP(t)}{dt} = P(t)A$$

Since P(0) = I, we have

$$P(t) = e^{tA} \tag{4}$$

To carry out our analysis, it is necessary to explicitly determine the individual substitution probability $P_{ij}(t)$ by using the specific value decomposition of the right-hand side of Eq. (4). By decomposing A as

$$A = \sum_{i=1}^{4} \lambda_i \underline{u}_i \underline{v}_i'$$

we have

$$e^{tA} = \sum_{i=1}^{4} \exp(t\lambda_i)\underset{\sim}{u}_i \underset{\sim}{v}_i'$$

where $\det(\lambda_i I - A) = 0$, $A\underset{\sim}{u}_i = \lambda_i \underset{\sim}{u}_i$, $A'\underset{\sim}{v}_i = \lambda_i \underset{\sim}{v}_i$, $(\underset{\sim}{u}_i, \underset{\sim}{v}_j) = \delta_{ij}$ for i, j = 1, 2, 3, 4, and a tilde under a letter indicates a vector. We get

$$\lambda_1 = 0, \qquad \lambda_2 = -\beta,$$
$$\lambda_3 = -(\pi_Y\beta + \pi_R\alpha), \qquad \lambda_4 = -(\pi_Y\alpha + \pi_R\beta) \tag{5a}$$

$$\underset{\sim}{v}_1 = \begin{pmatrix} \pi_T \\ \pi_C \\ \pi_A \\ \pi_G \end{pmatrix}, \quad \underset{\sim}{v}_2 = \begin{pmatrix} \pi_R\pi_T \\ \pi_R\pi_C \\ -\pi_Y\pi_A \\ -\pi_Y\pi_G \end{pmatrix},$$
$$\underset{\sim}{v}_3 = \begin{pmatrix} 0 \\ 0 \\ 1 \\ -1 \end{pmatrix}, \quad \underset{\sim}{v}_4 = \begin{pmatrix} 1 \\ -1 \\ 0 \\ 0 \end{pmatrix} \tag{5b}$$

$$\underset{\sim}{u}_1 = \begin{pmatrix} 1 \\ 1 \\ 1 \\ 1 \end{pmatrix}, \quad \underset{\sim}{u}_2 = \begin{pmatrix} 1/\pi_Y \\ 1/\pi_Y \\ -1/\pi_R \\ -1/\pi_R \end{pmatrix},$$
$$\underset{\sim}{u}_3 = \begin{pmatrix} 0 \\ 0 \\ \pi_G/\pi_R \\ -\pi_A/\pi_R \end{pmatrix}, \quad \underset{\sim}{u}_4 = \begin{pmatrix} \pi_C/\pi_Y \\ -\pi_T/\pi_Y \\ 0 \\ 0 \end{pmatrix} \tag{5c}$$

where $\pi_Y = \pi_T + \pi_C$ and $\pi_R = \pi_A + \pi_G$.

Now, the numbers of transition-type differences, $S(j_1, j_2)$, and transversion-type differences, $V(j_1, j_2)$, between the j_1-th and j_2-th sequences are defined as follows:

$$S(j_1, j_2) = n_{\underset{j_1}{..T..}\underset{j_2}{C..}} + n_{\underset{j_1}{..C..}\underset{j_2}{T..}} + n_{\underset{j_1}{..A..}\underset{j_2}{G..}} + n_{\underset{j_1}{..G..}\underset{j_2}{A..}} \tag{6a}$$

$$V(j_1, j_2) = n_{\underset{j_1}{..T..}\underset{j_2}{A..}} + n_{\underset{j_1}{..T..}\underset{j_2}{G..}} + n_{\underset{j_1}{..C..}\underset{j_2}{A..}} + n_{\underset{j_1}{..C..}\underset{j_2}{G..}} + n_{\underset{j_1}{..A..}\underset{j_2}{T..}} + n_{\underset{j_1}{..A..}\underset{j_2}{C..}} + n_{\underset{j_1}{..G..}\underset{j_2}{T..}} + n_{\underset{j_1}{..G..}\underset{j_2}{C..}} \tag{6b}$$

where $n_{\underset{j_1}{..T..}\underset{j_2}{C..}}$ indicates the number of sites that have T in the j_1-th sequence and C in the j_2-th sequence irrespective of other sequences.

Let us consider a pair of sequences separated t million years ago. States of each site of these two sequences are denoted by x(t) and y(t), respectively. Under the assumption of stationarity, we have, for $i \neq j$,

$$\Pr(x(t) = i, y(t) = j) = \Pr(x(t) = i, y(t) = j \mid \text{variable site}) \cdot \Pr(\text{variable site}) = f \sum_{\ell=T,C,A,G} \pi_\ell P_{\ell i}(t) P_{\ell j}(t) \tag{7}$$

Since reversibility, i.e.,

$$\pi_\ell P_{\ell i}(t) = \pi_i P_{i\ell}(t),$$

can be easily proven, Eq. (7) becomes

$$\Pr(x(t) = i, y(t) = j) = f\pi_i \sum_{\ell} P_{i\ell}(t) P_{\ell j}(t) = f\pi_i P_{ij}(2t)$$

(Chapman–Kolmogorov equation)

Therefore, the average numbers of transition- and transversion-type differences are calculated as follows:

$$\overline{V(t)} = 2fr\pi_Y\pi_R[1 - \exp(-2\beta t)] \tag{8a}$$

$$\overline{S(t)} = 2fr\{(\pi_T\pi_C + \pi_A\pi_G) + (\pi_T\pi_C\pi_R/\pi_Y + \pi_A\pi_G\pi_Y/\pi_R)\exp(-2\beta t) - (\pi_T\pi_C/\pi_Y)\exp[-2t(\alpha\pi_Y + \beta\pi_R)] - (\pi_A\pi_G/\pi_R)\exp[-2t(\alpha\pi_R + \beta\pi_Y)]\} \tag{8b}$$

Furthermore, by using Eqs. (6a), (6b), (1a), and (1b), variances and covariances among differences are calculated as follows: For one pair of sequences,

$$\mathrm{Var}(V) = \bar{V}(1 - \bar{V}/r) \tag{9a}$$
$$\mathrm{Var}(S) = \bar{S}(1 - \bar{S}/r) \tag{9b}$$
$$\mathrm{Cov}(V, S) = -\bar{V}\bar{S}/r \tag{9c}$$

and for two different pairs of sequences $(j_1^{(1)}, j_2^{(1)})$ and $(j_1^{(2)}, j_2^{(2)})$,

$$\mathrm{Cov}\{S(j_1^{(1)}, j_2^{(1)}), S(j_1^{(2)}, j_2^{(2)})\} = r \sum_{\ell_1 \ell_1'} \sum_{\ell_2 \ell_2'} \{q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}\underset{j_1^{(2)}}{\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}} - q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}} q_{\underset{j_1^{(2)}}{..\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}}\} \tag{9d}$$

(ℓ_1, ℓ_1': transition; ℓ_2, ℓ_2': transition)

$$\mathrm{Cov}\{S(j_1^{(1)}, j_2^{(1)}), V(j_1^{(2)}, j_2^{(2)})\} = r \sum_{\ell_1 \ell_1'} \sum_{\ell_2 \ell_2'} \{q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}\underset{j_1^{(2)}}{\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}} - q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}} q_{\underset{j_1^{(2)}}{..\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}}\} \tag{9e}$$

(ℓ_1, ℓ_1': transition; ℓ_2, ℓ_2': transversion)

$$\mathrm{Cov}\{V(j_1^{(1)}, j_2^{(1)}), V(j_1^{(2)}, j_2^{(2)})\} = r \sum_{\ell_1 \ell_1'} \sum_{\ell_2 \ell_2'} \{q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}\underset{j_1^{(2)}}{\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}} - q_{\underset{j_1^{(1)}}{..\ell_1..}\underset{j_2^{(1)}}{\ell_1'..}} q_{\underset{j_1^{(2)}}{..\ell_2..}\underset{j_2^{(2)}}{\ell_2'..}}\} \tag{9f}$$

(ℓ_1, ℓ_1': transversion; ℓ_2, ℓ_2': transversion)

Least-Squares Fitting of the Data

Since our data consist of two classes of nucleotide sites, all of the variables, all of the parameters except t, and all of the statistics defined above must have a subscript or superscript k to designate the class. We finally reduce the data to the following forms:

$$V_k^{(i)} \equiv \frac{1}{7-i}\sum_{j=i+1}^{7} V_k(i, j) \tag{10a}$$

$$S_k^{(i)} \equiv \frac{1}{7-i}\sum_{j=i+1}^{7} S_k(i, j), \qquad i = 1, 2, \ldots, 6 \tag{10b}$$

where the superscript (i) denotes the i-th splitting in Fig. 1. These values are listed in Table 1.

From the central limit theorem, these statistics can be regarded as constituting the following vector, which follows a multivariate normal distribution:

$$\underset{\sim}{D} \equiv (V_1^{(1)}, \ldots, V_1^{(6)}, S_1^{(1)}, \ldots, S_1^{(6)}, V_2^{(1)}, \ldots, V_2^{(6)}, S_2^{(1)}, \ldots, S_2^{(6)})$$

The expression of the multivariate normal distribution is then

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

where $\bar{D} = (\overline{V_1(t_1)}, \ldots, \overline{V_1(t_6)}, \overline{S_1(t_1)}, \ldots, \overline{S_1(t_6)}, \overline{V_2(t_1)}, \ldots, \overline{V_2(t_6)}, \overline{S_2(t_1)}, \ldots, \overline{S_2(t_6)})$, and Ω is the variance–covariance matrix. The averages can be calculated by Eqs. (8a) and (8b), and the variances by Eqs. (9a–f), (10a), and (10b). The likelihood function is

$$L(\bar{\underset{\sim}{D}}, \underset{\sim}{D}) = \frac{1}{(2\pi)^{12}\sqrt{\det \Omega}} \cdot \exp\left\{-\frac{1}{2}(\underset{\sim}{D} - \bar{\underset{\sim}{D}})'\Omega^{-1}(\underset{\sim}{D} - \bar{\underset{\sim}{D}})\right\}$$

If we substitute the variance and covariance data for Ω, the approximate maximum likelihood estimate of the parameters of the model can be obtained by minimizing

$$R \equiv (\underset{\sim}{D} - \bar{\underset{\sim}{D}})'\Omega^{-1}(\underset{\sim}{D} - \bar{\underset{\sim}{D}}) \tag{11}$$

In our earlier works (Hasegawa 1984; Hasegawa et al. 1984a, 1985), as an approximation of this generalized least-squares method, we solved the least-squares problem by minimizing

$$\tilde{R} \equiv \sum_{k=1}^{2}\sum_{i=1}^{6}\left[\frac{1}{\mathrm{Var}(V_k^{(i)})} \cdot \left\{V_k^{(i)} - \overline{V_k(t_i; f_k, \beta_k)}\right\}^2 + \frac{1}{\mathrm{Var}(S_k^{(i)})} \cdot \left\{S_k^{(i)} - \overline{S_k(t_i; f_k, \alpha_k, \beta_k)}\right\}^2\right] \tag{12}$$

Since the effects of covariance terms are not negligible, we minimize R directly in this paper. In our earlier works (Hasegawa and Yano 1984; Hasegawa et al. 1984a, 1985), $n_{ijk\ell}$ instead of $q_{ijk\ell}$ was used in calculating Ω. Since the sample size is small, the covariances estimated in that manner are unstable. Therefore, in this work we calculate $q_{ijk\ell}$ iteratively by means of the Newton method discussed below by setting the values of the parameters as follows: Unless $i = j = k = \ell$, $q_{ijk\ell}$ is given by

$$q_{\substack{ijk\ell \\ IJKL}} = \begin{cases} f\sum_x \sum_y \pi_x P_{xi}(2t_I - t_J)P_{xj}(t_J) \cdot P_{xy}(t_J - t_K)P_{yk}(t_K)P_{y\ell}(t_K) & \text{for } I < J < K < L \\ f\delta_{ij}\sum_x \pi_x P_{xi}(2t_I - t_K)P_{xk}(t_K)P_{x\ell}(t_K) & \text{for } I = J < K < L \\ f\delta_{jk}\sum_x \pi_x P_{xi}(2t_I - t_J)P_{xj}(t_J)P_{x\ell}(t_J) & \text{for } I < J = K < L \\ f\delta_{k\ell}\sum_x \pi_x P_{xi}(2t_I - t_J)P_{xj}(t_J)P_{xk}(t_J) & \text{for } I < J < K = L \\ f\delta_{ij}\delta_{k\ell}\pi_i P_{ik}(2t_I) & \text{for } I = J < K = L \end{cases} \tag{13}$$

where δ_{ij} is Kronecker's delta and $\sum$ is the sum over T, C, A, and G.

Newton Method

To minimize R, the Newton method was carried out as follows. If $\underset{\sim}{\theta} \equiv (t_1, t_3, t_4, t_5, t_6, f_1, \alpha_1, \beta_1, f_2, \alpha_2, \beta_2)'$ the iteration algorithm of the Newton method is given by

$$\underset{\sim}{\theta}_{n+1} = \underset{\sim}{\theta}_n - \left(\frac{\partial^2 R}{\partial\underset{\sim}{\theta}\,\partial\underset{\sim}{\theta}'}\right)^{-1}_{|\underset{\sim}{\theta}_n}\left(\frac{\partial R}{\partial\underset{\sim}{\theta}}\right)_{|\underset{\sim}{\theta}_n} \tag{14}$$

Variances of the Estimates

From Eq. (11), R is given as a function of $\underset{\sim}{D}$ and $\underset{\sim}{\theta}$ by

$$R(\underset{\sim}{D}, \underset{\sim}{\theta}) = (\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta}))'\Omega^{-1}(\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta}))$$

where $\bar{\underset{\sim}{D}}(\underset{\sim}{\theta}) = E[\underset{\sim}{D} \mid \underset{\sim}{\theta}]$. One can obtain $\hat{\underset{\sim}{\theta}}(\underset{\sim}{D})$ by setting

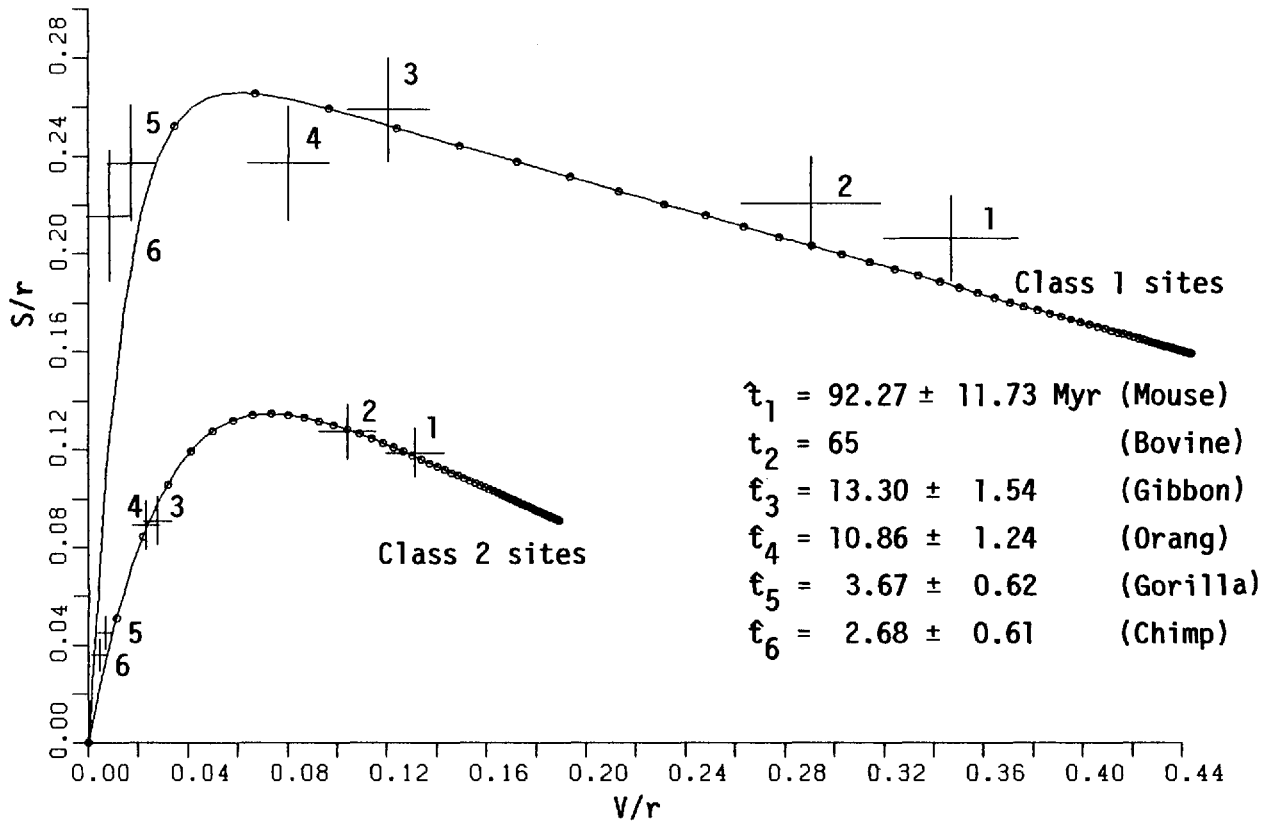

Fig. 2. Least-squares fitting of the relation between S/r and V/r. Vertical and horizontal lines indicate standard deviations of $S^{(i)}/r$ and $V^{(i)}/r$, respectively. The interval between neighboring small circles along the curve is 5 Myr

$$\frac{\alpha}{\partial \underset{\sim}{\theta}} R(\underset{\sim}{D}, \hat{\underset{\sim}{\theta}}) = 0. \tag{15}$$

Defining

$$R_{\underset{\sim}{\theta}}(\underset{\sim}{D}, \underset{\sim}{\theta}) \equiv \frac{\partial}{\partial \underset{\sim}{\theta}} R(\underset{\sim}{D}, \underset{\sim}{\theta}),$$

we expand $R_{\underset{\sim}{\theta}}(\underset{\sim}{D}, \hat{\underset{\sim}{\theta}})$ around $R_{\underset{\sim}{\theta}}(\bar{\underset{\sim}{D}}(\underset{\sim}{\theta}), \underset{\sim}{\theta})$ as follows:

$$\begin{aligned} R_{\underset{\sim}{\theta}}(\underset{\sim}{D}, \hat{\underset{\sim}{\theta}}) - R_{\underset{\sim}{\theta}}(\bar{\underset{\sim}{D}}(\underset{\sim}{\theta}), \underset{\sim}{\theta}) \\ \fallingdotseq A(\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta})) + B(\hat{\underset{\sim}{\theta}} - \underset{\sim}{\theta}) \end{aligned} \tag{16}$$

where

$$A \equiv \frac{\partial^2}{\partial \underset{\sim}{\theta}\, \partial \underset{\sim}{D}'} R(\bar{\underset{\sim}{D}}(\underset{\sim}{\theta}), \underset{\sim}{\theta})$$

and

$$B \equiv \frac{\partial^2}{\partial \underset{\sim}{\theta}\, \partial \underset{\sim}{\theta}'} R(\bar{\underset{\sim}{D}}(\underset{\sim}{\theta}), \underset{\sim}{\theta})$$

Since the left-hand side of Eq. (16) is zero,

$$\hat{\underset{\sim}{\theta}} - \underset{\sim}{\theta} = -B^{-1}A(\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta}))$$

Therefore, the variance and covariance matrix of the estimates is given by

$$\begin{aligned} \mathrm{Var}(\hat{\underset{\sim}{\theta}}) &= E[(\hat{\underset{\sim}{\theta}} - \underset{\sim}{\theta})(\hat{\underset{\sim}{\theta}} - \underset{\sim}{\theta})'] \\ &\fallingdotseq B^{-1}AE[(\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta})) \\ &\qquad \cdot (\underset{\sim}{D} - \bar{\underset{\sim}{D}}(\underset{\sim}{\theta}))']A'B^{-1} \\ &= B^{-1}A\Omega A'B^{-1} \end{aligned} \tag{17}$$

Results

Divergence Times in the Evolution of the Hominoidea

The results are shown in in Figs. 2 and 3. Our clock gives 92.27 ± 11.73, 13.30 ± 1.54, 10.86 ± 1.24, 3.67 ± 0.62, and 2.68 ± 0.61 Myr for the separation from the human line of mouse, gibbon, orangutan, gorilla, and chimpanzee, respectively (the number after ± is the standard deviation).

The estimate of the other parameters of the model are as follows: $\hat{f}_2 = 0.9491 \pm 0.0395$, $\hat{\alpha}_1 = 0.4483 \pm 0.1424$ Myr^{-1}, $\hat{\beta}_1 = 0.0082 \pm 0.0012$ Myr^{-1}, $\hat{f}_2 = 0.3847 \pm 0.0228$, $\hat{\alpha}_2 = 0.0684 \pm 0.0093$ Myr^{-1}, $\hat{\beta}_2 = 0.0062 \pm 0.0007$ Myr^{-1} (the number after ± is the standard deviation). The fact that $\hat{f}_1$ nearly equals unity shows that almost all of the third codon positions are variable.

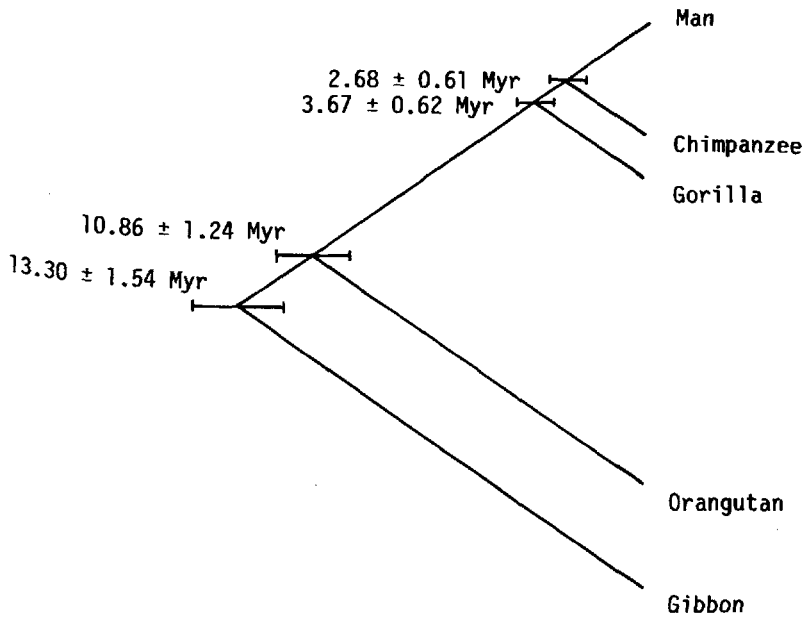

Fig. 3. Estimates of dates of separations during evolution of the Hominoidea. A horizontal line indicates the range of the standard error of the estimate

Simulation Experiment

To confirm the validity of our method, a computer simulation was carried out. A hypothetical ancestral sequence corresponding to node 1 in Fig. 1 was constructed according to the average nucleotide compositions of the respective classes of our data set of mtDNA. In the simulation the sequence evolves according to the Markov model in which α_i, β_i, f_i ($i = 1, 2$), and t_i ($i = 1, \ldots, 6$) are the estimates obtained by our analysis to give a set of seven contemporary sequences. Then the sequences are analyzed by our method, and the parameters of the model are estimated.

The simulation was performed 100 times, and the sample means and sample variances of the estimates were computed. The results are as follows: $t_1 = 91.80 \pm 11.30$ Myr, $t_3 = 13.24 \pm 1.16$ Myr, $t_4 = 10.79 \pm 0.88$ Myr, $t_5 = 3.65 \pm 0.48$ Myr, $t_6 = 2.73 \pm 0.43$ Myr, $f_1 = 0.9538 \pm 0.0266$, $\alpha_1 = 0.4547 \pm 0.1000$ Myr^{-1}, $\beta_1 = 0.0083 \pm 0.0009$ Myr^{-1}, $f_2 = 0.3852 \pm 0.0181$, $\alpha_2 = 0.0689 \pm 0.0076$ Myr^{-1}, $\beta_2 = 0.0062 \pm 0.0006$ Myr^{-1} (the number after $\pm$ is the standard deviation). These results are consistent with the estimates from the DNA sequence data, and the standard deviations calculated from the sample variances of simulation experiments, as well as those calculated from Eq. (17), indicate the degree of error in our estimate of the parameters.

Some Characteristics of the Relationship Between V and S

It is apparent in Fig. 2 that the number of transition-type differences S is not a monotonously increasing function of divergence time t, but has a maximum value and thereafter decreases. This is always the case if α is greater than β. This characteristic has not been pointed out by previous researchers, because their interest has been in counting the accumulated number of nucleotide substitutions, that is, transitions plus transversions (Kimura 1980, 1981; Takahata and Kimura 1981; Gojobori et al. 1982). In animal mtDNA, however, in which transition greatly predominates over transversion, this number is not adequate as a measure of genetic distance. Separate counting of transitions and transversions is preferable if possible, as is the case for direct sequence data.

The domain of convergence of the Newton method is narrow. To find a good initial parameter set, it is useful to have a good grasp of the characteristics of the relationship between $\bar{V}$ and $\bar{S}$,

$$\lim_{t \to 0} \frac{d\bar{S}}{d\bar{V}} = \frac{\alpha}{\beta} \cdot \frac{\pi_T\pi_C + \pi_A\pi_G}{\pi_Y\pi_R}$$

which is determined by α/β. Furthermore, for $\alpha > \beta$

$$\lim_{t \to \infty} \frac{d\bar{S}}{d\bar{V}} = -\frac{\pi_T\pi_C\pi_R/\pi_Y + \pi_A\pi_G\pi_Y/\pi_R}{\pi_Y\pi_R}$$

which is independent of the adjustable parameters. In Kimura's (1980) formulation, $d\bar{S}/d\bar{V}$ tends to $-\frac{1}{2}$ as t goes to infinity. His formula is a good approximation of ours for class 2 sites, where $d\bar{S}/d\bar{V}$ becomes -0.455 as t tends toward infinity, but not for class 1 sites, where $d\bar{S}/d\bar{V}$ becomes -0.288.

As t goes to infinity, $\overline{V(t)}$ and $\overline{S(t)}$ tend to $2fr\pi_Y\pi_R$ and $2fr(\pi_T\pi_C + \pi_A\pi_G)$, respectively, which values are dependent on the parameter f. To obtain a good

estimate of f, distantly related pairs of sequence data, in which the number of transition differences decreases as t increases, are needed. In our data set, the bovine–primate and mouse–(bovine, primate) pairs are important in this respect. On the other hand, to obtain a good estimate of α and β, closely related pairs of sequence data, in which the number of transition differences increases as t increases, are needed. In our data set, the human–chimpanzee and gorilla–(human, chimpanzee) pairs are important in this respect. We cannot obtain a good estimate of α, β, and f from a single pair of sequences, and sequence data from many species of organisms, both closely related and distantly related, are needed. Sequence data from intermediately related pairs of species increase the accuracy of the estimates. The statistical procedure developed in this paper takes into account the information contained in a set of sequence data more fully than any of the primitive methods of simple pairwise comparison of sequences. When mtDNAs from an Old World monkey and from a New World monkey are sequenced, the accuracy of our estimate will increase.

Average Rate of Nucleotide Substitutions in mtDNA

Our present study shows that in analyzing direct sequence data of mtDNA, transitions and transversions must be treated separately, because the rates of these two kinds of substitutions differ considerably. In analyzing restriction endonuclease mapping data of MtDNA, however, such a separate treatment is impossible. Therefore, it should be useful in interpreting the restriction mapping data to estimate the average rate of nucleotide substitutions in mtDNA irrespective of transition or transversion.

Since the previous studies estimated the rate of nucleotide substitutions in mtDNA without paying full attention to the fact that a considerable number of the transition-type differences in the class 1 sites, even those between human and chimpanzee, represent multiple hits (Brown et al. 1979, 1982; Nei 1982), their estimates are bound to be lower than ours. These clocks, which proceed slower than the real clock, have been used in dating relatively recent events; for example, divergences among human races have been dated based on restriction endonuclease fragment patterns of mtDNA (Cann et al. 1982; Nei 1982; Johnson et al. 1983). Therefore, these datings must be reexamined by our new molecular clock of mtDNA.

In a short time interval t, exp(x) in Eqs. (8a) and (8b) may be approximated by 1 + x. From these equations the average rate of nucleotide substitutions, that is transitions plus transversions, is therefore given by Hasegawa (1984)

$$(r_1 + r_2)^{-1} \sum_{k=1,2} 2r_k\hat{f}_k\{(\pi_T{}^k\pi_C{}^k + \pi_A{}^k\pi_G{}^k)\hat{\alpha}_k + \pi_Y{}^k\pi_R{}^k\hat{\beta}_k\}$$

the calculated value of which is $(25.4 \pm 6.1) \times 10^{-9}$ per site per year (the value after $\pm$ is the standard deviation). This is the average substitution rate of the segment of 899 nucleotides of mtDNA used in constructing our clock. Although regions outside this segment have evolved faster or slower than the segment, it seems reasonable to assume that the rate estimated above is representative of the average nucleotide substitution rate of the whole mitochondrial genome. The above estimate is much larger than the rates of 2.5×10^{-9} per site per year, estimated by Nei (1982), and 10×10^{-9} per site per year, estimated by Cann et al. (1982). Of the previous estimates, the rate of $10–20 \times 10^{-9}$ per site per year, estimated by Brown et al. (1982), is the closest to our value. The divergence times among human races estimated by the previous studies must be revised. The revised divergence times based on the molecular clock of mtDNA are more recent than the estimates obtained from the polymorphism of proteins coded for by nuclear genes (Hasegawa 1984). Because mtDNA is more susceptible to transfer between populations, the divergence time estimated from the mtDNA clock may indicate the time when mtDNA transfer last happened between two groups.

Discussion

The Date of Mammalian Divergence

Our datings of the splittings among hominoids are heavily dependent on the assumption that the divergence between bovines and primates occurred 65 Myr ago. Since the holocaust at the end of the Cretaceous is likely to have been responsible for the mammalian divergence, we think that the date of 65 Myr ago is closer to the truth than previous estimates.

Michael Novacek (personal communication) pointed out that the value of 90 Myr that we adopted for the divergence in the earlier paper (Hasegawa et al. 1984a) is unrealistically high, and suggested a range between 65 (first appearance of primates and ungulate groups in the fossil record) and 75 Myr ago for the split-off from the last common ancestor of primates and bovines. If the older limit of 75 Myr ago is taken, our clock gives 106.46 ± 13.54, 15.35 ± 1.78, 12.53 ± 1.43, 4.23 ± 0.71, and 3.09 ± 0.71 Myr ago for the separations from the human line of mouse, gibbon, orangutan, gorilla, and chimpanzee, respectively. Although there is some uncertainty as to the date of the mammalian divergence,

this divergence event seems to be the most reliable reference with which to calibrate the molecular clock of the various references used thus far.

Uniformity of the Rate of the Molecular Clock

The uniformity of the evolutionary rate of mtDNA among different lineages can be examined by a relative rate test (Wilson et al. 1977). From the data in Table 1, no significant difference is observed among the numbers of changes between the mouse and any one of the primates and bovines. Neither is any significant difference observed among the numbers of changes between bovines and any one of the primates. Furthermore, no significant difference is observed among the numbers of changes between the gibbon and other hominoids, and so on. One might notice that the number of transversion differences observed between gibbon and orangutan in the class 1 sites, 34, differs considerably from the 26 such differences observed between gibbon and the gorilla–chimpanzee–human trio. However, this discrepancy is not significant if the distribution is Poisson. The number of transition differences observed between mouse and bovines in the class 1 sites, 39, is also not significantly different from the 46–53 such differences between mouse and primates. Although the possibility of a small deviation from uniformity of the nucleotide substitution probability is not excluded, this test shows that our data indicate an approximate uniformity at least among primates and bovines.

Based on nuclear DNA hybridization data, some authors have contended that the nucleotide substitution rate was much higher along the lineages of mouse and rat than along other mammalian lineages (Kohne 1970; Kohne et al. 1972). However, their studies were based on questionable estimates of divergence time, as pointed out by Sarich and Wilson (1973) and by Wilson et al. (1977). Furthermore, Sarich and Cronin (1980) and Ferris et al. (1983) suggested that the rates of nucleotide divergence are similar for primates and rodents. Therefore, it is possible that the rate of mtDNA divergence in rodents does not differ from that for other mammalian orders.

At present, we tentatively think that the nucleotide substitution rate of rodents does not differ from that of other mammalian orders, and that, as our analysis indicates, rodents diverged from other placental mammals 92.27 ± 11.73 Myr ago, before the mammalian radiation among most of the extant placental mammalian orders that took place some 65 Myr ago. Since rodents are unique among placental mammals in that all attempts of paleontologists to relate them to other groups of mammals have been in vain (Colbert 1980), our hypothesis of an earlier rodent divergence may not be unreasonable.

In any case, the molecular clock hypothesis as applied to the whole mammalian class is still controversial. It will be desirable to test whether rodent DNA has evolved more rapidly than that of other placental mammals when an outside reference such as marsupial mtDNA is obtained. Even if the mouse line has evolved more rapidly than the others, it does not invalidate our approach in estimating divergence times among the Hominoidea.

Although the constancy of our clock with respect to absolute geological time has yet to be proven directly, future sequencing of mtDNAs from various families of primates and from various mammalian orders will clarify the accuracy and applicability of our clock in estimating divergence times among mammals. It has been proposed that the South American monkeys descended from African monkeys, not from North American prosimians, when the South American continent was close to the African continent some 35–38 Myr ago (Ciochon and Chiarelli 1980). When mtDNA from a new World monkey is sequenced, the validity of our clock will be tested.

Splittings of Orangutan and Gibbon from Human Line

In our earlier paper (Hasegawa et al. 1984a), we assumed that the 14.5-Myr-old specimen *Sivapithecus* was ancestral to the orangutan but not to humans (Raza et al. 1983), and that *Micropithecus,* which is some 20 Myr old, was ancestral to the gibbons but not to humans (Simons 1981). Assuming 90 Myr ago for the date of the splitting between primates and ungulates and using a least-squares method by minimizing $\tilde{R}$, represented by Eq. (12), we obtained for the divergence dates of the orangutan and of the gibbons from the human line 15.9 ± 2.9 and 19.1 ± 3.6 Myr ago ($n_{ijk\ell}$ values instead of $q_{ijk\ell}$ values were used in calculating Ω), respectively, which are consistent with the above interpretation of the fossil evidence. However, when we take 65 Myr for the date of mammalian divergence as in this paper, the estimated dates of the orangutan and gibbon divergences contradict the above interpretation of the fossil evidence.

Peter Andrews (personal communication) pointed out that the 14.5 Myr ago splitting time between humans and the orangutan (Raza et al. 1983) is based on the identification of a fragmentary fossil that is not well dated. Furthermore, he pointed out that *Micropithecus* had not been shown to be ancestral to the gibbons. *Micropithecus* is now recognized as belonging to a primitive group lacking the

synapomorphies (derived characters that are shared) of the Hominoidea (Andrews 1981). Our datings in this paper, 10.86 ± 1.24 and 13.30 ± 1.54 Myr ago for the orangutan and gibbon divergences, respectively, are in accord with those of Andrews and Cronin (1982), 10 ± 3 Myr ago for the orangutan divergence and 12 ± 3 Myr ago for the gibbon divergence, and also approximately in accord with those of Sarich and Cronin (1977), 9–11 and 11–13 Myr ago for the orangutan and the gibbon divergences, respectively.

Possibility That Australopithecus afarensis is Not an Ancestor of Humans That Evolved After the Human–Ape Splitting

There is a widely believed hypothesis that *Australopithecus afarensis,* which lived some 3.7 Myr ago at Laetoli in Tanzania and at Hadar in Ethiopia, is our ancestor and evolved after the human–ape splitting (Leakey et al. 1976; Johanson et al. 1978; Johanson and White 1979; Cronin et al. 1981; White et al. 1981). However, our dating of the human–chimpanzee splitting by the molecular clock of mtDNA is 2.68 ± 0.61 Myr ago, which is more recent that when *A. afarensis* lived. Two factors may be taken into consideration to explain this. First, the dating of the Hadar hominids is still controversial, and they could be younger than 3.4 Myr (Sarna-Wojcicki et al. 1985). Second, the divergence between primates and ungulates may have happened 75 Myr ago rather than 65 Myr ago, and if this is the case, then our clock gives 3.09 ± 0.71 Myr ago for the human–chimpanzee separation. Considering these uncertainties, the possibility that the hypothesis is compatible with our clock cannot be discounted. However, this compatibility rests on fragile ground, and it may be worthwhile to examine the validity of the hypothesis.

Since *A. afarensis* walked upright on two legs, despite the similarity of its brain capacity, dentition, and other features to those of apes, most paleoanthropologists believe it to be the first hominid. However, our molecular clock of mtDNA suggests that the human–ape splitting may have occurred more recently than when *A. afarensis* lived.

Bipedalism is widely believed to have been the first step in hominization (Leakey et al. 1976; Day and Wickens 1980; White 1980), and any fossil primates that walked upright have been readily accepted as our immediate ancestors. If *A. afarensis,* which walked upright, is not an ancestor that evolved after the human–ape splitting, as is suggested by our molecular clock, then the following two explanations of the origin of bipedalism are possible: (1) *A. afarensis,* an upright biped, was a common ancestor of humans and the chimpanzee (and probably the gorilla). The chimpanzee (and the gorilla) lost bipedalism after splitting from the human line. (2) *A. afarensis* was not an ancestor of any living primate. Bipedalism evolved independently in at least two lineages, the *A. afarensis* line and the human line.

The first possibility was pointed out by Gribbin and Cherfas (1982). Although no indication has been found that the chimpanzee had a bipedal ancestor, this does not necessarily exclude the first possibility.

Zihlman (1979) pointed out that the pygmy chimpanzee *Pan paniscus* has many morphological features in common with *A. africanus,* which is believed to have descended from *A. afarensis.* The pygmy chimpanzee is the species most closely related to the common chimpanzee *P. troglodytes* among extant hominoids (Zihlman et al. 1978; Ferris et al. 1981a, b; Brown et al. 1982; Sibley and Ahlquist 1984). Furthermore, Feldesman (1982a, b) noticed that *A. afarensis* clearly resembles *P. paniscus* in proximal ulnar morphology. The above arguments might be compatible with the first possibility, that knuckle-walking of extant chimpanzees and gorillas evolved from bipedality.

Oxnard (1975, 1984) pointed out that bipedalism might have evolved not once or even twice, but perhaps several times during the evolution of the Hominoidea. Therefore, the second possibility also appears likely. He claims that the australopithecines, including *A. afarensis, A. africanus,* and *A. robustus,* were not structurally closely similar to humans, and that they were adapted at least in part to an arboreal environment.

Although it is likely that the australopithecines were capable of bipedalism (but probably in a biomechanical mode quite different from that employed by humans), they might also have been quadrupedal, especially when climbing in trees. It is now being recognized that although *A. afarensis* could walk bipedally, it kept its balance more like a chimpanzee does than a human does (Stern and Susman 1981, 1983). Thus, the possibility that *A. afarensis* was not an ancestor of humans that evolved after the human–ape splitting cannot be ruled out at present.

It is unknown whether the last common ancestor of human and chimpanzee was like the living chimpanzee or the living human. However, it seems to have been widely assumed implicitly that the common ancestor of the two species was more like the chimpanzee than the human. There has been a tendency to view hominid features as specialized and those of apes as unspecialized. Any fossil hominoids that bear some resemblance to humans have been readily considered to be human ancestors that evolved after the human–ape splitting. Ramapithecines had long been believed to be ancestral to hu-

mans based on this type of reasoning. However, they are now believed to be ancestors of the living orangutan (Andrews 1982; Andrews and Cronin 1982; Pilbeam 1982).

Recently Alan Walker pointed out that the orangutan, which had been widely believed to be a highly specialized ape as compared with the chimpanzee and gorilla, may actually be the living hominoid that bears the most extensive resemblance among contemporary descendants to the last common ancestor of all the living great apes (Lewin 1983). In this sense, the orangutan may be a living fossil.

Schwartz (1984) pointed out that humans share uniquely few morphological features with either the chimpanzee or the gorilla, whereas many features are shared by humans and the orangutan. He concluded that humans and the orangutan may be the closest relatives among the living hominoids. Although the molecular evidence shows that his conclusion is clearly wrong (Goodman 1962, 1963; Sarich and Wilson 1967; Ferris et al. 1981a; Andrews and Cronin 1982; Brown et al. 1982; Hasegawa and Yano 1984; Sibley and Ahlquist 1984), his suggestion leads us to the following important point. Although brain capacity has increased very much along the human lineage, not only the orangutan but also the human may be living fossils with respect to various morphological features, whereas the chimpanzee and the gorilla may have specialized quickly after they diverged from the human line. If this is the case, it is possible that some fossil hominoids that were ancestral to the chimpanzee or gorilla but not the human have been assigned to human ancestors because of some of their residual features. It is now clear that the dating of the branching events from the fossil record alone is a highly difficult job in this circumstance, and that the molecular record is useful for this purpose.

A theory of human origin must be a theory of chimpanzee and gorilla origins too (Zihlman 1979), and to clarify our origin, paleoanthropologists must seek not only our ancestors but also fossil creatures ancestral to the chimpanzee or the gorilla but not to humans. However, no fossil assigned to be ancestral only to the chimpanzee or gorilla has yet been unearthed.

Possibility of Interspecies Transfer of Mitochondrial DNA between Proto-human and Proto-chimpanzee

Since mtDNA is inherited maternally, a molecular clock based on it can give only information on maternal lineages. Also, datings of species separation from mtDNA data may sometimes be in error because of the possibility of introgression of these independently segregating organelles from one species to another. This may be not only a weakness of mtDNA analysis, but also a strength, because in such a case the mtDNA clock could provide information on the ecological relationship between two species.

It has recently been found that mtDNA can pass across the species boundary in the mouse (Ferris et al. 1983), in the aquatic frog (Spolsky and Uzzell 1984), and in *Drosophila* (Powell 1983), and it is not impossible that such an event happened among early hominoids. If it did occur, the time derived from our clock should reflect it. The sequences of nuclear DNA, when they become available, should make the situation clear. At present the possibility must be considered that our clock reflects a transfer of mtDNA through hybridization between a proto-human and proto-chimpanzee after the former had developed bipedalism (Fig. 4).

When two closely related animal species are geographically contiguous, fertile interspecies hybrids sometimes arise at the boundary zone. Such cases are well known in primates, e.g., between anubis baboons (*Papio anubis*) and hamadryas baboons (*P. hamadryas*) (Nagel 1973; Shotake 1981; Sugawara 1982) and between redtail monkeys (*Cercopithecus ascanius*) and blue monkeys (*C. mitis*) (Aldrich-Blake 1968; Macdonald 1984).

Intergroup transfer of males is far more common among social primates than that of females, and in anubis baboons and Japanese and rhesus monkeys, which are among the most frequently studied monkeys, females typically remain in their natal group (e.g., see Moore 1984). In such a social system, interspecies transfer of mtDNA would seem difficult even if interspecies hybridization occurs frequently. In contrast, in societies of chimpanzees and gorillas, intergroup transfer of females and not of males is routine. Therefore, it appears possible that interspecies transfer of mtDNA happened between a proto-human and a proto-chimpanzee.

If interspecies transfer of mtDNA between proto-human and proto-chimpanzee did indeed occur, it is tempting to speculate in which direction the transfer occurred. The lesser intraspecies polymorphism of human mtDNA compared with that of chimpanzee (Ferris et al. 1981b) suggests that the transfer occurred from proto-chimpanzee into proto-human.

Interspecies transfer of mtDNA has been observed between the fruit fly *Drosophila pseudoobscura* and its sibling species *D. persimilis* (Powell 1983). Although male F_1 hybrids between the two species are sterile, F_1 females, the sex that must be fertile to pass mtDNA, are fertile. Thus even if there exists a barrier to interspecies hybridization, the introgression can occur (Takahata and Slatkin 1984).

Natural interspecies transfer of mtDNA is ob-

served from *Mus domesticus* into *M. musculus* (Ferris et al. 1983), and from *Rana lessonae* into *R. ridibunda* (Spolsky and Uzzell 1984). The extent of mtDNA divergence between *M. domesticus* and authentic *M. musculus* is about 5%, and that between *R. lessonae* and authentic *R. ridibunda* is about 8%. Since the average substitution rate of mtDNA is estimated to be 0.0254 per site per million years, the interspecies transfer of mtDNA occurred $0.05/(2 \times 0.0254) \approx 1$ Myr ago in mice and $0.08/(2 \times 0.0254) \approx 1.5$ Myr in aquatic frogs after the species separated. Therefore, if such an introgression occurred between proto-human and proto-chimpanzee 2.68 ± 0.61 Myr ago, then the human–ape splitting may date back to some 5 Myr ago, as suggested by Sarich and Wilson (1967) and by Sarich and Cronin (1977).

Sibley and Ahlquist (1984) calibrated the molecular clock based on their nuclear DNA–DNA hybridization data by assuming that the orangutan diverged from the human line 13–16 Myr ago. Since our molecular clock of mtDNA gives 10.86 ± 1.24 Myr ago as the date for the orangutan divergence, we think that the 13 Myr date is nearer the actual value than 16 Myr is, provided mtDNA transfer did not occur between the orangutan and the African apes. If the 13 Myr date is used for the orangutan splitting, their data give 6.3 Myr ago as the date for the human–chimpanzee separation, although the amount of error inherent in their data cannot be estimated.

Though the relevant data are limited, the hypothesis of a late divergence between humans and apes is also suggested by the amino acid sequence of proteins coded for by nuclear DNA (Goodman et al. 1983), and by the rarity of synonymous nucleotide substitutions in hemoglobin genes between humans and the African apes (Liebhaber and Begley 1983; Scott et al. 1984).

After submission of this paper, we learned that $\psi\eta$-globin genes, which are pseudogenes of the β-globin gene family, from human, chimpanzee, gorilla, owl monkey (New World monkey), and lemur (prosimian) have been sequenced (Chang and Slightom 1984; Goodman et al. 1984; Harris et al. 1984). Goodman and his coworkers (1984) contended that the nucleotide substitution rate was slower along the hominoid lineage than along the New World monkey lineage. However, the difference is not statistically significant, and we think that the nucleotide substitution probability per unit time interval of the $\psi\eta$-globin gene has been approximately uniform at least among the Anthropoidea. Assuming that the divergence between the Hominoidea and the New World monkeys occurred 38 Myr ago, our preliminary analysis of the $\psi\eta$-globin data gives dates of 5.2 ± 0.5 and 5.8 ± 0.6 Myr ago (the number after $\pm$ is the standard deviation) for the chimpanzee and gorilla separations, respectively, from the human line (unpublished data). These datings seem to contradict those from the mtDNA data, and this discrepancy may reflect mtDNA transfer between a proto-human and proto-chimpanzee.

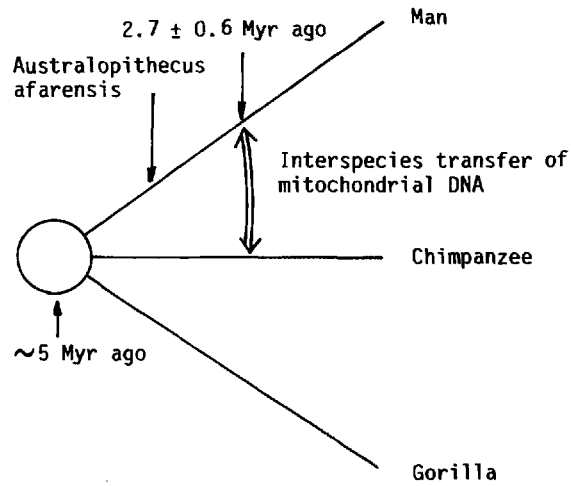

Fig. 4. A possible model of interspecies transfer of mtDNA between proto-human and proto-chimpanzee

The DNA sequence data presently available for setting our molecular clock are limited and the clock cannot always determine which one of the various possibilities discussed in this paper is the truth. Therefore, future sequencing of DNA, particularly nuclear DNA, in conjunction with future fossil findings should throw more light on the origin and evolutionary history of our species. In this paper, we have demonstrated that chimpanzee and human are far more closely related genetically than is generally believed. A molecular approach can be expected to remain an important tool for elucidating the origin and evolution of mankind.

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