Mitochondrial DNA Evolution in the Genus *Equus*[1]

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Employing mitochondrial DNA (mtDNA) restriction-endonuclease maps as the basis of comparison, we have investigated the evolutionary affinities of the seven species generally recognized as the genus *Equus*. Individual species' cleavage maps contained an average of 60 cleavage sites for 16 enzymes, of which 29 were invariant for all species. Based on an average divergence rate of 2%/Myr, the variation between species supports a divergence of extant lineages from a common ancestor ~3.9 Myr before the present. Comparisons of cleavage maps between *Equus przewalskii* (Mongolian wild horse) and *E. caballus* (domestic horse) yielded estimates of nucleotide sequence divergence ranging from 0.27% to 0.41%. This range was due to intraspecific variation, which was noted only for *E. caballus*. For pairwise comparisons within this family, estimates of sequence divergence ranged from 0% (*E. hemionus onager* vs. *E. h. kulan*) to 7.8% (*E. przewalskii* vs. *E. h. onager*). Trees constructed according to the parsimony principle, on the basis of 31 phylogenetically informative restriction sites, indicate that the three extant zebra species represent a monophyletic group with *E. grevyi* and *E. burchelli antiquorum* diverging most recently. The phylogenetic relationships of *E. africanus* and *E. hemionus* remain enigmatic on the basis of the mtDNA analysis, although a recent divergence is unsupported.

Introduction

The horse family, Equidae, comprising a single genus, *Equus*, represents a group with seven closely related species, notable for their rapid rate of chromosomal divergence and recent speciation (Bush et al. 1977; Ryder et al. 1978). The paleontological derivation of this genus is well documented, and its members are believed to have diverged within the past 3–5 Myr (Simpson 1951; Lindsay et al. 1980; M. F. Skinner, personal communication).

Some early studies of the mitochondrial DNA (mtDNA) molecule centered on its mode of transmission (Dawid 1972; Dawid and Blackler 1972). Toward that end, Hutchison et al. (1974) established that inheritance of mtDNA is maternal in *Equus*. Since then, mtDNA has been shown to be a powerful tool for probing relationships among related taxa with recent times of divergence (Avise et al. 1979; Brown et al. 1979; Brown 1980; Giles et al. 1980; Brown and Simpson 1981; Ferris et al. 1981*a*, 1981*b*; Cann 1982; George 1982; Densmore et al. 1985).

While the horse family has an abundant fossil record and has often been used to illustrate morphological evolution, controversy still remains regarding the phylogenetic

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relationships within this family, particularly among extant forms. Much of the controversy centers on implications drawn from morphological studies of dental, cranial, and postcranial specimens (Groves 1974; Churcher and Richardson 1978; Dalquest 1978; Bennett 1980; Eisenmann 1980; Harris and Porter 1980; Groves and Willoughby 1981).

We provide a molecular analysis of the genus *Equus* using mtDNA as an evolutionary probe. Comparison of mtDNA restriction-endonuclease cleavage maps of the seven extant species provides estimates of the extent of genetic diversity among the different mitochondrial genomes, of the phylogenetic relationships among equid taxa, and of the divergence times among these taxa.

Material and Methods

Tissues

mtDNA was purified from tissue obtained at necropsy, at castration (for *Equus caballus*), or from placentae (table 1). All animals studied, except the domestic horses and donkey, were part of the collection of the Zoological Society of San Diego. Specimens obtained from the Zoological Society of San Diego were all identified as individuals and are traceable to wild-caught animals either through registered studbooks (*E. przewalskii, E. africanus somalicus, E. hemionus onager, E. h. kulan,* and *E. grevyi*) or from the curatorial records of the San Diego Zoo (*E. burchelli antiquorum* and *E. zebra hartmannae*). Photographic records or location of museum voucher specimens are retained by the authors. In most cases the mtDNAs were prepared from frozen samples that had been stored at −70 C.

Preparation and Cleavage Mapping of mtDNA

mtDNA was prepared from tissues as described by Brown et al. (1979) and George (1982). The 17 restriction endonucleases employed, with their single letter codes, are listed in the legend to figure 1. All enzymes were obtained from New England Biolabs (Beverly, Mass.) or Bethesda Research Laboratories (Rockville, Md.) and used according to the supplier's directions. DNA fragments were labeled at the ends with ^{32}P according to the procedures given by Brown (1980). The methods for gel electrophoresis of DNA fragments and for mapping the cleavage fragments have been described (Brown et al. 1979; Brown 1980; George 1982). In most cases the smallest routinely scored fragment was ~180 bp long.

Alignment of the Cleavage Maps

The alignment of the cleavage maps was achieved by the use of sites that appear to be homologous among these equids. A large percentage of identical sites is seen in the region between 80 and 100 map units (fig. 3).

Calculation of Sequence Divergence from Map Comparisons

Estimates of the degree of sequence difference between pairs of mtDNAs were obtained by comparison of cleavage maps, employing equations (10) and (16) of Nei and Li (1979).

Phylogenetic Analysis of the Cleavage Maps

Phylogenetically informative endonuclease sites (sites not held in common by all examined taxa) were treated as discrete character states and utilized to produce trees according to the algorithms of Farris (1972) and Ferris et al. (1981*b*) based on the parsimony principle of minimum mutational length. Especially useful for this

purpose was the PAUP program of D. Swofford (Illinois Natural History Survey). The PAUP program also utilizes the Wagner distance method as described by Farris (1972). Confirmation of certain Wagner trees was performed manually.

Results

Genome Sizes

The genome sizes of the mtDNAs as determined by agarose gel electrophoresis of restriction-digest fragments are listed in table 1. The genome sizes for *Equus przewalskii* and *E. caballus* were found to be identical, as were the genomes of *E. hemionus onager* and *E. h. kulan.* The overall mean average length for all taxa was 16,560 ± 150 bp.

Cleavage-Site Differences among *E. przewalskii* and *E. caballus* Individuals

mtDNA cleavage maps were constructed for three different maternal lineages of *E. przewalskii* and five *E. caballus* individuals (two Arabian horses of different lineages, a quarter horse, a Morgan, and a Peruvian Paso). An average of 60 sites per genome were mapped (fig. 1). No differences were found among the maps of the three *E. przewalskii* maternal lineages, although one lineage represents a direct descendant of the last wild-caught *E. przewalskii* mare. No differences were observed between the quarter horse and the South American Peruvian Paso, nor between the two Arabian horses.

Restriction maps of the domestic horse, *E. caballus,* and the Mongolian wild horse, *E. przewalskii,* were similar but not identical (fig. 1). Two or three restriction-site differences distinguished the cleavage maps of all *E. caballus* cleavage-map individuals examined from the *E. przewalskii* restriction map. The most divergent *E. caballus* mtDNA cleavage maps were those of Arabian horses, thought to be one of the oldest breeds of domestic horses (Simpson 1951).

The percent sequence difference between *E. przewalskii* and *E. caballus* individuals was found to range between 0.27% and 0.41%. The Arabian horses and the Morgan showed the largest difference (0.55%). The tree presented in figure 2 shows the average

Table 1
Source of Material and Mitochondrial DNA Genome Sizes

Linnean Designation (*n*)	Common Name	Tissues Used	Base Pair Length[a]
Equus przewalskii (3)	Mongolian wild horse	Placenta, liver, spleen, heart	16,640 ± 560
E. caballus (5)	Domestic horse	Testis, spleen	16,640 ± 560
E. africanus somalicus (1)	Somali wild ass	Spleen	16,850 ± 520
E. hemionus onager (1)	Persian onager	Spleen	16,420 ± 600
E. h. kulan (1)	Transcaspian kulan	Spleen	16,420 ± 600
E. grevyi (1)	Grevy's zebra	Liver, spleen	16,480 ± 460
E. burchelli antiquorum (2)	Damara zebra	Liver, spleen	16,450 ± 260
E. zebra hartmannae (2)	Hartmann's mountain zebra	Liver, spleen	16,540 ± 280

[a] Size estimates were obtained from agarose gel electrophoresis of single and double restriction-endonuclease digestion of the different mitochondrial genomes. *Hin*cII fragments of phage ϕx174 RF DNA (Sanger et al. 1978), *Eco*RI fragments of phage λDNA (Thomas and Davis 1975), and *Hin*dIII fragments of phage PM2 DNA (Parker et al. 1977) were used as size standards.

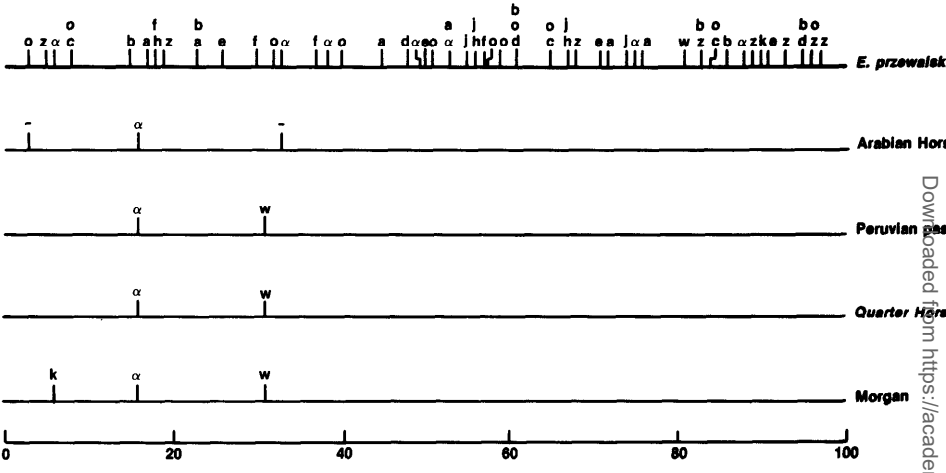

FIG. 1.—Cleavage-site differences among *Equus przewalskii* and *E. caballus* individuals. The following 16 restriction endonucleases were used to construct the cleavage maps: a, *Eco*RI; b, *Hin*dIII; c, *Hpa*I; d, *Bgl*II; e, *Xba*I; f, *Bam*HI; g, *Pst*I; h, *Pvu*II; j, *Sac*I (*Sst*I); k, *Kpn*I; l, *Xho*I; m, *Ava*I; o, *Hinc*II; w, *Bst*EII; z, *Fnu*DII (*Tha*I); and α, *Acc*I. *Sac*II (*Sst*II), designated β, was also used but was not mapped. All the enzymes recognize 6-bp sequences, except *Fnu*DII (*Tha*I), which recognizes a 4-bp sequence. The figure shows the complete cleavage map for *E. przewalskii* (61 sites) and only those sites that differ in *E. caballus*. Two Arabian horses of different stock had identical cleavage maps, but, as indicated by the minus sign (−), lacked the *Hinc*II (o) site at position 3 and *Acc*I site (α) at position 33. The Arabian horses also lacked the additional *Bst*EII (w) site at position 31, which is found in other domestic horses. The restriction endonucleases *Pst*I, *Ava*I, and *Xho*I did not cut these mitochondrial DNAs.

percent sequence differences among *E. przewalskii* and *E. caballus*. Overall, the amount of divergence presented here is small and not much greater than the 0.36% divergence reported for mtDNA differences found among the human racial groups (Brown 1980; Cann et al. 1984).

Comparison of the Cleavage Maps

mtDNA was prepared for representatives of the seven extant equid species described in Material and Methods. The cleavage maps are presented in figure 3. The

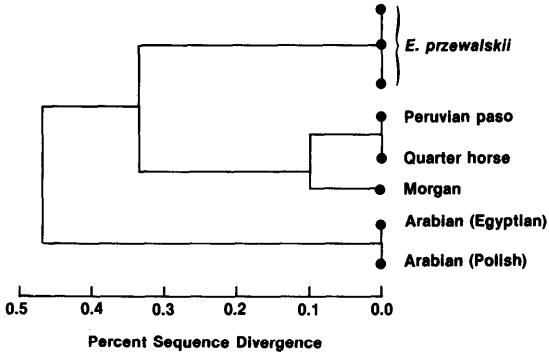

FIG. 2.—Percent sequence divergence among *Equus przewalskii* and *E. caballus*. The percent sequence divergence was determined by using algorithms as described by Nei and Li (1979). The tree shows the average percent sequence differences.

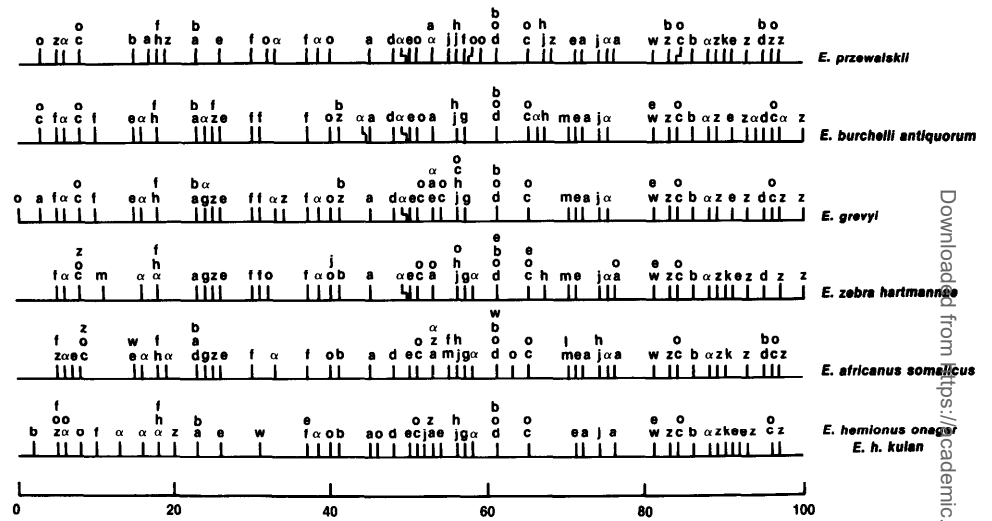

FIG. 3.—Mitochondrial DNA (mtDNA) cleavage maps of the genus *Equus*. The maps contain an average of 60 sites. The restriction endonucleases are designated by a single letter code (see legend to fig. 1). *E. przewalskii* is described in figure 1; *E. burchelli antiquorum* and *E. grevyi* mtDNAs are not cut by the restriction endonucleases *Xho*I or *Kpn*I; *E. zebra hartmannae* mtDNA is not but by *Xho*I; *E. africanus somalicus* mtDNA is cut by all enzymes employed; *E. hemionus onager* and *E. h. kulan* mtDNAs are not cut by *Ava*I or *Xho*I. The 29 invariant sites (position and site) are 6α; 8o; 18f and h; 23a; 26e; 37f; 40o; 45a; 50e; 51o; 53a; 56h and j; 61b, o, and d; 65c and o; 71e; 74j; 81w; 83z; 84c and o; 86b; 88α; 89z; and 93z. Fourteen highly conserved sites (found in five of the six species) are 5f; 8c; 16; 23b; 30f; 41b; 48d; 57g; 72a; 75α; 91e; 95d; 96o; and 97z.

E. caballus map is shown in figure 1. Again, more than 60 sites per genome have been mapped using 16 different restriction endonucleases. The maps show many sites in common among the different mitochondrial genomes. Of the 118 restriction-endonuclease sites mapped in one or more of the six species shown in figure 3, 29 occur in all six species. These invariant sites also occur in the *E. caballus* cleavage maps (fig. 1). There are 14 highly conserved sites that occur in five of the six species shown in figure 3. The identity and location of these invariant and highly conserved sites are listed in the legend to figure 3. Additionally, 44 sites were found to occur in only one of the six species and 31 were found to be phylogenetically informative. The phylogenetically informative sites will be described more fully in a later section.

Two examined taxa had identical restriction maps: the half-asses *E. h. onager* and *E. h. kulan*. Additionally, the *E. hemionus* map also contains the *Bst*EII (w) site at position 31, which is found only in the domestic horses, excluding the Arabian horses (fig. 1).

Estimates of Percent Sequence Differences and Divergence Times

Pairwise comparisons of sequence differences based on the cleavage maps (Nei and Li 1979) are presented in table 2. Sequence divergence was found to range from 0% (*E. h. onager–E. h. kulan*) to 7.8% (*E. przewalskii–E. hemionus*). Estimates of times of divergence are based on a pairwise divergence rate of 0.02 nucleotide substitutions/Myr (Brown et al. 1979). The divergence-time data suggest that the common ancestor to all extant forms was present ~3.9 Myr before the present (MYBP) and that the speciations leading to the lineages whose surviving members include *E. prze-*

Table 2
Estimation of Percent Sequence Differences and Divergence Time

	Eb	Eg	Ez	Ea	Eh	Ep
A. Restriction-Site Data						
Eb	(61)	0.828	0.732	0.703	0.672	0.672
Eg	53	(67)	0.775	0.746	0.703	0.688
Ez	45	50	(62)	0.729	0.683	0.666
Ea	45	50	47	(67)	0.719	0.688
Eh	41	45	42	46	(61)	0.656
Ep	41	44	41	44	40	(61)
B. Sequence Difference and Divergence Time						
Eb	—	3.3	5.6	6.4	7.3	7.3
Eg	1.6	—	4.5	5.2	6.4	6.9
Ez	2.8	2.2	—	5.7	7.0	7.5
Ea	3.2	2.6	2.8	—	6.0	6.9
Eh	3.6	3.2	3.5	3.0	—	7.8
Ep	3.6	3.4	3.8	3.4	3.9	—

NOTE.—Panel A shows the fraction of restriction sites in common among the six cleavage maps (numbers above the diagonal) shown in figure 3. The fraction of sites in common was determined by using eq. (10) of Nei and Li (1979). The numbers along the diagonal (in parentheses) represent the total number of restriction sites per map. The numbers below the diagonal show the number of restriction sites in common among the different cleavage maps. Panel B shows the percent sequence difference (above the diagonal) as determined from eq. (16) of Nei and Li (1979). The estimates of divergence times, in Myr (shown below the diagonal) were calculated by assuming a 2% divergence/Myr (Brown et al. 1979). Eb = *Equus burchelli antiquorium;* Eg = *E. grevyi;* Ez = *E. zebra hartmannae;* Ea = *E. africanus somalicus;* Eh = *E. hemionus onager;* and Ep = *E. przewalskii.*

walskii (and *E. caballus*), *E. hemionus,* and *E. africanus* took place within the next 0.5 Myr. The divergence-time estimates also suggest that the common ancestor of zebras was present ~2.8 MYBP and that *E. grevyi* diverged from *E. burchelli* 1.6 MYBP.

Comparison of Evolutionary Trees

As previously mentioned, 31 phylogenetically informative restriction-endonuclease sites were found among the six cleavage maps presented in figure 3. The 31 phylogenetically informative sites are listed in table 3. These sites served as the basis for the generation of trees a and b shown in figure 4. These sites were also used to provide a phylogenetic analysis of other published trees on equid evolution that are not based on mtDNA data (trees c–f, fig. 4). Table 3 also lists the minimum number and the most probable kinds of mutational events (loss or gain) required to account for the variation seen at each site for trees a and b of figure 4.

Trees a and b (of fig. 4) each require a minimum of 55 mutational events. The two trees differ in the relative placement of African and Asian wild asses (*E. africanus* and *E. hemionus,* respectively) with the wild horse, *E. przewalskii,* and the three zebra species. Thus, the mtDNA mapping data fail to discriminate the evolutionary branching order of the ancestors of *E. africanus* and *E. hemionus* from the ancestor of true horses, i.e., *E. przewalskii* and *E. caballus.*

Despite the discrepancy shown in trees a and b, tree a must be slightly favored over tree b. This favoritism is based on the following observations: As shown in table 3, tree a has more single mutational events than does tree b (10 vs. 9, respectively);

Table 3
Thirty-one Phylogenetically Informative Sites in *Equus* mtDNA

Position and Site[a]	Character State[b]						Events per Tree[c]	
	Eb	Eg	Ez	Ea	Eh	Ep	a	b
76a			+	+	+	+	L	L
95b				+		+	LL	LL
51c		+	+	+	+		LL	LL
96c	+	+		+	+		LL	LL
15e	+	+		+			GL	GG
53e		+			+		GG	GG
81e	+	+	+		+		LL	LL
10f	+	+			+		GG	GL
31f	+	+	+				G	G
24g		+	+	+			GL	LL
67h	+		+			+	LLL	LL
90k			+	+	+	+	L	L
70m	+	+	+	+			G	LL
3o	+					+	LG	LG
32o			+			+	GG	GG
54o		+	+				GL	GL
56o		+	+				GL	GL
5z				+	+	+	L	L
8z			+	+			GL	GG
20z					+	+	L	LL
25z	+	+	+	+			G	LL
41z	+	+					G	G
53z				+	+		LL	LL
100z	+	+	+				G	G
18α			+	+	+		LL	LL
24α	+	+					G	G
33α		+		+		+	LLL	LG
38.5α		+	+		+	+	LL	LL
49.5α	+	+	+			+	LL	LL
53α		+		+		+	LLL	LG
58α			+	+	+		LL	LL
Total mutations ...							55	55

NOTE.—L = loss; G = gain. Species abbreviations are as given in table 2.

[a] Restriction-endonuclease sites are represented by a single letter code given in the legend to fig. 1.

[b] Plus symbol (+) indicates the presence of a site.

[c] Minimum number of mutations and probable nature of the events (see Templeton 1983) for the two best trees presented in fig. 4.

Table 2B shows that the average estimated percent sequence divergence for *E. africanus* versus the three zebra species is 5.8% while the average for *E. hemionus* versus the zebras is 6.9%; and an unpublished observation (M. George, Jr., and O. A. Ryder) shows that the *Sst*II (*Sac*II) restriction-endonuclease digest pattern (or morph) for the zebras and *E. africanus* are identical, while the *E. hemionus* and *E. przewalskii–E. caballus Sst*II patterns are distinct from both each other and the zebra–*E. africanus* pattern.

When trees c–f of figure 4 were analyzed using the parsimony method, again using the 31 sites listed in table 3, they were found to be less parsimonious than trees a and b. They all required additional mutational events, and the number of events

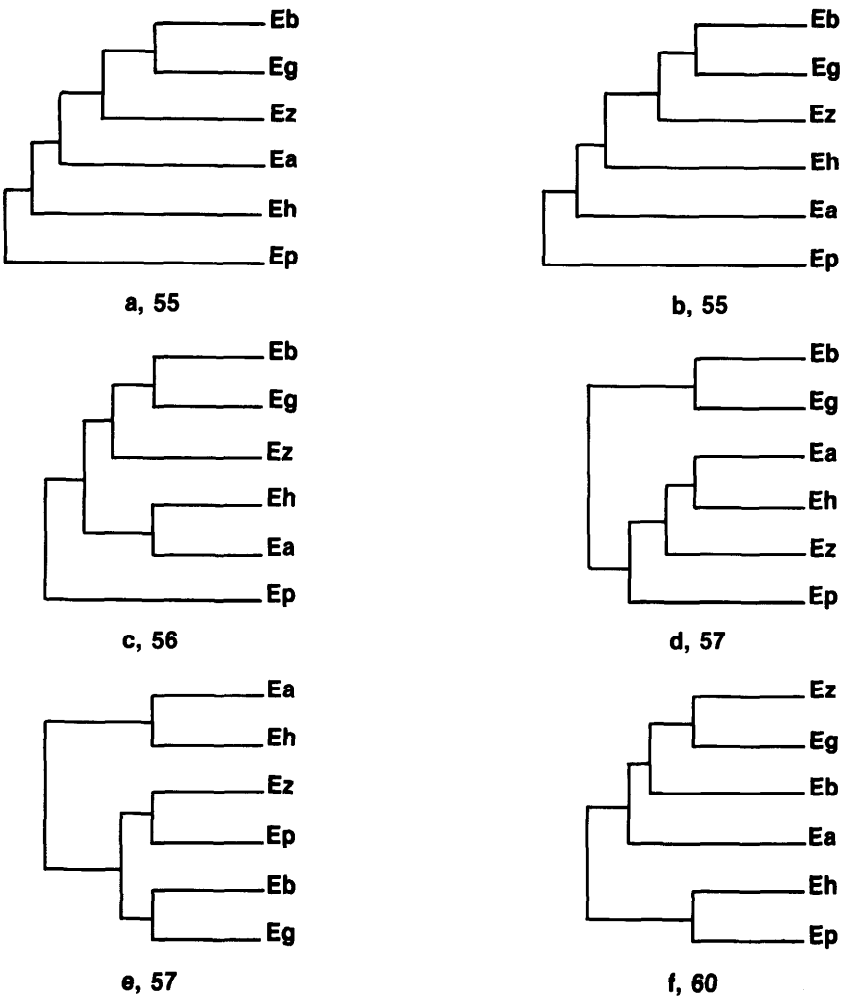

FIG. 4.—Six alternative trees relating mitochondrial DNA cleavage maps of six *Equus* species. Mitochondrial DNA cleavage-map data were used to produce the a and b trees. The protein and chromosomal work of Ryder et al. (1978, 1979) were used to produce the c tree. Morphological studies of dental, cranial, and postcranial material by Bennett (1980), Eisenmann (1980), and Harris and Porter (1980) were used to generate the respective trees designated d, e, and f. The minimum number of cleavage-site changes required to fit these topologies is also given. The species' abbreviations are given in the legend to table 2.

ranged from 56 to 60 (fig. 4). However, the three best trees (a–c) associate the zebras as a monophyletic unit. Data from trees a–c also suggest that there are at least three major clades in equid evolution, one that groups the zebras, a second that groups *E. africanus* and *E. hemionus,* and a third that associates the true horses, *E. przewalskii* and *E. caballus* as a unit. However, as stated previously, the *E. africanus–E. hemionus* clade remains enigmatic.

Is Parallelism High in *Equus* mtDNA?

An examination of the extent of parallelism and back mutations in *Equus* mtDNA shows that the 31 phylogenetically informative sites listed in table 3 require 55 mutational events to generate trees a and b of figure 4. The extent of parallelism was

estimated to be 44% (55 mutations − 31 sites = 24; 24/55 = 44%). In a study of *Mus domesticus* mtDNA comparisons that reported 73 mutations at 60 sites, the extent of parallelism was 18% (Ferris et al. 1983). An earlier study by Ferris et al. (1981*a*) comparing hominoid mtDNAs reported 67 mutations at 42 sites, which gave an estimate of parallelism of 37%.

Discussion

Systematics of the Equidae

The evolution of monodactyly in Equidae is one of the classic examples of morphological evolution (Simpson 1951). However, the phylogenetic systematics of the extant Equidae is in a state of controversy. Although several recent studies employing morphometric analysis of cranial and dental characters (Eisenmann 1980) or of cranial, dental, and postcranial characters (Bennett 1980; Harris and Porter 1980) have appeared, the phylogenies derived from these studies are congruent neither with each other nor with earlier suggested phylogenetic interpretations (e.g., Dalquest 1978). Extant equids are held to constitute a single genus, *Equus* (Bennett 1980; Eisenmann 1980; Harris and Porter 1980), perhaps including as many as six subgenera (Groves and Willoughby 1981) or at least two genera (Dalquest 1978).

In the light of these well-recognized difficulties in understanding the phylogenetic systematics of extant and recently extinct equids, it is perhaps safe to say that Simpson's (1951) statement—"*Equus* . . . has developed many distinct forms. The tangled threads of this fabric have not been fully unraveled"—still holds true.

Chromosomal Analysis of *Equus* Species

Analysis of banded karyotypes of extant equid taxa have demonstrated the extensive nature of the karyotype rearrangements that have occurred since their divergence from a common ancestor (Ryder et al. 1978). The extent of the chromosomal rearrangements described precluded a quantitative approach to establish cladistic relationships among equid karyotypes. However, certain phylogenetic affinities were implied by the presence of shared derived characters (e.g., particular chromosomal morphs). For example, *E. przewalskii* and *E. caballus* were highly similar karyotypically, as were *E. burchelli antiquorum* and *E. grevyi*. All three extant zebra species shared a particular chromosome morph. *E. hemionus* and *E. africanus* appeared more karyotypically similar to each other than to other equids.

Additionally, *E. h. onager* and *E. h. kulan* share a Robertsonian-type chromosomal polymorphism (Ryder et al. 1978; O. A. Ryder, unpublished observations) and are interfertile (Pohle 1972). Although these forms are also rather similar morphologically, distinct subspecific status was proposed by Groves and Mazak (1967).

Molecular Studies of the Equidae

Relatively few molecular studies have been conducted to provide additional data for equid phylogenetic analyses. Studies of equid hemoglobins (Kitchen and Easley 1969; Clegg 1974; Ryder et al. 1979) have to date provided only minimal phylogenetically useful data, although comparative nucleotide sequence data, particularly of alpha-globin loci, should be useful. Kaminski (1979) has utilized electrophoretic and immunological analyses of equid serum esterases and Ryder et al. (1979) have utilized serum protein electrophoresis to make phylogenetic inferences. Kaminski's 1979 work agrees well with mtDNA tree a of figure 4. The Ryder et al. (1979) protein work is represented by tree c of figure 4 and is the third most parsimonious tree.

The more recent molecular studies have centered on the placement of the extinct "zebra" species, *E. quagga,* within the Equidae (Higuchi et al. 1984; Lowenstein and Ryder 1985; Miller 1985; A. Higuchi, personal communication). Though the quagga has been extinct for >100 years, Higuchi et al. (1984) were able to isolate and clone two small DNA fragments (a total of 229 bp) from a dried quagga skin sample. The DNA fragments were identified as being of mitochondrial origin. The quagga mtDNA fragments and homologous mtDNA fragments from *E. zebra* and *E. burchelli* were sequenced. Their sequencing data showed that, of the 229 bp compared, the quagga differed from *E. zebra* by 12 bp, but only 1 bp difference was found when the quagga mtDNA was compared with that of *E. burchelli.* The extremely close relationship seen between the quagga and *E. burchelli* was also supported by the radioimmunoassay work of Lowenstein and Ryder (1985), who, using serum proteins isolated from a similar quagga skin sample, showed that the quagga is six times more closely related to *E. burchelli* than it is to either *E. zebra* or *E. grevyi.* Thus, *E. quagga* appears to be a "variant" of *E. burchelli.*

A Consensus Phylogeny?

By combining the cleavage-map data presented here and the data generated from other studies of the Equidae, a "consensus" phylogeny now seems possible. That phylogeny is best represented by tree a of figure 4. The molecular evidence presented by Higuchi et al. (1984) and Lowenstein and Ryder (1985) indicate that *E. quagga* is a zebroid most similar to *E. burchelli.* The morphological studies of Eisenmann (1980) and Harris and Porter (1980) also support this pairing. There has been little to no dispute over the close relationship that exists between *E. przewalskii* and *E. caballus*; thus the addition of *E. caballus* to the *E. przewalskii* branch should be easily accepted.

Our data also indicate that the three extant zebra species represent a monophyletic group. Additional support for this observation is derived from protein studies (Ryder et al. 1978; Kaminski 1979) and morphological studies (Harris and Porter 1980). The work of Harris and Porter (1980), however, suggests a close relationship between *E. zebra* and *E. grevyi,* while most other studies show a close affinity between *E. burchelli* and *E. grevyi* (see fig. 4).

The principal enigma in this consensus tree concerns the relative placement of *E. hemionus* and *E. africanus.* None of the studies to date, including our own, can clearly define their branching order. Identification of the correct phylogeny will probably require DNA sequencing studies. However, the data presented here support tree a of figure 4.

Although these molecular studies failed to strongly discriminate the branching order of *E. africanus* and *E. hemionus,* much has been confirmed and gained. We now have additional genetic markers and another measure of the genetic diversity found in this family. The estimated time since the extant Equidae diverged from a common ancestor, as derived from the mtDNA mapping data (i.e., ~3.9 MYBP), agrees well with fossil evidence and thus serves to reinforce the conclusions of other studies suggesting that mtDNA initially accumulates 2% nucleotide substitutions/Myr. These estimates of divergence time should also stimulate those who are interested in the speciation events that gave rise to this diverse family.

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LITERATURE CITED

AVISE, J. C., R. A. LANSMAN, and R. O. SHADE. 1979. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus *Peromyscus*. Genetics **92**:279–295.

BENNETT, D. K. 1980. Stripes do not a zebra make, part I: A cladistic analysis of *Equus*. Syst. Zool. **29**:272–287.

BROWN, G. G., and M. V. SIMPSON. 1981. Intra- and interspecific variation of the mitochondrial genome in *Rattus norvegicus* and *Rattus rattus:* restriction enzyme analysis of variant mitochondrial DNA molecules and their evolutionary relationships. Genetics **97**:125–143.

BROWN, W. M. 1980. Polymorphism in mitochondrial DNA of humans as revealed by restriction endonuclease analysis. Proc. Natl. Acad. Sci. USA **77**:3605–3609.

BROWN, W. M., M. GEORGE, JR., and A. C. WILSON. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA **76**:1967–1971.

BUSH, G. L., S. M. CASE, A. C. WILSON, and J. L. PATTON. 1977. Rapid speciation and chromosomal evolution in mammals. Proc. Natl. Acad. Sci. USA **74**:3492–3496.

CANN, R. L. 1982. The evolution of human mitochondrial DNA. Ph.D. thesis. University of California, Berkeley.

CANN, R. L., W. M. BROWN, and A. C. WILSON. 1984. Evolution of the human mitochondrial DNA: a preliminary report. Pp. 157–165 in B. BONNE-TAMIR, T. COHEN, and R. N. GOODMAN, eds. Human genetics, part A: the unfolding genome. Liss, New York.

CHURCHER, C. S., and M. L. RICHARDSON. 1978. Equidae. Pp. 379–421 in V. J. MAGLIO, and H. B. S. COOKE, eds. Evolution of African mammals. Harvard University Press, Cambridge, Mass.

CLEGG, J. B. 1974. Horse hemoglobin polymorphism. Ann. NY Acad. Sci. **241**:61–69.

DALQUEST, W. W. 1978. Phylogeny of American horses of Blancan and Pleistocene age. Ann. Zool. Fennici **15**:191–199.

DAWID, I. B. 1972. Evolution of mitochondrial DNA sequences in *Xenopus*. Dev. Biol. **29**:139–151.

DAWID, I. B., and A. W. BLACKLER. 1972. Maternal and cytoplasmic inheritance of mitochondrial DNA in *Xenopus*. Dev. Biol. **29**:152–161.

DENSMORE, L. D., J. W. WRIGHT, and W. M. BROWN. 1985. Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (genus *Gnemidophorus*). Genetics **110**:689–707.

EISENMANN, V. 1980. Les chevaux (*Equus* sensu lato) fossiles et actuels: cranes et dents jugales supérieures. Centre National de la Recherche Scientifique, Paris.

FARRIS, J. S. 1972. Estimating phylogenetic trees from distant matrices. Am. Nat. **106**:645–668.

FERRIS, S. D., W. M. BROWN, W. S. DAVIDSON, and A. C. WILSON. 1981*a*. Extensive polymorphism in the mitochondrial DNA of apes. Proc. Natl. Acad. Sci. USA **78**:6319–6323.

FERRIS, S. D., R. D. SAGE, E. M. PRAGER, U. RITTE, and A. C. WILSON. 1983. Mitochondrial DNA evolution in mice. Genetics **205**:681–721.

FERRIS, S. D., A. C. WILSON, and W. M. BROWN. 1981*b*. Evolutionary tree for apes and humans based on cleavage maps of mitochondrial DNA. Proc. Natl. Acad. Sci. USA **78**:2432–2436.

GEORGE, M., JR. 1982. Mitochondrial DNA evolution in Old World monkeys. Ph.D. thesis. University of California, Berkeley.

GILES, R. E., H. BLANC, H. M. CANN, and D. C. WALLACE. 1980. Maternal inheritance of human mitochondrial DNA. Proc. Natl. Acad. Sci. USA **77**:6715–6719.

GROVES, C. P. 1974. Horses, asses and zebras in the wild. Ralph Curtis, Hollywood, Fla.

GROVES, C. P., and V. MAZAK. 1967. On some taxonomic problems of the Asiatic wild asses: with the description of a new subspecies (Perissodactyla, Equidae). Z. Saugetierkunde **32**: 321–357.

GROVES, C. P., and D. P. WILLOUGHBY. 1981. Studies on the taxonomy and phylogeny of the genus *Equus*. I. Subgeneric classification of the recent species. Mammalia **45**:321–355.

HARRIS, A. H., and L. S. W. PORTER. 1980. Late Pleistocene horses of Dry Cave, Eddy County, New Mexico. J. Mammal. **61**:46–65.

HIGUCHI, A., B. BOWMAN, M. FREIBERGER, O. A. RYDER, and A. C. WILSON. 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature **312**:282–284.

HUTCHISON, C. A., J. E. NEWBOLD, S. S. POTTER, and M. H. EDGELL. 1974. Maternal inheritance of mammalian mitochondrial DNA. Nature **251**:536–538.

KAMINSKI, M. 1979. The biochemical evolution of the horse. Comp. Biochem. Physiol. **63B**: 175–178.

KITCHEN, H., and C. W. EASLEY. 1969. Structural comparison of the hemoglobin of the genus *Equus* with those of ruminants. J. Biol. Chem. **214**:6533–6542.

LINDSAY, E. H., N. D. OPDYKE, and N. M. JOHNSON. 1980. Pliocene dispersal of the horse *Equus* and late Cenozoic mammalian dispersal events. Nature **287**:135–138.

LOWENSTEIN, J. M., and O. A. RYDER. 1985. Immunological systematics of the extinct quagga (Equidae). Experentia **41**:1192–1193.

MILLER, J. A. 1985. Telling a quagga by its stripes. Sci. News **128**:70.

NEI, M., and W. H. LI. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**:5269–5273.

PARKER, R. C., R. M. WATSON, and J. VINOGRAD. 1977. Mapping of closed circular DNAs by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis. Proc. Natl. Acad. Sci. USA **74**:851–855.

POHLE, C. 1972. Statistisches zur Haltung von Halbeseln (*Equus hemionus*) in Tiergarten. Zool. Garten, N.F. **42**:189–203.

RYDER, O. A., N. C. EPEL, and K. BENIRSCHKE. 1978. Chromosome banding studies of the Equidae. Cytogenet. Cell Genet. **20**:323–350.

RYDER, O. A., R. S. STARKES, M. C. STARKES, and J. B. CLEGG. 1979. Hemoglobin polymorphism in *Equus przewalskii* and *E. caballus* analyzed by isoelectric focusing. Comp. Biochem. Physiol. **62B**:305–308.

SANGER, F., A. R. COULSON, T. FRIEDMANN, G. M. AIR, B. G. BARRELL, N. L. BROWN, J. C. FIDDES, C. A. HUTCHISON III, P. M. SLOCOMBE, and M. SMITH. 1978. The nucleotide sequence of bacteriophage ϕX174. J. Mol. Biol. **125**:225–246.

SIMPSON, G. G. 1951. Horses. Oxford University Press, New York.

TEMPLETON, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution **37**:221–244.

THOMAS, M., and R. W. DAVIS. 1975. Studies on the cleavage of bacteriophage lambda DNA with EcoRI restriction endonuclease. J. Mol. Biol. **91**:315–328.

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