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Recent African origin of modern humans revealed by complete sequences of hominoid mitochondrial DNAs

(molecular clock/synonymous substitution rate/multiregional hypothesis/"out of Africa" hypothesis)

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ABSTRACT **We analyzed the complete mitochondrial DNA (mtDNA) sequences of three humans (African, European, and Japanese), three African apes (common and pygmy chimpanzees, and gorilla), and one orangutan in an attempt to estimate most accurately the substitution rates and divergence times of hominoid mtDNAs. Nonsynonymous substitutions and substitutions in RNA genes have accumulated with an approximately clock-like regularity. From these substitutions and under the assumption that the orangutan and African apes diverged 13 million years ago, we obtained a divergence time for humans and chimpanzees of 4.9 million years. This divergence time permitted calibration of the synonymous substitution rate (3.89×10^{-8}/site per year). To obtain the substitution rate in the displacement (D)-loop region, we compared the three human mtDNAs and measured the relative abundance of substitutions in the D-loop region and at synonymous sites. The estimated substitution rate in the D-loop region was 7.00×10^{-8}/site per year. Using both synonymous and D-loop substitutions, we inferred the age of the last common ancestor of the human mtDNAs as 143,000 ± 18,000 years. The shallow ancestry of human mtDNAs, together with the observation that the African sequence is the most diverged among humans, strongly supports the recent African origin of modern humans, *Homo sapiens sapiens*.**

Light will be thrown on the origin of man and his history (1).

With recent advances of DNA technology and its introduction into evolutionary biology, we are in a position to shed new light on the history of man. Extensive analyses of cleavage sites and sequences of mitochondrial DNA (mtDNA) (2, 3) have shown that the African is the most variable of the ethnic groups and have supported an earlier suggestion (4) that the last common ancestor (LCA) of contemporary human mtDNAs existed some 200,000 years ago. These results have been taken as evidence that modern humans originated in Africa and migrated to Eurasia, replacing *Homo erectus* and the Neanderthals with no or little gene exchange. However, this notion was strongly disputed by proponents of the multiregional hypothesis (5), who claim that modern humans originated simultaneously in various geographic regions and that regional continuity in human characters has evolved over the past 1 million years (Myr). Under this hypothesis, the age of the LCA is unlikely to be as low as 200,000 years. The main controversy, therefore, has centered around the estimated age of the LCA and the reliability of the mitochondrial molecular clock (constancy of nucleotide substitution rates) on which it is based.

To resolve close relationships among humans, recent studies (6–9) have focused on sequence analysis in the rapidly evolving displacement (D)-loop region (≈1100 bp) of mtDNA. While the region is informative on sequence relatedness within a species, it is apparent that African apes are too distantly related to humans to permit calibration of the D-loop clock. The high substitution rate in the D-loop region results mainly from an extremely high rate of transitions, and to calibrate a D-loop clock, a method which uses the more slowly accumulating transversions was proposed (8). However, since there are only a few transversions in the D-loop region among humans, this method does not appear to calibrate the D-loop clock accurately.

Sequence data from the D-loop region alone are insufficient for the accurate estimation of substitution rates and divergence times of hominoid mtDNAs. We have therefore analyzed seven complete mtDNA sequences from three humans and four nonhuman hominoids.§ By selecting silent sites appropriate for a molecular clock and estimating the age of the LCA more accurately, we are able to discuss the origin of modern humans.

MATERIALS AND METHODS

African Sequence. Human mtDNA (SB17 in ref. 7) was isolated from a placenta obtained from an African from Uganda. Our analysis of a D-loop segment (482 bp) from 193 humans of various ethnic origins revealed that this individual had the most diverged sequence, which coalesced directly into the root of a phylogenetic tree (9). Ten sets of nonbiotinylated and 5′-biotinylated primers were designed to amplify overlapping DNA fragments (≈2 kb) covering the whole mitochondrial genome. Polymerase chain reaction (PCR) was carried out by a conventional procedure. The PCR products were mixed with magnetic beads (Dynabeads M-280 Streptavidin; Dynal). Both strands were then prepared as single-stranded DNAs by alkali denaturation and magnetic separation as described in the manufacturer's protocol and used as templates for sequencing reactions. Sequencing primers were made at ≈200-bp intervals, so that overlapping sequences could be obtained.

Japanese Sequence. The complete mtDNA sequences of 10 Japanese patients with neuromuscular disorders have been determined (10). For the present analysis, we selected the one (DCM1 in ref. 10) that is most distantly related to the European (11) in terms of nucleotide sequence and is not associated with any disease-specific mutations.

European Sequence. The European sequence (11) is unique in differing by transversions at as many as seven sites where all other human sequences have identical bases: G instead of T at 3423 (*ND1*) and 14,199 (*ND6*), and G instead of C at 9559 (*COIII*), 13,702 (*ND5*), 14,272 (*ND6*), 14,365 (*ND6*), and 14,368 (*ND6*) (numbering corresponds to ref. 11). Consequently, the percentage of transitions in European vs. African

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Abbreviations: D-loop, displacement loop; Myr, million years; LCA, last common ancestor.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D38112–D38116).

(86.4%) or Japanese (84.4%) is lower than in African vs. Japanese (95.1%) or in humans vs. either of the two chimpanzees (average of 92.5%). Inaccuracies in the published European sequence at the seven sites provide one possible explanation for these differences. To examine this possibility, we determined the nucleotides at the seven sites from an additional six individuals (two Europeans, two Japanese, one African, and one Papuan). These six sequences, as well as the 10 Japanese sequences (10), have no transversions at these sites. Also, four species of nonhuman hominoids have identical bases at four of the seven sites, and all other substitutions are transitions. These observations suggest that the bases at the seven sites of the European sequence are incorrectly identified. Thus, we have used an edited version of the European sequence, obtained by replacing bases at the seven sites with those shared by other humans.

Other Hominoid Sequences. Partial mtDNA sequences from common and pygmy chimpanzees, gorilla, and orangutan were determined previously (12–14). The remaining sequencing was completed in the following way. mtDNAs of common chimpanzee and Bornean orangutan purified from Epstein–Barr virus-transformed cell lines were cloned into plasmid pUC19 and sequencing templates were prepared by the alkali denaturation method. Nucleotide sequences of mtDNAs of gorilla and pygmy chimpanzee were determined directly from PCR amplifications from genomic DNA. While most of the 10 primers designed for human mtDNA gave sufficient amplification, species-specific primers were also used. All sequencing reactions were performed by the dideoxynucleotide chain-termination method using [α-^{32}P]dCTP and the 7-deaza Sequenase kit (United States Biochemical). Nucleotide sequence was determined for both strands.

RESULTS AND DISCUSSION

Phylogenetic Analysis. Tables 1 and 2 show the numbers of nucleotide differences in all pairs of the seven sequences. The African and Japanese sequences differ from the European sequence at 93 and 49 nucleotide sites, respectively, much larger differences than published previously. Among the interspecific comparisons involving humans, the smallest number of differences observed was between humans and chimpanzees, which reinforces our previous conclusion (12) that the closest relatives of humans are chimpanzees. An exception is the 12S rRNA sequence, in which human sequences are slightly more similar to the gorilla than to the chimpanzees. However, this exception may be due to mere chance, since the number of sequence differences is <40.

The substitution rates and base compositions differ substantially among regions. When the numbers of substitutions at synonymous sites are plotted against those at nonsynonymous sites (Fig. 1*a*) or those in the RNA genes (12S rRNA, 16S rRNA, and an assembly of 22 tRNA genes) (Fig. 1*b*), a linear relationship is observed in all comparisons except those involving the orangutan. The deviation of the orangutan comparisons most likely reflects uncorrected multiple-hit synonymous substitutions. In contrast, an approximately linear relationship is observed between nonsynonymous and RNA gene substitutions in all comparisons (Fig. 1*c*), indicating that these substitutions have accumulated at a constant rate during the evolution of hominoids.

The number of substitutions at nonsynonymous sites and that in the RNA region were added for each pair of sequences and the sums were used in constructing the UPGMA (unweighted pair-group method with arithmetic mean) tree (Fig. 2*a*). To evaluate the reliability of the UPGMA tree, we also applied the MP (maximum parsimony) and ML (maximum likelihood) methods to a data set consisting of the RNA genes as well as first and second codon positions of the protein genes. Both the MP tree (Fig. 2*b*) and the ML tree (Fig. 2*c*) gave exactly the same topology as the UPGMA tree, even though the data sets used were different; in particular, substitutions at first codon positions include synonymous substitutions of leucine codons.

Table 1. Nucleotide differences in the protein-coding genes (11,262 sites) among hominoid mtDNAs

	Synonymous changes					Nonsynonymous changes						
Sequences compared*	CT1	AG1	CT3	AG3	V3	CT1	CT2	AG1	AG2	V1	V2	V3
European–Japanese	2	0	12	9	0	3	0	7	0	0	0	0
European–African	2	0	18	16	2	1	1	13	2	1	0	0
African–Japanese	2	0	22	19	2	4	1	12	2	1	0	0
C. chimp–P. chimp	27	0	220	116	23	17	20	45	8	4	4	1
European–C. chimp	78	5	563	231	54	20	46	72	17	18	6	1
Japanese–C. chimp	78	5	567	229	54	23	46	77	17	18	6	1
African–C. chimp	78	5	566	227	56	21	47	75	19	19	6	1
European–P. chimp	74.5	5	567	212	50.5	24.5	52	60	13	16	6	2.5
Japanese–P. chimp	74.5	5	575	210	50.5	27.5	52	65	13	16	6	2.5
African–P. chimp	74.5	5	570	206	52.5	25.5	53	63	15	17	6	2.5
European–Gorilla	100	4	647	245	110.5	37	65	73	20	22	14	9.5
Japanese–Gorilla	98	4	645	242	110.5	38	65	78	20	22	14	9.5
African–Gorilla	100	4	645	239	112.5	38	66	74	22	23	14	9.5
C. chimp–Gorilla	83.5	3	624	238	104	38.5	59	89	23	24	12	11
P. chimp–Gorilla	86	3	619	214	102.5	40	65	81	18	22	10	12.5
European–Orangutan	106.8	4	675	239	242	66.2	137	147	28	88	30	27
Japanese–Orangutan	107.3	4	677	236	242.5	64.7	137	152	28	88	30	26.5
African–Orangutan	106.8	4	678	232	244.5	65.2	138	155	30	87	30	26.5
C. chimp–Orangutan	106.9	5	704	240	233.5	74.1	134	137	28	92	30	32.5
P. chimp–Orangutan	108	5	704	217	234.2	74	141	130	25	90	28	31.8
Gorilla–Orangutan	104.2	4	696	232	242	69.8	140	143	27	84	36	33

Numbers of A ↔ G (AG) and C ↔ T (CT) transitions and transversions (V) are shown for the 13 assembled protein-coding genes. The first, second, and third codon positions are indicated by suffixes 1, 2, and 3 to AG, CT, or V. Synonymous and nonsynonymous differences are distinguished. We excluded from the analysis intergenic sequences, overlapping sequences of ATPase subunits 8 and 6 and of *ND4L* and *ND4*, termination codons, and all sequences that were deleted in one or more species. The differences are relative to the light strand of mtDNA, on which all the protein genes, with the exception of *ND6*, are encoded. For the *ND6* gene, synonymous CT transitions [e.g., CTR (Leu) to TTR (Leu)] are counted as synonymous AG transitions.

*C. chimp and P. chimp, common and pygmy chimpanzees, respectively.

Table 2. Nucleotide differences in the RNA genes and D-loop region among hominoid mtDNAs

	12S rRNA† (945 sites)			16S rRNA (1554 sites)			tRNAs (1504 sites)			D-loop region‡ (1105 sites)		
Sequences compared*	CT	AG	V	CT	AG	V	CT	AG	V	CT	AG	V
European–Japanese	0	3	0	2	1	0	0	0	0	6	4	0
European–African	1	4	1	2	3	0	3	2	0	13	6	2
African–Japanese	1	3	1	4	2	0	3	2	0	15	8	2
C. chimp–P. chimp	5	5	1	17	14	6	18	9	4	63	27	19
European–C. chimp	24	8	3	47	25	9	37	18	4	75	40	31
Japanese–C. chimp	24	11	3	49	26	9	37	18	4	77	38	31
African–C. chimp	23	10	2	49	26	9	38	18	4	77	38	38
European–P. chimp	21	7	2	55	27	13	36	19	2	75	44	34
Japanese–P. chimp	21	10	2	55	28	13	36	19	2	73	44	34
African–P. chimp	22	9	1	57	28	13	37	19	2	74	42	36
European–Gorilla	19	11	3	67	33	16	52	30	7			
Japanese–Gorilla	19	14	3	67	34	16	52	30	7			
African–Gorilla	20	13	4	65	34	16	53	28	7			
C. chimp–Gorilla	25	9	6	61	26	17	50	30	9			
P. chimp–Gorilla	22	10	5	58	30	21	51	33	7			
European–Orangutan	44	31	6	78	53	33	86	48	21			
Japanese–Orangutan	44	34	6	78	54	33	86	48	21			
African–Orangutan	45	31	5	78	52	33	87	48	21			
C. chimp–Orangutan	49	31	7	75	48	34	84	48	21			
P. chimp–Orangutan	48	28	6	76	45	40	88	49	21			
Gorilla–Orangutan	43	34	9	83	55	41	88	47	22			

Numbers of A ↔ G (AG) and C ↔ T (CT) transitions and transversions (V) are shown for the 12S rRNA gene, the 16S rRNA gene, the 22 assembled tRNA genes, and the D-loop region. The nucleotide sequences of the D-loop region from gorilla (14) and Bornean orangutan (present study) are not included in the analysis, since they show deletions of 213 bp and 155 bp, respectively, when aligned with the remaining five sequences over a total length of 1153 bp. (Human, common chimpanzee, and pygmy chimpanzee have deletions of 31, 40, and 32 bp, respectively.)

*C. chimp and P. chimp, common and pygmy chimpanzees, respectively.

†Nucleotide sequence for Bornean orangutan was determined in this study; those for other nonhuman hominoids were previously reported (13).

‡Nucleotide sequences for common and pygmy chimpanzees have been taken from a previous paper (14).

Calibration of the Molecular Clock. To calibrate the substitution rate at nonsynonymous sites and in the combined RNA genes, we used the UPGMA estimates of branch lengths. By associating the node separating the orangutan and the African apes with the species divergence time based on the fossil record, we estimated the substitution rate as 0.35×10^{-8}/site per year. We used neither MP nor ML estimates, because the data set used for these methods includes synonymous substitutions. Assuming that the orangutan and African apes diverged 13 Myr ago (19), we obtained the divergence times of gorilla (T_g), human (T_h), between common and pygmy chimpanzees (T_c), and the LCA of humans (T_{LCA}) as 6.56 ± 0.26, 4.87 ± 0.23, 2.33 ± 0.17, and 0.45 ± 0.07 Myr ago, respectively (standard errors were estimated by the method of ref. 20). The T_h and T_c values are close to our previous estimates (T_h = 4.7 ± 0.5 and T_c = 2.5 ± 0.5 Myr ago in ref. 12), although T_g here is a little smaller. It should be noted that even if we use 16 Myr for the orangutan divergence time (21), T_h is still <6 Myr.

Abundance of A ↔ G Transitions Within Human mtDNAs. Interspecific comparisons of hominoid mtDNAs show more C ↔ T than A ↔ G transitions (C ↔ T/A ↔ G ratio = 2.27) and many more transitions than transversions (22). The low G content (13%) in these mtDNAs appears to account for this C ↔ T transition abundance. Among human mtDNAs, however, A ↔ G transitions are in general more abundant than C ↔ T transitions (C ↔ T/A ↔ G ratio = 0.82): the C ↔ T/A ↔ G ratio is 1.89 in the D-loop region and 1.32 at synonymous sites, while it is 0.80 in the RNA genes and 0.38 at first codon positions in the protein genes.

The two most frequent amino acid changes are Ala ↔ Thr and Ile ↔ Val (60–80% of the total amino acid changes among humans), both of which reflect A ↔ G substitutions at first codon positions. Since these amino acid changes seem to be conservative in terms of chemical properties, they might be selectively neutral and occur rapidly (23). The number of Ala ↔ Thr and Ile ↔ Val changes increases rapidly when species divergence times are short but tends to level off at longer times (data not shown). This is in contrast to the total numbers of other amino acid changes, which show an approximately linear increase. The curvilinear relationship for Ala ↔ Thr and Ile ↔ Val suggests either an enhanced rate of A ↔ G substitutions in the human and chimpanzee lineage or saturation in comparisons of more distantly related species. In either case, Ala ↔ Thr and Ile ↔ Val substitutions overestimate T_c and T_{LCA} (see Fig. 2*a*).

Rate of Synonymous Substitutions. We analyzed synonymous differences at third codon positions, where transitions in 2-fold degenerate codons and all substitutions in 4-fold degenerate codons are synonymous. We excluded synonymous differences at first positions in leucine codons for technical reasons. However, since the number of these differences is relatively small (Table 1), it is unlikely that this exclusion affects the result. Although synonymous differences approach saturation rapidly, correction of multiple-hit substitutions is feasible within the human and chimpanzee clade. In addition, there are a large number of synonymous differences even within humans (Table 1). It appears that synonymous substitutions provide the best molecular clock within the human and chimpanzee clade.

We compared 3754 third codon positions and found nearly 40 synonymous differences between the African and non-African sequences. Both numbers are much larger than in previous studies (2, 6–9). To infer the actual number of synonymous substitutions per site (K_s), we first estimated the number of substitutions from transitional differences at third positions in 2-fold degenerate codons and all differences in 4-fold degenerate codons separately and then averaged the estimates, weighted by the relative frequencies of the two kinds of sites. The K_s between humans and chimpanzees is 0.381, with 95% confidence intervals (±2 SE) ranging from 0.329 to 0.433. To calibrate the synonymous substitution rate, we used

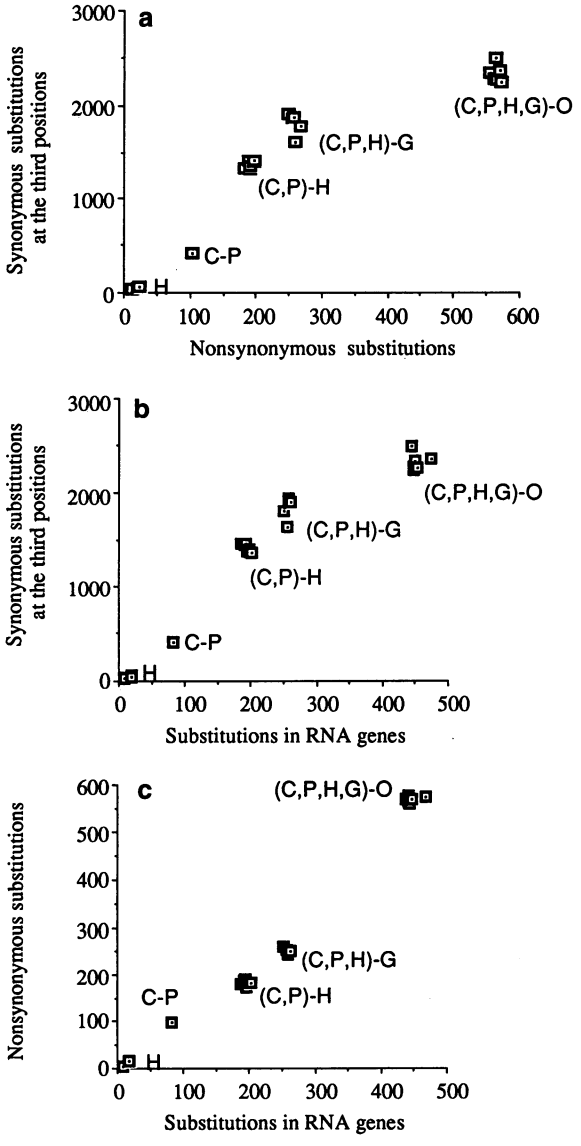

FIG. 1. Estimated numbers of nonsynonymous substitutions, synonymous substitutions at the third codon positions, and nucleotide substitutions in the RNA genes were plotted against each other. Points designated by H, C-P, (C, P)-H, (C, P, H)-G, and (C, P, H, G)-O represent the numbers in the comparisons within humans, between common and pygmy chimpanzees, between chimpanzees and humans, between gorilla and chimpanzees and humans, and between orangutan and the others, respectively. Substitutions for each of the RNA genes (12S rRNA, 16S rRNA, and an assembly of 22 tRNAs) and for each codon position of the grouped 13 protein genes were calculated separately by taking account of the base composition and empirical transition and transversion rate (15). The average content (%) for each region is listed in the order of A, T, G, and C as follows: assembly of 22 tRNAs (35, 27, 15, and 23%), 16S rRNA (35, 22, 17, and 26%), 12S rRNA (33, 22, 19, and 26%), first codon positions (32, 20, 20, and 28%), second codon positions (20, 41, 11, and 28%), third codon positions (37, 16, 4, and 43%), and D-loop region (30, 23, 14, and 33%).

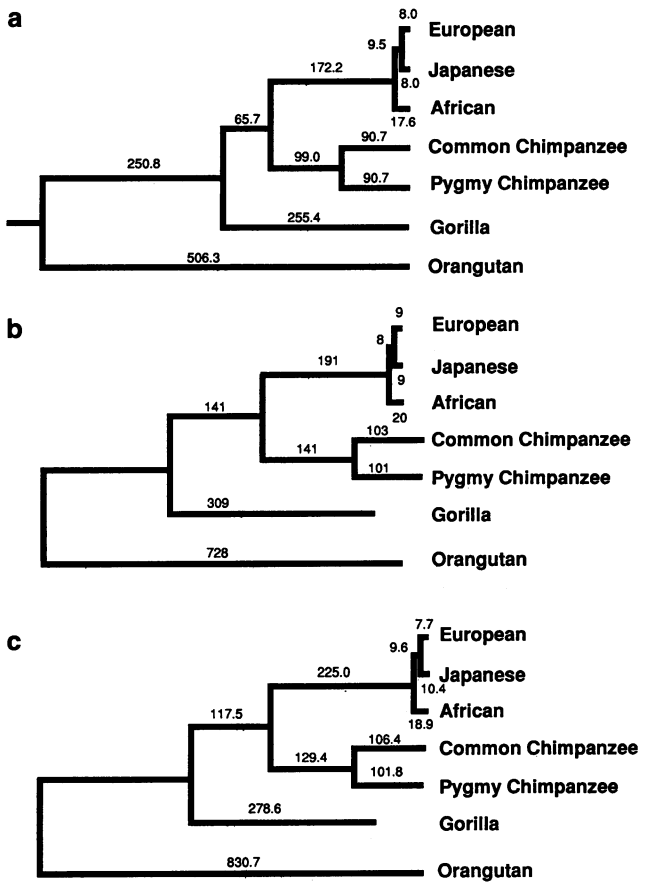

FIG. 2. Phylogeny of the seven sequences. Numbers represent branch length in terms of estimated numbers of substitutions. (*a*) UPGMA unweighted pair-group method with arithmetic mean tree (16). (*b*) MP (maximum parsimony) tree (17). (*c*) ML (maximum likelihood) tree (18).

the divergence time between humans and chimpanzees (T_h) of 4.9 Myr ago, estimated from Fig. 2*a*. The estimated rate [$k_s = K_s/(2T_h)$] is 3.89×10^{-8}/site per year.

Substitution Rate in the D-Loop Region. About 23% of the total sequence differences between African and European mtDNAs are found in the D-loop region (Table 2), which encompasses only 7% of the genome and encodes no genes.

Despite extensive analyses of D-loop sequence data (6–9), an accurate estimation of the substitution rate has remained problematic. We calculated the overall ratio of the per-site substitutions in the D-loop region to the per-site synonymous substitutions in pairwise comparisons of all human mtDNAs and obtained a value of 1.8. Thus, assuming that the D-loop region has evolved 1.8 times faster than synonymous sites, we obtained 7.00×10^{-8}/site per year as the D-loop substitution rate ($k_d = 1.8 \times k_s$). In the D-loop region, most substitutions occur in two hypervariable segments (I and II) (6). Our estimates of the substitution rate for segment I (378 bases; positions 16,024–16,401 in the numbering system of ref. 11) and for segment II (380 bases; positions 29–408) are, respectively, 10.3×10^{-8} and 7.39×10^{-8}/site per year.

Age of the LCA. Based on the synonymous substitutions and all substitutions in the D-loop region, we computed divergence times within humans (Fig. 3). The age of the LCA (T_1) is estimated as 143,000 ± 18,000 years. Although the estimated age of the LCA is not much different from that in ref. 2, the reliability of our estimate in terms of the standard error has increased substantially. As to the age of the common ancestor between the European and Japanese mtDNAs (T_2), our estimate is 70,000 ± 13,000 years. These estimates of divergence times are somewhat older than those inferred from gene frequency data, which place the first division between Africans and non-Africans at 117,000 years ago and the divergence between Europeans and Asians at 55,000 years ago (24). That gene divergence predates population divergence is the rule rather than the exception, particularly when the divergence time (in terms of generations) between populations is short relative to the number of breeding individuals in each population (16).

We note that the upper 95% confidence limit (divergence time + 2SE) of the age of the LCA is as low as 179,000 years.

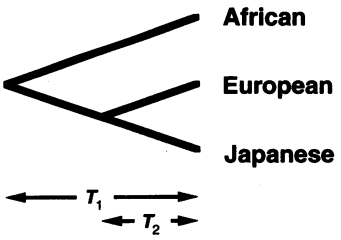

FIG. 3. Phylogeny and divergence times among humans. Estimates of divergence times T_1 and T_2 and their variances, $V(T_1)$ and $V(T_2)$, were determined from substitutions at synonymous third codon positions and the D-loop region by

$$T_1 = \frac{[(D_{AE} + D_{AJ})L_d + (S_{AE} + S_{AJ})L_s]}{4(k_dL_d + k_sL_s)} = 143{,}133$$

$$T_2 = \frac{(D_{EJ}L_d + S_{EJ}L_s)}{2(k_dL_d + k_sL_s)} = 70{,}358$$

$$V(T_1) = \frac{8[(L_d^2(VD_{AE} + VD_{AJ}) + L_s^2(VS_{AE} + VS_{AJ})]}{L_dk_d + L_sk_s} = 328{,}090{,}207$$

$$V(T_2) = \frac{4(L_d^2VD_{EJ} + L_s^2VS_{EJ})}{L_dk_d + L_sk_s} = 162{,}515{,}017,$$

where L denotes length (bp); k, substitution rate; D and S, distance; and V, variance. Subscript d designates the D-loop region, and s, synonymous third codon positions. Subscripts A, E, and J represent the African, European, and Japanese sequences, respectively. Standard errors for (T_1) and (T_2) are 18, 113 and 12,748, respectively. Values used: $L_d = 1105$, $L_s = 3646$, $k_d = 7.00 \times 10^{-8}$, $k_s = 3.89 \times 10^{-8}$, $D_{EJ} = 0.91 \times 10^{-2}$, $D_{AE} = 1.94 \times 10^{-2}$, $D_{AJ} = 2.32 \times 10^{-2}$, $S_{EJ} = 5.69 \times 10^{-3}$, $S_{AE} = 9.75 \times 10^{-3}$, $S_{AJ} = 11.77 \times 10^{-3}$, $VD_{EJ} = 0.84 \times 10^{-5}$, $VD_{AE} = 1.83 \times 10^{-5}$, $VD_{AJ} = 2.20 \times 10^{-5}$, $VS_{EJ} = 1.57 \times 10^{-6}$, $VS_{AE} = 2.58 \times 10^{-6}$, and $VS_{AJ} = 3.21 \times 10^{-6}$.

While such an estimated age of the LCA by no means implies that modern humans emerged at that time, the age expected according to the multiregional hypothesis (5) should be as old as 1 Myr if gene exchanges among local populations were limited. If the recent redating of Asian *H. erectus* is correct (25), the expected age of the LCA may be even older than 1.8 Myr. Only when gene exchanges occur frequently among local populations and the total number of breeding individuals in the whole population is kept as small as about 10,000 does the multiregional hypothesis become compatible with the estimated age of the LCA (26). However, it is difficult to explain how such a small number of individuals could occupy vast areas in Africa and Eurasia over the last 1 Myr while maintaining an evolutionary status as a single species. A more likely explanation is that the age of the LCA indicates that modern humans originated much less than 1 Myr ago without integrating the substantially diverged *H. erectus* genes. This, together with the premise that the genetic diversity in the oldest parental population is greater, provides support for the recent African origin of modern humans.

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