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Large sequence divergence among mitochondrial DNA genotypes within populations of eastern African black-backed jackals

(Canidae/genetic variability/intraspecific phylogeny)

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ABSTRACT **In discussions about the relative rate of molecular evolution, intraspecific variability in rate is rarely considered. An underlying assumption is that intraspecific sequence differences are small, and thus variations in rate would be difficult to detect or would not affect comparisons among distantly related taxa. However, several studies on mammalian mitochondrial DNA (mtDNA) have revealed considerable intraspecific sequence divergence. In this report, we test for differences in the rate of intraspecific evolution by comparing mtDNA sequences, as inferred from restriction site polymorphisms and direct sequencing, between mtDNA genotypes of the eastern African black-backed jackal, *Canis mesomelas elongae*, and those of two other sympatric jackal species. Our results are unusual for several reasons. First, mtDNA sequence divergence within several contiguous black-backed jackal populations is large (8.0%). Previous intraspecific studies of terrestrial mammals have generally found values of <5% within a single population, with larger divergence values most often occurring among mtDNA genotypes from geographically distant or isolated localities. Second, only 4 mtDNA genotypes were present in our sample of 64 jackals. The large sequence divergence observed among these mtDNA genotypes suggests there should be many more genotypes of intermediate sequence divergence if they had evolved in sympatry. Finally, estimates of the rate of mtDNA sequence evolution differ by approximately 2- to 4-fold among black-backed jackal mtDNA genotypes, thus indicating a substantial heterogeneity in the rate of sequence evolution. The results are difficult to reconcile with ideas of a constant molecular clock based on random fixation of selectively neutral or nearly neutral mtDNA sequence mutations.**

Past research on mitochondrial DNA (mtDNA) variability has focused primarily on small vertebrate or invertebrate species, many of which have limited dispersal abilities (1–4). The results of these studies have shown that mtDNA variation is often geographically partitioned such that the sequence divergence among mtDNA genotypes from different populations increases as a function of distance or the extent of geographic barriers between populations. However, recent studies on species with great mobility such as red-winged blackbirds or deer (5–7) find a lack of distinct geographic structure with respect to mtDNA variation. In this study, we assess mtDNA variation, as inferred from restriction site polymorphisms and direct sequencing, in a highly mobile medium-sized carnivore, the eastern African black-backed jackal, *Canis mesomelas elongae*. In general, mammalian carnivores have larger home ranges and tend to disperse over greater distances than comparable-sized herbivores or smaller terrestrial vertebrates (8, 9). Thus, patterns of genetic variability within canid species are expected to be less highly structured than within smaller, less mobile vertebrates.

Debate about the relative rate of molecular evolution usually centers on DNA or protein sequence differences among discrete taxa with divergence times ranging from 5 to 1300 million years (10–13). Intraspecific variability in rate is rarely considered and is implicitly assumed to be insignificant. We compare here restriction site patterns and DNA sequences of black-backed jackal mtDNA genotypes to those of two other jackal species. As a result, we can estimate the relative rates of sequence evolution among black-backed jackal mtDNA genotypes and thus test the notion of intraspecific rate constancy.

MATERIALS AND METHODS

Blood samples of 64 free-ranging black-backed jackals were collected from six eastern African localities (Fig. 1). The area covered by these localities includes approximately 15% of the total range of the eastern African subspecies (14, 15). Samples from two "outgroup" species of jackals, *Canis aureus* (golden jackal) and *Canis adustus* (side-striped jackal), also were obtained from several of these localities. Approximately 10 ml of blood were drawn from each individual, and leukocytes were isolated by erythrocyte lysis and centrifugation. Total genomic DNA was isolated from the leukocytes and digested with a panel of the following 17 restriction enzymes: *Bam*HI, *Bcl* I, *Bgl* II, *Bst*EII, *Cla* I, *Dra* I, *Eco*RI, *Eco*RV, *Fnu*DII, *Hin*dIII, *Hpa* I, *Nco* I, *Pst* I, *Sst* I, *Sst* II, *Xho* I, and *Xmn* I. The resulting DNA fragments were separated electrophoretically and transferred to nylon membranes, which were probed with radiolabeled mtDNA from the domestic dog *Canis familiaris*. mtDNA fragments were then visualized by autoradiography (Fig. 2). As determined by comparison with molecular weight standards, the total length of the mtDNA genome in all three jackal species is approximately 16,800 ± 200 base pairs (bp). No variation in total sequence length was detected among jackal mtDNA genotypes.

The fraction of shared restriction sites was inferred from the pattern of loss and gain of restriction fragments (e.g., ref. 17). For example, if the restriction fragment patterns in two mtDNA genotypes differed in that one genotype had a restriction fragment that was the sum of two unique fragments in the second genotype, one site change was inferred. However, when restriction patterns differed by a greater number of fragments, we reconstructed the site changes among all observed mtDNA genotypes so as to minimize the total site changes needed to relate them. Thus, our estimates of site differences are the minimal number of site changes likely to have occurred between genotypes. The proportion of shared restriction sites was used to estimate sequence

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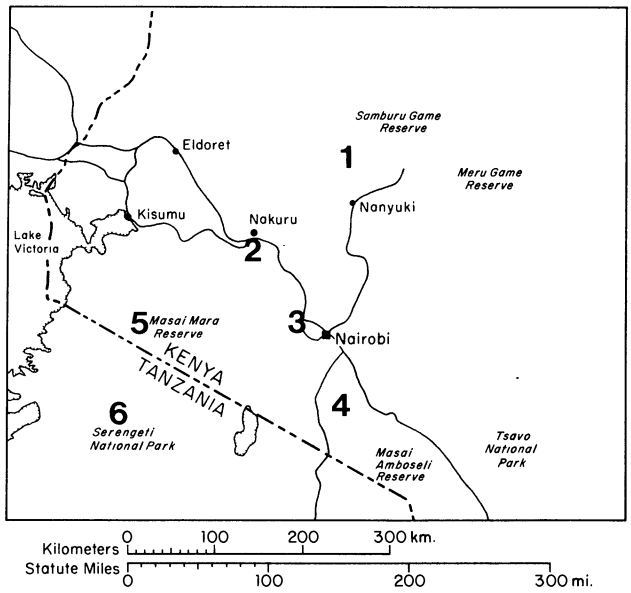

FIG. 1. Map of localities in Kenya and Tanzania where blood samples of black-backed jackals, *C. mesomelas elongae*, and the two outgroup species, *C. aureus*, the golden jackal, and *C. adustus*, the side-striped jackal, were obtained (see Table 1).

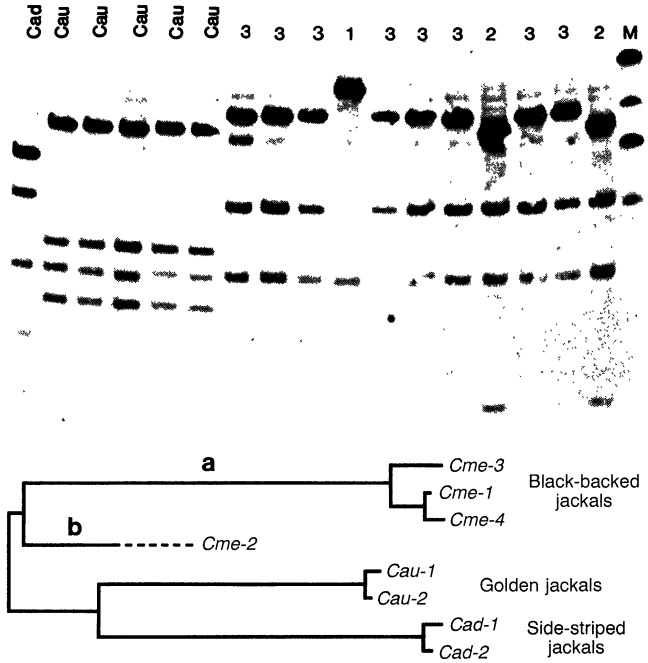

FIG. 2. (*Upper*) Examples of mtDNA restriction fragment length polymorphisms generated by *Xmn* I digests of genomic DNA from black-backed jackals (genotypes *Cme-1*, *-2*, and *-3*) and the two outgroup species, the golden jackal (genotype *Cau*) and the side-striped jackal (genotype *Cad*). In the marker (M) lane, fragment sizes are 23.1, 9.4, 6.6, and 4.4 kilobases from top to bottom. (*Lower*) A cladistic tree based on the presence/absence of mtDNA restriction sites in the three jackal species, generated by using the PAUP program by Swofford (16). Restriction site data on the golden and side-striped jackal were used to root the tree. Branch lengths indicate the relative amount of change in each lineage; "a" and "b" refer to the lineage lengths of the two black-backed jackal genotype groups from a common mtDNA ancestor. The dashed line in lineage b indicates the amount of relative change that occurred in that lineage when direct sequence data from cytochrome *b* are used to build the phylogenetic tree. Tree length = 105; consistency index = 0.886; *F* (normalized) = 0.097.

divergence among mtDNA genotypes (18). Direct sequence information was obtained from two mtDNA genotypes of black-backed jackals and one mtDNA genotype from each of two outgroup species by polymerase chain reaction amplification of 349 bp from the cytochrome *b* gene (19, 20). The products of the amplification reaction were sequenced directly (21) by using a commercial kit (Sequenase; United States Biochemical). The sequence divergence between mtDNA genotypes was corrected for multiple hits according to the Jukes–Cantor model (22). The standard error of sequence distance estimates based on restriction site data and sequence data was calculated as described by Nei *et al.* (23).

We used two methods to assess rate variation among black-backed jackal mtDNA genotypes. First, we analyzed character state data on the presence/absence of restriction sites and DNA sequences, using the PAUP program developed by Swofford (16). Data on the golden and side-striped jackals were used to root the tree. The length of the branches leading to the black-backed jackal mtDNA genotypes were compared as an estimate of the relative change occurring in each lineage. Second, we used distance data as described by Beverley and Wilson (24), in which estimates of sequence divergence are compared to an outgroup taxon, the golden jackal, to calculate the lineage length of ingroup taxa. To determine the statistical significance of departures from rate constancy, we used the nonparametric test of Templeton (25) on the restriction-site tree obtained from the PAUP program and the approach of Wu and Li (26) on the analogous sequence tree. For the distance data, we used the PHYLIP program by Felsenstein and compared the sum-of-squares of two phenetic trees, one that assumes a molecular clock and one that does not (27). Given certain assumptions, the ratio of the sum of squares of these two trees is distributed as an *F* statistic and thus provides a statistical test of rate constancy (28). It should be noted that the distance estimates used in this paper are not strictly independent, and thus one of the assumptions of this test is not fulfilled.

RESULTS AND DISCUSSION

Intraspecific Variability of mtDNA Genotypes. Only 4 mtDNA genotypes were detected among 64 mtDNA samples of black-backed jackals from six localities (Table 1 and Fig. 1). This number appears low because surveys of other vertebrate species of comparable sample size and geographic distribution have found 11–34 discrete mtDNA genotypes (17, 29). Sequence divergence among black-backed jackal mtDNA genotypes (*Cme-1* to *Cme-4*) as determined by restriction site analysis shows a discontinuous pattern; *Cme-1*, *-3*, and *-4* form a closely-related group that is distinguished by approximately 8.0% sequence divergence from *Cme-2* (Table 2 and Fig. 2). A large level of sequence divergence between black-backed jackal mtDNA genotypes is also indicated by the direct sequence data (Table 2). This degree of sequence divergence is as great as that among distinct canid species (R.K.W., unpublished data) and the largest recorded for a terrestrial mammal at a single locality, although an 8.7% mtDNA sequence divergence has been reported in a fish species (17, 29). However, in the latter study, as in others where large levels of intraspecific sequence divergence have been observed, either the divergent mtDNA genotypes do not cooccur and are separated by distance or geographic barriers (e.g., mountain ranges, rivers, or inhospitable habitats) or they cooccur only in distinct hybrid zones (5, 6, 17, 30–34). Moreover, in the present study, there are no mtDNA sequence divergence values that fall between 8.0% and 1.2% (Table 2). In past studies of terrestrial vertebrates of similar sample size, small sequence divergence values are commonly followed by intermediate values of sequence divergence, a

Table 1. Sampling localities and sample size in parenthesis of black-backed jackals and the two outgroup species *Canis aureus* (golden jackal) and *C. adustus* (side-striped jackal) (mtDNA genotype frequencies for the black-backed jackal are provided)

	C. mesomelas elongae mtDNA genotype frequency					
Locality	*1*	*2*	*3*	*4*	*C. aureus*	*C. adustus*
1	0.00 (0)	0.36 (4)	0.64 (7)	0.00 (0)	(0)	(2)
2	0.30 (9)	0.00 (0)	0.70 (21)	0.00 (0)	(20)	(3)
3	0.20 (2)	0.20 (2)	0.60 (6)	0.00 (0)	(0)	(1)
4	0.56 (5)	0.11 (1)	0.11 (1)	0.22 (2)	(0)	(1)
5	0.67 (2)	0.00 (0)	0.33 (1)	0.00 (0)	(0)	(0)
6	0.00 (0)	0.00 (0)	1.00 (1)	0.00 (0)	(0)	(0)
Total	0.28 (18)	0.11 (7)	0.58 (37)	0.03 (2)	(20)	(7)

result that is consistent with the predictions of the nearly neutral theory of gene evolution (3, 17, 29).

The cooccurrence of discordant mtDNA genotypes in several populations of black-backed jackals may reflect the unique dispersal abilities of large carnivores. Black-backed jackals have been observed to disperse as far as 200 km in a single year and can exist in a variety of habitats (14, 35). They are less likely to be stopped by physical or habitat barriers than are small terrestrial vertebrates, which view their environment in a more coarse-grained manner. Avise *et al.* (3) outline the consequences of various dispersal abilities on the pattern of microgeographic variation. They predict that mtDNA genotypes in species that disperse well will not show phylogeographic partitioning in which the most distinct mtDNA genotypes are separated by great distance or pronounced geographic barriers. This prediction appears to be realized among eastern African jackals because the most divergent mtDNA genotypes cooccur at several localities.

The observed mixture of divergent mtDNA genotypes at several localities could be the result of interspecific or subspecific hybridization. The former seems unlikely because no black-backed jackal mtDNA genotypes are closely related to genotypes in the golden or side-striped jackal, which are the only species for which hybridization is likely (14, 36). Another eastern African subspecies of black-backed jackal is reported from interior Somalia, but morphologic differences are minor and inconsistent (37). No apparent barriers to gene flow separate these populations at present or historically (38, 39). Moreover, past studies of mtDNA hybrid zones in other vertebrates have identified distinct allozyme or morphologic phenotypes on either side of the hybrid zone (5, 6, 17, 30–34). We measured morphologic variation of dental and external features and protein variation at 33 allozyme loci and did not find morphologic characters or alleles that are unique to any black-backed jackal mtDNA genotype or population (40). For example, when allozyme variability is used to calculate Nei's modified genetic distance, the value is only 0.002 between populations of *Cme-2* and *Cme-3* individuals, yet it ranges from 0.083 to 0.10 when these mtDNA genotype groups are compared to the other jackal species. Finally, levels of protein heterozygosity in the black-backed jackal are not increased relative to that of other species as might be expected in a hybrid zone (40). These data strongly suggest that interbreeding between the two mtDNA genotype groups is occurring freely and that recent hybridization between historically distinct black-backed subspecies is not a likely explanation of our results.

A possible source of genetically different immigrants is from southern Africa, where three subspecies have been described based on morphologic criteria (15). If the source of either mtDNA genotype group is from southern Africa and we assume usual rates of mtDNA sequence evolution (10), then the sequence divergence data suggest that the separation of the southern and eastern African populations would have occurred at least 4 million years ago. This date precedes the first appearance in the fossil record of a putative black-backed jackal ancestor by 1 million years (41). Moreover, the absence of intermediate mtDNA genotypes suggests that the potential hybridization event would have been very recent and that other hybridization events occurred rarely, if at all, over the last 4 million years. This possibility of hybridization cannot be discounted until samples are obtained and analyzed from southern Africa.

A final hypothesis is that the four genotypes evolved in sympatry in eastern Africa. However, the absence of mtDNA genotypes intermediate in sequence divergence seems diffi-

Table 2. Estimated sequence divergence (above diagonal) and fraction of shared restriction sites (below diagonal) between black-backed jackal and outgroup mtDNA genotypes

	Cme-1	*Cme-2*	*Cme-3*	*Cme-4*	*Cau-1*	*Cau-2*	*Cad-1*	*Cad-2*
Cme-1	—	0.081 (0.145)	0.012	0.002	0.119* (0.148)†	0.124*	0.128* (0.163)†	0.126*
Cme-2	0.62	—	0.082	0.084	0.068 (0.118)	0.067	0.086 (0.114)	0.085
Cme-3	0.93	0.62	—	0.010	0.109	0.113	0.129	0.128
Cme-4	0.99	0.61	0.94	—	0.118	0.122	0.133	0.132
Cau-1	0.50	0.68	0.53	0.50	—	0.001	0.097	0.095
Cau-2	0.49	0.68	0.52	0.49	0.99	—	0.095	0.094
Cad-1	0.48	0.61	0.47	0.46	0.58	0.58	—	0.002
Cad-2	0.48	0.62	0.47	0.47	0.58	0.58	0.99	—

Fifty-one to 64 restriction sites were scored in each mtDNA genotype. Values in parentheses are based on direct sequencing of 349 bp from the mitochondrial cytochrome *b* gene. mtDNA genotypes: *Cme-1* to *Cme-4*, *C. mesomelas elongae* (black-backed jackal); *Cau-1* and *Cau-2*, *C. aureus* (golden jackal); and *Cad-1* and *Cad-2*, *C. adustus* (side-striped jackal).

*These values are significantly different from the corresponding distance value for *Cme-2* ($P < 0.05$, two-tailed *t*-test). Their SEM is 0.017.

†These values are significantly different from the corresponding distance value for *Cme-2* ($P < 0.05$, two-tailed *t*-test). Their SEM is 0.021.

cult to explain in a large panmictic population. Theoretical models and the empirical data suggest that mtDNA genotypes that share recent ancestry should be most abundant, followed in declining frequency by more distantly related mtDNA genotypes (29). Avise *et al.* (42) use computer simulations to model the branching of mtDNA matrilines. Assuming a constant mutation rate, as predicted by the nearly neutral theory of molecular evolution, and a Poisson distribution of new lineages at each branch node, they conclude that after approximately $4N$ generations, where N equals the original number of matrilines, all genotypes in an interbreeding population will have a high probability of sharing a recent common ancestor. Given that the maximum number of original matrilines did not exceed the effective female population size in the area we surveyed [50,000 females, assuming a density of one jackal per square kilometer (43)] and that population size was not substantially greater in the past, then all black-backed jackals would be expected to share a common mtDNA ancestor <200,000 generations ago. Yet *Cme-2* is separated from the other mtDNA genotypes by 8.0% sequence divergence, a value that predicts a common mtDNA ancestor approximately 2 million generations ago [assuming a generation time of 2 years and a constant rate of 2% sequence divergence per million years (1, 2, 14)]. Empirical studies and the neutral theory-based expectations of Avise *et al.* (2, 3, 29, 42) would predict many more mtDNA genotypes intermediate in sequence divergence between 1.2% and 8.0% within the eastern African black-backed jackal if these mtDNA genotypes had evolved in sympatry.

Intermediate mtDNA genotypes may have been missed in our limited sample of black-backed jackals. Yet, intermediate genotypes, if they exist, would likely be rare. For example, because our frequency data approximate a geometric frequency distribution, we estimate that a fifth genotype, if present, would have a frequency of 0.018; a sixth, 0.008; and a seventh, 0.003. Given our sample size, genotypes with a frequency of >0.047 would likely be found 95% of the time. Thus, while the presence of additional genotypes is possible, they are likely to be extremely rare. Such coincident rarity of several intermediate genotypes seems unlikely, given a random phylogenetic branching process, although models have not been developed to specifically answer this question.

Relative Rates of mtDNA Sequence Evolution in Black-Backed Jackals. Significant intraspecific heterogeneity in the rate of mtDNA sequence evolution is implied by differences in sequence divergence values between each of the black-backed jackal mtDNA genotypes and the two outgroup taxa (Table 2 and Fig. 2). The actual disparity in rate among mtDNA genotypes can be calculated if divergence values are corrected so that only the distances from a common mtDNA ancestor are considered (24, 25). In Table 3, lineage lengths are estimated for both restriction site data and direct sequence data based on character state and distance analyses (Fig. 2). These results suggest that the ratio of lineage lengths leading to the two genotype groups differs by a factor of 2–4, depending on the approach used to estimate lineage lengths. Thus, there is a minimum of a 2-fold difference in the rate of sequence evolution between *Cme-1* and *Cme-2*; *Cme-2* is the more slowly evolving lineage. Finally, all relative rate tests indicate a significant departure from a constant molecular rate (Table 3).

Table 3. Lineage lengths from the common ancestor of the black-backed jackal mtDNA genotypes to the present-day mtDNA genotypes *Cme-1* (a) and *Cme-2* (b) (see Fig. 2)

	Nucleotide sequence			Restriction sites		
Analysis	a	b	a/b	a	b	a/b
Character state	33	13	2.54*	34	8	4.25†
Distance	0.097	0.048	2.02‡	0.044	0.022	2.00§

Lengths are measured in sequence or restriction site changes for character state data or in sequence divergence for distance data.
*Significant at the $P < 0.001$ level with test of Wu and Li (26).
‡Significant at the $P < 0.05$ level with test of Felsenstein (27).
†Significant at the $P < 0.01$ level with test of Templeton (25).
§Significant at the $P < 0.01$ level with test of Felsenstein (27).

The restriction site data and sequence divergence data differ in magnitude (Table 2). The values of the latter are approximately 3–6.4% larger than comparable values based on restriction site data. This difference might reflect our minimum estimate of the site changes that have occurred between mtDNA genotypes as well as perhaps a more rapid rate of cytochrome *b* sequence evolution relative to the average rate of sequence evolution in the mtDNA genome.

Our results parallel the recent work of DeSalle and Templeton (44), who document interspecific levels of sequence divergence among Hawaiian *Drosophila*. These authors found that the rate of mtDNA sequence evolution differed by 2- to 3-fold among species of *Drosophila* on islands where founder events are likely to have given rise to new species. In contrast, much less disparity in rate was found among species on other islands where founder events were less likely to be the source of new species. Their results are consistent with the nearly neutral theory of gene evolution as interpreted by Ohta (45) in which the rate of evolution is strongly influenced by population size. However, our results can not easily be explained as a modification of this model because population sizes of the more slowly evolving mtDNA genotype are likely to have been significantly larger over long periods of time only if maternal lineages of the *Cme-2* mtDNA genotype tended to leave relatively more offspring. Unless such an effect on fitness was determined by mitochondrial genes, it would probably not correspond with the inheritance of a specific mtDNA genotype, given the recombination of nuclear genes that takes place in large panmictic populations. Alternatively, the *Cme-1*, *-3*, and *-4* mtDNA genotype group may have become abundant only recently, having existed previously in isolation at small population size and hence having a greater rate of sequence evolution than *Cme-2*. Again, the latter seems unlikely considering the dispersal abilities of black-backed jackals, the long time period over which this isolation would need to be maintained, and the cooccurrence of two divergent mtDNA genotypes at localities separated by approximately 250 km.

A second alternative hypothesis is that the black-backed jackals are paraphyletic. Conceivably, the mtDNA genotype group that appears to be evolving more rapidly (*Cme-1*, *-3*, and *-4*) diverged prior to the divergence of the more slowly evolving one (*Cme-2*) and those in the other two jackal species. If this is true, then lineage lengths in the black-backed jackal might be more equal if a more distantly related outgroup is used. As recently discussed by Pamilo and Nei (46), the gene tree indicated by the mtDNA comparisons may not coincide with the tree representing the phylogenetic relationship of species. However, given that the maximum sequence divergence between mtDNA genotypes is 13–16% (Table 2), paraphyly would necessitate the persistence of the *Cme-1*, *-3*, and *-4* genotype lineage for >6–8 million years. This is improbable given the assumptions of the neutral theory (29, 42). Moreover, because jackal-like species first appear in Africa approximately 3 million years ago, the 6 million year date suggests an origin of the *Cme-1*, *-3*, and *-4* genotype group outside of Africa prior to the first fossil appearance of many other related canids such as the coyote and gray wolf (47, 48). Accordingly, the *Cme-1*, *-3*, and *-4* mtDNA genotype lineage might be ancestral to those found in many extant canids. The implications of this hypothesis are troubling because they suggest that mtDNA-based phylogenies may bear little resemblance to the species phylogenies

due to the retention of primitive mtDNA genotypes. This hypothesis is presently being tested by analysis of sequence data from more distantly related species.

In conclusion, this study suggests that the assumption of rate constancy may be violated at the intraspecific level. Thus, calculations of the divergence time between two mtDNA genotypes based on a single rate may be incorrect unless intraspecific rate constancy is demonstrated by use of an outgroup. Similarly, interspecific divergence patterns may be strongly biased by differences in sample size or diversity of mtDNA genotypes within each species. However, large inequities in rate may not be common because several studies have shown that sequence divergence correlates linearly with divergence time for taxa with divergence times <10 million years (49). Moreover, extensive data on mtDNA sequence variation in humans suggests that there is a uniform rate of sequence evolution and that approximately 71% of the genetic diversity falls within the range predicted by the neutral theory (50–52). The results of this study provide an exception to past generalizations about mtDNA sequence variability and evolution and suggest a need for more studies on large vertebrates with good dispersal capabilities, such as large carnivores, whales, ungulates, and some species of birds and fishes.

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