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

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ABSTRACT

Restriction endonuclease analysis has revealed extensive mtDNA polymorphism in two species of rats, *Rattus rattus* and *Rattus norvegicus*. Sequence divergence values for the eight detected *R. norvegicus* variants range from 0.2% to 1.8% and for the eight *R. rattus* variants, from 0.2% to 9.6%. Three of the most closely related *R. norvegicus* mtDNA's appear to differ by deletions/insertions of about 4 base pairs apiece. Restriction sites for seven enzymes have been mapped for 11 of these variants. The 31 intraspecific and 41 interspecific variant sites appear to be evenly distributed on the mtDNA molecule outside of the rRNA cistrons. The location of sites present in all the DNAs suggests that the rRNA genes and possibly the light strand origin of replication may be more highly evolutionarily conserved than other parts of the molecule. The sequence divergences among the mtDNAs of animals whose geographic origins are separated by major barriers, such as oceans, were significantly greater than those among animals found within large land masses, such as the continental United States. Dendrograms (phenograms), which have been constructed to depict the relationships among the various DNAs, indicate that East Asian members of the *R. rattus* species are more closely related to American rats of this species than to other Asian *R. rattus* animals from Sri Lanka. Moreover, it appears that *R. norvegicus* comprises a group taxonomically distinct from any of the *R. rattus* subspecies.

IT is now widely recognized that more than one type of mtDNA may occur within a mammalian species. The first observations of this phenomenon were made in horses and humans (POTTER *et al.* 1975), sheep and goats (UPHOLT and DAWID 1977) and in laboratory rats independently in a number of laboratories FRANCISCO and SIMPSON 1977; FRANCISCO, VISSERING and SIMPSON 1977; SACCONE *et al.* 1977; HAYASHI *et al.* 1978; BUZZO, FOUTS and WOLSTENHOLME 1978). In each case, differences were found between individual animals in mtDNA restriction endonuclease cleavage sites. More recently, such intraspecific mtDNA polymorphism has been observed in a number of additional mammalian species (AVISE, LANSMAN and SHADE 1979; AVISE *et al.* 1979), avian species of (GLAUS

et al. 1980), and in Drosophila as well (SHAH and LANGLEY 1979), indicating that the phenomenon is a widespread and perhaps universal feature of animal mtDNA.

Two kinds of mtDNA, types A and B (FRANCISCO and SIMPSON 1977), have been reported to occur in the laboratory rat *Rattus norvegicus*.[1] The presence of such mtDNA polymorphism in a laboratory animal has allowed certain aspects of this phenomenon to be more intensively investigated. For example, in each of over 200 offspring of heterologous crosses between rats of the two mtDNA types, the mtDNA variant transmitted is that of the maternal parent (BUZZO, FOUTS and WOLSTENHOLME 1978; HAYASHI *et al.* 1978b; KROON, DEVOS and BAKKER 1978; FRANCISCO, BROWN and SIMPSON 1979). Furthermore, direct sequence analysis has demonstrated that these mtDNAs differ by nucleotide sequence rather than by differential base methylation (DEVOS *et al.* 1980; CASTORA, ARNHEIM and SIMPSON 1980; CASTORA, BROWN and SIMPSON 1980; BROWN *et al.* 1980).

Clearly, there exist many potential applications for such mtDNA variants to the study of mitochondrial genetics in animal systems. However, at present little is known about the range of this diversity or its phenotypic consequences. The extent of sequence divergence, 1 to 2% (FRANCISCO, BROWN and SIMPSON 1979), between these two laboratory rat mtDNA's suggested that additional mtDNA types might be found in wild populations of the *R. norvegicus* species. We felt that such mtDNAs would offer an opportunity to obtain information on these points. For example, the locations of the variant restriction sites on such DNA's would provide information on how the variation affects the different genic regions of the molecule. In addition, we hoped that, by correlating the geographic distribution of different mtDNA variants with the evolutionary relationships they bear to one another, it would be possible to gain information on the history of the dispersion of the species. In this respect, results from a number of laboratories, AVISE, LANSMAN and SHADE on Peromyscus (1979), AVISE *et al.* on Geomys (1979), and HAYASHI *et al.* (1979), BROWN and SIMPSON (1979a,b) and BROWN *et al.* (1980) on wild Rattus, have recently demonstrated the usefulness of mtDNA restriction analysis in determining the relationships among populations in several rodent species.

We report here our results on the variation of mtDNA within and between two rat species, *Rattus norvegicus* and *Rattus rattus*. We have constructed restriction maps for the variant DNAs that permit, for the first time, an examination of the relative extent to which different regions of the mtDNA molecule are subject to genetic variability. The maps also permit comparison of the changes that occur at the intra- and interspecific levels. In addition, the relationships we have determined among the various mtDNA samples have allowed several inferences to be drawn on the evolutionary history of different populations of rats in the two species.

[1] In order to extend the nomenclature system developed for the *R. norvegicus* mtDNA variants (types A, B, C, etc.) to other Rattus species, we have prefixed the type designations by a letter that designates the particular species, *e.g.*, NA for *R. norvegicus* type A, RD for *R. rattus* type D, etc.

MATERIALS AND METHODS

Sources and preparation of wild rat mtDNAs: Frozen livers and kidneys and occasional live animals of both species were obtained from STEPHEN FRANTZ, Director of the New York State Rodent Control Evaluation Laboratory (U.S. and Puerto Rican *R. norvegicus*; Floridian and Puerto Rican *R. rattus*). JAMES PATTEN, Curator of Mammals, Museum of Vertebrate Zoology, University of California, Berkeley, provided us with frozen livers and kidneys of Californian *R. rattus*. T. H. YOSIDA, Head, Dept. of Cytogenetics, National Institute of Genetics, Misima, Japan, provided frozen livers of Hong Kong, Japanese and Sri Lankan *R. rattus* and Japanese *R. norvegicus*. In terms of the correct identification of species, it might be well to point out that all three of these individuals are rodent specialists, particularly on the genus Rattus. Tissues of individual animals were homogenized and nuclei were removed as described by FRANCISCO, BROWN and SIMPSON (1979). Mitochondria were pelleted at 12,000 × g, and washed once prior to isolation of mtDNA by CsCl-ethidium bromide gradient centrifugation. Both the lower- and upper-band fractions for each individual were saved. Ethidium bromide was removed by first dialyzing the DNA samples against STE buffer (FRANCISCO, BROWN and SIMPSON 1979) for a few hr and then extracting them twice with STE saturated phenol.

Restriction endonuclease analysis: Digestions and agarose gel electrophoresis were performed as described by FRANCISCO, BROWN and SIMPSON (1979), normally on lower-band material. When yields of lower-band DNA were too small to allow for detection of restriction fragments by ethidium staining, digestion products from upper- and /or lower-band material were transferred from the gel to nitrocellulose filters (SOUTHERN 1975) and hybridized to a mtDNA probe labeled with [^{32}P] by nick translation. All steps were carried out as described by JEFFREYS and FLAVELL (1977), except that our hybridization conditions were 4 × SSC, 50% formamide at 42°. Fragments were visualized by autoradiography.

In the case of *Hinf* I digestions, fragments generated from about 10 ng of lower-band mtDNA were labeled at their 3′ termini, using DNA Polymerase I large-fragment and α[^{32}P]dATP (BROWN 1980). The fragments produced were resolved on a 30 cm 5% polyacrylamide gel run in E buffer (FRANCISCO and SIMPSON 1977), which was dried and autoradiographed. Denaturing gels were run in the same manner except that 8 M urea was included in the gel matrix.

Cleavage maps: Restriction maps for the laboratory rat mtDNA types NA and NB constructed in this laboratory and references to those constructed in other laboratories, as well as the methodological details of our mapping procedures, can be found in FRANCISCO, BROWN and SIMPSON (1979). Using laboratory rat mtDNA maps, we were able to position the newly detected restriction sites in both the *R. norvegicus* and *R. rattus* variants. The additional *Hind* II site in type NC_1 was positioned by comparing the *Hind* II/*Sst* I fragments from this DNA with those of type NB, and the additional *Hha* I site in type ND was positioned by comparing *Hha* I/*Bam* HI fragments with those of types NA and NB.

Sst II sites, which are identical in all the DNAs of this study, were mapped in *R. norvegicus* by double digestion with *Bam* HI and *Eco* RI. These sites were then used to position the *Bam* HI, *Sst* I and *Hha* I sites in the *R. rattus* variants, which in turn were used to position sites for *Eco* RI, *Hind* II and *Hind* III, all by combination digestion procedures. The fragments generated from the different *R. rattus* variants by each enzyme pair were directly compared by running them side-by-side on the same slab gel. Thus, evidence as to whether a site varied or was conserved between two DNA's was obtained by direct restriction fragment comparison.

The few sites that could not be located by combination digests were mapped by partial digestion product sizing and by hybridization to cloned *R. norvegicus Bam* HI fragments (CASTORA, ARNHEIM and SIMPSON (1980). These techniques were also used to confirm many of the site locations determined by the double digestion protocol. The positions of the *Sst* I, *Sst* II and *Bam* HI sites relative to the D loop in type RD DNA were confirmed by examination of glyoxal-fixed (BROWN and VINOGRAD 1974), restricted, replicative forms in the electron microscope. The conservation of a restriction fragment in single digest comparisons of *R. norvegicus* with the *R. rattus* variants was taken as evidence that the flanking restriction sites were also con-

served. Other sites that, on the basis of their map locations, appeared as though they might be conserved between the two species were designated as "conserved" or "variant" on the basis of comparisons of fragments produced in double digests with appropriate enzymes.

Sequence divergence and tree construction: Sequence divergence was estimated from the fraction of conserved sites, as defined by NEI and LI (1979), using the cleavage-site method of UPHOLT (1977) and NEI and LI (1979). The results with 4- and 6-base pair (bp) recognition site enzymes and with *Hind* II were weighted according to the number of cleavage sites produced. As a first approximation, *Hind* II was treated as a 5-bp enzyme since its recognition site specificity (GTPyPuAC) lies at a point between that of a 6-bp and a 4-bp enzyme.

Construction of trees of the phenetic type (phenograms) and calculation of co-phenetic correlations were done with the aid of the computer programs of F. JAMES ROHLF, Dept. of Ecology and Evolution, SUNY, Stony Brook.

Materials: Suppliers for enzymes and reagents were as previously indicated (FRANCISCO, BROWN and SIMPSON 1979). DNA polymerase I large fragment was from Boehringer/Mannheim, and α-[^{32}P]ATP (50–300 Ci/mmol) was from New England Nuclear.

Definition of terms: In order to avoid possible confusion in the understanding of our classification system of the various mtDNA types, it might be well to define certain terms. We use the term *restriction morph* to mean a restriction fragment pattern produced by gel electrophoresis of mtDNA from an individual animal after digestion with a single given restriction endonuclease (or a given pair of nucleases in the case of a double digest). Each isolated mtDNA preparation is tested against a variety of restriction enzymes, each producing a restriction morph to which we assign a lower-case Roman number. We then employ the term *type* (or *subtype*) to indicate the particular spectrum of restriction morphs produced by a given mtDNA. Thus, the terms *restriction morph* and *type* (or *subtype*) are not synonomous. For example, the actual restriction pattern of each morph is shown in Figure 1 and the mtDNA types in Table 1. It can be seen that type NA and NB mtDNA differ from one another in that, although *Hind* III (as well as the *Bam* HI *Sst* I pair) produces hte same morph in both cases, each of the other enzymes used produces a different morph for each of the two DNA's.

It should also be pointed out that no necessary relationship exists between the morphs produced by different enzymes or by the same enzyme in different species, even though the morph numbers are identical. For example, the *Hind* II-i morph is not related to the *Eco* RI-i *morph*, nor is the *R. norvegicus Eco* RI-*i morph* related to the *R. rattus Eco* Ri-i *morph*.

Finally, we differentiate mtDNA subtypes from types by the extent of sequence divergence, as discussed later.

RESULTS

mtDNA polymorphism in R. norvegicus: The mtDNA isolated from each wild rat was digested with six different endonucleases, and the restriction morphs obtained for *R. norvegicus* animals, along with those of the laboratory types for comparison, are displayed in Figure 1A. (See MATERIALS AND METHODS for a definition of terms used in this paper.) Twelve different morphs were produced by the digestion of 21 samples with these enzymes. We have chosen to present the morphs as a composite schematic, rather than try to align photographs of the many original gels. It can be seen that, while only a single restriction morph was obtained with some enzymes such as *Hind* III, other enzymes such as *Eco* RI produced three or four different morphs. Any restriction morph could be related to at least one other generated by the same enzyme by the gain or loss of a single site.

The relationships between the restriction morphs and the individual animals are shown in Table 1A. The series of restriction morphs that characterize each

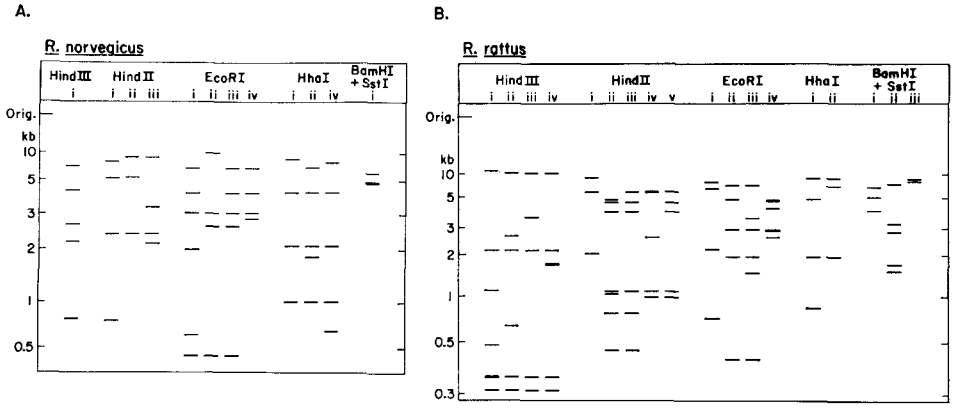

FIGURE 1.—Restriction morphs of *R. norvegicus* and *R. rattus* mtDNAs. The lower-case Roman numerals heading the columns designate particular restriction morphs found in one or more individuals. These designations are arbitrary; restriction morphs bearing identical numerals, but produced by different enzymes (*e.g.*, *Hha* I-iii and *Eco* RI-iii), are not necessarily derived from the same DNA. *Sst* I and *Bam* HI were used in combination, because many of the mtDNA's were cut only once by either enzyme alone; thus, a possible change in a restriction site of either DNA would fail to be revealed. Heavy lines indicate two or more bands that are poorly or incompletely resolved. Fragments smaller than 0.5 kb in diagram A and 0.3 kb in diagram B have not been included.

DNA is given. The last column shows the designation of the DNA types found. Thus far, we divide the *R. norvegicus* mtDNA's into four major types: NA and NB (the laboratory types), and NC and ND. Subscripts designate subtypes of these (see DISCUSSION). *Hinf* I data will be considered in a subsequent section.

Perhaps the most salient feature of the table is the prevalance of the iii-i-ii-i-i restriction morph series, which characterizes type NC mtDNA throughout the U.S. and Puerto Rico. One additional morph, *Hind* II-iii, was found in the U.S. samples. Interestingly, this appeared only in the mtDNA's of four of the five animals trapped in the Bedford-Stuyvesant section of Brooklyn. These DNAs have been designated as a subtype of NC (NC_1).

mtDNA polymorphism in R. rattus: The different restriction morphs obtained from *R. rattus* mtDNAs are shown in Figure 1B. For this species, 18 different morphs were observed in 26 samples with the same six enzymes used in the *R. norvegicus* survey. In general, fewer fragments were conserved in the various morphs produced by a given enzyme. In addition, certain morphs could frequently not be related to any other morph by a single site change. Thus, more diversity appears to exist among the *R. rattus* samples than among the *R. norvegicus* samples.

Table 1B shows the geographical origin of each *R. rattus* animal, together with its restriction morph spectrum. Five types of mtDNA were found, namely, RA, RB, RC, RD and RE. Again, a single type was prevalent in the U.S. and Puerto Rican samples. The morph spectrum observed in the Sri Lanka animals is an unusual finding. Each enzyme yielded a unique restriction morph, suggesting less conservation of sequence relative to the other Rattus mtDNAs.

TABLE 1

Distribution of restriction morphs in R. norvegicus *and* R. rattus *individuals*

Sample origin	mtDNA* type	No. of animals	Restriction morph†					
			*Eco*RI	*Hha*I	*Hind*II	*Hind*III	*Bam*HI +*Ssi*I	*Hinf*I
A. *R. norvegicus*								
Laboratory	NA		i	i	i	i	i	i
Laboratory	NB		ii	ii	ii	i	i	ii
Troy, N.Y.	NC‡	1	iii	i	ii	i	i	
Boston, Mass.	NC‡	1	iii	i	ii	i	i	
San Juan, P.R.	NC‡	1	iii	i	ii	i	i	
Brooklyn, N.Y.	NC_1	4	iii	i	iii	i	i	iii
Brooklyn, N.Y.	NC_2	1	iii	i	ii	i	i	iv
Ponce, P.R.	NC_2	1	iii	i	ii	i	i	iv
Camden, N.J.	NC_3	1	iii	i	ii	i	i	v
Cohoes, N.Y.	NC_4	1	iii	i	ii	i	i	vi
Harrisburg, Pa.	NC_4	1	iii	i	ii	i	i	vi
New York, N.Y.	NC_5	1	iii	i	ii	i	i	iii
Chesapeake, Va.	NC_5	1	iii	i	ii	i	i	iii
Omaha, Neb.	NC_5	1	iii	i	ii	i	i	iii
Houston, Tex.	NC_5	2	iii	i	ii	i	i	iii
Ponce, P.R.	NC_5	1	iii	i	ii	i	i	iii
Japan	ND	1	iv	iii	ii	i	i	
B. *R. rattus*								
Sri Lanka	RA	5	i	i	i	i	i	
Hong Kong	RB	2	ii	ii	ii	ii	ii	
Japan	RC	3	iii	ii	iii	ii	ii	
Alameda Co., Cal.	RD‡	4	iv	ii	iv	iv	iii	
Alameda Co., Cal.	RD_1	7	iv	ii	iv	iv	iii	ii§
Tampa, Fla.	RD_1	1	iv	ii	iv	iv	iii	ii
Chesapeake, Va.	RD_2	1	iv	ii	iv	iv	iii	iv
San Joaquin Co., Cal.	RD_3	1	iv	ii	iv	iii	iii	i
San Juan, P.R.	RD_4	1	iv	ii	iv	iv	iii	v
Ft. Lauderdale, Fla.	RE	1	iv	ii	v	iv	iii	iii§

* Changes in nomenclature have been made since a symposium presentation of this work (BROWN *et al.* 1980).

† No relationship is implied among samples bearing the same designation in the different species.

‡ Subtype not assigned since *Hinf* I analysis was not performed.

§ Morph assignment changed since BROWN *et al.* (1980).

Hinf *I-revealed variants*: The probability of detecting additional variants of the previously described mtDNA types should be increased by the use of an enzyme that makes more cuts than are made by the enzymes previously used, since such an enzyme would survey a larger proportion of the genome. Thus, we turned to *Hinf* I, which cuts these DNAs at 26 to 32 sites. Four subtypes of *R. norvegicus* type NC were revealed in this manner: NC_2, NC_3, NC_4 and NC_5. The characteristic *Hinf* I morphs for NC_4 and NC_5 (vi and iii, respectively) are shown in Figure 2A. As expected, morphs of these two DNAs are more similar to one another than to those of either of the laboratory types (morphs i and ii. Figure 2A). Subtypes NC_2, NC_3 and NC_5 are of particular interest; their restriction morphs are shown

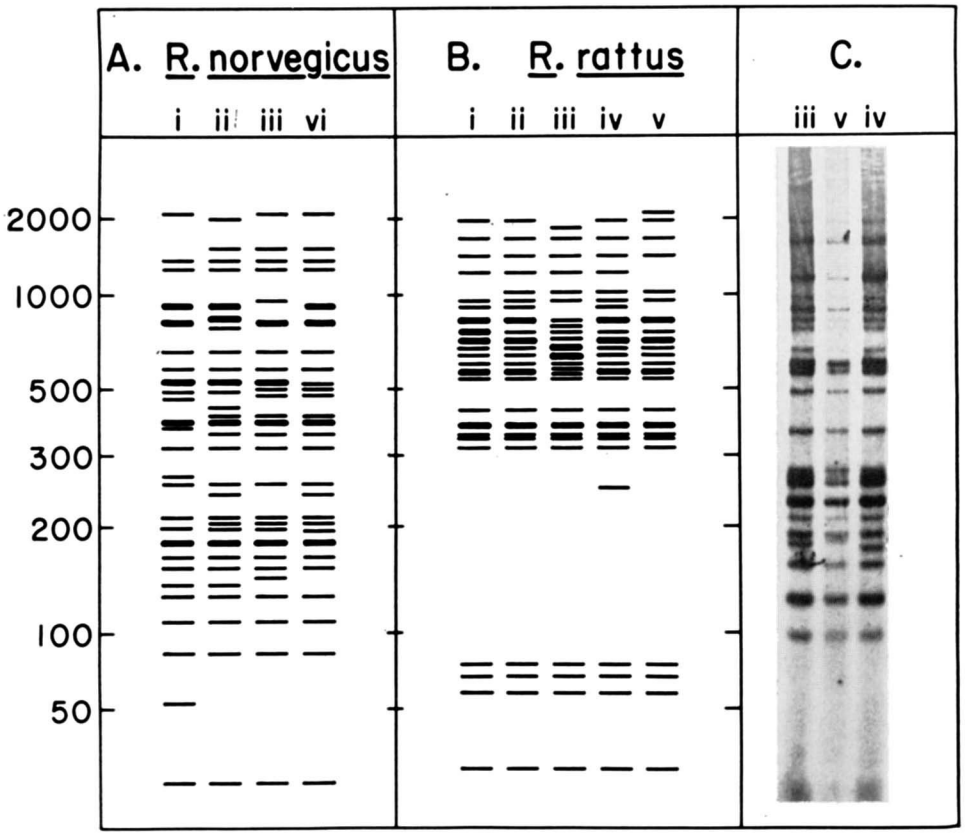

FIGURE 2.—Restriction morphs obtained by *Hinf* I digestion of certain *R. norvegicus* and *R. rattus* mtDNAs. mtDNAs from various type NC (and laboratory types NA and NB) *R. norvegicus* individuals and from various type RD and RE *R. rattus* individuals were subjected to *Hinf* I analysis. The lower-case Roman numerals heading the columns designate restriction morphs found in one or more individuals. Heavy lines indicate poorly or incompletely resolved fragments, as observed in ethidium bromide stained gels. Fragments smaller than 0.03 kb would not have been detected. Frame C, autoradiograph; morphs iii, iv and v correspond to *R. norvegicus* subtypes NC_5, NC_2 and NC_3, respectively. Morph iii appears in both frames A and C for comparison. Four fragments larger than 1000 bp are not shown in frame C. These fragments are identical in mobility in all three morphs. Mobilities in frame C do not correspond to the bp size scale on lefthand axis. However, the variant bands in frame C are located at approximately 185 on the scale.

as an autoradiograph (Figure 2, iii, v, iv, respectively). Only one fragment varies in mobility in the three subtypes, and this fragment appears to differ in size by increments of 4 (or possibly 3) bp. Because of the identical mobilities of all the other corresponding fragments in the three DNAs, the variant fragments very likely correspond to one another. Thus, the very small differences among these fragments suggest that they differ by deletions/insertions.

We have considered alternative explanations. One possibility is that the difference among the three fragments lies in the occurrence of an additional cleavage

site in NC_2 and NC_5, 3 to 4 bases and 6 to 8 bases, respectively, from the fragment terminus. We consider this unlikely for two reasons. First, it is improbable that we should find three such identical evolutionary differences occurring so close to one another in DNA molecules obtained from animals trapped at random from widely separated geographic areas. This view is strengthened by our failure to detect evolutionarily "hot" regions in the mtDNA molecule, as will be seen later. Second, since *Hinf* I possesses a 5-bp recognition site (GANTC) and since the size differences between the fragments are 3 or 4 bp each, one must postulate the existence of three overlapping *Hinf* I recognition sites, a view difficult to support considering the sequence of this site.

Mobility differences due to variation in the tertiary structure of these fragments were ruled out since the same differences were observed in gels run under denaturing conditions. These results also eliminated the possibility that the variant fragments reflect different size classes of D-loop. The possibility that the tracking dye, bromphenol blue, differentially affected the mobility of these fragments by moving at the same rate was considered and discounted; in these gels, bromphenol blue migrates considerably faster than the bands in question.

With respect to *Hinf* I studies on *R. rattus*, three subtypes, RD_1, RD_2 and RD_4, were revealed within previously indistinguishable American samples (morphs ii, iv and v, Figure 2B). RD_1 and RD_2 appear to differ only by the presence of an additional *Hinf* I site in RD_2. RD_4 appears to differ from RD_1 by the presence of one additional band of approximately 200 bp. This probably does not result from an insertion in RD_4, since an insertion of this magnitude would have been detected as a decrease in electrophoretic mobility of one of the restriction fragments of this DNA type. No such mobility changes were detected. More likely, the band is generated by the presence of a site in one of the fragments of the *ca.* 800 bp multiplet with the generation of an additional fragment in the *ca.* 550 bp multiplet.

One of the *R. rattus* subtypes, RD_1, showed some geographic localization in its prevalence in the Alameda County animals. These DNAs, however, were indistinguishable from those in the Tampa, Florida, sample. The *R. norvegicus* subtypes NC_2, NC_4 and NC_5 also appeared to be widespread in geographic distribution.

Restriction maps: Our goal in mapping restriction sites was to be able to scrutinize the mtDNA molecule for distinctive evolutionary features, such as exceptionally divergent or conserved regions. Furthermore, we wished to compare the intraspecific with the interspecific evolution of the molecule. We began by mapping the sites for seven restriction enzymes on the mtDNA variants of both species (Figure 3). Since, for these purposes, the correct determination of whether or not a site varied between different DNAs was critical, sites that mapped in similar locations on two DNAs were designated as conserved or variant only when additional evidence could be obtained from comparisons of restriction fragment mobilities in analyses of single or double digests. The designation of each mapped site as conserved or variant, both intraspecifically and interspecifically, could be confirmed in this manner. It should be understood, of course, that absolute proof

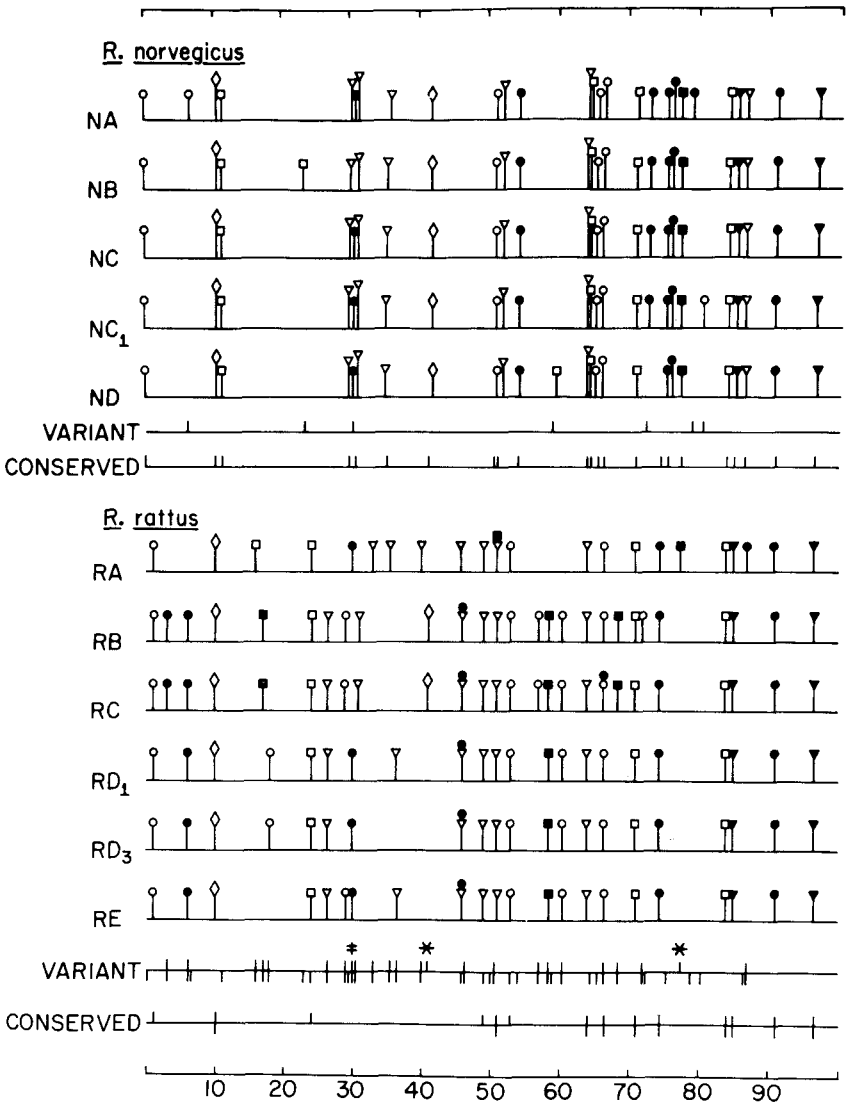

FIGURE 3.—Restriction maps of various *R. norvegicus* and *R. rattus* mtDNA types and subtypes. Although the maps are actually circular, they are presented in linear form for more convenient comparison. The scale begins at the origin of H-strand replication (D-loop) and proceeds from left to right in the direction of D-loop expansion. Positions at the sites that vary or are conserved within each species are indicated on the horizontal axes beneath each set of maps. Downward-pointing indicators on the axes beneath the *R. rattus* maps indicate sites that vary or are conserved between the mtDNAs of the two species. *, site present in all *R. norvegicus* DNAs, but in only some *R. rattus* DNAs. ‡, sites present in certain *R. norvegicus* and certain *R. rattus* mtDNAs. (●) *Eco* RI; (▼) *Sst* II; (■) *Sst* I; (○) *Hind* II: (▽) *Hind* III; (□) *Hha* I; open diamond, *Bam* HI.

of the identity of sites that ostensibly map in the same position can come only from sequencing studies.

Some uncertainty still exists regarding the conservation of particular restriction sites in certain DNA pairs, namely the *Hind* III site near position 30 in type RA, which was considered to be different from that in types RB and RC, and the *Hind* III site at position 34 in type RA, which was considered to differ from that in types RD and RE. Confirmatory evidence for these designations could not be obtained; thus, it is conceivable that the sites are conserved in these DNA pairs, although we think this unlikely.

The locations of the intraspecific variant sites occurring in *R. rattus* (Figure 3, indicators above *R. rattus* "Variant" axis) are more-or-less evenly distributed outside the region containing the rRNA cistrons (approximately 82.5 to 100 map units; SACCONE *et al.* 1977; BATTEY and CLAYTON 1978). Only one of the intraspecific variant sites falls within the rRNA coding region. Although fewer data are available for the mtDNA's of *R. norvegicus*, the overall pattern of intraspecific variation is not altered when the variant sites occurring in this species are taken into account. A similar distribution is noted for the interspecific variant sites (downward indicators on the *R. rattus* "Variant" axis). Thus, intraspecific and interspecific site variation seems to differ only in extent and not in kind. Ten sites are conserved in all the DNAs, clustering in the rRNA region and in a region to the left, extending from 64 to 74 map units. The rRNA genes therefore, appear to be more highly conserved than the remainder of the genome. While the origin of replication of the H strand does not appear to be exceptionally conserved, confirming the results of UPHOLT and DAWID (1977), the light strand origin, which occurs at about map position 67 (MARTENS and CLAYTON 1979), contains a *Hind* II site that is conserved in all these DNAs, and occurs in mouse mtDNA as well (PARKER and WATSON 1977; MARTENS and CLAYTON 1979). Two other sites conserved in all these DNAs, as well as in mouse mtDNA (PARKER and WATSON 1977), are located at positions 73 and 83, the latter falling within the large rRNA gene. Some sites that vary intraspecifically are nevertheless conserved interspecifically between certain DNA pairs (see legend to Figure 3).

Sequence divergence: In order to quantify the extent of variation among the various mapped DNAs, we have employed the method based on the fraction of conserved restriction sites described by UPHOLT (1977) and NEI and LI (1979). This method is more reliable than those based on restriction fragment conservation (UPHOLT 1977; NEI and LI 1979) in comparing less closely related DNAs (UPHOLT 1977) such as some of our more divergent samples.

Sequence divergences, expressed as percent base substitution, resulting from pairwise comparisons of these DNAs are presented in matrix form in Table 2A. The wide spectrum of intraspecific divergences in *R. rattus*, from 0.4% to 9.6%, is particularly notable. By contrast, sequence divergence estimates for *R. norvegicus* pairs range from 0.4% to 1.8%. As might be expected, all interspecific differences were higher than any intraspecific difference, ranging from 13.7–18.4%.

The additional data from the *Hinf* I analyses were used in calculating sequence

divergences for the more closely related DNAs (Tables 2B and 2C). Since the *Hinf* I sites were not mapped, the cleavage fragment method of UPHOLT (1977) was employed. It can be seen that pairs with divergences as low as 0.2% occur in *R. rattus*, and as low as 0.3% in *R. norvegicus*.

Relatedness of DNAs: The inter- and intraspecific relationships among these DNAs can best be depicted in the form of a tree or dendrogram. Using the matrices in Table 2 and applying the unweighted pair-group method (UPGMA; SNEATH and SOKAL 1973) we have constructed one type of dendrogram, a phenogram, that depicts present-day similarities between DNAs without reference to how these similarities came into being (SNEATH 1974). The trees so generated are shown in Figure 4. Tree A reveals four major taxa: *R. norvegicus*, Sri Lankan *R. rattus* (RA), East Asian *R. rattus* (RB and RC), and American *R. rattus* (RD and RE). Interestingly, the East Asian *R. rattus* appear more closely related to the American animals of this species than to the Sri Lankan rats. In general, however, the variants from within major geographic areas are more closely related to one another than to DNAs of more distant geographic origin. The close relationships and less extensive sequence divergeneces between the subtypes in each species are apparent from trees B and C.

TABLE 2

Sequence divergence values for different mtDNA pairs

A.

	RA	RB	RC	RD_1	RD_3	RE	NA	NB	NC_5	NC_1	ND
RA	—										
RB	9.6	—									
RC	9.6	0.7	—								
RD_1	5.7	4.5	4.4	—							
RD_3	5.3	4.2	4.0	0.4	—						
RE	5.3	3.4	3.4	1.0	1.4	—					
NA	13.3	18.4	18.0	16.4	16.1	16.4	—				
NB	15.1	17.8	17.3	17.1	16.8	17.1	1.6	—			
NC	13.5	17.5	17.1	15.4	15.1	15.4	0.7	0.9	—		
NC_1	14.1	18.2	17.8	16.1	15.9	16.1	1.2	1.4	0.4	—	
ND	13.8	17.8	16.7	15.7	15.2	15.7	1.6	1.8	0.9	1.3	—

B.

	NA	NB	$NC_{2,3,5}$*	NC_1	NC_4
NA	—				
NB	2.8	—			
NC_5	1.8	2.3	—		
NC_1	2.1	2.7	0.3	—	
NC_4	1.7	2.2	1.0	0.3	—

C.

	RD_1	RD_2	RD_3	RD_4	RE
RD_1	—				
RD_2	0.2	—			
RD_3	0.3	0.3	—		
RD_4	0.3	0.5	0.6	—	
RE	2.1	2.3	2.2	2.3	—

* Since the only differences observed between NC_2, NC_3 and NC_5 appeared to be due to small deletions and insertions of DNA, they were considered as a single subtype for the purpose of sequence-divergence calculations.

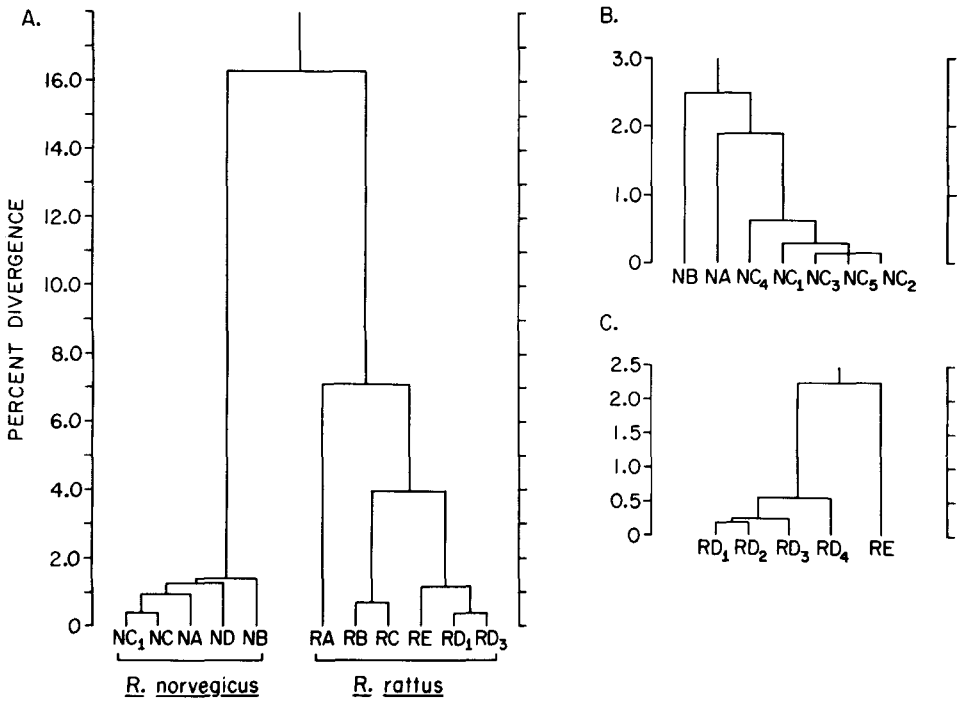

FIGURE 4.—Phenograms depicting relationships between various *R. rattus* and *R. norvegicus* mtDNAs. The trees were constructed using the following matrices: A, table 2A; B, Table 2B; C, Table 2C.

DISCUSSION

Extent of mtDNA polymorphism: Sequence divergences for the intraspecific variants detected in this study range from 0.2% to 9.6%. In an attempt to deal with this wide range of variation, we have categorized individual DNAs whose sequences diverge by less than 1% as subtypes of more general types whose sequences diverge by more than 1%. Since divergences in the neighborhood of the lower figure have also been found between human mtDNA's (BROWN 1980), it is probable that mtDNAs with this degree of relatedness are widespread in occurrence.

The upper figures for intraspecific mtDNA sequence divergence in this study study are higher than any previously reported. The East Asian and Sri Lankan animals for which this value was obtained apparently represent subspecies on the verge of speciation. This view is suggested by the observation that crosses between these rats produce sterile offspring, whereas crosses of either East Asian or Sri Lankan rats with Oceanian (American) rats result in the production of fertile offspring (YOSIDA 1977). In view of the wide range of sequence divergences observed and since closely related subtypes very likely exist within most types, the number of distinct mtDNA variants found within a mammalian species may be quite high.

The sequence divergences observed for some *Rattus rattus* pairs were four to

five times higher than those observed for any of the *R. norvegicus* pairs. This may be a reflection of the more limited sampling area covered in the *R. norvegicus* study. Alternatively, *R. norvegicus* may be a more recently evolved species (PRATT and BROWN 1976), or may have undergone a severe population constriction in the more recent past. Presently, we are surveying European and Asian populations of this species in order more completely to understand this difference.

With respect to the types of changes in mtDNA that occur within a species, the three variants that differ by putative deletions/insertions are of considerable interest. Such intraspecific variants have not previously been described. Their detection in this study probably results from their fortuitous occurrence within a fragment small enough to reveal mobility differences due to length changes of 4 bp. The fact that identical morphs are obtained from the three types with *Sau* 3A, which cuts them at at least nineteen sites, indicates that the sequence divergence among these DNAs is less than 0.2% and that, conceivably, the deletions are the only difference among them. Additional studies, aimed at better defining the nature of the differences among these DNAs, are in progress.

Intra-individual homogeneity of mtDNA: In view of the extensive intraspecific heterogeneity that occurs in these species, one might expect that some individuals possessing more than one type of mtDNA (heteroplasmic individuals) might be found. Such animals could conceivably arise as either the result of crosses between rats of different mtDNA types, or as the result of mtDNA mutation. However, heteroplasmic individuals have not been observed in this study, and this probably results from two factors. First, very little, if any, of an animal's mtDNA is derived from its male parent; searches for low levels inherited from the male parent have been unsuccessful (HAYASHI *et al.* 1978; FRANCISCO, BROWN and SIMPSON 1979; AVISE *et al.* 1979). In our case, after analyzing crosses between laboratory rats of differing mtDNA type, we have concluded that paternally derived molecules, if they occur at all, comprise less than 0.02 per cent of the overall mtDNA composition of adult tissues (BROWN and SIMPSON 1979a). Heteroplasmic individuals therefore will not result from crosses that are heterologous with respect to mtDNA type. The second factor is the rapid rate at which variant mtDNA's would be expected to sort out through vegetative segregation (BIRKY 1978; UPHOLT and DAWID 1977; AVISE *et al.* 1979). Thus, mutations occurring in the female germ line will give rise to occasional heteroplasmic individuals, but, as has been estimated by UPHOLT and DAWID (1977), within 20 to 200 generations the descendants of such animals will be essentially homoplasmic. The intra-individual heterogeneity that results from the appearance of a new mtDNA variant type, therefore, will not persist, and the heteroplasmic individuals that arise from such events probably occur at frequencies too low to have been detected by our sampling procedure. Moreover, even if such an animal had been analyzed, it is probable that the new variant type would differ from its predecessor by only a single nucleotide. The probability of distinguishing a DNA with this small extent of sequence divergence by the methods employed would be very low.

The effects of evolutionary change on mitochondrial genes: The extensive divergences found within *R. rattus* mtDNA, which in some cases are comparable to the divergence between rabbit and human β-globin mRNA (KAFATOS *et al.* 1977), can be regarded as a manifestation of the rapid rate of evolution of the molecule (DAWID 1972; BROWN, GEORGE and WILSON 1979). However, it is difficult to reconcile this rapid rate of evolution with the genetic functions that animal mtDNA is thought to possess. One escape from this difficulty is the possibility that much of the variation is confined to noncoding regions that might be evolving much more rapidly than the coding parts of the molecule (DAWID 1972). However, because the size of animal mtDNA only slightly exceeds that necessary to fill its coding functions (SCHATZ and MASON 1974; ATTARDI *et al.* 1979), such noncoding regions would probably be short in length and few in number. If this were the case, we would anticipate that the majority of variant restriction sites would be clustered in a few such regions. The relatively random distribution of variant sites on the molecule, however, indicates that such evolutionarily "hot" regions do not occur. Moreover, alignment of known human (BARRELL, BANKIER and DROUIN 1979) and putative mouse (BATTEY and CLAYTON 1978) protein-coding regions with our "variation map" indicates that many of the variant sites fall within protein structural genes. Hence, the mitochondrially encoded proteins may well be evolving rapidly. As one would not expect this of these essential proteins (BROWN, GEORGE and WILSON 1979), they may prove to be particularly interesting from the standpoint of molecular evolution.

An alternative escape route is the possibility that, owing to the extensive degeneracy of the genetic code, most of the evolutionary mutations in the mtDNA of these animals are silent, not affecting the primary structure of the encoded proteins. Indeed, CASTORA, ARNHEIM and SIMPSON (1980) and DEVOS *et al.* (1980) noted, in their determination of the sequence of a 169-bp stretch within types NA and NB mtDNA, that none of the three nucleotide replacements that occur would result in amino acid substitutions. However, this extent of sampling is insufficient to support the silent mutation hypothesis, and in view of the large sequence divergences found between some mtDNAs, we do not currently favor this possibility above others.

The extensive intraspecific sequence divergences found raise another problem. Most of the known mitochondrial protein gene products are subunits of respiratory enzymes, in which they are tightly complexed with other subunits of nuclear genetic origin (SCHATZ and MASON 1974). One might expect that the formation of such tight complexes would place strict primary structural requirements on both sets of subunits. For the mitochondrially encoded subunits to evolve rapidly, then, we might expect those regions of the nuclear encoded subunits that complex with them also to evolve rapidly. We encounter difficulty with this scheme, however, when we try to explain the production of viable offspring from crosses between animals whose mtDNAs are widely divergent in sequence, such as between the Sri Lankan (type RA) and East Asian (types RB and RC) rats. As mitochondrial genes are inherited only from the female parent, while genes for the nuclear encoded subunits are inherited from both parents, we would expect that the for-

mation of "hybrid" respiratory complexes with the nuclear encoded subunits of the male parent would be essential for survival of such offspring. If the nuclear genes for these subunits are in fact evolving rapidly in "tandem" with the mitochondrial genes, it is difficult to see how this could occur in cases where the parental mitochondrial genomes have diverged to such an extent. Any one of several plausible hypotheses might obviate these difficulties: (1) It is possible that functional complex formation does not have a strict dependence on the primary structure of the proteins involved or, more probably, that the variation in the mitochondrially encoded proteins occurs outside of binding domains. This would eliminate the necessity of having the nuclear and mitochondrial gene products evolve in tandem. (2) Only the female parental alleles for the nuclear-encoded proteins are expressed, either by inactivation of male parental alleles or by the selection of "homologous" subunits at the level of assembly of the respiratory complexes. Studies in progress should shed further light on these problems. (3) As discussed previously, the mtDNA sequence variations do not affect the sequences of mtDNA encoded proteins.

This study, as well as Dawid's hybridization study (1972), indicates that the mitochondrial rRNA genes evolve more slowly than the bulk of the molecule. In addition, the single restriction site that we have mapped in the L-strand replication origin is very highly conserved, suggesting that this region also may evolve more slowly. In accord with this view, Martens and Clayton (1979) have noted that extensive sequence homology is found in this region with the replication origins of SV40 and ϕX174 viral DNAs.

Phylogenetic considerations: For the phenograms of Figure 4 to depict evolutionary relationships, the ultrametric property must hold (Sneath 1974). This would require mtDNA to evolve at both a constant rate and in a divergent fashion. According to the analysis of Brown, George and Wilson (1979), the rate of evolution of mtDNA appears to be reasonably constant, but whether this holds for the short periods of evolutionary time that apply to much of our data is not clear. Since, in addition, no test for divergence of mtDNA evolution has yet been performed, we must regard evolutionary conclusions based on these phenograms as tentative. Nevertheless, two considerations justify an attempt at evolutionary interpretation. First a major source of intraspecific convergent evolution, recombination, must be severely limited by maternal inheritance. Second, Sneath (1974) suggested that the co-phenetic correlation (Sokal and Rohlf 1962) of a UPGMA phenogram can serve as a measure of the ultrametric property, and the values of this parameter, as calculated for the trees of Figure 4 are quite high: 0.99 for A, 0.97 for B and 0.95 for C. Thus, particularly with respect to data set A, some reasonable, albeit tentative, evolutionary speculations seem possible.

Both the North American (RD and RE) and East Asian *R. rattus* (RB and RC) appear to have descended from a lineage distinct from that of the Sri Lankan animals, and *R. norvegicus* has descended from a lineage separate from that of any *R. rattus* groups. On the basis of extensive karyological data, Yosida and his colleagues (Yosida *et al.*, 1974) have suggested that Sri Lankan and Oceanian *R. rattus* (apparently similar to our American *R. rattus*) have descended from a

common ancestor different from that of the East Asian animals. This phylogeny is inconsistent with the one we have postulated, and it may require several different kinds of approaches to resolve this discrepancy.

Recently, HAYASHI *et al.* (1979) have suggested, on the basis of a less extensive mtDNA analysis, that *R. norvegicus* is more closely related to Asian type *R. rattus* (type RB) than to other *R. rattus* groups. This taxonomy was based on restriction maps for the *R. rattus* types that disagree with ours at most of their assigned sites. We consider the "comparison-combination digestion" mapping protocol we have employed to be highly reliable, for both determining the order of restriction fragments on the maps and determining whether particular sites vary or are conserved between different DNA pairs. Thus, we feel that our Figure 3 maps are correct and that our taxonomy, as shown in the phenograms of Figure 4, is not incorrect on these grounds. Further support for this view comes from the fact that we obtain the same taxonomy from a matrix of sequence divergences calculated on the basis of restriction *fragment* comparisons, which do not require mapping. Finally, the classical scheme of ELLERMAN (1941), based on morphological data, is in accord with our results, but in disagreement with the scheme of HAYASHI *et al.* (1979).

Geographic distribution of variant mtDNAs: In those locales in which the populations were sampled more extensively, such as Alameda Co., Calif., and Brooklyn, N.Y., a single variant type was found to predominate. This phenomenon has been observed previously (AVISE, LANSMAN and SHADE 1979; AVISE *et al.* 1979) in rodent populations of other species, and is thought to result from the "clonal" nature of mtDNA propagation. In this study, for samples other than those from the same locale, the correlation of geographic closeness with a small extent of sequence divergence is not maintained. AVISE, LANSMAN and SHADE (1979) have also observed this in some *Peromyscus maniculatus* samples. For *R. rattus* and *R. norvegicus*, this probably results from the "scrambling" of mtDNA clones by dispersion, since the rate at which both these species are spread by man is thought to be high. Thus, any given mtDNA clone could arise from either the mutational generation of a new variant or the introduction of a pre-existing variant from another locale.

In spite of the scrambling of mtDNA clones over large geographic areas, significant differences are found for mtDNAs whose origins are separated by major geographic barriers, such oceans. These barriers probably serve as obstacles to the introduction of more diverse mtDNA types. The mtDNA differences found between the United States and Asian animals are therefore most likely due to differences in the founding populations for these areas. If this supposition is correct, then by comparing the mtDNA makeup of different major geographic areas it should be possible to retrace the history of dispersion of the two species examined here.

The sequence divergence of the two laboratory types of *R. norvegicus*, NA and NB, is similar to that for distantly separated (U.S. and Japanese) animals of this species. Therefore, the laboratory rat population may have been derived from animals of diverse geographic and genetic backgrounds. This interpretation would

caution againstthe use of outbred rat strains in studies in which the possible inherent genetic diversity of these animals could play a role. In order to gain more information on this point, a survey of European *R. norvegicus*, which is thought to be the probable source of the laboratory rat (ALTMAN and KATZ 1979), is presently underway.

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