CLONAL DIVERGENCE of MITOCHONDRIAL DNA versus POPULATIONAL EVOLUTION of NUCLEAR GENOME

Received June 29, 1984; August 23, 1984

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ABSTRACT: We sequenced the 110 5'-terminal nucleotides of mouse 16S mitochondrial ribosomal RNA from European and South Asian species of the genus Mus. The 16 sequences we determined showed large differences, mainly base substitutions, up to 25% between species and 4% within species. From these data the phylogenies of the individual mitochondrial molecules were drawn using parsimony and maximum-likelihood computation modes, both methods giving identical dendrograms. The trees so obtained show large discrepancies from those of the species themselves as inferred from protein-electrophoresis data. These unexpected results are principally in the divergence displayed by two sister-species whose mitochondrial lines do not show any common segment within the tree, whereas their nuclear genomes are very similar to one another.

These discrepancies could be due to the different nature of the two data sets: protein variation as measured by gene frequencies reflects average populational divergence, whereas changes in mitochondrial DNA as appreciated by nucleotide sequence of individual molecules reflect clonal divergence that can go faster and occur before the populational divergence.

Our results warn against the use of mtDNA sequences of a limited number of individuals to reconstitute phylogenies of closely related species, as well as to estimate divergence time by the measure of rates of nucleotide substitution.

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INTRODUCTION

The mitochondrial genome of animals differs in several interesting ways from the nuclear genome : 1) no recombination between the mitochondria within a mammalian cell has so far been detected, 2) the mitochondria are maternally inherited (Giles et al., 1980), 3) they accumulate nucleotide substitutions 5 to 10 times faster than nuclear DNA (Brown et al., 1979). For these reasons, mitochondrial DNA analysis should prove to be a good tool for phylogenetic studies, especially for studies on closely related species. In this paper, we report sequencing of the 110 5' terminal nucleotides of mitochondrial 16S rRNA of 16 mice belonging to different taxonomic units of the genus Mus between which the phylogenetic relationships are well documented, principally through protein electrophoresis (Bonhomme et al., 1984). This provides us with the possibility of comparing evolutionary events from nuclear and mitochondrial standpoints. The results obtained with the two data sets show important differences which are discussed in this paper.

MATERIAL AND METHODS

Animals: The genus Mus presents an array of related species at every stage of differentiation, among which the house-mouse complex of species is the best known. It displays taxonomical units which range from good sympatric species to parapatric semi-species interacting along a hybrid zone. For convenience, and since its taxonomy is far from being unambiguous, it is described in terms of biochemical groups (see Bonhomme et al., 1984). Probable latin binomials corresponding to these groups are given below. The

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Evolutionary Theory 7:81-90 (October, 1984)
The editors thank two referees for help in evaluating this paper.

individuals analysed here were either trapped in the wild or were laboratory descendants of wild-caught animals maintained as separate lines in our laboratory for a few generations. The following animals were used:

3 from M. musculus domesticus (Mus 1), 3 from M. m. musculus (Mus 2A), 1 from M. m. castaneus (Mus 2C), 3 from M. spretus (Mus 3), 2 from M. spicilegus (known also as M. hortulanus) (Mus 4B), 2 from M. spretoides (ex. spicilegus southern form, sometimes referred to as M. abbotti) (Mus 4A), 2 from M. cervicolor, 1 from M. caroli and 1 from M. (Nannomys) minutoides. We also analyzed an African murid, Myomys daltoni, representing a distantly related genus (out-group).

The geographical origin of each individual is given in Table 1.

Chemicals and enzymes: The primer (a 13-mer oligonucleotide, Panabières et al., 1982) was synthesized by Collaborative Research. Reverse transcriptase was kindly supplied by Dr. J. Beard, Life Sciences . T4 polynucleotide kinase was obtained from P.L. Biochemicals. Gamma-labelled (^{32}P)ATP was purchased from Amersham International. Dimethyl sulfate, hydrazine and piperidine were from Aldrich, Eastman and Sigma, respectively.

mtRNA preparation: Crude mitochondria were purified from the liver of an individual mouse by cycles of differential centrifugation in 0.25M sucrose, and after adding SDS and Proteinase K (Attardi et al.,1969), RNA was extracted by phenol metacresol (Liarakos et al., 1973).

cDNA synthesis and sequencing: 20 ug of total RNA was mixed with a 100-fold molar excess of primer (Panabières et al., 1982), previously labelled at its 5' terminus with (^{32}P)ATP using T4 polynucleotide kinase (Chaconas and Van de Sande, 1980). After reverse transcription (3 hr at 20°C in a 20 ul mixture with ten units of reverse transcriptase) labelled products were separated according to their size on a 10% polyacrylamide slab gel. The 140-nucleotide-long run-off product was retrieved from the gel by elution and DNA sequencing was performed according to Maxam and Gilbert (1980).

Calculations: The programs we used for the construction of an estimate of the phylogeny were DNAPARS (a program using the parsimony criterion) and DNAML (a maximum likelihood program) as described by Felsenstein, 1981).

RESULTS and DISCUSSION

Nucleic acid sequences: Table 1 shows nucleic-acid sequences of the last 110 nucleotides of the 5' terminus of the 16s rRNA. As a control, we used the same method for the determination of human (HeLa cells) and murine (mouse strain C57B16) sequences. Both of them were identical to those already published (Anderson et al., 1981 and Van Etten et al., 1981, resp.). DNA sequencing from M. caroli and M. minutoides could not be performed as these samples yielded a run-off product a hundred fold less radioactive than the others, while Myomys daltoni yielded a ten fold more radiactive one. The reasons why three murine samples yielded so different signals whereas the same intensity was obtained for man and mouse are discussed in the Appendix 1.

Phylogenetic dendrogram: Evolutionary trees can be derived using different computational methods. Properly speaking cladistic methods rely on the knowledge we have of the ancestral state of a given character. The usual method of taking an external group (here such as M. cervicolor or Myomys) to ascertain primitive character states fails if, as in our case, there are many incompatibilities due to a large amount of homoplasy (parallel, reverse and/or convergent evolution). This is even more true when comparing our data to widely divergent species whose mitochondrial sequences are available in the literature such as man, rat and cow : we were unable to align these sequences properly. On the other hand, since we have no evidence for the constancy of substitution rates,

		10	20	30	40	50	60	70	80	90	100	110
Mus 1 a	Lab. strains	ACGCCATGAT	CAAGATATCG	ACGATCTACA	TGCTTAAAGA	AAGAGGTTAT	GAAAATCATC	CTATTTACAA	AACTAAATAT	ATTATCATAT	TAATATAAAC	ACATCCCGAT
Mus 1 b	Greece	.....	.....	.....	.....	.....	.....	.....	C	.....	.....	.....
Mus 1 c	France	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mus 2A a	Yugoslavia	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mus 2A b	Bulgaria	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mus 2A c	Yugoslavia	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mus 2C	Indonesia	.....C....	..C.....	.....	.....	.....	T.....	.....T.	.....	.....	.....	..T.....
Mus 3a	Spain	.T.....	.....	.....	.....	.....	.....	.A.....	C	T...CT.C..	.....	....A.....
Mus 3b	France	.T.....	.....	.....	.....	.....	.....C.	.A.....	C	T...CT.C..	.....	....A.....
Mus 3c	France	.T.....	.....	.....	.....	.....	.....C.	.A.....G	C	T...CT.C..	.....	...CA.....
Mus 4A a	Bulgaria	.....	.....	.....	.....	.....	.....C.	AG.....	.....	T....T....	.....	...CA ....
Mus 4A b	Bulgaria	.....	.....	.....	.....	.....	.....C.	AGG.....	.....TA	T....T....	....T.....	...CA.....
Mus 4B a	Bulgaria	.T.....	.....	.....C...	.....	.....	....T...C.	.A.....T.	.....	T.....C	....T.....	.....
Mus 4B b	Bulgaria	.T.....	.....	.....C...	.....	.....	.(A)....C.	.A.....	.....	T.....C	.....d..	.....
Mus cervi.a	Thailand	----------	--.....	.....C CATA.....	.....	.....	....T.T.CT	AA.....	...C....TA	.....TT...	C..C.C...A	.T..AA....
Mus cervi.b	Thailand	.T...C...A	.GC.....	.....G.C CATA.....	T.....	----------	----------	----------	----------	----------	----------	----------
Myomys	Centrafrica	.T.....A	AG.....C..	.....TA.A.	ddd.....	.....	.....ACAT	TATC.....	...A.....C	..A....C..	.....CTT	.dd..TAC.A

TABLE 1: Nucleotide sequences of the 5' end of mouse 16S mitochondrial rRNA

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purely phenetic methods seem unjustified and too rough for dealing with detailed information at a series of nucleotide sites.

Therefore, we preferred to use parsimony and maximum-likelihood approaches as available in the computer program package written by J. Felsenstein (1981). For the likelihood approach (DNAML) several runs were made with either the whole sequence or the data restricted to the variant sites. No major differences showed up and a very good convergence was obtained with the most parsimonious tree (DNAPARS), which required a minimum of 57 base substitutions for its construction (upper panel of Figure 1). Out of these 57 changes, we observed 31 single events, 11 double events (i.e. two successive independent mutations occuring at the same position) and one four-fold multiple event. Among these multilple events, in which is contained any observable homoplasy of the data set, at least ten events led to parallelism whereas no sign of convergence or reversion was detected. This homoplasy exemplifies the fact that simple pairwise comparison methods account inadequately for multiple substitutions, as was previously shown for human mtDNA sequences (Aquadro and Greenberg, 1983) and for restriction analysis of deermice mtDNA (Lansman et al., 1983). Due also to this homoplasy, the exact branching point of the most divergent mouse species (Mus cervicolor) could not be accurately localized by the maximum likelihood method, given that no intermediate species allowed us to ascertain the number and the intermediate stages of multiple events on the branch leading to this taxon. Longer sequences also could help to find this branching point. However, as far as the house-mouse species complex is concerned (Mus 1, 2A, 2C, 3, 4A and 4B), the same topology was observed in all runs. The following discussion will therefore deal principally with these six groups.

Variability of 16 S ribosomal 5' terminus: The last 120 nucleotides of the 5' end of this molecule seem to be a hot spot within a highly conserved sequence region, as revealed by a comparative analysis of widely divergent species. In the 31 mutational events within the house-mouse species complex, the ratio of transitions/transversions (16/15 = 1.1) is much smaller than expected according to current literature (Brown et al., 1982; Brown and Simpson, 1982; Greenberg et al., 1983; Cann et al., 1984). These results are discussed in more detail in Appendix 2.

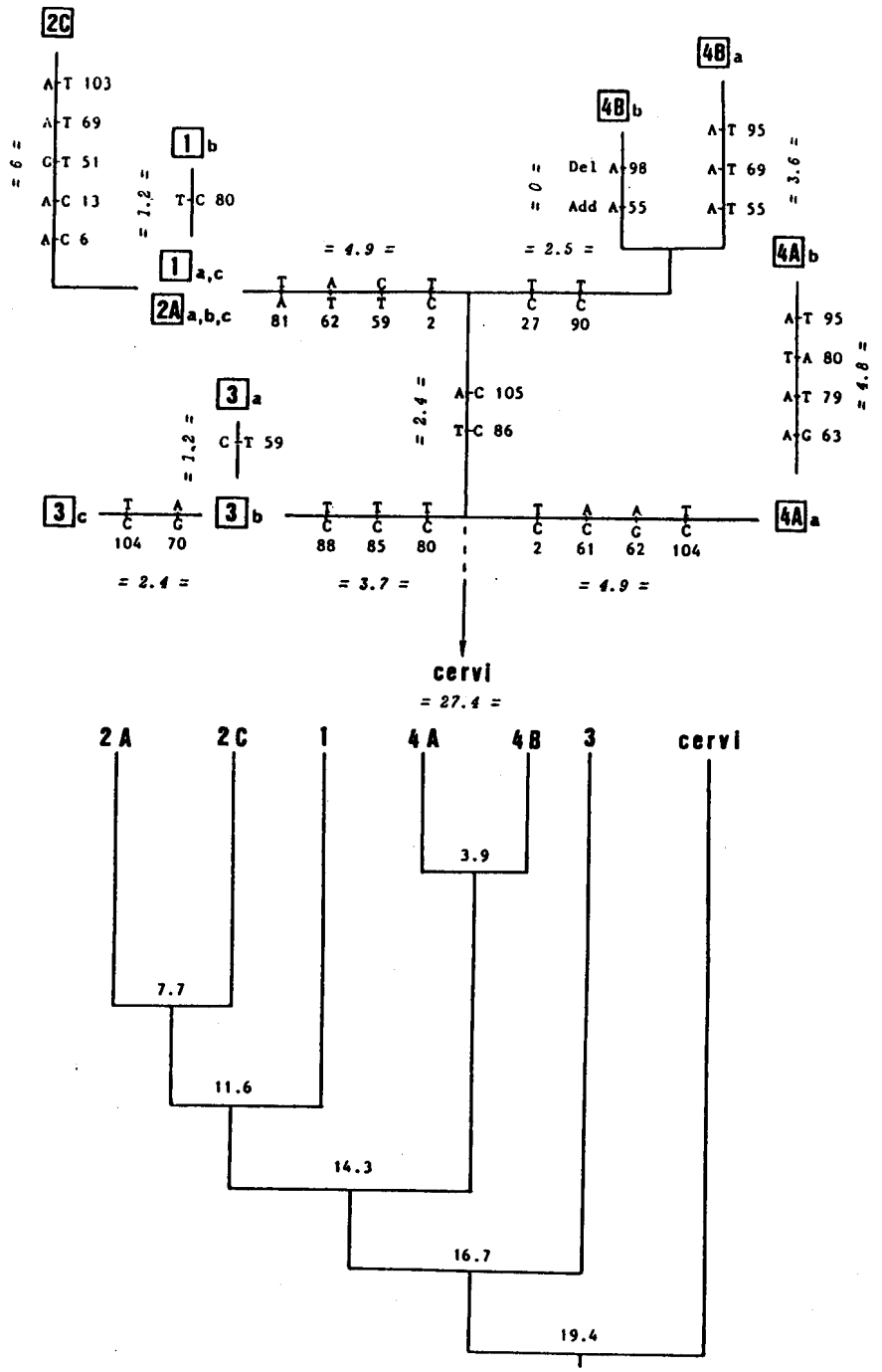

Figure 1 : Upper panel: phyletic relationships between individual clones of mtDNA within six biochemical groups of the Mus musculus species complex.

The topology of the tree is the one yielded by all computer runs. Individual mice are labelled according to table I. The explicit point mutations (change from left to right nucleotide) are manually reconstituted from the minimal number of mutation at each site given by the DNAPARS program. Their order on each branch is arbitrary. Branch lengths given to the side are those from the DNAML program output, and are strictly proportional to the number of mutations proposed, except for the branch leading to 4Bb since Del 98 and Add 55 were excluded from the data set for computational simplicity.

Lower panel: phyletic relationships between six biochemical groups of mice of the Mus musculus species complex (from Bonhomme et al., 1984).

This tree was obtained by average clustering of a distance matrix . Branch length are proportional to the number of genes out of 42 protein loci studied differing between two randomly chosen haploid genomes in each species.

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Interspecific differentiation: We found large interspecific variation, up to 25% nucleotide substitutions. The sequences of Mus cervicolor are widely divergent from the ones of all the other species, in keeping with the electrophoretic results which have established it as a distantly related species.

As for Mus 4A and Mus 4B, their ribosomal mtDNA 5' terminus appear to be as divergent from each other as from Mus 1, 2A or 3. This has been confirmed by restriction-enzyme analysis of the whole molecule (Yonekawa, 1983, pers. comm.).

On the other hand the ribosomal mtDNA 5' terminus of Mus 2C, in the individual sequenced here, seems to have evolved much further than that of Mus 1 or Mus 2A. This is not reflected by restriction enzyme analysis of the whole molecule (Ferris et al., 1983a), which showed these three groups to be aproximately equidistant (3 - 4 % nucleotide divergence).

More surprising is that the mtDNA sequences of Mus 1 and Mus 2A are nearly identical (one nucleotide difference in one individual). These groups differ greatly from one another from a nuclear (Selander et al., 1969 ; Sage, 1981; Bonhomme et al., 1978, 1984) and mitochondrial standpoint (Yonekawa et al., 1981 ; Ferris et al., 1983b). Nevertheless Mus 2A and Mus 1 are known to share a narrow hybrid zone in central Europe through which a limited amount of introgression exists. Restriction enzyme analysis (Yonekawa et al., 1982 ; Ferris et al., 1983a, 1983b) has shown that some Mus 2A mice from Denmark possess Mus 1 type mitochondrial DNA whereas their nuclear genome is almost unchanged from that of Mus 2A type. We can exclude the possibility that a similar phenomenon affects our samples, since mitochondrial introgression of Mus 1 into Mus 2A was not found in the regions of origin of our samples (Boursot et al., 1984) and mtDNA restriction analysis of the same wild-derived strains as used in this study (P. Boursot, unpublished) has confirmed that they belong to Mus 2A. Therefore the reason why the 5'ends of mitochondrial ribosomal RNA of Mus 1 and Mus 2A are identical are not related to interpopulational hybridization.

Thus, it appears that the mtDNA coding for the 5' end of the 16S rRNA may have different rates of evolution in different lineages. Such contrasting rates of evolution within small regions of the mtDNA are not detected when investigating the whole molecule through restriction analysis. Therefore, as we point out in Appendix 2, while data such as ours are adequate to infer phylogenetic relationships between mtDNA clones, they may not be accurate for estimates of the amounts of divergence of the whole molecule.

Mitochondrial versus nuclear evolution: Protein-electrophoresis data at 42 loci has established the phylogenetic relationships of the taxa used in the mtDNA analysis as proposed by Bonhomme et al. (1984). We can therefore compare a phylogeny of the Mus complex based on nuclear genetic data with the topology of the network obtained with the 16S ribosomal mtRNA 5'end (fig. 1). Several differences are clearly apparent between the two trees:

Mus4A and Mus 4B are among the closest groups we know from the standpoint of nuclear genome (as well as from other criteria like morphology or ecology), yet they display a very large divergence in their ribosomal mtDNAs and no common segment shows up in the mitochondrial tree.

This discrepancy can be explained in the following manner: an "electrophoretic dendrogram" reflects population divergence of gene frequencies while a "mitochondrial dendrogram" reflects clonal divergence of individual DNA sequences. In a given population, several mitochondrial sequences coexist. For instance, a newly arisen sequence and one of its distant ancestors (several mutational steps apart) may occur side by side, as exemplified by Mus 3 as well as Mus 4A (fig. 1 upper panel). Whatever the process of speciation, the set of mitochondrial genomes present in an ancestral population before splitting into two daughter populations is the result of an older clonal evolution. If one of these daughter-groups is a new small geographical isolate undergoing founder effect it may very well inherit only one sequence, and this sequence may be old or young. Even without founder effect, two daughter species inheriting the same set of mitochondrial sequences may eventually lose by drift and/or selection different preexisting clones so that the two related ancestral sequences may differ as much as two mitochondria in a

given present species. This phenomenon will be even more sensitive when the daughter-species were produced by partition of a large and long established species that has already differentiated its mtDNA on a geographical basis (such a case has been exemplified by Lansman et al., 1983, in Peromyscus without substantial differentiation of nuclear gene frequencies).

Thus, the electrophoretic divergence as measured by genetic distances derived from gene frequencies starts at close to zero at the time of speciation, whereas phylogenies of individual mitochondrial molecules can trace back to earlier events. mtDNA is likely to register events occuring before population division more easily than nuclear sequences as a result of two phenomena that reinforce each other. Firstly, mtDNA is non-recombinant (or relatively so) and therefore differences established among lines within a population will not be cancelled out by recombination. Secondly, rates of mtDNA evolution are higher than that of nuclear DNA (Brown et al., 1979) and therefore many different mitochondrial clones may be produced in a relatively short time. A lack of congruence between phylogenies based on mtDNA or nuclear data may therefore be the result of these intrinsically different properties. Our model is appears in figure 2.

Another possible explanation to account for the great divergence between 4A and 4B and their lack of a common segment within the tree would be an interspecific exchange of mtDNA, Mus 4A receiving the DNA of a more anciently diverged species, one that would still have to be found. Although we cannot exclude this possibility (interspecific mitochondrial exchange has already been reported in drosophilids: Powell, 1983), it seems less parsimonious to us since it involves hypothetical events with hypothetical species for which there is at present no evidence.

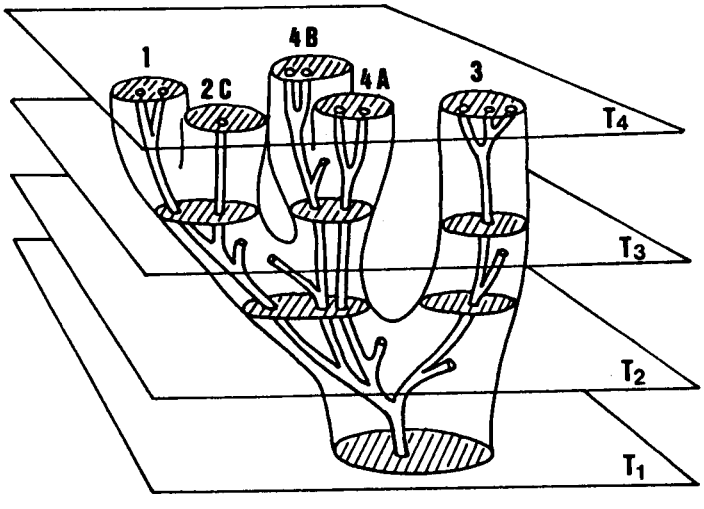

Figure 2: This figure illustrates the model developed in the text. The main tree symbolizes the nuclear genome, while internal ramifications represent mitochondrial clones.

Time scale is arbitrary.

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CONCLUSION

Our study implies that, if intraspecific polymorphism is high, one can infer the phylogeny of the molecules themselves rather than that of the species which carry them. This is particularly clear with mtDNA sequences, which can be much older than the species themselves. For this reason also, the rates of nucleotide substitution currently estimated for these molecules may be considerably over-estimated. Therefore, one has to be cautious when inferring species phylogenies based on particular molecules from a few individuals. The near future will bring interesting comparisons when analogous data for nuclear genes becomes available.

ACKNOWLEDGEMENTS

We are particularly grateful to J.M. Blanchard for his constant encouragement and advice during this work, F. Dabonneville for maintaining mice strains, P. Boursot for confronting these results with those of endonuclease analysis, M. Lathrop for help in the computer analysis, L. Van Valen for discussing the possibility of a cladistic analysis and J. Felsenstein, D. Grantham and J. Antonovics for helpful comments on the manuscript.

APPENDIX 1

Accuracy of the DNA sequencing method: Our sequencing method involves reverse transcriptase, which is commonly used for cDNA cloning procedures. To our knowledge, introduction of systematic errors during reverse transcription has never been hitherto reported, except under very special conditions (Traboni et al., 1983). In fact, the main problem encountered in using this enzyme is that cDNA elongation is easily blocked by RNA secondary structures, yielding incomplete products. Anyway, this does not interfere with our results, as we purified full- length run-off products before sequencing, as shown in Figure 3 .

The probe we used for priming cDNA synthesis was a mixture of three 13-mer oligonucleotides differing from one another by a single substitution (Panabières et al., 1982). Determination of our sequences showed that only one primer was selected for the hybridization to 16S RNA, which means that a single base change within the primer prevents quantitative hybridization. On the other hand, as this primer did not fit exactly to 16S rRNA, it is also possible that single base substitution will increase hybrid stability. Indeed, we observed that two samples (M. caroli and M. minutoides) yielded a much lower radioactive signal, while Myomys daltoni yielded a ten-fold more radioactive one (Figure 3). Our explanation is that single base changes occurred within the RNA region involved in hybridization with the probe, leading to a dramatic destabilization of the heteroduplex in the first case and to the enhancement of its stability in the other case.

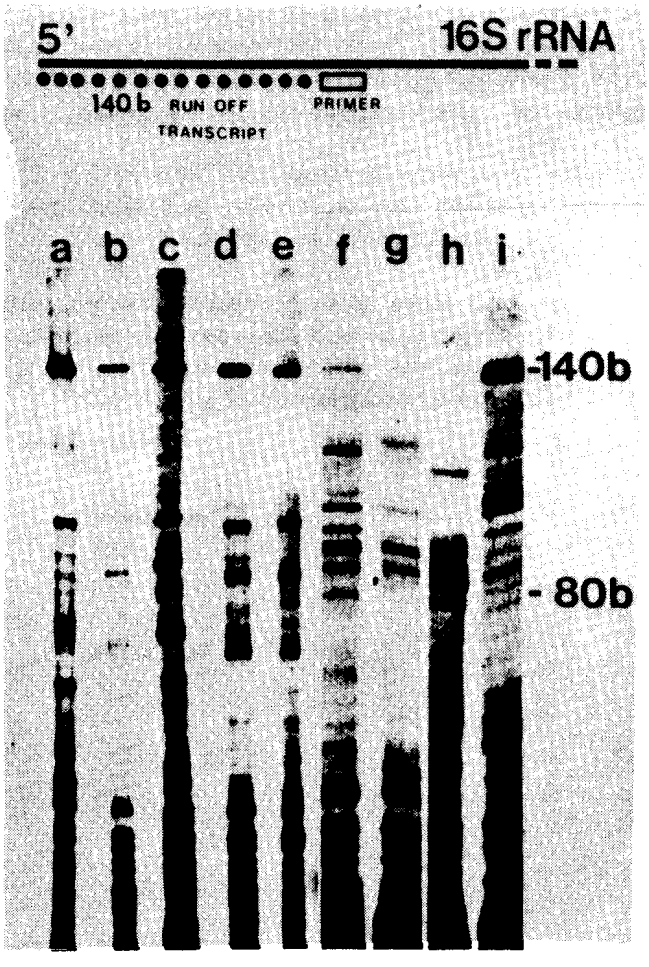

Figure 3: Sequence analysis of mitochondrial 16s rRNA by primer extension. Lane a: Mus 1; lane b: Mus 2C; lane c: Mus 3; lane d: Mus 4A; lane e: Mus 4B; lane f: Mus cervicolor; lane g: Mus caroli; lane h: Mus minutoides; lane i: Myomys daltoni.

APPENDIX 2

Variability of 16S ribosomal 5' terminus: A comparative analysis of 16S RNA among widely divergent species (man, cow, mouse, rat and Paramecium aurelia) has revealed a high degree of conservation of these structures (C. Branlant, unpublished results). However, this comparison showed also that the 5' 120 last nucleotides display a much lower homology than the rest of the molecule. The number of intraspecific changes in our samples ranges from 1 (Mus 1) to 4 (Mus 4A). On the other hand, 42 positions out of 110 are potential targets for mutational changes among the murine species analyzed. When comparison is extended to Myomys and man, this number goes up to 70. As far as the base composition is concerned, one can observe a high percentage of A+T residues, as shown in Table 2, particularly in rodent samples. Thus, if this feature is of any biological importance for the function of the ribosomal molecule, mutational events affecting the A+T content will be counterselected.

Table 3 shows the respective numbers of the different types of mutational events that were required to build the most parsimonious tree (among the 57 steps, the 26 ones leading from the house-mouse species complex to M. cervicolor are of course non-oriented, since the root of the tree cannot be located precisely). Thus, excluding cervicolor, the more frequent events are T/C (42%) and A/T (32%), and the ratio of transitions over transversions is 1.1 to 1, which is unusually low for mitochondrial DNA changes: previous analyses estimated this ratio as 11:1 (from African hominoids - Brown et al.,1982), 8:1 (from two rat species - Brown and Simpson, 1982) and 24:1 (from seven human samples - Greenberg, Newbold and Sugino, 1983). More interesting is the restriction analysis of the 112 human samples made by Cann et al. (1984), which showed that tRNAs and rRNAs are the least variable regions of the mitochondrial genome, and evolve mainly by transversions.

From these observations, we suppose that the 16S rRNA 5' terminus is a hot spot much more variable than the rest of the molecule, and that there may be a biological constraint that would maintain the proportion of A+T residues, thereby lowering the apparent frequencies of transitions.

Therefore, we feel that our data set provides enough information to infer the topology of the phyletic relationships between the cytoplasmic lineages, although it does not allow us to draw general conclusions about the nature and the rate of substitutions that have accumulated on to the whole mitochondrial genome.

	Base				Percentage
	A	G	C	T	A + T
Mus 1	47	13	19	31	71
Mus 3	49	13	19	29	71
Mus cervicolor	49	13	18	30	72
Myomys	52	12	14	27	75
Rattus (1)	51	11	25	20	66
Cow (2)	48	15	23	21	64
Man (3)	41	14	30	20	58

(1) Saccone et al. 1981
(2) Anderson et al., 1982
(3) Anderson et al., 1981

TABLE 2: Base content of the 110 last nucleotides of the 5'end of the 16S mitochondrial rRNA.

Substitution type			
Transitions	A-G / G-A	3	16
	C-T / T-C	13	
Transversions	A-T / T-A	10	15
	A-C / C-A	4	
	G-T / T-G	1	
	G-C / C-G	4	

TABLE 3: Frequencies of mutational events in the House Mouse species complex.

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