African Haplogroup L mtDNA Sequences Show Violations of Clock-like Evolution

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A set of 96 complete mtDNA sequences that belong to the three major African haplogroups (L1, L2, and L3) was analyzed to determine if mtDNA has evolved as a molecular clock. Likelihood ratio tests (LRTs) were carried out with each of the haplogroups and with combined haplogroup sequence sets. Evolution has not been clock-like, neither for the coding region nor for the control region, in combined sets of African haplogroup L mtDNA sequences. In tests of individual haplogroups, L2 mtDNAs showed violations of a molecular clock under all conditions and in both the control and coding regions. In contrast, haplogroup L1 and L3 sequences, both for the coding and control regions, show clock-like evolution. In clock tests of individual L2 subclades, the L2a sequences showed a marked violation of clock-like evolution within the coding region. In addition, the L2a and L2c branch lengths of both the coding and control regions were shorter relative to those of the L2b and L2d sequences, a result that indicates lower levels of sequence divergence. Reduced median network analyses of the L2a sequences indicated the occurrence of marked homoplasy at multiple sites in the control region. After exclusion of the L2a and L2c sequences, African mtDNA coding region evolution has not significantly departed from a molecular clock, despite the results of neutrality tests that indicate the mitochondrial coding region has evolved under nonneutral conditions. In contrast, control region evolution is clock-like only at the haplogroup level, and it thus appears to have evolved essentially independently from the coding region. The results of the clock tests, the network analyses, and the branch length comparisons all caution against the use of simple mtDNA clocks.

Introduction

It is more than 15 years since Cann, Stoneking, and Wilson (1987) published their watershed study in which the divergence of mtDNA molecules in different populations was used to derive a model for the evolution of modern humans. They concluded that modern humans arose in Africa ~200,000 years ago, migrated from there, and replaced archaic humans without admixture. Many aspects of this study have been challenged, as well as defended (a comprehensive analysis and discussion of the main issues can be found in Relethford [2001]).

A key issue for the approach used by Cann, Stoneking, and Wilson (1987), and for subsequent studies of this type, is whether the human mitochondrial genome has evolved in a clock-like manner. The use of a mtDNA clock to time the major events in human evolution and population dispersal has been questioned on several grounds (Gibbons 1998; Ayala 1999; Glazko and Nei 2003). Recent results support the operation of more complex, non-clock evolutionary processes in numerous taxa, including primates (e.g., Zeng et al. 1998; Grissi et al. 2000; Yoder and Yang 2000; Soltis et al. 2002; Yi, Ellsworth, and Li 2002; Glazko and Nei 2003). The more general issue of whether human mtDNA evolution fits the neutral model has also been investigated. The available evidence indicates that the evolution of most, and perhaps all, mitochondrial genes has been nonneutral (Gerber et al. 2001; Rand 2001; Mishmar et al. 2003; Moilanen and Majamaa 2003; Moilanen, Finnilä, and Majamaa 2003; Nielsen and Yang 2003; Elson, Turnbull, and Howell 2004).

Key words: mitochondrial DNA, molecular evolution, phylogenetic analysis, molecular clock, human evolution.

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The availability of large sets of complete human mtDNA sequences (e.g., Ingman et al. 2000; Herrnstadt et al. 2002) provides the foundation for comprehensive and critical tests of the molecular clock model. Ingman et al. (2000) analyzed a set of 53 complete mtDNA sequences of diverse ethnic origin. They observed that coding region sequences evolved according to a molecular clock, but that control region evolution was not clock-like. Torroni et al. (2001) analyzed a small set of complete sequences from the African L2 mtDNA haplogroup, and they observed a violation of clock-like evolution. In view of the reliance of phylogeographic studies of human evolution and population dynamics on an mtDNA clock, especially one based on control region evolution, the accuracy of such a clock is a subject that will benefit from further analysis and refinement.

We have recently analyzed a set of 560 mtDNA coding region sequences (Herrnstadt et al. 2002) and shown that selection has influenced the evolution of the human mitochondrial genome (Elson, Turnbull, and Howell 2004). As a follow-up to those studies, we report here our tests of clock-like evolution in African haplogroup L mtDNA sequences. The results are complex, and they argue against any simple mtDNA clock for timing events during human evolution. In addition, this is the most extensive analysis thus far of African mtDNA coding region sequences.

Experimental Procedures

mtDNA Sequences

The complete sequences of 93 African haplogroup L mtDNAs were determined as described in detail elsewhere (Herrnstadt et al. 2002; Herrnstadt, Preston, and Howell 2003). The coding region sequences of 56 of these mtDNAs were published in Herrnstadt et al. (2002), and they are published on the MitoKor Web site

Table 1
African mtDNA Sequences Analysed

Clade L0a (5): 149, 560, 585, 586, 587[a]
Haplogroup L1b (12): 158, 293, 192, 215, 379, 386, 514, 588, 589, 590, 591, 592
Haplogroup L1c (9): 173, 194, 207, 328, 593, 594, 595, 596, 597
Haplogroup L2a (30): 142, 156, 162, 165, 172, 193, 195, 199, 208, 223, 233, 380, 382, 388, 389, 401, 421, 434, 561, 562, 563, 564, 565, 566, 567, 569, 570, 571, 576, 577
Haplogroup L2b (5): 175, 222, 385, 568, 574
Haplogroup L2c (5): 572, 574, 575, TORR02, TORR03
Haplogroup L2d (3): 153, 160, TORR01
Haplogroup L3b (6): 104, 108, 155, 164, 189, 309
Haplogroup L3d (3): 140, 381, 413
Haplogroup L3e (13): 136, 159, 163, 166, 180, 196, 211, 216, 217, 242, 387, 582, 583
Haplogroup L3f (5): 578, 579, 580, 581, 584

NOTE.—The 96 African mtDNA sequences analyzed here are listed according to their three-digit MitoKor database numbers. For the sequences with numbers of 560 or less, the coding region sequences have been reported previously (Herrnstadt et al. 2002). Sequences with numbers >561 have not been reported. Three of these sequences (TORR01, TORR02, and TORR03) were published by Torroni et al. (2001). These African mtDNA sequences have been assigned to haplogroup L subclades on the basis of the nomenclature of Salas et al. (2002).

[a] These sequences may be further classified as L0a2, L0a1a, L0a1a, L0a1, and L0a1a, respectively (see fig. 2 of Salas et al. 2002).

(http://www.mitokor.com/science/560mtdnas.php). In addition to the 93 MitoKor sequences which are provided in the Supplementary Material online, the one haplogroup L2d and the two haplogroup L2c mtDNA sequences analyzed by Torroni et al. (2001; see their fig. 2) were included in the present analyses.

These analyses involved 11 African mtDNA haplogroup L subclades (table 1), and the sequences were assigned to the different subclades on the basis of the phylogenetic studies of Salas et al. (2002). L0a sequences were previously included in paraphyletic haplogroup L1, but L0 is now considered a separate clade that includes the ancestral node of modern humans (see the discussion on p. 1094 of Salas et al. [2002]). Clades L1b and L1c remain members of haplogroup L1. Superclade L3 includes both sub-Saharan haplogroups as well as the Eurasian M and N superhaplogroups (Salas et al. 2002). In this report, the analysis is limited to sequences of the former (paragroup L3A lineages in the terminology of Salas et al. [2002]), and we designate this group of sequences as "haplogroup L3."

The mtDNA control region is the contiguous sequence that spans nucleotides 1–576 and 16024–16569, whereas the coding region spans nucleotides 577–16023, although it does contain some small regions that do not encode an RNA or protein product. All mtDNA alleles are expressed as the nucleotide on the L-strand and all sequence changes are relative to the outgroup sequence.

The haplogroup L mtDNAs analyzed here were obtained from African Americans, not directly from African populations. In a similar fashion, the African haplogroup L2 mtDNA sequences analyzed by Torroni et al. (2001) were obtained from inhabitants of Puerto Rico, whereas the comprehensive analyses of Salas et al. (2002) involved samples from a number of populations, including African Americans (see also Salas et al. 2004). There is no evidence that these studies are biased by this sampling scheme.

Background and General Approach of the Present Study

According to Kimura's model of neutral evolution (1968), the rate at which mutations become fixed in the population equals the rate of mutation ($k = u$), because the vast majority of sequence changes are neutral with respect to selection. As a consequence, the rate process of substitution should be the same among all lineages (that is, sequence divergence should be clock-like). It was further assumed by Kimura that this single substitution rate operated as a simple Poisson process. As pointed out by Zheng (2001), however, the molecular clock model requires no more than the same process of substitution—irrespective of its complexity—in all lineages. A molecular clock can operate in the presence of selection, although the conditions required are restricted and unlikely to have occurred during human evolution.

Donnelly (1991) has drawn some important distinctions among the operational rates of mutation that bear directly on tests of an mtDNA clock (see also the related discussion on p. 1607 in Sigurðardóttir et al. [2000]). In his terminology, the molecular clock stipulates that $k_s = u$, where k_s is the rate at which mutations are fixed at the species level. A third parameter is k_a, the rate of sequence evolution along an ancestral lineage, and it is the rate of evolution that is tested here and that was tested in the clock analyses of Ingman et al. (2000) and Torroni et al. (2001). The present study is thus designed to test whether the rate of divergence k_a within haplogroup L mtDNAs has been clock-like in the specific sense of a single substitution process among all lineages. Intraspecific analyses have been used by other investigators to test the neutral model of evolution (e.g., Fu and Li 1993; Templeton 1996; Huelsenbeck and Rannala 1997; Gerber et al. 2001).

Likelihood Ratio Tests of Clock-like Sequence Evolution

Likelihood ratio tests (LRTs) were used to determine if the divergence of mtDNA sequences was consistent with a molecular clock (Felsenstein 1988; Huelsenbeck and Rannala 1997; Zhang 1999; Posada 2001). LRTs were carried out with Tree-Puzzle version 5.0 (Strimmer and von Haeseler 1996; available at http://www.tree-puzzle.de). In brief, the clock test involved computation of maximum-likelihood branch lengths for a set of sequences under two conditions. In the first condition, evolution was constrained to a single mutation rate process for all branches (clock) and, in the second, rates were allowed to vary among branches (non-clock). For statistical analysis, the differences between the clock and non-clock maximum-likelihood phylogenetic trees (multiplied by 2) are assumed to fit a χ^2 distribution with $n - 2$ degrees of freedom, where n is the number of sequences analyzed (Felsenstein 1988).

In our "default" analyses, the Tree-Puzzle clock tests were performed with the Neighbor-Joining algorithm, the HKY model of nucleotide substitution (Hasegawa, Kishino, and Yano 1985), the "accurate (slow)" estimation procedure, and a uniform rate of mutation at all sites (Strimmer and von Haeseler 1996). Tests were also performed with the Tamura-Nei (TN) model of nucleotide substitution, which is

a more general model of substitution than the HKY model. More importantly, clock tests were also performed under the condition of site variability of divergence rates, because branch lengths are underestimated with uniform substitution rates and, as a result, molecular clock tests can become too conservative (Cunningham, Zhu, and Hillis 1998; Zhang 1999; Posada 2001). When rate heterogeneity is incorporated into the clock test, Tree-Puzzle models substitution rates as i.i.d. "draws" (changes are independently and identically distributed) from a discrete gamma distribution that is parameterized by α, the shape distribution parameter (Yang 1994). When α approaches infinity, then all sites evolve at the same rate.

A human mtDNA sequence, usually from the L0a subclade, has been used as the outgroup for the studies reported here, rather than a nonhuman primate mtDNA sequence. For comparison, Ingman et al. (2000) used gorilla and chimpanzee mtDNA outgroup sequences in their clock tests, whereas Torroni et al. (2001) used an L0a outgroup sequence. There are two main reasons for our choice of an intraspecific outgroup. First, a distant outgroup sequence is more likely to be affected by mutational saturation and the clock test can become too conservative as a result (Bromham et al. 2000). There are longstanding concerns that primate sequences are too diverged from human sequences to function as informative outgroups (e.g., Wheeler 1990, Maddison, Ruvolo, and Swofford 1992). Second, there is evidence that the substitution process in mtDNA is not homogenous among different primates (e.g., Excoffier and Yang 1999; Meyer, Weiss, and von Haeseler 1999; Weiss and von Haeseler 2003). With a nonhuman primate outgroup sequence, therefore, failure of a clock test could result from failure of the ingroup sequences (in this case, human) to evolve according to a molecular clock or from inhomogeneity of sequence evolution between the two species.

For some mtDNA sequence sets, L1b and L1c outgroup sequences were used, in addition to an L0a outgroup. The results of the clock tests, in all cases, were insensitive to the choice of the outgroup sequence.

LRTs tend to inflate type I error, which for our analyses would involve a rejection of the null hypothesis (clock-like evolution) when it is true (Posada 2001), and a standard Bonferroni correction was used here because each sequence set was analyzed under multiple conditions. In our tests, the molecular clock model was not supported if P was less than 0.0050 for any individual test in order to preserve a "family" significance cut-off of 0.0500.

Reduced Median Networks

Reduced median networks were constructed, and reticulations resolved, as described earlier (Bandelt et al. 1995), with Network 3.1 (http://fluxus-engineering.com). This approach is better suited to analyses of human mtDNA sequences, especially control region sequences with their high levels of homoplasy, than standard phylogenetic approaches that constrain the sequences to simple bifurcating trees (Bandelt et al. 1995; Posada and Crandall 2001).

Results

Initial Studies

For our initial tests of an African mtDNA clock, likelihood ratio tests (LRTs) were carried out with the 56 African mtDNA sequences that were analyzed previously (Herrnstadt et al. 2002; Elson, Turnbull, and Howell 2004). Evolution in the coding region did not show a marked departure from a clock-like process (table 2). In contrast, evolution in the control region showed a significant violation of clock-like evolution when site variability of mutation rates was incorporated into the model of evolution. Clock-like evolution in the coding region was not expected on the basis of our analyses of selection (Elson, Turnbull, and Howell 2004). To obtain further insight into this apparent discrepancy, therefore, we subjected these coding region sequences to standard neutrality tests (Rozas and Rozas 1999), and we again obtained evidence for the operation of selection. The following values were obtained: -1.86 (Tajima's D test; $P < 0.05$); -3.69 (Fu and Li's D^* test; $P < 0.02$); and -3.57 (Fu and Li's F^* test; $P < 0.02$). The pairwise mismatch distributions are "jagged" (data not shown), the typical finding for sequences from African populations, and the negative values thus indicate selection, rather than recent population expansion.

We have now obtained the sequences of an additional 40 African mtDNAs, and we also used the larger combined sequence set for clock tests (table 2). Marked violations of clock-like evolution were now observed both in the coding and control regions, a result that is more compatible with those from the neutrality tests. Not only did these tests involve a larger number of sequences, but the larger sequence set included subclades (L2c and L3f) that were not represented in the smaller set. These results, and the previous evidence that haplogroup L2 subclades evolve at different rates (Torroni et al. 2001), suggest clock violations may be more severe in some African mtDNA clades than in others. It also appears that negative selection against slightly deleterious mutations, by itself, has not been sufficiently "strong" to produce clock violations with the models of evolution tested. To further investigate clock-like evolution in human mtDNAs, LRTs have been performed with the individual African mtDNA haplogroups and subclades.

Analysis of African Haplogroup L2 mtDNA Sequences

As the first step in our analysis of African haplogroup L mtDNAs, clock tests were carried out with haplogroup L2 sequences (table 3). The control region showed a clear-cut violation of clock-like evolution under all test conditions. The clock tests that incorporated site-variable substitution rates, rather than a uniform rate, yielded quartet puzzling trees with both better statistical support and poorer fits to a molecular clock. These results are not surprising in view of the high levels of homoplasy in the mtDNA control region (see further results below). The haplogroup L2 clock tests for the coding region segments also showed a marked departure from clock-like evolution under all test conditions (table 3).

African haplogroup L2 consists of four main subclades (e.g., Torroni et al. 2001; Salas et al. 2002). Torroni et al. (2001) concluded that L2b and L2d mtDNAs

Table 2
Molecular Clock Tests of Haplogroup L mtDNAs

Sequences	Outgroup/Model[a]	Region[b]	Ts/Tv[c]	ML(nc/c)[d]	Delta[e]	P[f]
African (55)[g]	149/HKY/UNI	Control	10.33	−2977/−3013	73.87	0.0365
African (55)	149/HKY/GAM	Control / 0.02	11.67	−2852/−2937	168.18	<0.0001
African (55)	149/HKY/UNI	Coding	23.76	−24377/−24409	62.79	0.1681
African (55)	149/HKY/GAM	Coding / 0.08	24.24	−24433/−24469	70.45	0.0547
African (92)[h]	149/HKY/UNI	Control	12.20	−3647/−3719	142.77	<0.0001
African (92)	149/HKY/GAM	Control / 0.04	14.10	−3276/−3354	157.17	<0.0001
African (92)	149/HKY/UNI	Coding	22.03	−25920/−25992	142.56	0.0004
African (92)	149/HKY/GAM	Coding / 0.09	22.56	−25761/−25826	129.72	0.0039
L1 + L3	149/HKY/UNI	Control	8.93	−2681/−2716	68.82	0.0260[i]
L1 + L3	149/HKY/GAM	Control / 0.02	10.15	−2582/−2629	93.76	0.0001[i]
L1 + L3	560/HKY/UNI	Control	7.88	−2737/−2781	88.68	0.0003
L1 + L3	560/HKY/GAM	Control / 0.02	9.63	−2574/−2608	67.41	0.0337
L1 + L3	585/HKY/UNI	Control	7.82	−2728/−2760	64.86	0.0527
L1 + L3	585/HKY/GAM	Control / 0.02	9.48	−2567/−2601	67.70	0.0319
L1 + L3	586/HKY/UNI	Control	9.03	−2775/−2823	95.10	0.0001
L1 + L3	586/HKY/GAM	Control / 0.02	10.28	−2592/−2634	82.80	0.0013
L1 + L3	587/HKY/UNI	Control	7.82	−2723/−2755	64.66	0.0546
L1 + L3	587/HKY/GAM	Control 0.02	9.48	−2571/−2605	67.36	0.0340
L1 + L3	149/HKY/UNI	Coding	29.51	−23827/−23851	46.73	0.5250[i]
L1 + L3	149/HKY/GAM	Coding / 0.07	29.52	−23798/−23821	46.57	0.5314
L1 + L2bd + L3	149/HKY/UNI	Control	10.14	−3107/−3163	111.39	<0.0001[i]
L1 + L2bd + L3	149/HKY/GAM	Control / 0.02	12.34	−2819/−2877	115.59	<0.0001[i]
L1 + L2bd + L3	149/HKY/UNI	Coding	24.46	−24704/−24735	60.37	0.3208[i]
L1 + L2bd + L3	149/HKY/GAM	Coding / 0.08	24.83	−24663/−24693	60.18	0.3269[i]

[a] The outgroup sequence is shown first. The numbers 149, 560, 585, 586, and 587 refer to L0a outgroup sequences, whereas 158 and 173 refer to L1b and L1c outgroup sequences, respectively. The model of substitution is shown next, followed by the model of rate heterogeneity (UNI = uniform; GAM = site variable under the assumption of a gamma distribution of rates).

[b] For those clock tests that incorporated rate heterogeneity in the model of evolution, the estimated shape distribution parameter (α) is shown after the mtDNA region analyzed.

[c] Ratio of transitions to transversions.

[d] This column shows the maximum-likelihood values obtained, rounded to the nearest integer, for the quartet puzzling trees (non-clock/clock model of evolution).

[e] Delta = twice the difference between the maximum-likelihood values for the "no-clock" and "clock" quartet puzzling trees.

[f] This column shows the significance level for the two models of evolution, clock-like and non-clock-like. If $P < 0.005$, it is concluded that the analyzed sequences do not show clock-like evolution with the models tested.

[g] In these tests, we analyzed the haplogroup L mtDNAs that were used previously (Hernnstadt et al. 2002; Elson, Turnbull, and Howell 2004), although the control regions are now included in the clock tests. This sequence set contains 56 haplogroup L sequences, and it includes sequences with numbers $\leq$560 (table 1). Sequences 149 and 560 belong to the L0a clade, and number 149 was used as the outgroup sequence for the clock tests, the results of which are shown here (thus explaining why $n = 55$, rather than 56). The test results were essentially identical when the other L0a sequence (number 560) was used as the outgroup or when the TN substitution model was used (data not shown).

[h] In these tests, the complete sets of haplogroups L1, L2, and L3 were analyzed (n = 91) sequences were analyzed with an L0a outgroup. The topologies of the clock and non-clock trees were identical in all of these tests, thus fulfilling one criterion for robust tests of a molecular clock.

[i] The results of tests with the HKY and TN models of substitution were essentially the same, so the results for the latter are not shown.

[j] Clock tests were also carried out with the other four L0a outgroup sequences (numbers 560, 585, 586, and 587) and essentially identical results were obtained.

were more highly diverged relative to the L2a and L2c sequences. This possibility was furthered investigated through analysis of the relative branch lengths of the haplogroup L quartet puzzling trees (table 4). The branch lengths of the coding region sequences fit the pattern observed by Torroni et al. (2001). Thus, the L2a and L2c coding region sequences showed essentially the same mean branch lengths, which were shorter than those for the L2b and L2d sequences. In addition, the L2a control region sequences were markedly less diverse than those of the L2b, L2c, or L2d sequences, irrespective of the model of rate substitution (table 4). The mean branch lengths of the control regions for the L2c sequences were shorter than those of the L2b and L2d sequences. Because we lacked a consistent distinction between the L2a/L2c and L2b/L2d clade pairs, we subjected four sequence subsets to clock tests: L2a, L2bcd, L2ac, and L2bd (table 3).

(1) The L2a control region sequences showed clock-like evolution under conditions of both uniform and site-variable substitution rates. In the coding region, the evolution of the L2a sequences showed a significant violation of the molecular clock under all test conditions.

(2) The L2bcd control region sequences showed clock-like evolution with a uniform rate of substitution, but a marked violation with site-variable rates of substitutions. The fit of the evolution for the L2bcd coding region sequences to a molecular clock was relatively poor, but the deviation did not reach statistical significance.

(3) L2ac control region sequences, in contrast to L2a sequences only, showed a significant departure from clock-like evolution. This result is in accordance with the longer branch lengths of the L2c sequences (table 4). The L2ac coding region sequences showed non-clock evolution under all test conditions.

(4) The L2bd subset of sequences showed clock-like evolution in both the control and coding regions.

These results confirm that subclades L2b and L2d have a similar pattern of evolution and that evolution was clock-like in both the control and coding regions. Subclade L2c sequences also seem to have undergone "clock-like"

Table 3
Molecular Clock Tests of African Haplogroup L2 mtDNAs

Sequence Set[a]	Outgroup/Model	Region	Ts/Tv	ML(nc/c)	Delta	*P*
L2abcd	149/HKY/UNI	Control	29.54	−2411/−2480	136.16	<0.0001[b, c]
L2abcd	149/HKY/GAM	Control / 0.02	29.55	−2294/−2368	147.58	<0.0001[c]
L2abcd	158/HKY/UNI	Control	28.03	−2410/−2480	140.38	<0.0001
L2abcd	173/HKY/UNI	Control	16.07	−2414/−2482	136.14	<0.0001
L2abcd	149/HKY/UNI	Coding	16.01	−22955/−22994	77.72	0.0007[b, c]
L2abcd	149/HKY/GAM	Coding / 0.10	16.20	−22946/−22985	77.77	0.0007[c]
L2abcd	158/HKY/UNI	Coding	15.40	−22902/−22940	75.86	0.0011
L2abcd	173/HKY/UNI	Coding	15.87	−22942/−22980	76.22	0.0010
L2a	149/HKY/UNI	Control	27.33	−1951/−1968	35.24	0.1968[b, c]
L2a	149/HKY/GAM	Control / 0.02	29.31	−1897/−1916	37.81	0.1265[b, c]
L2a	149/HKY/UNI	Coding	24.51	−21902/−21928	53.23	0.0040[b, c]
L2a	149/HKY/GAM	Coding / 0.12	24.76	−21899/−21925	53.49	0.0037[b, c]
L2bcd	149/HKY/UNI	Control	26.83	−2002/−2008	11.50	0.4865[b, c]
L2bcd	149/HKY/GAM	Control / 0.02	29.55	−1982/−2006	47.08	<0.0001[b, c]
L2bcd	149/HKY/UNI	Coding	13.62	−22060/−22070	19.76	0.0717[b, c]
L2bcd	149/HKY/GAM	Coding / 0.08	13.82	−22057/−22068	21.67	0.0414[b, c]
L2ac	149/HKY/UNI	Control	29.45	−2075/−2108	66.37	0.0007
L2ac	149/HKY/GAM	Control / 0.02	29.45	−2063/−2107	86.43	<0.0001
L2ac	149/HKY/UNI	Coding	16.98	−22167/−22196	59.44	0.0044
L2ac	149/HKY/GAM	Coding / 0.21	16.98	−22165/−22195	59.51	0.0044
L2bd	149/HKY/UNI	Control	21.98	−1901/−1905	7.09	0.4194[b]
L2bd	149/HKY/GAM	Control /0.02	26.31	−1882/−1885	5.60	0.5874[b]
L2bd	149/HKY/UNI	Coding	16.18	−21853/−21858	9.81	0.1994[b]
L2bd	149/HKY/GAM	Coding / 0.06	16.51	−21851/−21856	10.57	0.1584[b]
L2a′	149/HKY/UNI	Control	27.54	−1657/−1663	12.38	0.3361[d]
L2a′	149/HKY/UNI	Coding	29.52	−21328/−21336	14.83	0.1903[d]
L2a″	149/HKY/UNI	Control	24.35	−1863/−1870	13.79	0.6816[d]
L2a″	149/HKY/UNI	Coding	19.55	−21633/−21651	36.46	0.0040[d]

[a] The clock test results are displayed as in table 2.
[b] Clock tests were also performed with the other four L0a outgroup sequences. Essentially identical results were obtained, and those results are not shown here.
[c] The results of tests with the HKY and TN models of substitution were essentially identical, and only the former are shown.
[d] Essentially identical results were obtained when site-variable mutation rates (GAM) were incorporated into the model of evolution tested.

evolution, but the sequence divergence in both coding and control regions is less than the values for the L2bd sequences. The L2a sequences yield distinct and complex results. Sequence divergence in the coding region shows a departure from clock-like evolution. In contrast, divergence in the control region did not show a violation of clock-like evolution. However, during the analyses of the L2a sequences, it was noted that the topologies of the coding region and control region quartet puzzling trees were different (data not shown). Clock tests are generally insensitive to tree topology, but a high level of homoplasy in the control region might lead to markedly erroneous tree topologies and, as a result, to biased clock tests. Therefore, we supplemented these clock tests with reduced median network analysis (Herrnstadt et al. 2002).

Reduced Median Network Analysis of Haplogroup L2 mtDNA Sequences

The coding region sequences were used to construct a reduced median network of the entire haplogroup L2 sequence set (fig. 1) in which informative sites were used to derive the topology (see also Herrnstadt et al. 2002). Other than an unresolved reticulation at the most ancestral node, the topology of this network is straightforward and there are three noteworthy results.

(1) Salas et al. (2002), who used the HVS1 segment of the control region to construct African haplogroup L phylogenetic trees, found that the L2d1 and L2d2 subclades branch from a common L2d ancestor (see their fig. 2). In contrast, our coding region network shows that these two subclades are separate branches off the L2 ancestral node. A similar branching pattern was noted in the quartet puzzling trees (data not shown) and these results suggest that they are distinct L2 subclades.

(2) The L2a sequences form two star-like subclusters (see below) that we designate L2′ and L2a″. The L2a″ sequences descend from nodal sequence #563 and carry coding region polymorphisms at nucleotides 3918, 5285, 15244, and 15629. These subclusters appear to have arisen early during L2a evolution. On the basis of network analyses of control region sequences, other investigators have identified L2a subclusters (e.g., see fig. 5 of Pereira et al. 2001 and fig. 6 of Salas et al. 2002). Those subclusters appear to be different from the "deeper" evolutionary event that emerges from our analysis of coding region sequences.

(3) A number of homoplasies occur in these sequences. On the basis of our analyses here and of our previous network analysis of 560 coding region sequences (Herrnstadt et al. 2002), 25 of the coding region substitutions shown in figure 1 are homoplasic. For example, the substitution at nucleotide 13708 (which also occurs in European mtDNAs) has arisen on three separate occasions among the haplogroup L2 sequences analyzed here. In addition to multiple forward mutations at the same site, reversion events were also observed.

At the next stage of the analysis, complete L2b, L2c, and L2d sequences (coding plus control regions) were

Table 4
Branch Lengths of Haplogroup L mtDNAs

Subclade[a]	Coding Region	Control Region	Control / Coding
L1b (12)	0.00198 ± 0.00005	0.00756 ± 0.00117	3.8 (UNI)
	0.00202 ± 0.00005	0.02784 ± 0.00135	13.8 (GAM)
L1c (9)	0.00239 ± 0.00012	0.01402 ± 0.00295	5.9 (UNI)
	0.00243 ± 0.00013	0.04474 ± 0.00598	18.4 (GAM)
L2a (30)	0.00195 ± 0.00007	0.01656 ± 0.00059	8.5 (UNI)
	0.00200 ± 0.00007	0.01502 ± 0.00102	7.5 (GAM)
L2a′ (12)	0.00203 ± 0.00007	0.01509 ± 0.00037	7.4 (UNI)
	0.00208 ± 0.00008	0.01247 ± 0.00063	6.0 (GAM)
L2a″ (18)	0.00190 ± 0.00010	0.01755 ± 0.00059	9.2 (UNI)
	0.00195 ± 0.00010	0.01672 ± 0.00105	8.6 (GAM)
L2b (5)	0.00271 ± 0.00013	0.02075 ± 0.00211	7.7 (UNI)
	0.00277 ± 0.00013	0.02420 ± 0.00384	8.7 (GAM)
L2c (5)	0.00202 ± 0.00012	0.01839 ± 0.00125	9.1 (UNI)
	0.00206 ± 0.00012	0.01832 ± 0.00194	8.9 (GAM)
L2d (3)	0.00326 ± 0.00266	0.02309 ± 0.00675	7.1 (UNI)
	0.00274 ± 0.00123	0.02998 ± 0.02109	10.9 (GAM)
L3b (6)	0.00202 ± 0.00006	0.01373 ± 0.00078	6.8 (UNI)
	0.00206 ± 0.00006	0.00989 ± 0.00129	4.8 (GAM)
L3d (3)	0.00202 ± 0.00065	0.01365 ± 0.00260	6.8 (UNI)
	0.00205 ± 0.00065	0.00995 ± 0.00488	4.9 (GAM)
L3e (13)	0.00175 ± 0.00011	0.01273 ± 0.00143	7.3 (UNI)
	0.00180 ± 0.00012	0.00828 ± 0.00256	4.6 (GAM)
L3f (5)	0.00206 ± 0.00022	0.01511 ± 0.00234	7.3 (UNI)
	0.00210 ± 0.00044	0.01218 ± 0.00401	5.8 (GAM)

[a] Branch lengths are expressed as mean ± 95% confidence intervals and they refer to the distance to the main branch of the non-clock quartet puzzling trees constructed with the complete set of haplogroup L sequences (N = 92; Table 2). Because of shared polymorphisms, mtDNA branch lengths are not completely independent and these confidence intervals should therefore be treated with caution. Both the trees with a uniform (UNI; first line) rate of substitution and with site-variable rates (GAM; second line) were analyzed; the HKY model of substitution was used. The branch lengths of the outgroup sequence (L0a/149) for the coding and control regions were 0.00247 and 0.01277, respectively, were obtained with the UNI trees. The corresponding values for the GAM trees ("Trees") in bold instances (site-variable rates) were 0.00254 and 0.03388.

analyzed (fig. 2). The topology becomes slightly more complex with the incorporation of the control region substitutions, but the overall structure of the network remains the same. In marked contrast, incorporation of the control region sequences yielded a complex L2a network with multidimensional hypercubes (data not shown). A complex pattern of reticulations was also obtained by Salas et al. (2002) in their L2a networks (see their fig. 6). Bandelt et al. (2002) have shown that networks with hypercubes can result from errors in the sequences analyzed, and we had removed a number of errors from our sequence database prior to the present studies (Herrnstadt, Preston, and Howell et al. 2003). For some of the control region "hot spots" we inspected the sequencing electropherograms for both strands to confirm the allele status and that the sites were not heteroplasmic. Finally, the control regions for eight L2a, one L2b, and one L2c mtDNA were sequenced independently with a different approach (the manual dideoxy chain termination method; see Herrnstadt et al. 2002 and references therein). In all 10 instances, the two sequencing approaches yielded identical results, and we conclude that the complex network topology was due to homoplasy, not to sequence errors.

We sought another approach that would provide phylogenetic information on control region evolution, especially at highly variable sites.. As a first step, we assumed that the topology of the coding region tree was the best representation of the evolution of L2a sequences. Onto this network, we then added all control region polymorphisms under an assumption of maximum parsimony for the sequences analyzed (see below), and the resulting network is shown in figure 3. The most striking result is the number of control region sites that have apparently undergone multiple homoplasic events.

(1) A G:A transition at nucleotide 143 arises in the L2a ancestral haplotype but subsequently undergoes reversion on five occasions. We are in the process of analyzing a set of ~300 complete haplogroup H mtDNAs, and we have not observed this mutation among those mtDNAs (unpublished data). One scenario is that mutation to the A allele at this site is rare and that, if it occurs, there is a high rate of back mutation to the G allele.

(2) A T:C transition at site 16086 occurs on three occasions. We do not detect this mutation in our set of haplogroup H mtDNAs and this site has not been observed to be hypervariable by other investigators (Excoffier and Yang 1999; Meyer, Weiss, and von Haeseler 1999).

(3) The L0a outgroup sequence carries C alleles at nucleotides 16189 and 16192, whereas the L2a ancestral sequence is predicted to carry C and T, respectively, at these sites. The 16189 site subsequently undergoes mutation on four occasions (three forward and one reverse relative to the outgroup sequence), whereas the 16192 site undergoes reversion on five occasions. Thus, both sites appear to have relatively high rates of mutation, a result that has been observed in previous studies (Excoffier and Yang 1999; Meyer, Weiss, and von Haeseler 1999; Howell and Bogolin Smejkal 2000) and in the L2a networks of Salas et al. (2002).

(4) The ancestral L2a sequence carries an A:G transition at nucleotide 16309 and there have subsequently been three reversion events and one additional forward mutation. Excoffier and Yang (1999) also found that this site has a high rate of substitution. Salas et al. (2002) concluded that the reticulations in their L2a network did not involve homoplasy at site 16309. However, they also noted that the six subclusters in their network could be "collapsed" into two main clusters, that one of these carries the 16309 substitution in all sequences, and that this alternative topology would involve multiple homoplasic events at the 16309 site in the second cluster (Salas et al. 2002, p.1,099). While precise comparisons are not possible because of the lack of coding region information for their mtDNAs, their alternative topology could be the same one that we obtain (fig. 3). In addition, Quintana-Murci et al. (2004) have recently carried out a network analysis of control region sequences with European haplogroup U7 mtDNAs. In a situation that is strikingly similar to our analyses, their U7 network contains several reticulations that indicate multiple homoplasic events at site 16309 (see their fig. 3).

(5) The ancestral L2a sequence carries a C:T transition at nucleotide 16519, which undergoes reversion on three occasions. These results are not surprising and this site has long been recognized to have a high mutation rate.

An anonymous reviewer pointed out that our approach did not, in one sense, use maximum parsimony and that it might therefore have inflated the number of homoplasies.

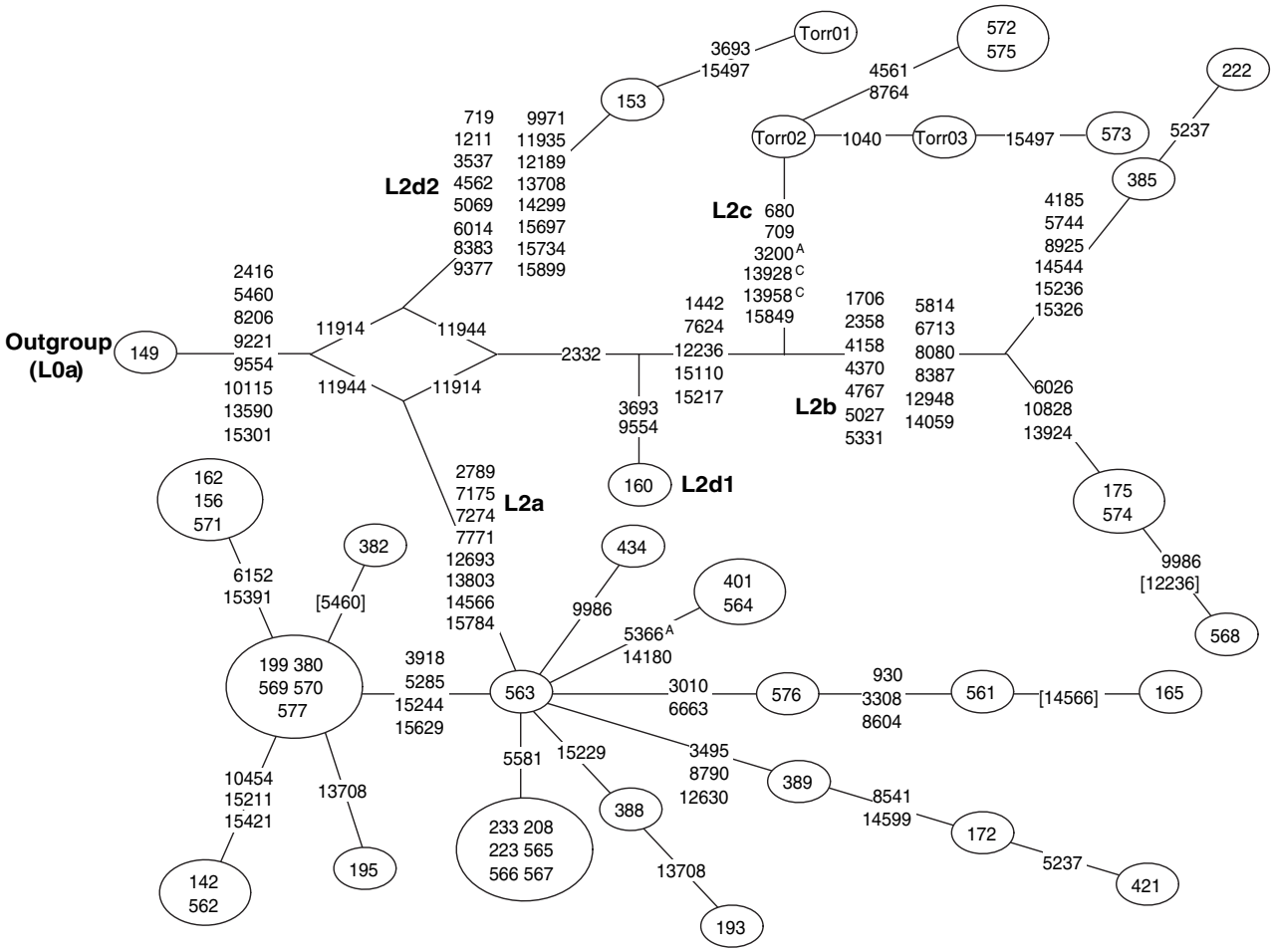

FIG. 1.—Reduced median network of coding region sequences for haplogroup L2 clades. All nucleotide changes are relative to the L0a outgroup sequence. Forward mutations (that is, to an allele state different from that of the outgroup) are shown in plain text while reversions (changes back to the allele state of the outgroup sequence) are enclosed by square brackets. Superscripts indicate that the sequence change involves a transversion and denote the replacement nucleotide in the L-strand sequence. This is a skeleton network in which private polymorphisms are not shown.

For example, the network in figure 3 shows six branches connecting to an ancestral node defined by sequences 434 and 563. In this network, a forward mutation at site 16189 occurs in three of these branches. If, however, there were a second and undetected ancestral node that differed by the *single* occurrence of the 16189 mutation, then this topology would reduce the apparent number of homoplasic events at this site. That is, there would be three "non-16189" branches descending from the 434/563 nodal sequence and three branches descending from the undetected "16189" nodal sequence. Similar scenarios can be devised for other apparently homoplasic sites. We have chosen to present the results as shown here, however, for two reasons. First, it seems risky to make assumptions about, or to draw conclusions from, network topologies that require undetected sequences. Second, while such a scenario could explain the results for one site, no single "undetected" ancestral node can explain the multiple homoplasmic sites in this network. Obviously, the further analysis of complete L2a mtDNAs will clarify the situation.

Based on these results, the two L2a subclusters identified in the networks were analyzed separately as L2a′ (12 sequences) and L2a″ (18 sequences). The L2a′ and L2a″ coding region sequences had similar mean branch lengths (table 4), although the latter set showed a clock violation in the LRTs (table 3). The L2a′ and L2a″ control sequences both showed clock-like evolution, but the mean branch lengths were different. As an independent approach to the branch length calculations, we also determined the mean pairwise differences (MPDs) for the two L2a subclades. For the L2a′ mtDNAs, the coding region and control region MPDs were 5.0 and 1.5, respectively, and the ratio of the two regions was 3.3. The respective MPD values for the L2a″ mtDNA were 8.2 and 4.6, and the ratio was 1.8. The results of the two approaches thus agree that the pattens of sequence divergence differ between these two subclades. Furthermore, they suggest that the evolutionary pathways of the coding and control regions are not tightly coupled.

Analysis of Haplogroup L1 and L3 mtDNA Sequences

A set of haplogroup L1 sequences (which belong to the L1b and L1c subclades) was used for clock tests (table 5). Evolution in the control region does not violate clock-like behavior, but the fit to a clock model is poor,

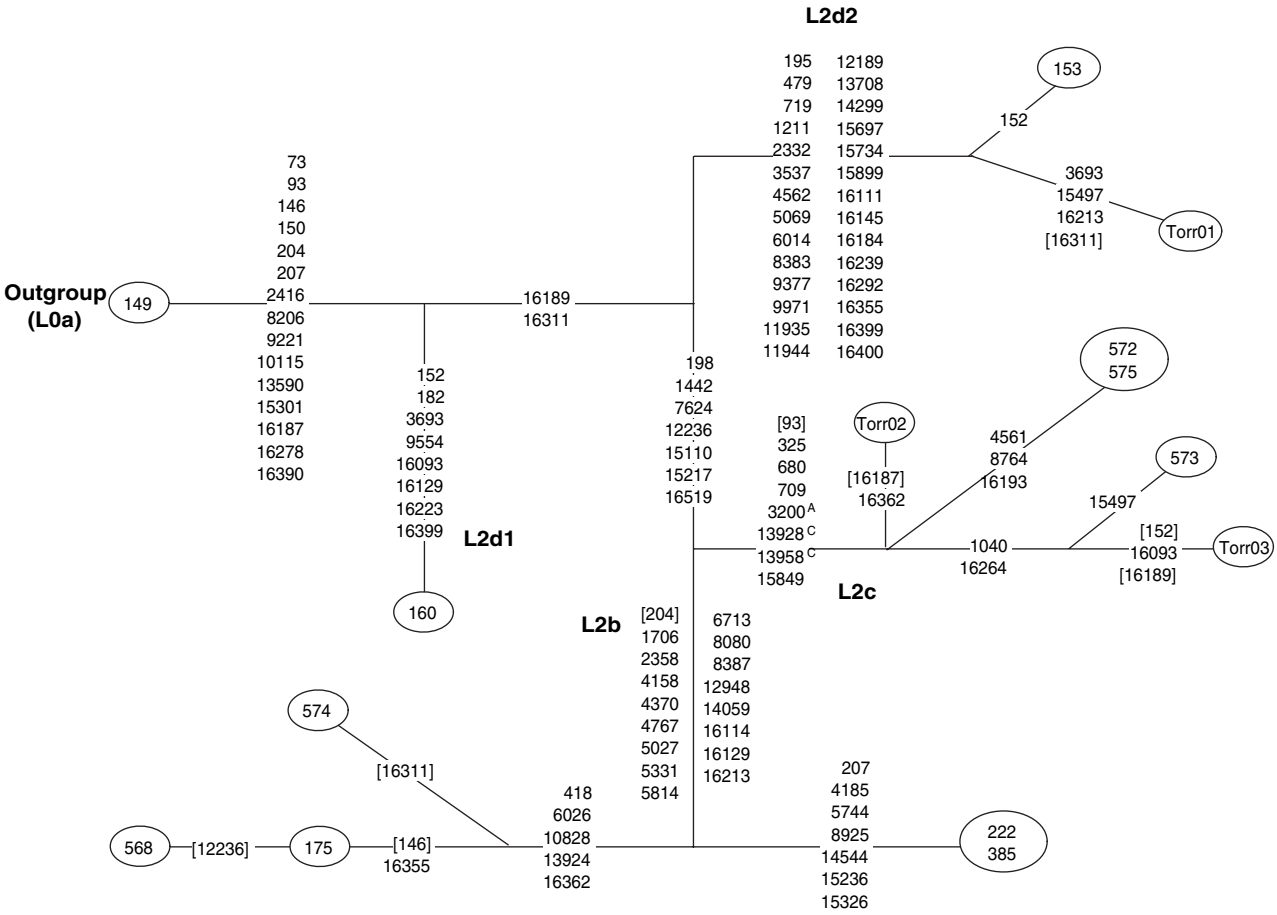

FIG. 2.—Reduced median network of complete mtDNA sequences from subclades L2b, L2c, and L2d. The data are displayed as in figure 1. Note the divergence between the L2d1 and L2d2 sequences. The control region sequences for 568 (L2b) and 572 (L2c), as well as ~ 3 kb of the coding region, were independently verified with a different methodology (see *Experimental Procedures*).

especially with the TN (rather than the HKY) model of substitution. In contrast, the evolution of the L1 coding region sequences fits very well with a molecular clock model. The results for the haplogroup L3 sequences are summarized in table 6, and no clock violations were observed for either of the mtDNA regions analyzed, irrespective of the model of substitution.

The estimated Ts/Tv ratios for the L2 control and coding regions were in the approximate ranges of 27–29 and 15–18, respectively. The equivalent ranges were 5–6 and 29 for the L1 sequences and 13–17 and 26–29 for the L3 mtDNAs. Thus, with an L0a outgroup sequence, each of the L haplogroups has a different Ts/Tv "fingerprint." Finally, we carried out reduced median network analyses with the L1 and L3 coding region sequences (data not shown). As expected, several sites have undergone multiple homoplasies (for example, those at nucleotides 16189, 16311, and 16519), but nothing so marked as the results obtained with the L2a sequences.

Analysis of Combined Sequence Sets

In the final set of clock tests, analyses of "pruned" haplogroup L sequence sets were carried out (table 2). In a combined set of L1 and L3 coding region sequences, no deviation from clock-like evolution was detected, whereas the tests with control region sequences were inconsistent. None of these tests showed a good fit to the clock model, and some of the tests showed a significant violation of the model (table 2). These results suggest that a small number of site differences in the outgroup sequence can "tip the balance" one way or the other for the L1 + L3 sequence set. Finally, we added the five L2b and the three L2d sequences to the L1 and L3 sequences and tested this set for clock-like evolution. Again, the coding region sequence set showed a good fit to a molecular clock model of evolution under all the test parameters that were varied. For the control region, in contrast, there was now a clear-cut violation of the clock model under all test conditions.

The branch lengths from the haplogroup L coding region and control region quartet puzzling trees were analyzed (table 4). A number of results emerged, in addition to those discussed previously for the haplogroup L2 sequences. For the coding region, the mean branch lengths are increased only slightly when site-variable substitution rates are incorporated. Thus, homoplasy in the coding region has minimal effects on tree construction. More importantly, the mean branch lengths are remarkably similar among the different haplogroups and subclades. The control regions present noteworthy differences. First,

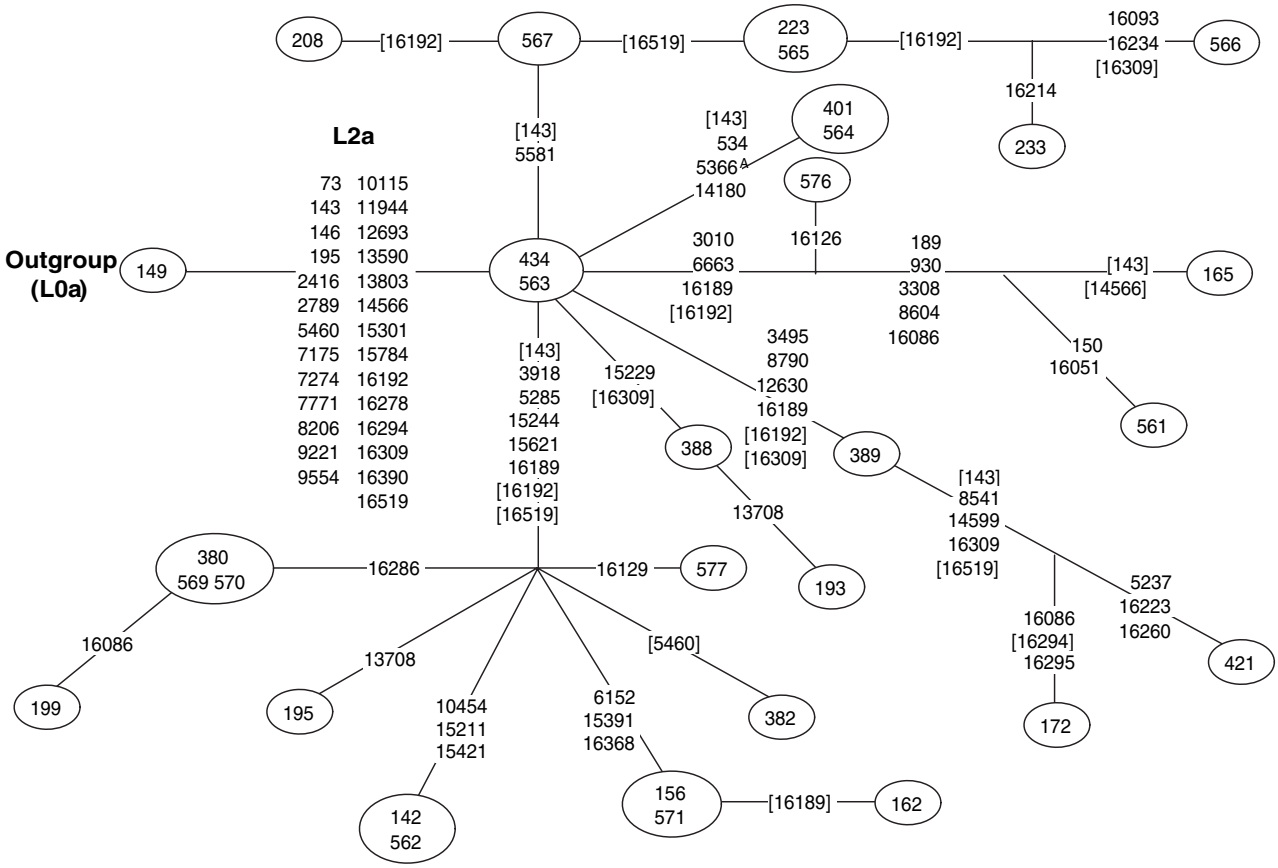

FIG. 3.—Network of complete L2a mtDNA sequences. The network was constructed as described in *Results* and it is based on the reduced median network of coding region sequences. Private polymorphisms in the coding region are not shown, but all control region substitutions are used for network construction. The control region sequences for 563, 564, 565, 566, 567, 569, 570, and 571 were independently verified with a different methodology (see *Experimental Procedures*). With the exception of 567, ~ 3 kb of the coding region sequence was also verified.

the branch lengths are more sensitive to the model of substitution rate, a result in accordance with the high levels of homoplasy in the control region. Second, in contrast to the coding region, there is a clear trend for control region branch lengths in which L1 > L2 > L3 when site-variable rates are incorporated into tree construction.

Discussion

The results presented here reveal a complex pattern of evolution in the African haplogroup L mtDNAs. Evolution in the haplogroup L1 and L3 coding regions has been largely clock-like, although the L2 sequences showed a marked deviation from clock-like evolution for both the coding and control regions. In contrast, clock-like evolution cannot be discerned for the control region beyond the haplogroup level of phylogeny. Thus, L1 and L3 control region sequences show clock-like evolution when analyzed separately, but when analyzed as a single set of sequences, the fit to a molecular clock was poor. Beyond the issue of molecular clock violations in haplogroup L mtDNA clades, our results also indicate striking differences between evolution in the coding region and that in the control region. We note, before discussing our results, that these clock tests have limitations.

(1) First, we did not analyze sequences from all haplogroup L subclades, such as those from subclades L1d, L1e, L1k, and L3g (Salas et al. 2002, 2004). In this

Table 5
Molecular Clock Tests of African Haplogroup L1 mtDNAs

Outgroup/Model[a]	Region	Ts/Tv	ML(nc/c)	Delta	*P*
149/HKY/UNI	Control	5.32	−2086/−2102	31.50	0.0489[b]
149/TN/UNI	Control	5.65	−2080/−2099	37.70	0.0096[b]
149/HKY/GAM	Control / 0.02	5.60	−2017/−2032	30.11	0.0681[b]
149/TN/GAM	Control / 0.02	6.16	−2013/−2031	35.74	0.0165[b]
149/HKY/UNI	Coding	29.43	−22354/−22363	16.73	0.6707[b,c]
149/HKY/GAM	Coding / 0.36	29.43	−22356/−22364	16.58	0.6802[c]

[a] The results are shown as in Table 2.
[b] Clock tests were also carried out with the other four L0a outgroup sequences and similar results were obtained.
[c] Clock tests with the HKY and TN models of substitution yielded essentially identical results.

Table 6
Clock Tests of African Haplogroup L3 mtDNAs

Outgroup/Model[a]	Region	Ts/Tv	ML(nc/c)	Delta	*P*
149/HKY/UNI	Control	16.11	−2181/−2194	27.82	0.4201[b, c]
149/HKY/GAM	Control / 0.02	17.40	−2081/−2094	26.08	0.5141[c]
158/HKY/UNI	Control	16.12	−2182/−2196	28.71	0.3751
173/HKY/UNI	Control	10.32	−2206/−2226	40.22	0.0489
149/HKY/UNI	Coding	27.66	−22353/−22368	29.76	0.3250[b, c]
149/HKY/GAM	Coding / 0.02	28.19	−22341/−22356	29.63	0.3308[b, c]
158/HKY/UNI	Coding	26.59	−22306/−22321	29.23	0.3499
173/HKY/UNI	Coding	27.52	−22345/−22360	29.36	0.3425

[a] The results are shown as in Table 2.
[b] Clock tests were also carried with the other four L0a outgroup sequences, and essentially identical results were obtained.
[c] Essentially identical results were obtained with the HKY and TN models of substitution.

regard, further analysis of larger sequence sets should be informative.

(2) Contemporaneously sampled mtDNA sequences were analyzed and, as a result, clock violations will not have been detected if the rate of divergence changes equally and simultaneously in all lineages (Drummond and Rodrigo 2000; Seo et al. 2002). Such an evolutionary scenario is plausible when all lineages occur in a single population and if population size changes at some point in time (thereby changing the efficiency of selection against slightly deleterious mutations). The evolutionary history of African mtDNAs is complex and not yet well understood (Salas et al. 2002, 2004), but there might have been such conditions at an early stage during the emergence and dispersal of modern humans. In short, there might have been changes in mtDNA "clock speed" that we were unable to detect with our approach.

(3) It is possible that, for those mtDNA sequences for which a violation was obtained, there is a molecular clock but that the appropriate model of sequence evolution was not within the interrogated parameter space. In this regard, Yang, Goldman, and Friday (1994) compared different models of sequence evolution to a multinomial model that was unconstrained except for the i.i.d. condition. In analyses with a segment of the mtDNA coding region from humans, chimpanzees, gorillas, and orangutans, they found that the HKY model of substitution provided an appropriate description of evolution (that is, the maximum-likelihood value of the phylogenetic tree was not significantly different from that obtained with the multinomial model), but only when site variability was incorporated. In more recent studies, which added the gibbon mtDNA sequence for this region, Whelan, Liò, and Goldman (2001) observed that neither the HKY nor the REV (general time-reversible Markov process) models with uniform rates of substitutions were adequate. They concluded that those models are inadequate to fully describe the pathway of mtDNA evolution because they do not incorporate the effects of selection. That conclusion would be more compelling had they obtained similar results with tests that incorporated site variability (as did Yang, Goldman, and Friday 1994).

(4) Finally, we used LRTs for these analyses, and subsequent clock tests with Bayesian methods (Suchard, Weiss, and Sinsheimer 2003) should also be carried out. These limitations notwithstanding, the results reported here provide new insights into the evolution of the human mitochondrial genome.

We noted marked clock violations among haplogroup L2 subclades. What accounts for the complex pattern of evolution in the haplogroup L2 sequences? The departure from clock-like evolution might result from the operation of selection on the haplogroup L2a and L2c sequences; that is, their lower extents of divergence might reflect the effects of negative selection. Alternatively, there might have been some marked demographic event(s) that influenced the evolution of these sequences. The net substitution rate of nonneutral mutations, incorporating the rates of both mutation and fixation, is in most evolutionary scenarios a function of effective population size (but see Cherry 1998). The finding that the L2 subclades evolve at different rates could reflect geographic subdivision and different subpopulation sizes producing different rates of fixation of slightly deleterious mutations. The analyses of Salas et al. (2002) indicate that haplogroup L2a is the most prevalent and geographically widespread mtDNA clade in Africa and that it has a complex history of population dispersals in sub-Saharan Africa. On the basis of the network analyses of coding region sequences, our studies provide the first evidence for two well-separated L2a subclusters (fig. 1), a result that might reflect very early population subdivision. However, from tests of sequences from the two L2a subclades, we still obtained a clock violation for the L2a″ coding region sequences. Furthermore, for both the two L2a subclades, as well as for the four L2 subclades, there were obvious disparities between the rates of coding region and control region evolution. While population effects might have contributed to the clock violations among the haplogroup L2 mtDNAs, the complexity of the results argues against a simple explanation.

Why is clock-like evolution more prevalent in the coding regions of the haplogroup L sequences than in the control region? Schierup and Hein (2000) have shown that violations of clock-like evolution can be caused by recombination, but the preponderance of evidence argues that human mtDNA is clonal and that it behaves as a single linkage group (Elson et al. 2001 and references therein). Under the restriction of clonality, selection in the coding region should be "reported" by the control region as a consequence of hitchhiking. However, it seems unlikely that the marked clock violations in the control region reflect the effects of selection in the coding region, where

evolution is more often clock-like. The rapid sequence divergence of the control region is often seen as evidence for a lack of selection in this segment of the mitochondrial genome, but perhaps selection has acted directly on the control region. The marked site variability of substitution rates in the control region (e.g., Excoffier and Yang 1999; Meyer, Weiss, and von Haeseler 1999) suggests the operation of selection, at least on a large fraction of the sites. However, there is also evidence both of asymmetrical mutation rates in the forward and reverse directions and of nonindependence of mutations, so one might need to consider a scenario where non-clock evolution in the control region is not caused by, or not solely by, the effects of selection. Thus, this might be a situation where the models of evolution tested here are inadequate for the complex evolutionary processes in the control region. A major challenge for future studies is to tease out the factors that underlie the complex process of control region evolution.

Phylogeographic analysis of mtDNA sequences is widely used to study human evolution and population dispersal. For example, it has been used to study the peopling of the Americas (reviewed in Eshleman, Mahli, and Smith 2003) and the spread of agriculture and language from the Near East to the European heartland (see especially Richards 2003). Such studies, in addition to their assumptions about population structure and history (Knowles 2004), rely at present on a simple mtDNA control region clock. Based on the results presented here, such control region clocks are highly suspect (see also the related comments on pp. 1105–1106 in Salas et al. 2002). It might be relatively "safe," however, to develop and use an mtDNA coding region clock, although the results with the haplogroup L2 sequences caution that any such clock will not be a panacea.

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