Chapter 5

MITOCHONDRIAL DNA: VARIATION IN HUMANS AND HIGHER PRIMATES

Rodney L. Honeycutt and Ward C. Wheeler

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I. INTRODUCTION

The mitochondrial DNA (mtDNA) genome has become a model genetic system, receiving considerable attention over the past 10 years from both molecular and evolutionary geneticists. As a result, animal mtDNA is one of the best known segments of eukaryotic DNA in terms of the patterns and processes of DNA sequence change, genome organization, and functional/structural constraints at the molecular level. The reason for this molecule receiving such extensive attention is due to the ease of isolating an intact genome and the characteristics of the molecules which lend themselves well to experimentation. From the start, man and other higher primates have been a focal point for investigations of mtDNA, and a large portion of baseline data on the organization and evolution of animal mtDNA has been derived from a primate perspective. In this review, we will focus primarily on those aspects of mtDNA characteristics which have evolutionary and systematic implications, and will provide a brief account of the molecular biology of mtDNA. Several excellent reviews on the details of transcription, replication, and molecular genetics of mtDNA have been published and we refer readers to these for more complete information.[1-6] In addition, we will examine mtDNA variation in humans and other higher primates and discuss several evolutionary problems which have been addressed using mtDNA.

II. GENETIC PROPERTIES OF MAMMALIAN MITOCHONDRIAL DNA

The mode of inheritance, simple design, and high rate of sequence divergence of mitochondrial DNA (mtDNA) are properties which have stimulated considerable interest from both evolutionary and molecular biologists. Mitochondrial DNA has proven to be an effective genetic marker for the examination of geographic variation within a species, the effects of population structure on genetic variation, speciation, and microevolution.[7-33] Additionally, several unique properties of mtDNA related to copy number and inheritance have provided a challenge to population geneticists who must explain patterns of mtDNA variation with current population genetics theory.[34-39] On the other hand, the molecular biologists sees the molecule as a means of examining functional and structural constraints relative to genome organization and the mechanisms of molecular evolution. The mtDNA molecule can provide a link between two rather divergent fields of biology.

A. MOLECULAR STRUCTURE AND FUNCTION

Aside from coelenterates,[40] animal mtDNA is a duplex, closed-circular DNA molecule which can be separated from nuclear DNA by buoyant density centrifugation (Figure 1).[3,4] The size, organization, and sequence composition of the mtDNA genome depict simplicity in design and efficiency in the utilization of sequences. Although the mitochondrial genome size varies among animals (15,700 to 19,500 bp), most of the detected variation occurs in the noncoding region.[3,4,41] As can be seen in Table 1, mammalian mtDNA reveals a somewhat narrower range of genome size than that seen for other animals, in general, with the average genome consisting of 16,400 base pairs (bp). In contrast to nuclear DNA, detailed sequence analyses of both vertebrates and invertebrates indicate that the mtDNA genome is composed primarily of coding sequences (approximately 90%), lacks split genes (no introns), has repetitive sequences confined to the "control" or noncoding region, and reveals a small number of intergenic sequences.[1,42-52] Both the gene content and order within the vertebrate mtDNA genome are highly conserved (Figure 2), and the major features are as follows: (1) The coding regions consist of two ribosomal RNA genes (12S, 16S, rRNAs), 22 tRNAs, cytochrome C oxidase subunits I, II, III, ATPase subunit 6, cytochrome b, seven genes encoding subunits of the NADH dehydrogenase complex (ND1-4, 4L, 5, 6) and one gene, A6L, which codes for a subunit of the ATP synthetase complex.[1,53,54]

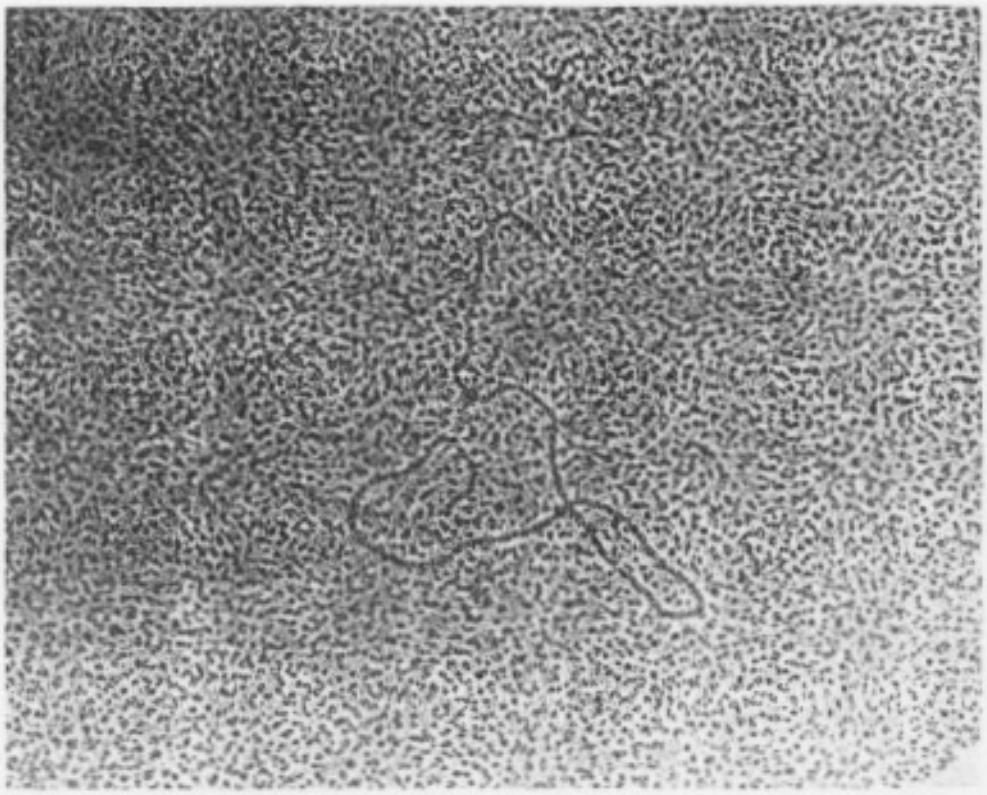

FIGURE 1. Electron micrograph of mt from the mole-rat *Crytpomys hottentus*.

(2) There is a G + T strand bias in vertebrates with the complementary strands designated as heavy (H) or light (L).[1,4,55] The gene distribution along these two strands is asymmetrical with 8 tRNA genes and ND6 encoded on the L strand and the remaining genes on the H strand. (3) Some adjacent genes overlap and in many cases termination codons are absent.[1] The tRNA genes bracket most genes in the genome, and probably play a role in processing of primary transcripts.[1] (4) There is one major noncoding region or D-loop region, located between $tRNA^{Pro}$ and $tRNA^{Phe}$.[1,42,43,52]

1. Genetic Code

The mitochondrial genetic code of animals differs from the "universal" code seen in the nuclear genome. An examination of human mtDNA sequences has revealed several changes of codon usage in the mitochondrial DNA genome.[1,56] These changes are as follows: (1) UGA is a tryptophan rather than termination codon; (2) AUA is a codon for methionine rather than isoleucine; (3) AGA and AGG are both termination and not arginine codons. Aside from the above changes in the genetic code, fewer tRNAs are in the mtDNA genome, and a bias for codons ending in A through U rather than G has been found in animals.

2. Control Region

The control or D-loop region in vertebrates has a functional relationship to both transcription and replication.[2,4-6] Transcription for both the heavy (H) and light (L) strands seems to be initiated from promoters in the control region. The process is continuous resulting in polycistronic RNAs which are later processed to yield tRNAs, and mRNAs.

TABLE 1
Genome Size and Intraspecific Variation in Mammals

Species	Number of specimens	Source of specimens	Restriction enzymes	Sites	δ[a]	p[b]	Genome[c] size	Ref.
Primates								
Homo sapiens	21	Caucasoids, Mongoloids, and Negroids	18	244	0.004	0.025	16,569	1,12
Homo sapiens	6	Caucasoids, Mongoloids, and Negroids	19		0.003			16
Homo sapiens	147	Caucasoids, Mongoloids, Negroids, and Australoids	12	467	0.003	0.066		14
Pongo pygmaeus	5	Borneo and Sumatra	25	62	0.050	0.046	16,500	16, 20
Pan troglodtyes	10	Captive; possibly two subspecies	25	60	0.013	0.029	16,500	16, 110
Pan paniscus	3	Captive	25	54	0.010	0.013	16,500	16
Gorilla gorilla	4	Captive	25	51	0.006	0.010	16,400	16, 20
Cercopithecus aethiops		Cell line (BSC-1)					16,400	110
Macaca fuscata	10	Japan	18	49	0.013	0.028	17,000	22
Macaca mulatta	2	Captive					16,500	110
Papio papio	1	Captive					16,500	110
Miopithecus talapoin		Primary Cells					16,500	110
Lagothrix cana		Primary Cells					16,300	110
Galago senegalensis		Liver					16,500	110
Rodentia								
Peromyscus maniculatus	135	North America; 15 subspecies	8	80	0.020	0.096	16,000	26
Peromyscus polionotus	68	Southeastern U.S.	8	48	0.011	0.042	16,000	10
Peromyscus leucopus	14	Eight states	8	48	0.014	0.037	16,000	10
Sigmodon hispidus	134	Georgia; 3.2 hectare Oldfield	2	11		0.017	16,500	24
Rattus rattus	26	North America, Puerto Rico, East Asia	6		0.040			186

Rattus norvegicus	21	North America, Puerto Rico, Japan	6		0.010			186
Mus domesticus	7	Europe	11		0.009		16,300	17
Mus molossinus	2	Japan	11		0.000		16,300	17
Spalax ehrenbergi	65	Israel; 4 chromosomal races	16		0.047		16,600	187
Geomys pinetis	87	Southern U.S.	6		0.018			7
Crytomys hottentus	5	South Africa; 3 localities	15	113	0.016			188
Crytomys natalensis	5	South Africa; 2 localities	15	131	0.017			188
Georychus capensis	6	South Africa; 3 localities	15	113	0.115			188
Heterocephalus glaber	49	East Africa Kenya; 9 localities	12	209	0.001	0.001		125
Artiodactyla								
Sus scrofa		Four breeds plus Japanese wild boar	17	52	0.016	0.016	16,350	189
Odocoileus virginianus	68	West Texas and South Carolina	10	41	0.013	0.018	16,600	190
Ovis aries	2	Domestic	3		0.020			191
Capra hircus	3	Domestic	3		0.010			191
Perissodactyla								
Equus caballus + prezwalski	8	Domestic	16	63	0.034	0.006	16,640	21
Cetacea								
Tursiops truncatus	5	Atlantic, Gulf of Mexico, Australia	6	143	0.015			192

[a] δ calculated from Nei and Li.[97]
[b] *p* calculated as in Hudson.[193]
[c] Genome sizes in parentheses determined from direct sequencing.

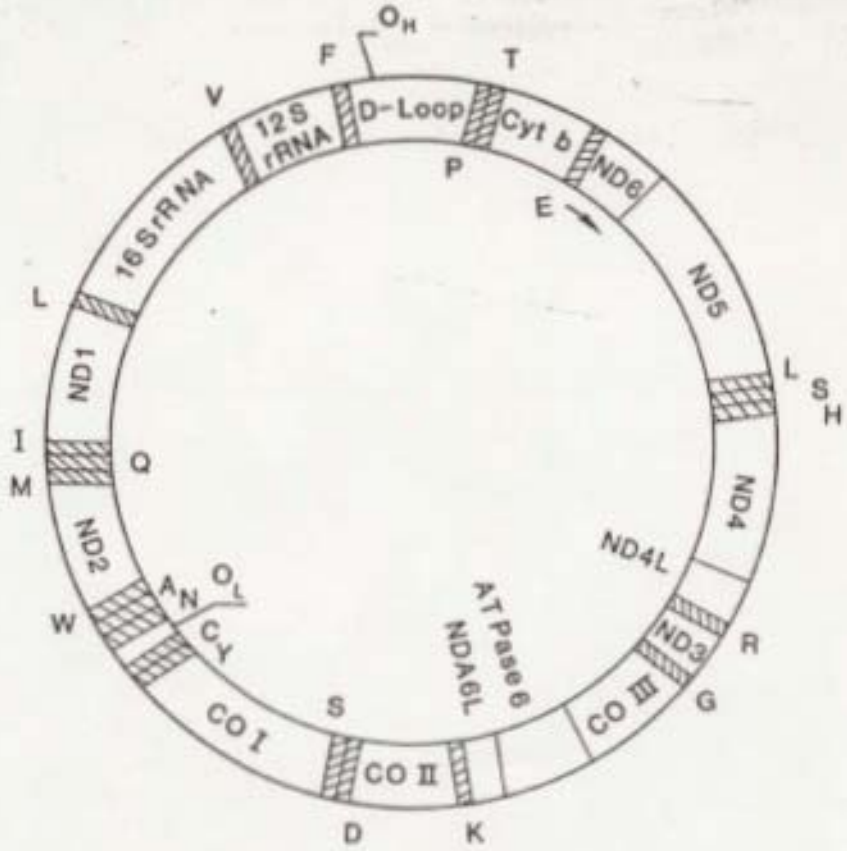

FIGURE 2. Organization of the human mitochondrial genome constructed from several references.[1,4,43,54] Heavy (H) and light (L) strands are the outer and inner circles, respectively. O_H and O_L denote the origin of replication for the two strands. Gene designations are as follows: ribosomal genes, 12S and 16S rRNA; tRNAs proline (P), threonine (T), glutamic acid (E), leucine (L), histidine (H), arginine (R), glycine (G), lysine (K), aspartic acid (D), serine (S), tyrosine (Y), cysteine (C), asparagine (N), alanine (A), tryptophan (W), methionine (M), glutamine (Q), isolencine (I), valine (V), and phenylalanine (F). Cyrochrome oxidase subunits (COI, II, III); cytochrome b (cyt b); ATPase 6 subunit (ATPase 6): ATP synthetase complex (NDA6L); NADH dehydrogenase complex (ND1-4, 4L, 5, 6). All genes encoded on the L strand are placed inside the circle and the direction of L strand transcription is indicated by the arrow next to ND6.

Replication of the complementary H and L strands of mtDNA occurs in an asymmetric, continuous, and unidirectional manner with initiation sites located in different regions of the genome.[1,5,57] Heavy (H) strand replication begins at a site (O_H) in the control region with the formation of a displacement loop (D-loop) by the synthesis of a short piece of DNA, 7S DNA, complementary to the L-strand (Figures 2 and 3). This 7S DNA displaces the parent or homologous H strand, and new H-strand synthesis proceeds away from the rRNA genes. The origin for L-strand synthesis (O_L) is located at a point approximately two thirds of the genome length from the origin of H-strand synthesis. L-strand synthesis does not occur until H-strand synthesis reaches the L-strand origin, and then proceeds in the opposite direction.

The D-loop of several mammals and other vertebrates have been examined in an effort to determine the structural and functional constraints placed on this region of the mtDNA genome.[1,4,57-63] Several observations have been made in these studies (Figure 3). First, 7S DNA in humans exists in four discrete size classes with length heterogeneity occurring at the 5′ end. In contrast, size heterogeneity in *Mus* maps to both the 3′ and 5′ ends of 7S DNA.[60,63] Second,

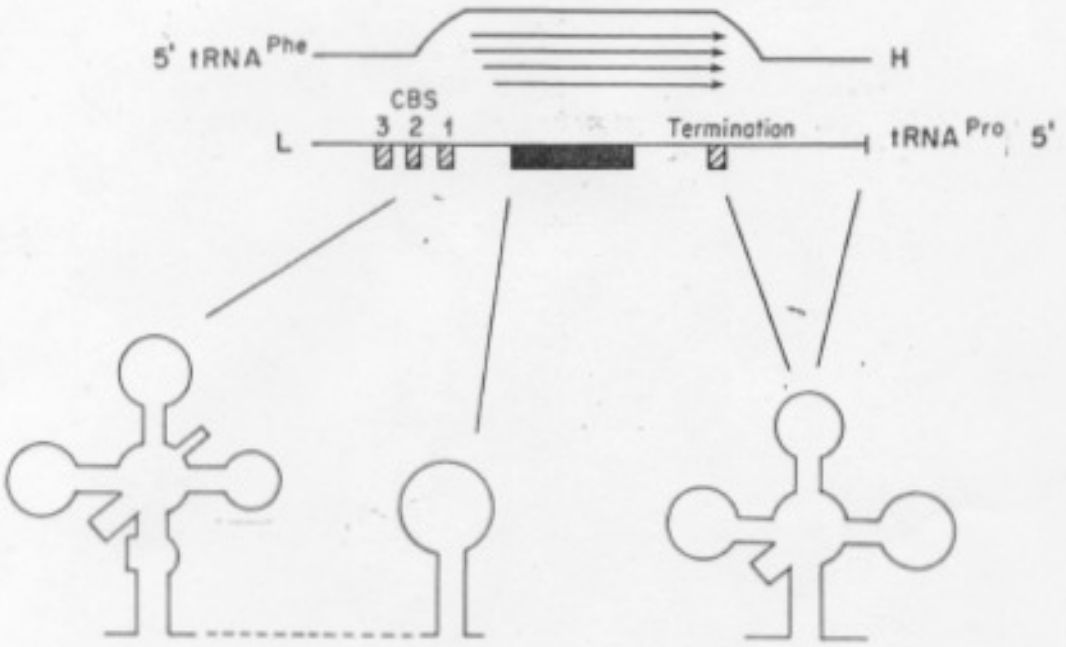

FIGURE 3. Structure of human D-Loop region compiled from several references.[4,59,61,63] The arrows denote the four 7S DNA size classes,[60] and the cloverleaf structures depict possible secondary structures found in the 5' and 3' domains.[59] CBS 1,2,3, the central black box, and termination region represent domains of conserved sequences. tRNAs for proline and phenylalamine are labeled, $tRNA^{Pro}$ and $tRNA^{Phe}$ respectively.

at the nucleotide sequence level the D-loop is the fastest evolving region in mammalian mtDNA. An examination of sequence variation between individual humans has revealed an average nucleotide divergence of 1.7% with most changes being the result of nucleotide substitutions biased toward transitions (96.1%) over transversions.[58] Unlike patterns of variation seen in *Rattus*,[59] only 8.9% of all changes in humans are caused by deletions or insertions.[58] Species level comparisons of sequence variation reveal considerable size variation ranging from 900 bp in most mammals to 2100 bp in amphibians.[4] Within primates, D-loop size also varies with deletions in four regions reducing the gorilla D-loop by 170 bp relative to humans. In terms of nucleotide substitutions, percent divergence increases with taxonomic distance as can be seen in comparisons of pygmy chimp to common chimp (8%), human to chimp (12%), and gorilla to human (13%). These same species level comparisons among all vertebrates as well as primates suggest that only partial homology is maintained relative to primary sequence. Finally, three domains of partial homology have been found in the D-loop region of mammals (Figure 3). These domains consist of a central conserved segment, three conserved blocks (CBS) at the 5' terminus and one conserved block at the 3' end which may be associated with D-loop termination.[4,61,63] In addition, the 5' and 3' domains have sequences capable of forming cloverleaf (secondary) structures, and these have been found in all vertebrates examined.[59] As a result, these regions may be under strong functional constraints.

3. Nuclear-Mitochondrial DNA Interactions

Although the mitochondrial genome autonomously replicates and encodes most RNA species (see Chang and Clayton[65] for an exception) and several key protein subunits used in either electron transport or ATP synthesis, most proteins required for mitochondrial metabolism, replication, transcription, and translation are encoded by the nucleus. There is no doubt that strong nuclear-mitochondrial genome interactions are required for cell metabolism, and these

interactions involve the transport of nuclear encoded proteins into the cytoplasm to the mitrochondrion.

Other interactions can be seen between proteins coded by both the nucleus and the mitochondria which are needed for proper functioning of processes such as electron transport, and phylogenetic studies of nuclear and mitochondrial gene evolution have born out the importance of such interactions. Sequence coevolution between the cytochrome c gene (nuclear) and the cytochrome oxidase II subunit gene (mitochondrial) has been demonstrated for mammals.[4,66,67,196] A phylogenetic analysis (based on parsimony) of nucleotide substitutions and amino acid replacements in the cytochrome oxidase II (CO II) gene of five mammals representing three orders (Primates, Artiodactyla, and Rodentia) indicated a fivefold rate increase in amino acid replacements along the primate lineage. A similar analysis of amino acid replacements in the cytochrome c gene showed a fourfold increase for primates as well. These two molecules are known to interact in electron transport, and it is on this basis that coevolution to maintain function has been suggested. Two questions remain unanswered about this coevolution. First, what caused the rate increase along the primate lineage? Second, is the rate increase restricted to the order primates, and if so, does the rate increase begin at the start of primate radiation or is it restricted to higher primates? The first question is more difficult to address, but the second question can be answered with a more extensive phylogenetic analysis of mammals, especially primates. The original primate observation represented by the human CO II gene sequence has now been expanded to include pygmy and common chimpanzee and a macaque.[68,196] Both chimpanzee species and humans show a similar rate increase of amino acid replacements relative to other mammals suggesting that the rate change is not restricted to the human lineage. Osheroff et al.[69] have also provided evidence for the localization of the rate increase. By examining homologous/heterologous reactions between cytochrome c and cytochrome oxidase, these authors observed more compatibility in heterologous reactions as the distance from humans increased. Current research is now being directed toward bracketing the primate radiation by sequencing the CO II gene from prosimians (galago), New World monkeys (wooly monkey), Old World monkeys (green monkey), and hominoids (gibbon, orangutan and gorilla) in order to further localize the rate increase.[68]

4. Mode of Inheritance

The inheritance of mtDNA appears to be clonal with the type of mtDNA transmitted to the next generation derived from the maternal egg cytoplasm. The confirmation of maternal inheritance of mtDNA has come from both breeding studies and pedigree analyses, where the mtDNA haplotype as defined by restriction endonuclease digestion patterns was known for both male and female parents. To date, all animals including invertebrates (*Drosophila*[70] and *Heliothis*[71]) and vertebrates (*Xenopus*,[72] *Equus*,[73] *Homo*,[74,75] *Rattus*,[76] and *Mus*[77]) which have been investigated in the above matter have shown maternal inheritance. In humans, maternal inheritance has been confirmed on two separate occasions by following mtDNA haplotypes over one to several generations.[74,75] Hybridization studies involving crosses between species which show distinct mtDNA restriction patterns have also demonstrated maternal inheritance.[71,77] These hybridization studies are very strong evidence, because in at least two cases, backcrossing of female F_1 hybrids bearing the mtDNA haplotype of one species to males of the other parental species was done for numerous generations. In the case of *Mus*,[77] the backcrossing experiments were reciprocal and involved six to eight generations, whereas in *Heliothis*[71] a nonreciprocal backcrossing experiment extended for 91 generations. These backcrossing experiments are sensitive to the ability to detect "paternal leakage" because they are designed to allow for maximum accumulation of the paternal mtDNA genome through the backcross generations, yet no evidence for paternal inheritance is seen. Thus, if the paternal mtDNA is transmitted to the zygote, it occurs in a low frequency relative to the maternal mtDNA (1 to 1000).

5. Recombination

Although there is evidence for the rearrangement of mtDNA gene order between phyla,[4,78] the duplication of both noncoding and coding regions within a given taxon,[79] and small deletions/insertions within particular regions of the mtDNA genome,[80] the contribution of intermolecular recombination to the production of this variation in animals has not been confirmed. Recombination has been verified for yeast mtDNA,[35] but cell hybrid experiments involving mammalian mitochondrial genomes[81] and detailed restriction endonuclease mapping of numerous organisms have not revealed any evidence for recombination. Therefore, mtDNA variation, unlike that seen for nuclear DNA, is primarily the result of mutation in the absence of recombination.

III. ESTIMATING GENETIC VARIATION IN MITOCHONDRIAL DNA

Patterns of mtDNA variation have been examined at several levels of divergence in order to diagnose the phylogenetic relationship among species or geographic races within a species and to understand the genetic structure of local populations. The genetic markers used to examine this variation have been of two basic types, nucleotide sequences and restriction endonuclease sites, with the latter providing an indirect estimate of the former.

A. DIRECT SEQUENCING ANALYSIS

Nucleotide sequencing techniques such as the Maxam and Gilbert[82] or Sanger[83] methods provide maximum resolution of intra- and interspecific genetic variation. The problem is that there have been fewer detailed systematic studies which have used nucleotide sequences to examine mtDNA divergence. Seven complete animal mtDNA genomes have been sequenced including *Homo sapiens*,[1] *Mus domesticus* (mouse),[43] *Bos taurus* (cow),[42] *Xenopus laevis*,[52] *strongylocentrotus purpuratus*,[197] *Rattus norvegicus*, and *Drosophila yakuba*.[44-49] Although these complete sequences provide information on conserved and variable features of mtDNA over long periods of evolutionary time as well as particular patterns of sequence change and genome organization, they are of limited value in examining the details of the rates of mtDNA evolution, and provide a limited amount of information on the expected distribution and frequency of nucleotide substitutions among closely related species and within populations. The only detailed studies of nucleotide sequence variation have been done on hominoid primates and the regions examined are as follows: (1) D-loop region — includes within species studies of a 900 base pair (bp) region from seven humans representing two ethnic groups, Caucasian and Africans, and a comparison of a similar size fragment in gorilla, chimpanzee, and man.[4,58,61,82] (2) Cytochrome oxidase subunit III — a *Sac*I/*Xba*I fragment representing approximately half of the CO III gene from five humans with 200 nucleotides from 248 clones sequenced in order to evaluate within and between individual variation.[84] (3) 896 base pair fragment — this region contains three tRNA genes ($tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$), and parts of ND 4 and 5 and was sequenced for human, chimpanzees, gorilla, orangutan, gibbon, four macaques, squirrel monkey, tarsier, and ring-tailed lemur,[13,199] (4) 12S rRNA genes — represented by an EcoRI/AvaI or *Kpn*I fragment in human, both chimpanzees, gorilla, and orangutan.[85] (5) Cytochrome oxidase subunit II gene — sequenced in human, both chimpanzees, and a macacaque.[68,196] Aside from the primate sequences, some small regions of the mitochondrial genome have been sequenced in related rodent species and artiodactyls.

B. RESTRICTION ENDONUCLEASE ANALYSES

By far the most extensively used method of estimated mtDNA variation has been the comparison of restriction endonuclease digestion patterns within and between species.[7-33] This

approach involves either the direct digestion of isolated mtDNA followed by end-labeling with ^{32}P-dNTPs and autoradiography,[12] the digestion of total DNA followed by Southern blot hybridization[86] using nick-translated mtDNA as a probe,[87] or ethidium staining.[88-90] The former approach provides higher resolution because the combination of agarose and acrylamide gel electrophoresis with end-labeling of mtDNA restriction endonuclease fragments can routinely reveal fragments below 300 bp whereas the Southern blot hybridization method cannot. This allows for the use of restriction endonucleases which recognize four- as well as six-base sites, thus permitting one to examine a higher percentage of the genome for restriction site variation.[33]

One approach used in restriction endonuclease analyses has been termed "high resolution" mapping and involves the comparison of four-base recognition site variation among individuals of a species to a known mtDNA sequence for that species. Detailed studies of inbred strains of *Mus*[17,18] and human racial variation[14,67,80,91-93] have taken advantage of this technique. Both these studies have provided valuable insight into not only the level of mtDNA variation occurring within a species but the distribution and frequency of changes within the mtDNA genome.

A considerably larger number of studies which have examined intra- and interspecific mtDNA variation have utilized primarily restriction endonucleases which recognize six bases, and the resulting patterns of overall restriction site variation for all enzymes have been used to determine the number of mtDNA haplotypes represented in the group under study as well as how the various haplotypes relate. Comparisons of individual haplotypes have involved either detailed composite restriction site maps or a comparison of the total number of similar versus different restriction fragments produced by a particular enzyme.

1. Genetic Distance

A variety of methods exist for estimating overall sequence divergence between mtDNA haplotypes from both restriction endonuclease site and fragment data.[94-99] The most widely used method is that proposed by Nei and Li.[97] In this method the nucleotide sequence divergence, δ, between haplotypes can be calculated from restriction endonuclease site data using their Equations 9 and 10. The estimate of δ is calculated separately for four-base and six-base enzymes and weighted relative to the number of cleavage sites produced by these two sets of enzymes. Alternatively, a similar estimate of δ can be determined for unmapped restriction fragment data using their Equations 20 and 21 with the assumption that fragments of similar electrophoretic mobility share homologous sites.

Several assumptions are necessary for the calculation of δ by the Nei and Li method as follows: (1) a constant G + C content across taxa compared; (2) the distribution of bases and base changes within mtDNA are distributed randomly with nucleotide substitutions following the Poisson process; (3) rate of substitution is constant over time; (4) probability of nucleotide substitution is the same for all sites; (5) identical sites have remained unchanged since divergence from a common ancestor. Not all these assumptions have been substantiated by nucleotide sequencing. For instance, not all sites have the same probability of substitution and individual haplotypes may share sites as a result of convergence rather than divergence from a common ancestor. Recently, Adams and Rothman[100] have analyzed the distribution and frequency of 54 restriction endonuclease cleavage sites in the sequenced human mtDNA genome, and have shown particular restriction sites to be distributed in a nonrandom manner. These data suggest that δ values may represent a biased estimate of nucleotide sequence divergence with the direction of the bias being unknown. Adams and Rothman recommend that one must discard enzymes that demonstrate a nonrandom distribution when estimating nucleotide sequence divergence using restriction endonucleases.

By comparing known nucleotide sequences among taxa to estimates of divergence derived from restriction endonucleases, one can further evaluate both the direction of bias provided by the Nei and Li estimate as well as test for a random versus nonrandom distribution of nucleotide substitutions. We have examined the 896 bp sequence[13] from human, chimpanzee, gorilla,

Table 2
Pairwise Comparisons of Restriction Sites Derived from 896 bp Sequence*

	Human	Chimpanzee	Gorilla	Orangutan	Gibbon
Human	0				
Chimpanzee	125	0			
Gorilla	115	109	0		
Orangutan	108	96	88	0	
Gibbon	89	87	81	80	0

* Shared sites below the diagonal of 165 sites total calculated from Brown et al.[13]

TABLE 3
Delta Values Compared to Percent Nucleotide Divergence for the 896-bp Fragment

	Human	Chimpanzee	Gorilla	Orangutan	Gibbon
Human	0	8.8	10.3	16.1	18.1
Chimpanzee	6.0	0	10.6	17.2	18.9
Gorilla	7.9	9.0	0	16.7	18.9
Orangutan	9.2	11.8	13.7	0	18.9
Gibbon	13.4	13.9	15.5	15.7	0

Note: Actual percent sequence divergence $\times 10^{-2}$ bp/bp above diagonal; below diagonal Nei and Li δ value $\times 10^{-2}$ bp/bp.

orangutan, and gibbon in order to address both the accuracy and distribution of site variation. Using the Pustell program[101] for DNA sequence analysis, the five homologous primate sequences were searched for every known restriction endonuclease cleavage site and the ensuing fragment sizes were estimated. A total of 169 sites were found for enzymes recognizing four, five, and six bases which averaged 4.6 bases per site. These sites, in some cases, overlapped, and sequences which were either identical or wholly subsumed in recognition sequences of other enzymes were not considered more than once. The number of shared fragments between all pairwise comparisons of taxa are presented in Table 2. The percent divergence estimates calculated from the data in Table 2 by the method of Nei and Li are compared to the actual percent sequence divergence calculated directly from the known sequence in Table 3. A regression of δ on the actual percent sequence divergence shows (Figure 4):

$$\delta = -0.00405 + 0.776 \text{ (\% sequence change)}$$

The Y intercept is very near zero, as expected since both should recognize the absence of differences. The interesting aspect to this analysis comes from the slope which is approximately three quarters and shows an underestimation of actual divergence by the Nei and Li value.

The distribution of variable base positions appears to be randomly distributed as a Poisson distribution, with a mean of +3.19 variable sites per each of 89 ten-base cells in the first 890 bases of the sequence. A chi-square test does not show statistical significance ($X^2 = 4.05$, 8 df), thus unlike the distribution of restriction endonuclease cleavage sites examined in the human mtDNA sequence, the distribution of nucleotide substitutions are randomly distributed. The actual δ value, however, does show an underestimate of divergence.

C. CONSTRUCTION OF MATERNAL PHYLOGENIES

The phylogenetic relationships among mtDNA haplotypes can be determined by two basic methods, phenetics or cladistics. The phenetic method involves the calculation of a distance

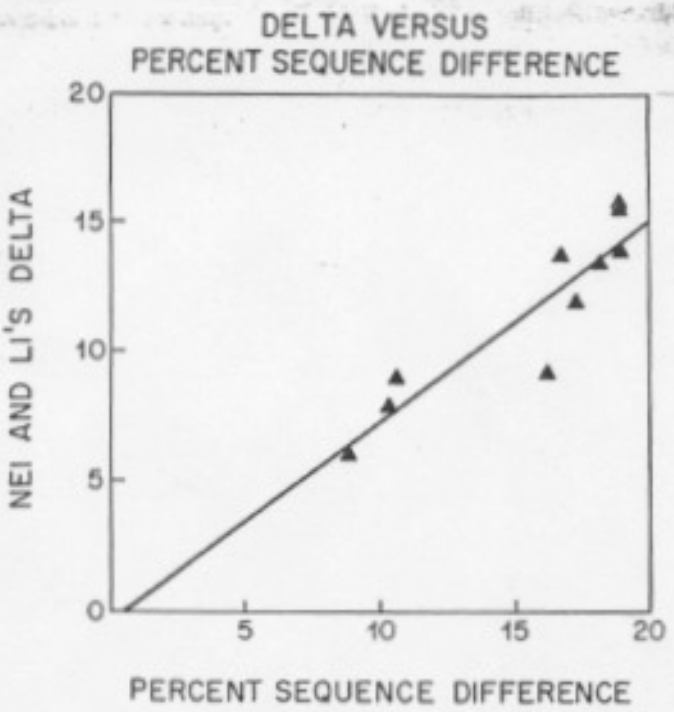

FIGURE 4. Regression analysis of δ values calculated from restriction endonucleases cleavage sites relative to actual percent sequence divergence for the 896-bp fragment[13] from six primate species.

estimate, such as δ, between haplotypes or groups of haplotypes, and clustering algorithms (e.g., UPGMA)[102] used to derive a phylogenetic tree. This approach has several inherent problems.[103-106] First, it assumes a constant rate of nucleotide substitution. Second, it does not consider convergent or parallel evolution when constructing trees. Third, it is difficult to evaluate or select between alternative phylogenies. Finally, there are some biases associated with the original distance estimates calculated from mtDNA data (see previous section).

The cladistic method treats mtDNA restriction endonuclease sites as individual characters and lacks many of the inherent difficulties and assumptions associated with the phenetic analyses. In such a comparison, restriction sites are coded as either present (a gain) or absent (a loss), and phylogenetic trees are produced using a parsimony criterion where overall tree length is used to compare alternative phylogenetic trees. The most parsimonious tree is considered the shortest tree relative to the distribution of site gains and losses along individual branches. There are several versions of the cladistic approach, however, which differ somewhat, and all have been used in mtDNA analyses. First, the simplest approach is to code sites as present or absent and assume no direction to change followed by a phylogenetic analysis using parsimony (PAUP).[20,21,107,108] Such a tree can be rooted at the midpoint of the two most divergent lineages or by outgroup which establishes polarity. Second, undirected networks can be constructed for each restriction endonuclease by linking haplotypes based on the minimum number of substitutions required to account for the differences.[7,10,15,23,26,32] These networks can then be combined so that composite haplotypes are related by total site gains or losses in a manner which produces a minimum-length, nondirectional phylogenetic network. Directionality can be assumed if the pleisiomorphic (primitive) haplotype can be verified by outgroup. Third, Templeton[105,106] has proposed the construction of separate phylogenetic trees for each restriction endonuclease using Wagner parsimony followed by the selection of a consistent overall phylogenetic hypothesis constructed from the individual trees using compatibility analysis.

Finally, DeBry and Slade[109] suggest the weighting of site gains and losses relative to the probability of each using Dollo parsimony. This approach may resolve problems associated with any asymmetries resulting from the frequency of site gains and losses.

IV. PATTERNS OF VARIATION IN MITOCHONDRIAL DNA

As indicated earlier, seven complete animal mitochondrial genomes have been sequenced as well as selected coding and noncoding regions in mainly primate and rodent species. These nucleotide sequence data together with "high resolution" restriction endonuclease mapping and comparisons of restriction site variation within and among animal species allow the examination of the mode and tempo of mtDNA evolution. The consensus of these studies is that the major mode of evolution is by nucleotide substitutions with length variation playing a minor role. Genome rearrangements have occurred but are restricted to differences between highly divergent groups. These rearrangements involve gene order differences among insects, vertebrates, echinoderms, and nematodes.[4,78]

A. NUCLEOTIDE SEQUENCE CHANGE

The most detailed comparative nucleotide sequencing study has involved the examination of variation at an 896-bp fragment found in 13 primate species, *Homo sapiens*, *Pan troglodytes*, *Pan paniscus*, *Gorilla gorilla*, *Pongo pygmaeus*, *Hylobates lar*, *Macaca fuscata*, *M. mulatta*, *M. fascicularis*, *M. sylvanus*, *Saimiri sciureus*, *Tarsius syrichta*, and *Lemur catta* which show a maximum divergence time of 55 million years (myr).[13,199] This study is important because it compares homologous coding regions (three tRNA and two protein genes) over a time span which can provide details of mtDNA evolution not obtained by comparing the more divergent mitochondrial genomes. Results from this study are as follows: (1) predominance of single base substitutions relative to base deletions/additions; (2) silent substitutions four to six times higher than amino acid replacements; (3) frequency of substitutions at codon positions highest at the third position and lowest at the second position; (4) mutation bias toward transitions (A↔G and C↔T) over transversions (A or G↔C or T) with transitions occurring at a frequency of 92 to 95% among recently diverged species; (5) transition to transversion ratios decrease with increase in divergence time possibly as a result of an increase in multiple substitutions at a nucleotide site; (6) rate of mtDNA sequence divergence estimated to be five to ten times higher than nuclear DNA.

Three additional studies of sequence variation among primate mitochondrial genomes substantiate the conclusions drawn from the 896 bp fragment. First, D-loop sequences from seven humans not only revealed nucleotide substitutions to be the predominant type of change but also a 32-fold bias of transitions relative to transversions.[58] Second, the 12S rRNA genes of human, chimpanzees, gorilla, and orangutan were shown to vary primarily by nucleotide substitution with 1 to 4 bp deletions/additions being restricted to seven locations.[85] Transitions occurred at a frequency between 87 to 100%. Third, preliminary nucleotide sequence data on the cytochrome oxidase subunit II gene (CO II), parts of the ATPase 6 and Nd A6L genes and three tRNAs ($tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Ser(I)}$ of human and pygmy chimpanzee revealed similar patterns to those observed in the 896-bp fragment (Table 4).[68] Transitions accounted for 88 to 100% of all nucleotide substitutions with most substitutions being silent and most frequent at first and third condon positions.

High resolution restriction endonuclease mapping of 112 human mtDNA also provides insight into the patterns of mtDNA variations.[67] If one compares the ordering of protein coding regions relative to the amount of variation found at a given region, the results from high resolution mapping between individual humans approximates nucleotide sequence comparisons of human relative to the cow and mouse mtDNA (Table 5). The restriction endonuclease mapping, however, does underestimate variation in particular instances. The transition to

TABLE 4
Sequence Comparisons between Human and Pygmy Chimpanzee[a]

Gene	Divergence	Transitions	Transversions	Silent	Replacements	Codons 1	Codons 2	Codons 3
CO II	9.7	88	12	81	19	14	8	78
ATPase6	8.5	97	3	59	41	32	16	52
NDA6L	5.5	100			100	60	40	
$tRNA^{Asp}$	11.7	87	13					
$tRNA^{Lys}$	5.7	100						
$tRNA^{Ser}$	4.2	100						

Note: All values given are percentile.

[a] Unpublished data from Honeycutt and Brown.[68]

TABLE 5
Variation in Human Mitochondrial DNA Protein Coding Regions

Region[a]	Size (bp)	Restriction sites[b]	Nucleotides[c]
CO I	1542	2	1
CO III	784	1	2
Cyth	1141	4	3
NDI	956	5	4
ATPase6	680	6	5
CO II	684	7	6
ND4L	297		7
ND4	1378	4	8
ND3	346	9	9
ND5	1812	8	10
ND2	1042	3	11
ND6	525		12
A6L	207		13

Note: Ranking is from most conservative to most variable.

[a] Abbreviations for genes same as in Figure 2.
[b] Based on number of inferred mutations estimated by high resolution restriction endonuclease mapping of 112 human mtDNAs by Cann el al.[57]
[c] Based on comparisons of nucleotide sequences of human mt DNA genome to those of mouse and cow as in Brown.[4]

transversion ratio (2.5:1) was found to be considerably less than suggested by nucleotide sequencing. Aquadro and Greenberg[58] have also shown estimates of nucleotide diversity in the D-loop region to be several-fold higher when estimated with nucleotide sequencing as opposed to restriction endonuclease mapping.

1. Rates

The rates of nucleotide substitution in the mammalian mitochondrial genome can be evaluated by species comparisons using several analytical approaches. In terms of rate estimates, information from primates has set the standard by which all other estimates are made. The first primate comparisons involved an examination of restriction endonuclease maps (oriented to the origin and direction of replication) for the mitochondrial genes of Guinea baboon, rhesus macaque, green monkey, and human.[110] A plot of estimates of nucleotide

sequence change (δ) relative to divergence times for pairwise comparisons of primates and other mammals revealed a five- to tenfold rate increase for mtDNA divergence relative to single copy nuclear DNA (scnDNA). The initial slope suggested a rate constant of approximately 0.02 substitutions per base pair per million years; however above 15 myr the rates of mtDNA divergence became similar to or less than scnDNA. Subsequent DNA annealing experiments and nucleotide sequencing have confirmed rate estimates from restriction mapping. Pairwise sequence estimates of 0.2 to 1.9% are similar to those estimated for the same taxa restriction endonucleases, and both techniques suggest a similar rate constant of 2% per million years.[13,20]

Although restriction map data of 145 human mtDNA suggest that at least 70% of genetic diversity found in regions of the mitochondrial genome can be accounted for by neutral mutation theory,[111] coding regions do differ with respect to the magnitude of nucleotide substitutions (Table 5). The mitochondrial tRNA genes evolve 100 times faster than the nuclear counterparts (1.7% per million years) yet relative to other mitochondrial genes, the tRNAs have one of the lowest rates of substitution (1/2 rate of protein genes) with only rRNA genes evolving at a slower rate.[13] The rRNA genes in the mitochondrion, however, are also quite divergent relative to nuclear rRNA genes over the same time periods.[67,85] As can be seen in Table 5, the mtDNA protein genes show significant variation in rates as well.

By far the most divergent parts of the mitochondrial genome are the noncoding regions.[4,58,59,61,62,67] Rates of D-loop divergence relative to protein genes have been estimated to be 1.4 to 5 times higher depending on the method of analysis. In addition, intergenic sequences also show high rates of nucleotides substitutions and deletions/additions.[4,80]

B. HETEROPLASMY

Although heteroplasmy (more than one mitochondrial genome within an individual) occurs less extensively than interindividual variation, several cases of length heteroplasmy (genome size differences resulting from deletions/additions) have been documented (Table 6).[87,112-122] On the other hand, site heteroplasmy has been reported only four times.[62,113,123-125] In one case, heteroplasmy involving a HaeIII recognition site was detected in a pedigree representing maternally related Holstein cows.[113,123] Two mtDNA haplotypes were observed in this maternal lineage suggesting the maternal ancestor to be heteroplasmic. Two other examples of site heteroplasmy were detected by sequencing cloned D-loop sequences from the same individual in the case of humans[62] and 14 maternally related Holstein cows.[122] Finally, one colony (17 individuals) of the naked mole-rat, *Heterocephalus glaber*, was found to be heteroplasmic for two genomes differing by the presence or absence of an extra HpaII site.[125] These individuals shared considerable variance relative to the copy numbers of the two genomes suggesting a rather rapid turnover rate for such mutations. These rodents are eusocial with only one breeding female and have overlapping generations so all 17 individuals may be assumed to be from the same mother.

One might explain the lack of site heteroplasmy as an artifact of sampling methods. Length heteroplasmy can be detected in any standard survey of mtDNA variation, because such heteroplasmy will be revealed by all restriction endonucleases. On the other hand, single-base substitutions (a site variation) are related to the restriction endonucleases used. Therefore, a study designed to examine both the frequency and turnover rate of site hetroplasmy would require the sequencing of more than one homologous mtDNA fragment from the same individual. One such study has been done for human mitochondrial DNA where 248 fragments cloned from five individuals were sequenced.[84] Only one within individual difference in 49 kb of sequence was found suggesting the concerted evolution of mtDNA sequences within individuals.

C. THE MOLECULAR CLOCK

Several authors have proposed the idea of a mtDNA molecular clock with the estimated

TABLE 6
Length Variation in Animal Mitochondrial DNA

Taxa	Size variation	Genome location	Heteroplasmy	Ref.
All animals	15,700—19,500 bp	Genome size		
within vertebrate species				
1. Mammalia				
Homo sapiens	4—9 bp	Intergenic region between CO II and $tRNA^{Lys}$ genes		131
Homo sapiens	6—12 bp	CBS-2 region near 5' end of D-loop	*	112
Homo sapiens	1—2 bp	D-loop		58, 62
Homo sapiens	6—14 bp	D-loop and noncoding regions at junction between genes		80
Bos taurus	1—10 bp	5' end of D-loop	*	113
Rattus norvegicus	6—8 bp	Between $tRNA^{Leu}$ and $tRNA^{Thr}$		193
Rattus norvegicus	30 bp			88
Mus musculus	5000 bp	Six tRNA and seven protein genes	*	114
Mus domesticus	12 bp	Either in D-loop or adjacent tRNAs		17
Cryptomys hottentotus	50—150 bp	Three size classes		115
Cryptomys hottentotus		Continuously varying, D-loop?	*	115
2. Reptilia				
Cnemidophorus tessilatus	1—370 bp	D-loop	*	116
Cnemidophorus tessilatus	30—35 bp	Near D-loop	*	116
Cnemidophorus exsanguis	4800 bp	D-loop tRNAs, rRNA genes		194
3. Amphibia				
Hyla cinerea	17,500—18,400 bp	Genome size, not mapped	*	117
Hyla gratiosa	18,800—18,900 bp	Genome size, not mapped	*	117
Rana esculenta	400—700 bp	D-loop	*	118
4. Pisces				
Amia calva	16,000—16,900 bp	Genome size, not mapped	*	117
Between vertebrate species				
1.Vertebrata	16,300—19,200 bp	Genome size		3,4
Xenopus to mammals	900—2100 bp	D-loop region between $tRNA^{Pro}$ and $tRNA^{Phe}$		64
Xenopus to mammals	27 bp	Region between $tRNA^{Thr}$ and $tRNA^{Pro}$		64
2. Mammalia				
All mammals examined	16,000—17,3000 bp	Genome size, possibly D-loop		3, Table 1
All mammals examined	1—3 bp	tRNA genes, some codon size deletions in protein genes, origins of L-strand replication		4, 13

Capra to *Ovis*	170 bp	D-loop		190
Gorilla to *Homo*	170 bp	D-loop		4, 61
Homo to *Bos, Mus, Rattus*	300 bp	D-loop		4
Homo, Pan, Gorilla, Pongo	1—4 bp	12S rRNA gene		85
Rattus norvegicus and *Rattus rattus*	6—8 bp	Between $tRNA^{Leu}$ and $tRNA^{Tyr}$		193
3. Reptilia				
Cnemidophorous sexlineatus to *Cnemidophorous gularis*	17,500—18,000 bp	Genome size		194
4. Amphibia				
Xenopus species	300—1100 bp	D-loop		107
Hyla crucifer to other *Hyla* species	3000 bp	Genome size, not mapped		25
Within invertebrate species				
1. *Drosophila*				
Drosophila melanogaster	18,100—19,900 bp	Genome size, A + T-rich region of control	*	70, 119
Drosophila simulans	470 bp repeat units	A + T-rich region of control	*	120
Drosophila mauritiana	470 bp repeat units	A + T-rich region of control	*	120, 121
Drosophila sechellia	470 bp repeat units	A + T-rich region of control	*	120
2. *Gryllus firmus*	220 bp tandem repeats	A + T-rich region of control	*	87, 122
Between invertebrate species				
Most Drosophila	16,200—16,700 bp	A + T-rich region of control		4, 41
Melanogaster subgroup	15,700—19,500	A + T-rich region of control, 470 bp repeats		41, 120, 121
Drosophila virilis to *Drosophila melanogaster*	1000—5000 bp	A + T -rich region of control		41
Drosophila pseudoobscura, Drosophila persimilis Drosophila miranda	100—200 bp	Defined by *Cla*I		195

Note: Asterick denotes regions which show heteroplasmy.

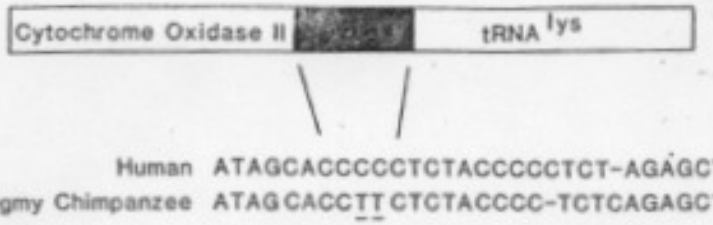

FIGURE 5. Comparison of intergenic sequence located between cytochrome oxidase subunit II and $tRNA^{Lys}$ for pygmy chimpanzee[68] and human.[131] Deletions denoted by blank spaces and nucleotide substitutions are underlines.

average rate of nucleotide substitution ranging from 2.5×10^{-9} to $25.4 + 6.1 \times 10^{-9}$ per site per year.[13,92,126-128] The accuracy of this mtDNA molecular clock, however, remains an open question as a result of several difficulties. First, aside from primates, a well substantiated calibration has not been provided, and the across the board estimate of 2% per million years proposed by Wilson et al.[33] must be considered with caution. These calibrations are sensitive to both the number[129] and quality of the time points, and the primate average rates even fluctuate (0.5 to 1.0% or 2% per base per myr) between researchers[4,13,33,110] depending upon the acceptance of a particular divergence time for hominoids. Second, Nei[130] has suggested that mtDNA does not provide a useful estimator of time since divergence especially when the populations under consideration diverged relatively recently. The standard error of δ can be larger than the expected value of δ in cases of recent divergence time as a result of stochastic errors of nucleotide substitutions. Third, mtDNA haplotypes may have been polymorphic implying that the acutal divergence time between two haplotypes may be older than the actual splitting of the populations.[14,27,34,38,130]

D. LENGTH VARIATION

Relative to invertebrates and other vertebrates, mtDNA length variation is rather restricted in mammals (Table 6) with most occurring in noncoding regions (either intergenic or D-loop). Cann and Wilson[80] reported 14 length variants among 112 humans involving deletions/additions of 6 to 14 base pairs. Three were found in two locations of the D-loop and the other 11 at seven sites in noncoding regions at junctions between genes. One such region (between cytochrome oxidase subunit II gene and $tRNA^{Lys}$) has been sequenced in 18 humans.[131] Normally, the region has two tandemly repeated 9 bp intergenic sequences (CACCCCCTCTACCCCCTCTAGAG). Two length variants were found. The first involved a deletion of one 9-bp sequence and was found almost exclusively in humans of East Asian origin, suggesting the probability of this deletion being used as a phylogenetic marker. The other variant involved two mutations, a transition, and addition of four cytosines producing a run of 11 cytosines (CACCCCCCCCCCCTACCCCCCTCTAGAG). This same region has been sequenced in pygmy chimpanzee,[68] and when compared to human, the pygmy chimpanzee intergenic sequence represents a size identical to the normal human sequence even though the two species differ by counterbalancing deletions/additions (Figure 5). The normal two repeats of the 9-bp sequence may be pleisiomorphic and support the idea of the 9-bp deletion type as being a synapomorphy for a group of individuals.

Other length variation occurs in mammals in coding regions such as tRNAs and rRNAs but the variation normally represents additions/deletions of only a few base pairs. The only exceptions are the unusual 5000-bp addition found in two *Mus musculus*[114] individuals and the 50 to 150 bp size difference found in the mole-rat, *Cryptomys hottentotus*.[115] This latter size variation consists of three size classes, and based on preliminary mapping data, this region is not located in the D-loop. The variation in *Cryptomys* is complicated by a continuously varying region, as well, which may map to the D-loop, and individuals show heteroplasmy in this region. Although there are exceptions, the large length variation in vertebrates and invertebrates maps

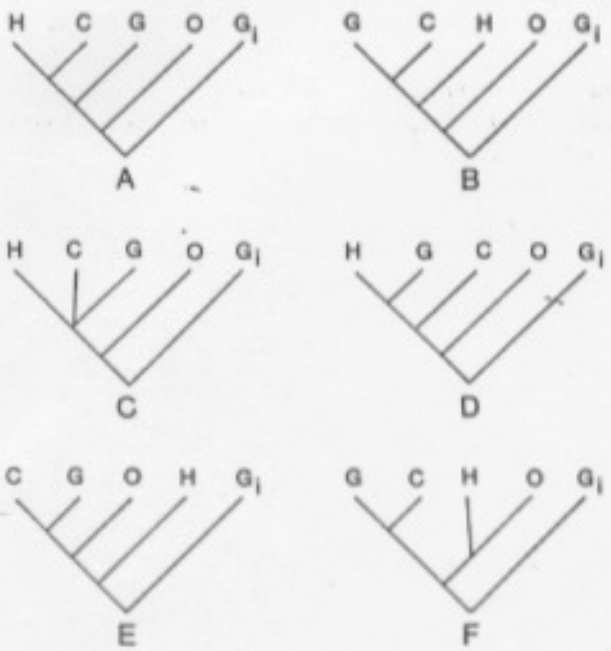

FIGURE 6. Alternative phylogenetic hypotheses for hominoid relationships derived from molecular, chromosomal, and anatomical data. Species designations are H (humans), C (chimpanzees), G (gorilla), O (orangutan), and Gi (gibbon).

to the D-loop and A + T rich control regions, respectively (Table 6), and most genome size variation among animals can be accounted for when one considers these regions.

V. HOMINOID CONTROVERSY

> "The earlier debate between physical anthropologists and molecular biologists over the pattern and timing of hominoid evolution is now basically settled."[131]

The debate of which Pilbeam speaks is the controversy over the phylogenetic relationships and divergence times for six primate taxa of the superfamily Hominoidea, human *(Homo sapiens)*, common chimpanzee (*Pan troglodytes*) pygmy chimpanzee *(Pan paniscus)*, gorilla *(Gorilla gorilla)*, orangutan *(Pongo pygmaeus)*, and gibbon *(Hylobates)*. This controversy has been in existence for approximately 20 years, and the debate has switched from arguments between anthropologists and molecular biologists to one among molecular biologists. The resolution of this controversy is an important issue in evolutionary biology for two major reasons. First, more empirical data have been collected on the hominoids than any other vertebrate, and these data span every available approach to systematics. Therefore, the hominoids stand as a basic test of both the theory and methodological approaches used in systematics. Second, the evaluation of particular hominoid traits such as locomotion (knuckle-walking and bipedality) cannot be interpreted without an unambiguous phylogenetic tree.

All available evidence points to the Old World monkeys, family Cercopithecidae, as being sister to the Hominoidea, and there is no disagreement over the placement of gibbons at the base of the hominoid radiations.[132] Comparative data from anatomical, chromosomal, and molecular studies, however, suggest several alternative phylogenetic hypotheses for the relationships of human, chimpanzee, gorilla, and orangutan (Figure 6). The derivation of these hypotheses need

to be addressed in light of the existing data on mtDNA variation as well as all characters used in previous studies. In order to appreciate the controversy, the strength of various phylogenetic hypotheses, and the contribution of mtDNA toward resolving phylogenetic relationships in hominoids, an overview of each type of character and how that character contributes to the controversy needs to be considered.

A. MOLECULAR DATA

Four phylogenetic hypotheses (Figure 6 A to D) have been proposed from molecular data, and all these hypotheses suggest a closer relationship of human, chimp, and gorilla relative to orangutan. The contradictions reside with the relationships among the former three species. Studies using albumin immunology,[133,134] amino acid sequencing of globin genes, [135] protein electrophoresis,[136] and nucleotide sequencing of pseudogenes[137] have been unable to resolve the human-chimp-gorilla trichotomy (Figure 6C). To the contrary, studies using DNA:DNA hybridization[138,200] of single copy DNA reveal a clear relationship between human and chimpanzee (Figure 6A), and this conclusion has been supported by more recent amino acid sequencing[139,140] studies of several proteins and restriction endonuclease studies of rRNA genes.[141]

There have been several interpretations of mitochondrial DNA data with the result that any one of four hypotheses (Figure 6 A to D) can be supported. The first study of primate mtDNA involved a comparison of aligned restriction endonuclease maps from six hominoid taxa.[20] The alignment was based on 11 invariant cleavage sites from an average of 50 sites per mitochondrial genome. Differences were found at a total of 121 positions, and the 42 phylogenetically informative positions were used to derive a phylogenetic tree based on parsimony. The shortest tree, based on the minimum number of mutations needed to derive the tree, suggested a closer relationship between chimpanzee and gorilla by a length of 67 mutations (Figure 6B). Alternative trees, however, required only one to four more mutations as follows: (1) Figure 6A — 68 mutations; (2) Figure 6B — 70; (3) Figure 6D — 71. The relatively small differences among alternative trees prompted Ferris et al.[20] to suggest that the human-chimp-gorilla triad represented a true trichotomy.

Templeton[105,106] re-analyzed the restriction endonuclease data of Ferris et al.[20] based on an algorithm which uses a combination of parsimony and compatibility analysis. From this analysis, Templeton suggested that the mtDNA data supported a closer relationship between chimpanzee and gorilla (Figure 6B). Templeton's analysis, however, has been strongly criticized as not providing a statistically significant resolution of the human-chimp-gorilla trichotomy.[130,142-144] In short, his interpretation may be based on false statistical accuracy and inappropriate methodology. Therefore, his results could be nothing more than a "red herring."

Two nucleotide sequencing studies of primate mtDNA have also been used to examine the phylogenetic relationships among the hominoids. An 896-bp fragment sequenced by Brown et al.[13] provided 90 phylogenetically informative sites which were used to derive a tree based on parsimony. The shortest tree (length 145) suggested a chimp + gorilla clade (Figure 6B), but again, the human + chimp (length 147; Figure 6A) and human + gorilla (length 148; Figure 6D) associations differed by only one to two mutational events. The unresolved trichotomy (Figure 6C), however, had a length of 157. Nei et al.[145] have calculated the standard errors of branching points derived from the 896-bp sequence and have shown the branching points separating human, chimp, and gorilla to be not statistically significant. Thus, one is left with an unresolved trichotomy.

Hixson and Brown[85] have sequenced the 12S rRNA gene from hominoids and found two trees (Figure 6 A and D) with a length of 133 events. The next shortest tree revealed a chimp + gorilla relationship (Figure 6B) with a length of 134. Other trees, such as the unresolved trichotomy (length 138) were much longer. In addition to examining nucleotide substitutions, these same authors provided a phylogenetic interpretation of deletions/additions. One deletion at position

73 was shared by chimpanzee and gorilla suggesting a closer relationship between these two taxa. As suggested by Hixson and Brown, the interpretation of this shared deletion may be polymorphic or represent convergence, and neither of these two factors can be eliminated without sequencing genes from more than one individual and comparing the hominoid sequence to an approproate primate outgroup.

B. CHROMOSOME DATA

Two phylogenetic hypotheses (Figure 6 A and B) have been suggested by chromosome banding.[146-148] If one considers differences in the resolving power of the various chromosomal analyses, there seems to be stronger support for a human-chimp association relative to gorilla. G-banding of late prophase chromosomes provides a high resolution of both G-positive and G-negative bands along the length of individual primate chromosomes.[148] A total of 1000 bands has been produced with this technique, and chimp and human have been shown to share three synapomorphies (chromosomes 2,7, and 9).

C. ANATOMICAL DATA

Comparative anatomical data have suggested several different alternative phylogenetic hypotheses as follows: (1) Figure 6 A or B — Andrews and Cronin;[149] Delson et al;[150] (2) Figure 6E — Delson and Andrews,[151] Kluge;[152] (3) Figure 6F — Schwartz.[153] The alternative phylogenies depicted in Figure 6 E and F are contradictory to all other comparative data as well as to some anatomical data. Kluge's[152] analysis included a treatment of molecular and chromosomal data followed by a re-evaluation of existing morphological characters. Although the resulting phylogeny supports a more traditional anatomical view of hominoid relationships, there are several problems with the basic approach used in this study. A systematic effort was made to discredit other forms of data relative to the new cladistic interpretation of morphological characters which requires one to ignore all molecular and chromosomal data as well as some anatomical evidence. The weakness of this morphological analysis relates to the fact that the analysis was based on a reinterpretation of past anatomical studies, and many questions relative to the accuracy of these interpretations remain unanswered. Schwartz's[153] phylogenetic hypotheses, on the other hand, is not substantiated by any data set, and must be regarded with considerable skepticism.

D. A CONSENSUS

If one considers the combined conclusions derived from chromosomal, molecular, and several anatomical studies, the African apes (chimp and gorilla) and humans form a monophyletic group relative to Asian apes (orangutan and gibbon). Thus, the family Pongidae can be viewed as paraphyletic. The relationships of human, chimp, and gorilla are somewhat more difficult to discern. The results from DNA:DNA hybridization, some nuclear gene studies, and high resolution chromosome banding provide the most unambiguous phylogenetic tree with human and chimp being closely aligned. The mtDNA studies, including restriction endonuclease analyses of the mtDNA genome plus selected sequencing of coding regions, reveal a chimp-gorilla clade relative to human, but minimum tree lengths among several alternative phylogenies differ by very small amounts. Therefore, these results must be considered equivocal.

Two divergence times, 20 to 30 myr for the Old World monkey-hominoid split and 13 to 16 myr for the African-Asian ape split, have been used to determine the average rate of nucleotide substitutions per site per year derived from several different molecular data sets. Overall, the molecular data suggest that the African ape-human split occurred within the past 4 to 8 myr. Low divergence times for the split have been provided by albumin immunological[122] distances (4 to 5 myr) and a calibration of mtDNA distances yield a low estimate as well (2.7 to 3.7 myr).[127] Nei[130] has also provided an estimate of divergence for the split between gorilla and the chimp-

human clade at 7.8 myr followed by the chimp-human separation at 6.6 myr using the 7.15×10^{-8} substitutions per site per year rate provided by Brown et al.[13] for mtDNA sequence divergence. These estimates are very similar to the 8 myr and 6.3 myr estimates provided by Sibley and Ahlquist[138] using DNA:DNA hybridization. According to Pilbeam,[132] these older time-since-divergence estimates are more compatible with the fossil record.

VI. HUMAN RACIAL VARIATION

The reconstruction of the evolutionary history of hominids has received considerable attention over the decades, yet a consensus has not been reached. Interpretation of ancestry, center of origin, mode, and tempo of evolution in the major hominid taxa is still controversial, and both fossils and molecules have been used to substantiate particular viewpoints. The controversies reside around two transitions, australopithicines to the genus *Homo* and early *Homo* types to anatomically modern *Homo sapiens*. Based on fossil evidence, the first transition began with the appearance of hominids in Africa 3.75 to 4 million years ago and ended with the appearance of *Homo* 2 to 2.5 million years ago.[132] There are two major questions regarding this transition which have been addressed with varying interpretations of fossils and dates. First, which australopithicines, if any, are ancestral to the genus *Homo*? Several alternative phylogenetic hypotheses have been proposed, and most of these hypotheses present the transition as anagenic change rather than involving branching or cladistic change.[154] If one were to interpret these hypotheses cladistically, they range from multiple or polyphyletic origins for the major hominid lineages to paraphyly for the genus *Australopithecus* with at least one species associated with the line leading to *Homo*. Second, can the transition be explained better by phyletic gradualism or punctuation? The answer to this question depends on the interpretation of morphological change and the age of particular taxa in the fossil record.[154,155]

The second transition begins with the appearance of early *Homo* followed by *Homo* erectus 1.6 million years ago and ending with modern *Homo sapiens* 100,000 to 125,000 years ago.[132] The date for the appearance of modern humans varies depending upon where the line is drawn, if at all, between anatomically "archaic" and modern *Homo sapiens*; therefore, the dates range from 40,000 to 100,000 years for modern humans with the dates for "archaic" humans ranging from 100,000 to 600,000 years.[34,132,154] The controversy surrounding this transition can be summarized with the following questions: (1) Where and when did the transformation from "archaic" to anatomically modern forms of *Homo sapiens* occur? (2) What conditions allowed for the diversification and spread of *Homo sapiens*? (3) What are the relationships of geographic populations of modern *Homo sapiens* and how can these relationships be explained with respect to human history? Aside from fossils which have provided a temporal and anatomical framework, morphological and genetic studies on extant populations of humans have been instrumental in addressing these questions. In this section we will discuss the past morphological and biochemical genetic studies on human populations, and relate hypotheses derived from these studies to more recent research on patterns of nuclear and mitochondrial DNA variation.

A. MORPHOLOGY

Although there are many morphological manifestations of geographical variation in Homo sapiens denoting patterns of differentiation among populations, the task of classifying the geographic variants into races has been a long and complicated matter with as few as three and as many as 32 races of man being recognized.[156-159] External morphological characteristics, such as skin pigmentation, facial structure, including eyes, nose, and lips, body build, and hair texture, delineate geographic subdivisions of *Homo sapiens*, but many of these traits may be subject to selection resulting in possible convergence of phenotypes in response to similar environmental conditions. Thus, the distinction of groups depends upon the characters used; hence, the divisions are rather arbitrary. The divisions do not become any less arbitrary when exomorphol-

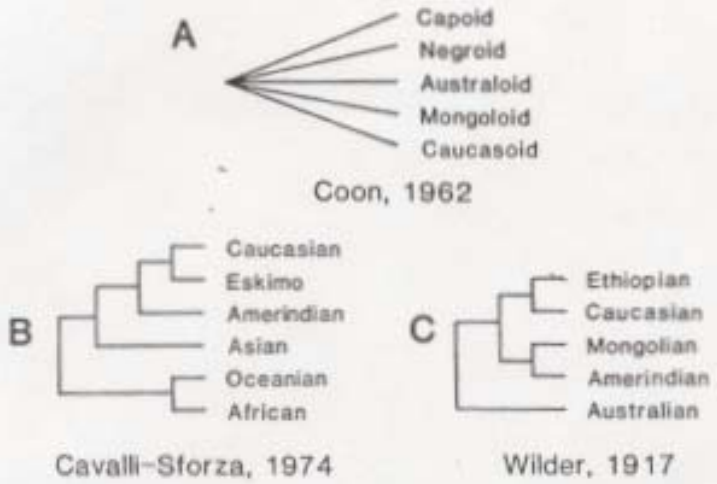

FIGURE 7. Relationships of human races based on morphology.[157,163,166]

ogical characters are used, making fossil humans rather difficult to assign to any particular extant geographic race.[160] The confusion as to how human populations should be subdivided using morphological criteria has created controversy over the utility of recognizing races of man. On the one hand, some scientists suggest that the classification of *Homo sapiens* has no biological basis,[161-162] and that such a classification is not necessary for understanding or appreciating the patterns and processes of human evolution. A less critical view is that the recognition of races is an abstract (as opposed to a biological) concept, but an exercise which can provide clues as to the past history of human evolution when the patterns of variation are examined in a careful and objective manner.[158,163] Regardless of what one's opinion is on whether or not to recognize human races, it is important to consider some of the hypotheses which have been proposed for the origin and groupings of geographic variants of *Homo sapiens* using morphological data, because these hypotheses provide an historical framework from which to view recent attempts using molecular characters.

The more traditional classification of *Homo sapiens* into groups or races have subdivided human populations according to five continents (Africia, Europe, Asia, Australia, and North America). The phylogenetic trees in Figure 7 represent three hypotheses of relationship based morphology. Coon's[157] hypothesis as to origin and evolution of modern *Homo sapiens* is by far the most widely cited work, and the views expressed by this hypothesis are considered contrary to most of the molecular evidence which will be discussed later. Basically, Coon separates modern *Homo sapiens* into five subspecies, Capoid, Negroid, Australoid, Mongoloid, and Caucasoid, using dentition and fossil evidence as the major criteria. The human populations and geographic regions assigned to each subspecies are as follows: (1) *Capoid*— Bushmen and Hottentots of South Africia; (2) *Negroid* — Negroes and Pygmies of Africa; (3) *Australoid* — Australian aborigines, Melanesians, Papuans, and some tribes of South Asia and Oceania; (4) *Mongoloid* — East Asiatics, Indonesians, Polynesians, Micronesians, American Indian and Eskimos; (5) *Caucasoid* — Europeans, Middle Eastern whites from Morocco to West Pakistan, India, and the Ainu of Japan. Coon further suggests that the development of human races may have taken place over several hundred thousand years, and that the transition from "archaic" to modern man occurred independently and in parallel lines from different geographic populations of *Homo erectus* which were present in both Africia and Asia at least 1 million years ago.[164] As does Wilder,[165] Coon suggests that Australoids, in morphological terms, represent the most pleisiomorphic (or basal) line of human races with the Mongoloids and Negroids being the most

differentiated or derived. The Caucasoids are considered more generalized relative to Mongoloids and Negroids as a result of possible intergradation between races. The difference between both Wilder's[165] and Cavalli-Sforza's[166] morphological treatment of human racial variation in contrast to Coon's hypothesis is that particular races are shown as being sister groups. Both studies show a sister group relationship between Amerindians and Asians whereas one study shows Asians and Caucasians to be related[166] and the other shows Caucasians to be more closely related to Negroids or Africans.[165] The alternative phylogenetic hypotheses of Wilder and Cavalli-Sforza are obviously only two of the many possible trees which could be proposed as one increases the number of geographic samples, but these two trees emphasize a major controversy which is the relationship among Caucasians, Asians, and Africans. This particular controversy continues with a consideration of molecular data.

What do the morphological and fossil data suggest about the center of origin for *Homo sapiens*? This question has been debated for over 100 years, and although other regions have been proposed, the consensus is that either Africa or Asia represents the center of origin for modern man.[167-169] Coon's[157] idea about the independent origin of races would suggest that at least three races probably arose in Europe and Asia, whereas more recent evidence suggests that the Asian forms of *Homo erectus* were probably not the ancestor to modern *Homo sapiens*.[14] Thus, one is left with a problem which cannot be convincingly resolved by interpretations of current fossil materials or morphology alone.

B. NUCLEAR GENES

The more traditional studies of genetic variation within and between populations have utilized two primary sources of data, human blood groups and allozymes. Early work on blood groups revealed qualitative differences among morphologically or geographically distinct populations of humans.[170] Cavalli-Sforza and Edwards[171] provided the first extensive quantitative analysis of human blood group loci by converting allele frequency data into genetic distances and constructing phylogenetic trees (Figure 8). The results of the blood group data suggest two major groups: Asiatic and Afro-European. The Asiatic group is considerably more heterogenous consisting of Asian, Australian-New Guinea, Eskimo, and Amerindian, whereas the African lineages are the most divergent. The phylogenetic relationships of human populations depicted by the blood group loci have been supported by a more extensive analysis of 58 histocompatibility loci.[166,172]

The advent of starch-gel electrophoresis and Harris'[173] discovery that human populations revealed both high levels of protein polymorphism (30% of loci) and heterozygosity (average 16%) stimulated a new round of genetic studies on human racial variation using genetic markers. Over the past 15 years, Nei and Roychoudhury, have contributed a considerable amount of information on allozyme variation within and between human populations and have provided some rather different interpretations relative to those derived from the blood group and histocompatibility loci.[128,130,163,174-177] These authors also converted allele frequency data into genetic distances, but used a different estimate, Nei's D,[178] based on the number of codon substitutions per locus that occurred after separation of two populations. These distance values were used to construct phylogenetic trees using clustering algorithms such as the unweighted pair group method of arithmetic averaging (UPGMA)[102] or Fitch-Margoliash.[179] Both methods have an inherent assumption of homogeneous rates of change between taxa. The phylogenetic relationships derived from this approach can be seen in Figure 8. In both cases, the Caucasians are now grouped with the Asian populations whereas the African populations are divergent. These trees, however, are not without anomalies, For instance, the Amerindians, which are historically associated with Asians, are clustered closer to Caucasians, and the placement of Australian aborigines and New Guineans differs between the two methods of tree construction. Another result not depicted in either figure is the association of the Ainu with Japanese rather than Caucasians as suggested by some morphological accounts.

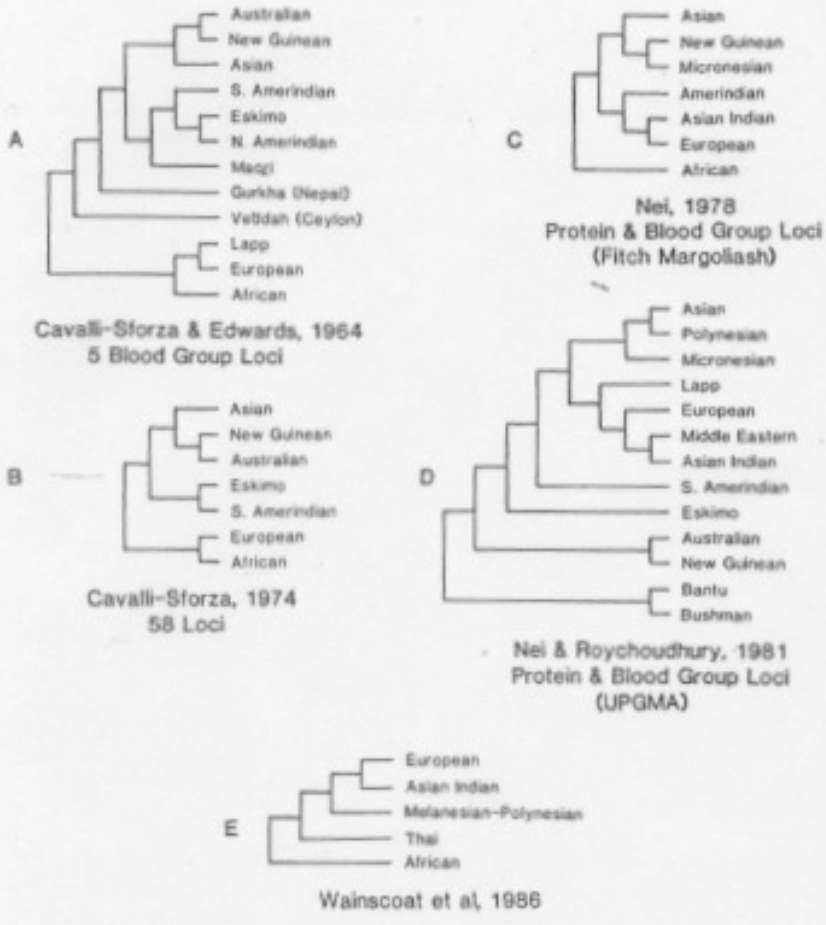

FIGURE 8. Relationships of human races based on nuclear gene data.[163,246,171,177,180]

If one examines the genetic distances and patterns of population variation in more detail, several other items can be gleaned from these genetic data. First, both blood group and protein loci indicate that the degree of racial or between-population divergence corresponds to that seen between local populations of animals rather than subspecies,[163] and only 9 to 10% of this variation can be used to distinguish between populations. Thus, the level of between-population variation as viewed through genetic distances is lower than within-population variation as revealed by the level of polymorphism. Second, some populations are highly divergent and have undergone more change than others. Although they form a group, the Australian aborigines and Papua New Guineans are separated from each other as well as from other populations by large distances and at the same time low levels of within-population variation. A similar level of divergence can be seen for the South Amerindians. Third, when analyzed separately, the genetic distance data for blood groups suggest a closer association of Caucasians with Asians (or Mongoloids) rather than Africans (or Negroids). Fourth, minimal divergence times calculated from genetic distance data suggest that Africans split from Caucasians and Asians first approximately 120,000 years ago with the split between Caucasians and Asians occurring approximately 60,000 years ago.[163,177]

Studies of nuclear gene variation within and between human populations has recently turned

to the examination of DNA sequence polymorphisms associated with both coding and noncoding regions. Wainscoat et al.[180] have examined patterns of restriction endonuclease site variation among five closely linked, yet polymorphic sites, in the β-globin gene cluster. Two restriction enzymes, *Hinc*II and *Hind*III, define these five sites and combinations of these sites along a chromosome are referred to as a haplotype. Although recombination can and does occur between sites, the resulting patterns of variation are analogous to those seen for mtDNA variation. Fourteen haplotypes out of a possible 32 were found for eight human populations representing three major geographic groups, Europeans, Asians, and Africans. The African populations were shown to possess two haplotypes in high frequency which were absent from all but one non-African population (where they occurred in low frequency). Conversely, two haplotypes which occurred in high frequency in all populations on non-Africans (except for one haplotype being absent in the Thai population) were either absent or in low frequency in African populations. The characteristics distinguishing the four major haplotypes which define particular groups cannot be explained by single events such as crossovers and base mutations. Based on this observation, Wainscoat et al. suggest that all four haplotypes have been in the human populations for a long period of time and predate racial divergence, whereas rare variants have arisen as a result of recent recombination. Genetic distances among the eight human populations were calculated from the haplotype data and analyzed by phenetic clustering (Figure 8). The major separation in the resulting phylogenetic tree is between Africans and Eurasians. This has led Wainscoat et al. and other authors to suggest that the β-globin gene data indicate an African origin for humans followed by subsequent migrations of small founder populations into other regions of the world. The combination of small numbers of individuals and drift in the founder populations are considered to be the reason for the loss by non-Africans of the high frequency haplotypes seen in Africans.

In criticizing these data, there is no doubt that Africans and non-Africans are divergent, yet an African ancestry for human racial groups is less clear. The ancestral haplotype is difficult to derive from the fourteen haplotypes observed among human populations, and Wainscoat et al.[180] have already indicated that the four common haplotypes cannot be accounted for by single events. Therefore, no directionality can be determined based on the current data, and without some outside reference or outgroup, it is just as likely that the founding events could have occurred in the opposite direction, Eurasia to Africa. Jones and Rouhani[181] realized this point and this is why their bottleneck hypothesis, proposed to account for the β-globin gene data, was founded on the assumption that the fossil evidence points to an African as opposed to an Asian origin for human populations.

As can be seen from the previous account of human morphological data, the nuclear gene data provide both conflicting and complementary information on human racial variation. Although each data set may differ with respect to how well populations group in relation to geography, all data sets suggest that groups of human populations must have experienced isolation throughout periods of their evolutionary history. The three broad geographic categories, Asian, Caucasian, and African, are supported by all the nuclear gene data. In addition, the divergent nature of Africans relative to non-Africans is substantiated. The relationships of the three groups, however, is more problematical. The blood group and histocompatibility loci suggest a close association of Caucasians with Africans, whereas the protein loci and β-gene cluster suggest a closer relationship between Caucasians and Asians. Therefore, unless one makes a choice as to which data set is to be given more weight, the site of origin for *Homo sapiens* cannot be unequivocally determined from the nuclear gene data.

C. MITOCHONDRIAL DNA

In recent years, human racial variation has been investigated using restriction fragment length polymorphisms of mitochondrial DNA. The research on mitochondrial DNA (mtDNA) has been more extensive, primarily because of the nature of the mitochondrial genome. As

indicated earlier, characteristics such as maternal and haploid inheritance, lack of recombination between maternal and paternal genomes, and a rapid rate of change as compared to nuclear DNA make mitochondrial DNA ideal for studying short-term evolution such as that seen in *Homo sapiens*. A nuclear counterpart to mtDNA would necessitate that restriction sites be linked with recombination playing a minimal or at least a predictable role in producing variation, and the nuclear DNA studies conducted on human populations have involved just such a system, the β-globin gene cluster.[180] To date, the major studies on both nuclear and mitochondrial DNA variation in humans have been done on a global scale with emphasis on worldwide surveys of variation using the major human groups as defined by geography. Nevertheless, several detailed mtDNA studies have been done on a regional scale and these studies have the potential of providing a more complete view of mtDNA variation, which may clarify some of the current conflicting data derived from the global studies.

In terms of mtDNA variation, there seems to be two opinions as to what the data mean relative to the origin of modern human races. Brown's[12] publication on mtDNA polymorphism in humans represents the beginning of studies on *Homo sapiens*, and is still the most widely cited paper on the topic as much for the technical aspects of the research as for the accounts of variation. This study examined mtDNA variation in 21 humans representing three major groups, Caucasoids, Mongoloids, and Negroids, and five geographic localities. Eighteen restriction endonucleases, six of which recognized 4 base sites and 12 six-base sites, were used to digest mtDNA and the site changes between individual mtDNA genomes were compared. Two conclusions were drawn from this study and have set the stage for argument. First, a qualitative analysis of the patterns of site gains and losses among mtDNA haplotypes found in the 21 humans suggested that group-specific mtDNA restriction endonuclease fragment patterns exist. Second, the average amount of nucleotide change in human mtDNA was estimated from the cleavage site data using the method of Nei and Li.[97] The pairwise nucleotide diversity, was shown to be 0.0036 substitutions per base pair for humans which is less than that calculated for other mammals (Table 1). Basically, this means that any two haplotypes differ by a very low number of substitutions. Using the rate of base substitution for mammalian mtDNA of 0.5 to 1.0% per million years and the average value of 0.18% base substitutions per bp for individual humans, Brown estimated that all humans may have been derived from a single mating pair that existed 180,000 to 360,000 years ago. Brown indicated that the lack of divergence suggested that humans may have evolved from "a small mitochondrially monomorphic population."[12] This statement was the beginning of the "Garden of Eden" concept for human evolution as painted by mtDNA variation.

Brown's original study has been expanded upon by examining mtDNA in 147 humans from five geographic regions, African, Asia, Australia, New Guinea, and Europe.[14] These studies used "high resolution mapping" which involves digesting mtDNA with a series of 12 restriction enzymes (most of which recognized 4 base sites), and comparing the fragment patterns to a known human mtDNA sequence. A total of 467 independent sites (195 of which were polymorphic) representing approximately 9% of the human mtDNA genome were surveyed. The 147 individual humans could be divided into 133 mtDNA haplotypes, and 93 phylogenetically informative sites were used to construct a genealogy for these 133 types. This genealogy was constructed using the PAUP (phylogenetic analysis using parsimony) program.[108] Figure 9 is an abbreviated version showing the major phylogenetic information derived from the analysis by Cann et al.[14] Several general observations can be made from not only the phylogenetic analysis but an examination of the degree of divergence among haplotypes as follows: (1) The average number of differences observed between any two humans was 9.5 sites, and the percent nucleotide sequence divergence (δ) for each pair of individuals was 0.32% which is similar to Brown's original estimate. (2) Africans were more variable than any other geographic sample with the intrapopulational variation of Africans (δ values among haplotypes) being greater than the variance among populations or geographic samples. (3) Most mtDNA

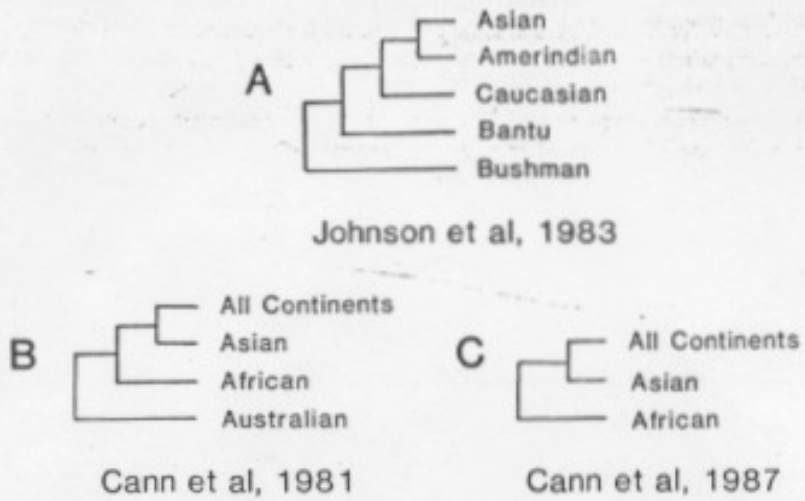

FIGURE 9. Relationships of human races from mtDNA.[14,23,92]

variants are shared among human populations. The only major geographic separation among the 133 haplotypes was the grouping of six African haplotypes, followed by two Asian haplotypes, and then a combination of the remaining 125 haplotypes. A phylogenetic arrangement of all haplotypes according to geographic locality produced a much longer and less parsimonious tree. An earlier analysis of basically the same data set, except for the New Guinea samples, suggested that the Australian aborigines have the most divergent mtDNA types followed by one African haplotype, and then a mixture of different combinations of haplotypes (Figure 9).[92] This analysis was done with UPGMA (a phenetic analysis) as opposed to the cladistic analysis and may explain the contradiction. Horai et al.,[182] using a larger number of Asians (116 Japanese), and the Africans and Caucasian samples from Cann et al.,[14] reported similar findings in terms of the general intermingling of human mtDNA haplotypes and the large number of nucleotide substitutions seen in Africans.

What interpretations were made from these findings? First, an African origin for the ancestral human mtDNA haplotype is inferred. Cann et al.[14] base this conclusion on the observation that the most divergent mtDNA haplotypes are found in humans of African descent. Second, the intermingling of geographic races or clusters throughout the tree implies that non-African geographic regions were colonized by multiple mtDNA lineages. Third, time-since-divergence estimates between mtDNA types were calculated under the assumption of a constant rate of nucleotide substitutions at 2 to 4% per million years. Using this approach, the age of the common ancestor to all mtDNA haplotypes was calculated to be 140,000 to 290,000 years old. Furthermore, they suggest that the migrations from Africa may have taken place 90,000 to 180,000 years ago. Finally, these researchers imply, as does Brown,[12] that the low levels of mtDNA divergence may be due to a population bottleneck resulting in the formation of all human mtDNA types from a single female.

What are some problems with Brown's[12] original conclusions and the interpretations and data reported by Cann et al.?[14] First, although the degree of divergence between any two human mtDNA haplotypes is low, the proportion of polymorphism, P, as calculated using Hudson's[183] method is similar to, if not higher than, other mammals (Table 1). This means that the mtDNA haplotypes may be similar, but there is a relatively high level of polymorphism with respect to the number of haplotypes in a population. The low level of divergence might be expected in a highly polymorphic and recently subdivided species. Second, recent authors have suggested that

the idea of all humans being derived from "Mother Eve" following a population bottleneck is in error. Avise et al.[34] developed a model which predicts the rate of survival and extinction of mtDNA lineages using techniques analogous to "male surname" survivorship studies. The results of this model suggest that similar patterns of mtDNA variation to those seen in human populations can occur without a population reduction, and that the single female, Eve, may have been one of thousands of females, whose mtDNA haplotypes did not survive to the present as a result of random lineage extinctions. Although the original haplotype may have been derived from a single female, the descendants of past human populations may all have a mtDNA haplotype which occurred in a large proportion of the ancestral population.[27] The approach used by Avise et al. has another implication that relates to the intermingling of human mtDNA haplotypes. Rather than reflecting either multiple invasions of geographic regions or intergradation between regions, a high number of similar mtDNA haplotypes among human races may reflect the retention of ancestral mtDNA lineages (or polymorphisms) which predate population subdivision. Third, δ values have large standard errors when populations are relatively recently derived, and the time since divergence estimates based on these δ values are not very informative.[130] Calculations of time since divergence from mtDNA haplotypes may also produce overestimates, because the separation of haplotypes may predate actual population subdivision as implied in the model provided by Avise et al.[34] Fourth, the African origin hypothesis must be viewed with caution. Part of the problem stems from the confidence one wants to place on the phylogenetic tree produced for the 133 haplotypes. The tree presented by Cann et al.[14] is one of thousands of possible trees, and the account of the particular analytical approach does not provide enough detail to appreciate the reason for presenting any one tree. It must also be noted that rooting the tree becomes problematical without an outgroup and is based on the midpoint between the two most divergent haplotypes. Thus, the determination of an ancestral mtDNA haplotype is not possible. Another problem arises from the actual human samples used in the analysis. All African, except one, are Afro-Americans. It seems a little premature to place too much weight on these findings without a more extensive study of other African populations which have been shown to be variable in both their nuclear and mtDNA genomes.[15,23,163,180]

Contrary to the conclusions of Cann et al.,[14] several studies on human mtDNA variation have proposed that Asia is central to the radiation of human ethnic groups.[11,15,23,32] In addition, individual mtDNA haplotypes were shown to cluster according to ethnic groups unlike the results of Cann et al.[14] (Figure 9). These authors used fewer restriction endonucleases, most of which recognize six base sites, and Southern blot hybridization which does not provide the resolution obtained by using enzymes which recognize four base sites and end-labeling such as the approach used by Cann et al. Thus, more variation (as low as 0.03% as opposed to 0.3%) can be detected by the latter method. In addition to discrepancies associated with methodological approach, the analytical approaches used by both groups are quite different with respect to both the production of phylogenetic networks and the interpretation of mtDNA variation. The primary approach used by Denaro et al.[15] was to relate all haplotypes produced by a particular restriction endonuclease by a network which involved the fewest number of site gains and losses to connect the haplotypes.[11,15,23,32] The haplotype from which all other haplotypes can be derived and the one resembling that possessed by other hominoid primates is considered the ancestral type. A composite phylogenetic tree can then be produced by combining the networks for all restriction endonucleases into an unrooted tree.

Using just the *Hpa*I patterns of variation produced for 235 individuals, 133 of which were Africans including Pygmies, San-Bushmen, and Bantu, 48 Asians, and 54 European and North American Caucasians, Denaro et al.[15] produced a network which suggested that Asians possessed the ancestral *Hpa*I types (*Hpa*I-1 and *Hpa*I-2) in high frequency with Africans revealing both these types in low frequency. The ancestral nature of *Hpa*I-1 was also supported by the presence of this type in the orangutan. All haplotypes unique to either Africans (*Hpa*I-

3). Asians (*Hpa*I-4,6), or Caucasians (*Hpa*I-5) could be derived from the two ancestral types. The higher frequency of the *Hpa*I-1 type in Asians and the low frequency in Africans (Bantu only) plus the fact that one unique Asian mtDNA type and all other types can be derived directly or indirectly from HpaI-1 prompted these researchers to suggest an Asian origin for human mtDNA types.

Johnson et al.[23] have expanded on the Denaro et al. study by using an additional four enzymes to analyze mtDNA variation in 200 humans representing Asians, Africans, European and North American Caucasians, and Amerindians. Thirty-five haplotypes were found, and a network analysis of these data showed 32 of these haplotypes to cluster according to ethnic groups. The remaining three haplotypes were shown to be central to the network and inferred as being ancestral with the Asian unique types closer to the ancestral types. Again, *Hpa*I was found to be the diagnostic enzyme with two of the three ancestral haplotypes differing by the presence or absence of a *Hpa*I site. When genetic distances or mean number of restriction site differences between two ethnic groups were used to produce a phylogenetic tree from the mtDNA data, Africans were shown to be the most divergent as in the study of Cann et al.[14] (Figure 9). The results of this analysis were interpreted as suggesting that the Bushmen lineage had undergone a rate increase, being twice as divergent as Bantus and three to five times as divergent as other groups. An alternative to this conclusion is that the African lineages may have diverged much earlier than the other lineages. Current research on more regional mtDNA variation in Japanese,[18,24] Tharu of Nepal,[11] and Amerindians[32] all reveal that the *Hpa*I type 1 occurs in higher frequency in Asians and their direct descendants. These authors use two facts, that the ancestral *Hpa*I type is in highest frequency in Asians and the *Hpa*I-3 type in Africans is not only in high frequency but unique, to suggest an Asian origin.

The contrasting views with respect to human mtDNA origins cannot be totally resolved, but there are several points of agreement. Both studies indicate that the overall level of nucleotide sequence divergence among human mtDNA haplotypes is low. In addition, Africans are shown to contain the most divergent and unique mtDNA haplotypes. The disagreement arises with how patterns of variation are evaluated and what these patterns mean in terms of determining the center of origin for human mtDNA types. The study by Cann et al.[14] is much more extensive with a large number of haplotypes differing by a small number of sites, whereas Denaro et al.[15] and Johnson et al.[23] place greater emphasis on patterns of variation produced by a small number of restriction endonucleases which discriminate a much smaller number haplotypes, but whose ancestral type has been established by outgroup comparison. Which approach is more valid? One might argue that the approach used by Denaro et al. and Johnson et al. provides the clearest separation of individuals by ethnic groups, and that the *Hpa*I patterns are more important phylogenetic markers. The example of Cann et al. could be seen as a case of not being able to see the forest for the trees. Conversely, the *Hpa*I phylogenetic marker might be considered an over simplistic interpretation of variation, which may become more complicated as detailed examinations of site differences produce more haplotypes and make the *Hpa*I patterns less clear. The main problem comes from how to weigh the overall patterns of variation seen in particular ethnic groups. Can one assume that older human populations have more divergent their mtDNA haploytypes should be? Or does this divergence relate more to the past history of human populations in a particular region? Given the theoretical consideration of mtDNA lineage survivorship, can an ancestral mtDNA type be determined from the existing data and does the exestence of primitive mtDNA types in high frequency suggest an older origin for such populations? One must be cautions about providing yes or no answers to any of these questions. The bottom line is that past events associated with population fluctuations, increased mutation rates, selection, and immigration may have all played a role in generating the complex patterns one sees today in human populations, and the question of origin may remain unresolved as far as mtDNA is concerned.

If we accept the conclusion that mtDNA variation may not be able to provide much

information on the origin of human ethnic groups, much less their relationships on a global scale, then what is the value of this molecule relative to human evolution? The answer may be that the molecule is more important when used to examine localized or regional problems of human origins and relationships. At least three regional studies have provided insight into relationships of particular human populations to broader ethnic groups as well as information on founding of regional populations. Brega et al.[31] have examined the question as to the ethnic affiliations of this population with Mongoloids. Wallace et al.[32] have provided data on mtDNA variation among Amerindians from the southwestern U.S. which suggest that local tribes were founded from small numbers of mtDNA lineages which subsequently diverged from other groups, and these lineages can be traced back to Asian ancestors. A similar regional analysis was done on Eastern Highlanders from Papua New Guinea.[30] The results of this study suggested that the Highlands constitute a single genetic unit which cannot be separated into linguistic or cultural groups with mtDNA, and that this region was founded by a least five maternal lineages.

D. A CONSENSUS

Discrete patterns of morphological and genetic variation are apparent in *Homo sapiens*. These patterns correlate, in part, to the current geographic distribution of human populations, and collectively suggest regional changes in human populations as a result of geographic separation. These regional differences break down into the five major continents and three broad ethnic groups, Caucasoid, Mongoloid, Negroid. Discrepancies arise when one evaluates patterns of variation using less inclusive categories, such as local populations or even individual humans. These discrepancies probably arise as a result of several factors acting independently or in concert. Selection obviously has to be considered with respect to morphology and maybe even genetic markers, such as blood groups, when considering geographic contradictions. Adaptation to local environmental conditions can increase divergence or convergence, and obscure ancestor-descendent relationships. Immigration between human populations has obviously occurred in the past as well as today, and the resultant mixing of populations can influence the patterns of genetic variation, especially at nuclear gene loci. The overall low level of nuclear and mitochondrial gene divergence among human populations may be the result of both immigration and the fact that modern *Homo sapiens* is relatively recently derived. Therefore, one would expect human populations to share polymorphisms. Isolation in combination with genetic drift can influence the rate of genetic divergence in small populations and this rate increases when a population undergoes a severe bottleneck. Such events can alter patterns of variation and increase divergence in both nuclear and mitochondrial genomes (Chakroborty and Nei[185]; Birkey et al.[36]). An example of the effects of such isolation can be seen in Australian aborigines, New Guineans, and Amerindians where a considerable amount of genetic divergence has occurred. One might even invoke a similar explanation to account for the larger divergence in African populations. Finally, different analytical approaches may also account for particular areas of disagreement. The nuclear gene data have been analyzed using phenetic approaches which have an inherent assumption of homogeneous rates of change and group on the basis of overall similarity. The pitfalls of analyzing genetic distance data with such approaches have been discussed at length, and the results of such analyses must be viewed with skepticism. The incongruence between relationships and geographic distribution may be partially explained by problems of analysis. Another analytical problem is that proper rooting of the phylogenetic tree for human races is critical to the interpretation of patterns of variation derived from the tree. All nuclear and mitochondrial gene studies suffer from a lack of an appropriate outgroup, the ability to determine ancestral states, and the use of midpoint rooting. The one exception is the *Hpa*I-1 mtDNA type which was suggested as being ancestral based on comparisons with other hominoids.

Where does all this leave us relative to the relationships among the three major ethnic groups, and the time and origin of human populations? We feel that genetic and morphological studies

of extant human populations cannot provide conclusive answers to these questions without some reference to fossil material. The effectiveness of the molecular data to provide information on divergence times depends on a proper point of calibration as well as a demonstration that the molecules are evolving in a clock-like manner. When dealing with divergence times involving periods of less than 1 million years, the calibration must be accurate and errors small if meaningful estimates are desired. Not only is there considerable disagreement as to the identification and date of key fossils, but the error or range of time estimates provided by both nuclear gene loci and mtDNA lead one to place little confidence in the dates. Nearly every source of data suggests that African populations are divergent from non-African ones. The problems arises in how to interpret this finding relative to the origin of human populations. If one excludes a consideration of selection, migration, and drift, then the magnitude of variation among the three broad ethnic groups supports the claim of an African origin. On the other hand, this claim can only be strengthened with reference to an outside group, fossil *Homo sapiens*.

VII. CONCLUSIONS

Detailed studies of primate mitochondrial DNA have provided valuable information on both the molecular structure and inheritance of mtDNA and the patterns of mtDNA variation. A comparison of primate taxa (hominoids) with known divergence times has allowed an evaluation of the rates of nucleotide substitutions in the mitochondrial genome relative to rates in nuclear DNA. These same comparisons have also provided insight into the possible relationships and divergence times of humans relative to African and Asian apes. In addition, the studies on human racial variation represent the most extensive mtDNA analysis of intraspecific variation, and provide a perspective on the levels of divergence one might expect as well as conditions which may influence levels of mtDNA variation. These data provide a baseline upon which to compare genetic variation in other natural populations.

Although the studies of primate mtDNA did not resolve all controversies, they do suggest several avenues for future study. First, the rate of nucleotide substitutions per site per year for mtDNA needs to be confirmed from sequencing studies of other vertebrates, especially mammals. Such studies should concentrate on groups such as rodents which not only have a reasonable fossil record for particular taxa but differ in generation time from primates. Artiodactyls are another group of mammals which have a good fossil record and could be used to test the primate rate. Second, the general observation of relatively low levels of both length variation and heteroplasmy in mammals needs to be investigated in more taxa. The rodents are definitely underrepresented, and the variation seen in *Cryptomys* points to the need for more extensive studies. Third, although particular species differ with respect to levels of intraspecific mtDNA variation (Table 1), few detailed studies on species which differ in life history strategies have been done. There is a lack of information on how patterns of mtDNA variation relate to environmental grain, and the spatio-temporal relationships of maternal lineages within a population are basically unknown. Until such studies have been done, the influence of environment vs. stochastic processes on mtDNA variation will remain unknown. Finally, the use of mtDNA as a genetic marker in behavioral and ecological studies has not been realized, yet this may be one of the most powerful uses of the molecule. Primate populations would make an ideal subject for such studies, especially in species which form social groups around matrilines.

ACKNOWLEDGMENTS

We wish to thank Kimberly Nelson and David Pilbeam for their helpful comments on the manuscript, and David Foran, James Hixson, Wesley Brown, and Rebecca Cann for providing us access to unpublished data and manuscripts in press. We are extremely grateful to Jane Harrison for typing this manuscript. Costs of this manuscript were defrayed by NSF grant BSR85-084790 to R.L.H. and NSF graduate fellowship to W.C.W.

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