EVOLUTIONARY RELATIONSHIPS AMONG FIVE SUBSPECIES OF *MUS MUSCULUS* BASED ON RESTRICTION ENZYME CLEAVAGE PATTERNS OF MITOCHONDRIAL DNA*

HIROMICHI YONEKAWA, KAZUO MORIWAKI[1], OSAMU GOTOH, JUN-ICHI HAYASHI, JUNKO WATANABE, NOBUMOTO MIYASHITA[1], MICHAEL L. PETRAS[2] AND YUSAKU TAGASHIRA

Department of Biochemistry, Saitama Cancer Center Research Institute, Kitaadachi-Gun, Saitama, 362 Japan, [1] *Department of Cytogenetics, National Institute of Genetics, Yata, Mishima, Shizuoka, 411 Japan* and [2] *Department of Biology, University of Windsor, Windsor, Ontario, Canada.*

Manuscript received October 21, 1980
Revised copy received May 1, 1981

ABSTRACT

The intra- and intersubspecific genetic distances between five subspecies of *Mus musculus* were estimated from restriction enzyme cleavage patterns or maps of mitochondrial DNA (mtDNA). The European subspecies, *M. m. domesticus* and Asian subspecies, *M. m. bactrianus*, *M. m. castaneus*, *M. m. molossinus* and *M. m. urbanus* were examined. For each subspecies, except *M. m. urbanus*, at least two local races from widely separated localities were examined. Intrasubspecific heterogeneity was found in the mtDNA cleavage patterns of *M. m. bactrianus* and *M. m. castaneus*. *M. m. molossinus* and *M. m. domesticus*, however, revealed no intrasubspecific heterogeneity. Four of the subspecies had distinct cleavage patterns. The fifth, *M. m. urbanus*, had cleavage patterns identical to those of *M. m. castaneus* with several enzymes. Estimates of genetic distances between the various races and subspecies were obtained by comparing cleavage maps of the mtDNAs with various restriction enzymes. Nucleotide sequence divergences of mtDNA between local races were estimated to be less than 0.4% in *M. m. bactrianus* and less than 0.3% in *M. m. castaneus*. The times of divergence of both subspecies were calculated to be $0.1–0.2 \times 10^6$ years. These values suggest that the intrasubspecific divergence began some $0.1–0.2 \times 10^6$ years ago. On the other hand, nucleotide sequence divergences between European subspecies *M. m. domesticus* and Asian subspecies *M. m. bactrianus* and *M. m. castaneus* were 7.1% and 5.8%, respectively. The times of divergence were calculated to be $2.1–2.6 \times 10^6$ years. Further, the nucleotide sequence divergence and time of divergence between *M. m. molossinus* and the other two Asian subspecies were comparable to those between *M. m. molossinus* and *M. m. domesticus* (about 3% and 1×10^6 years, respectively). These results suggest that *M. m. molossinus* is situated in a unique evolutionary position among Asian subspecies.

MICE of the species *Mus musculus* are believed to have originated in the southern part of western Asia and, from there, dispersed throughout the world (KEELER 1931). In the process, at least ten subspecies evolved; of these, all,

* Contribution no. 1368 from the National Institute of Genetics, Japan.

except two or three European representatives, are still widely distributed in Asia. The taxonomy of these subspecies is still under considerable discussion, and the nomenclature is far from standardized (SCHWARZ and SCHWARZ 1943; KLEIN 1975; MARSHALL 1977; MORSE 1978; BERRY 1980).

Protein polymorphisms have been used to determine genetic distances between populations of local races and of subspecies of the house mouse in Europe and North America. NEI (1975) has shown a correlation between genetic distances and taxonomic groupings. Because this approach requires many mice and assays of a large number of proteins by electrophoresis, it has not been applied to Asian subspecies of house mice; thus little is known about their phylogenetic relationships.

Recently, a powerful method, requiring only a few mice from a particular group, has been developed. This involves the determination of molecular heterogeneity of mitochondrial DNA (mtDNA) through digestion by restriction endonucleases. This procedure has already been applied successfully to elucidate evolutionary relationships in a variety of animals (UPHOLT and DAVID 1977; AVISE, LANSMAN and SHADE 1979a, b; BROWN, GEORGE and WILSON 1979; HAYASHI *et al.* 1979; SHAH and LANGLEY 1979; BROWN 1980), including those between laboratory house mice and wild mice (YONEKAWA *et al.* 1980).

In the present study, the electrophoretic patterns obtained from mtDNA after digestion with a number of restriction endonucleases were used to elucidate the evolutionary relationships among an European and four Asian subspecies of *Mus musculus*, *M. m. domesticus*, *M. m. bactrianus*, *M. m. castaneus*, *M. m. molossinus* and *M. m. urbanus*.

MATERIALS AND METHODS

Animals: The wild mice used were collected at localities shown in Figure 1 and listed in Table 1. Mice of the inbred strain SK/Cam were also used because this strain originated from three mice trapped on the island of Skokholm in England where the mice have been identified as *M. m. domesticus* (SCHWARZ and SCHWARZ 1943; STAATS 1976).

Enzymes and chemicals: Restriction endonucleases *Bam*HI, *Eco*RI, *Hind*II, *Hind*III and *Pst*I were purchased from Boehlinger, while *Bgl*I, *Hpa*I, *Hpa*II, *Hae*II and *Hae*III were obtained from the Miles Laboratories. DNase I and RNase T_1 came from Worthington Biochemical Corp. and Sanko Co. Ltd., respectively. RNase A and ethidium bromide were received from Sigma Chemical Co. Seakem agarose was from Marine Colloids Inc. The rest of the chemicals used were reagent grade.

mtDNA preparation from mice: The mtDNAs were prepared by either the SDS-phenol method or the cleared-lysate method. The former method was used for more than four mice and mainly for a litter of mice under inbreeding. Mitochondria and crude mtDNAs were prepared as described by YONEKAWA *et al.* (1978). mtDNAs were further purified by CsCl-ethidium bromide density-gradient centrifugation at 36,000 rpm for 40 hr (Hitachi, RPS-40T-2 rotor). The fractions containing closed circular and open ciruclar mtDNAs were collected separately. Both fractions were used for restriction analysis. Contamination of the open circular fraction with nuclear DNA could be efficiently eliminated by treating mitochondria with DNase I (0.2 mg/ml final concentration, 37°, 30 min). Conditions for removal of the dye and storage of mtDNA were described by YONEKAWA *et al.* (1980). For electron microscopic observation, the open circular fraction was used (YONEKAWA *et al.* 1978).

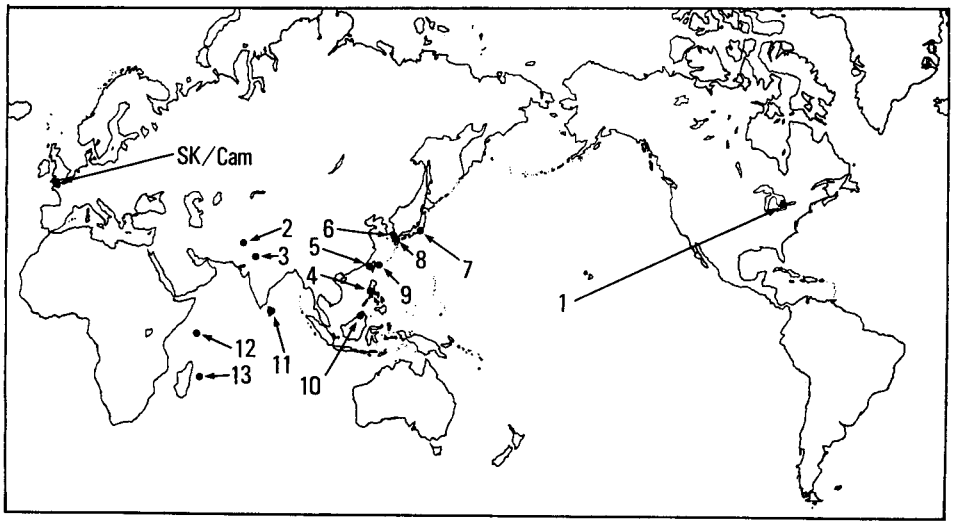


FIGURE 1.—Collection localities of wild mice: (1) *M. m. domesticus*, Windsor (Canada); (2) *M. m. bactrianus*, (Afghanistan); (3) *M. m. bactrianus*, Lahole (Pakistan); (4) *M. m. castneus*, Quezon City (Philippines); (5) *M. m. castaneus*, Taichung (Taiwan); (6) *M. m. molossinus*, Tsushima (Japan); (7) *M. m. molossinus*, Mishima (Japan); (8) *M. m. molossinus*, Hakozaki (Japan); (9) *M. m. molossinus*, Yonaguni Island (Japan); (10) *M. m. subsp.* A, B, Kota Kinabal (Borneo); (11) *M. m. urbanus*, Banderuwela (Sri Lanka); (12) *M. m. subsp.* D, Seychelles; (13) *M. m. subsp.* C, Mauritius.

The cleared-lysate method was used for fewer than three mice, mainly for wild mice. Liver mitochondria were incubated with 0.2 mg/ml DNase I at 37° for 30 min in 10 ml of 0.25 M surose, 50 mM $MgCl_2$ and 10 mffl Tris-HCl (pH 7.0). An equal volume of 0.1 M EDTA, 0.15 M NaCl and 10 mM Tris-HCl (pH 8.0) was added to the mixture, and then the mitochondria were spun down. The mitochondria were completely lysed by suspending the pellet in 2.5 to 3 ml of 0.6% sarcosyl, 10 mM EDTA and 10 mM Tris-HCl (pH 8.0). The lysate was heated at 60° for 10 min and then dialyzed against the above buffer. The volume of the lysate was adjusted to 3 ml by the same buffer,and 3 grams of solid CsCl and 0.2 ml of 4.6 mg/ml ethidium bromide were added to the lysate and mixed well. The following procedures were the same as those of the SDS-phenol method.

Restriction endonuclease digestion and gel electrophoresis: The mtDNAs were digested by incubating them at 37° for 1 hr with appropriate amounts of the enzymes under the conditions described by the suppliers (Boehlinger and Miles Laboratories). Agarose slab gel (1% agarose) and polyacrylamide gel (4% acrylamide) electrophoresis were carried out as described by HAYWARD and SMITH (1972) and HAYASHI *et al.* (1979). After electrophoresis, the gels were stained with 0.1 μg/ml ethidium bromide and photographed as described by YONEKAWA *et al.* (1978).

Determination of molecular weights of restriction fragments of the mtDNA: Molecular weights of restriction fragments of mtDNAs from *M. m. bactrianus*, *M. m. castaneus* (*M. m. urbanus*) and *M. m. molossinus* were determined by comparing their relative mobilities to those of standard restriction fragments, as described by YONEKAWA *et al.* (1980).

Mapping of cleavage sites on mtDNAs of three Asian subspecies: Mapping of cleavage sites on mtDNA molecules was carried out by the partial digestion or double digestion method. Sites mapped within 1% genome length were regarded as common sites.

Estimation of sequence divergence from the restriction enzyme cleavage maps: Nucleotide sequence divergence and time of divergence were calculated by the maximum likelihood method

TABLE 1

The subspecies of Mus musculus *used, their sources and the set of restriction enzyme cleavage patterns of mtDNAs obtained for each sample*

Subspecies		Condition	Sex and no. ♂	Sex and no. ♀	Sources	Restriction enzyme cleavage patterns* Bm	Ec	H2	H3	He2	Hp1	Ps	Bg	Hp2	He3
M. m. domesticus		Wild populations	25	3	Windsor, Canada‖	A	A	A	A	A	A	A	A	A	A
		SK/Cam (inbred strain)		1§	Skokholm Island, England¶	A	A	A	A	A	A	A	A	A	A
M. m. bactrianus		Under inbreeding in Ms†		1§	Afghanistan**	C	C	B	B	B	B	B	B	B	B
		Under inbreeding in Ms		1§	Lahole, Pakistan	C	C	B	B	B	B	B	B	C	C
M. m. castaneus		Under inbreeding in Ms		4§	Quezon City, Philippines	C	B	C	B	C	C	B	A	D	D
		Under inbreeding in Ms		2§	Taichung, Taiwan	C	B	C	B	C	C	B	A	D	E
M. m. molossinus		Wild populations	12	11	Mishima, Japan	D	B	A	B	C	A	B	A	A	F
		Wild populations	4	3	Hakozaki, Japan	D	B	A	B	C	A	B	A	A	F
		MOT (inbred strain established in Ngo‡)		1§	Tsushima, Japan	D	B	A	B	C	A	B	A	A	F
		Wild populations	3	1	Yonaguni Island	D	B	A	B	C	A	B	A	A	F
M. m. urbanus		Under inbreeding in Ms		1§	Banderuwela, Sri Lanka††	C	B	C	B	C	C	B	A	—	—
Mus musculus subsp.	A	Wild population		1	Kota Kinabal‡‡	C	C	B	B	B	B	B	B	C	—
	B	Wild population		1	Kota Kinabal, Borneo‡‡	C	D	C	B	C	C	B	A	D	—
	C	Under inbreeding in Ms		3§	Mauritius	A	A	A	B	A	A	A	A	A	A
	D	Under inbreeding in Ms		3§	Seychelles	A	A	A	B	A	A	A	A	A	A

* Abbreviations used are Bm: *Bam*HI, Ec: *Eco*RI, H2: *Hind*II, H3: *Hind*III, He2: *Hae*II, Hp1: *Hpa*I, Ps: *Pst*I, Bg: *Bgl*I, Hp2: *Hpa*II and He3: *HaeIII*. Letters refer to the types of cleavage patterns in order of discovery in each enzyme, as exemplified by the letters under the photographs in Figures 2, 3.

† Ms; K. Moriwaki, National Institute of Genetics, Mishima, Japan.

‡ Ngo; K. Kondo, Nagoya University, Nagoya, Japan.

§ Original dam(s) when the inbreeding started.

‖ Collected at four different localities near the city (see text).

¶ See Staats (1976); Gift from M. Nishimura of Wakayama Medical College.

** Exact localities are unknown because of difficulties with transporting the original mice.

†† See Phillips (1935).

‡‡ Gift from M. Harada of Osaka City University, Medical School, Osaka, Japan.

described by GOTOH *et al.* (1979). For computation of intersubspecific nucleotide sequence divergence, cleavage maps with six-base enzymes were used. For estimation of intrasubspecific nucleotide sequence divergence of *M. m. bactrianus* and *M. m. castaneus*, restriction patterns with two kinds of four-base enzymes (*Hpa*II and *Hae*III) were also used. In the latter case, as cleavage maps have not been constructed, a change of one restriction fragment in the pattern was counted as a change of one cleavage site.

Times of divergence were calculated on the assumption that the branch point between rat and mouse was 10^7 years ago (KOHNE 1970). This standard divergence time is tentative. If a better standard is obtained, our estimates of divergence time should be corrected by a constant factor.

RESULTS

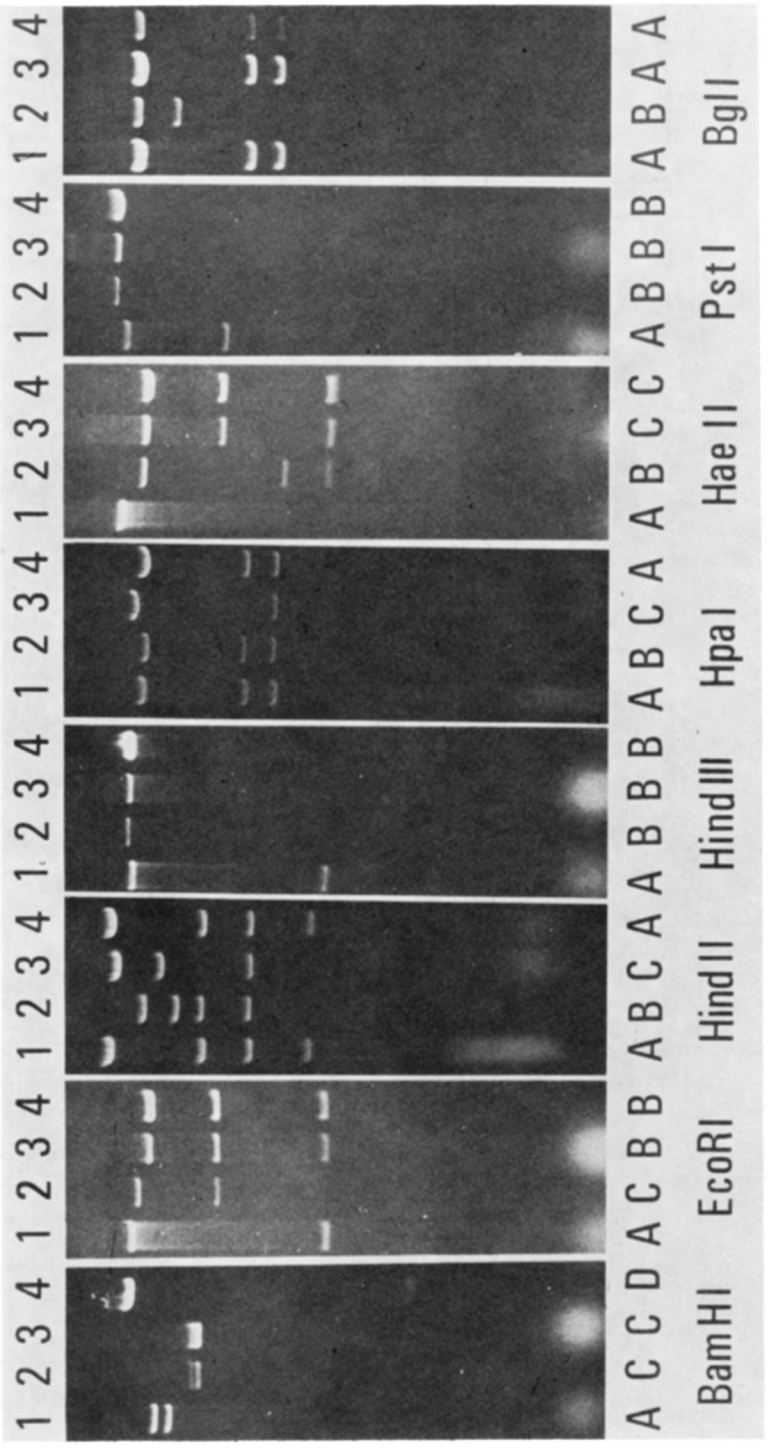
Figure 2 shows a comparison of the cleavage patterns of four subspecies of *Mus musculus* with eight restriction endonucleases: *Bam*HI, *Eco*RI, *Hind*II, *Hind*III, *Hae*II, *Hpa*I, *Pst*I and *Bgl*I. Four distinct patterns were seen in the five subspecies of mice studied. The cleavage patterns of mtDNA from *M. m. urbanus* were identical to those of *M. m. castaneus* with the eight enzymes used (Table 1).

The cleavage pattern observed with each enzyme was designated by a letter. (See Figure 2 and 3 for the features of each pattern.) By using such designations, a set of mtDNA cleavage patterns can readily be summarized for any group of organisms (Table 1).

Previous studies (YONEKAWA *et al.* 1980) showed no differences among the mtDNA cleavage patterns of 25 strains of laboratory mice, which were identical to the patterns of Canadian wild *M. m. domesticus* trapped at four different localities 10 to 30 km apart. The same cleavage patterns were also observed in mice of the inbred strain SK/Cam, which originated from European *M. m. domesticus*. In these studies two four-base enzymes (*Hpa*II and *Hae*III) were involved. No heterogeneity has been detected even with the four-base enzymes. These results suggest that intrasubspecific heterogeneity in *M. m. domesticus* mtDNA is very small, although minor heterogeneity can possibly be detected by increasing the sample size and the number of restriction enzymes (NEI and TAJIMA 1981).

Similar results were obtained for the four distinct races of *M. m. molossinus* (from distantly separated Japanese islands); that is, they showed no intrasubspecific variability in the cleavage patterns of mtDNAs with the same set of restriction enzymes as used for *M. m. domesticus*. The mtDNAs from different populations of *M. m. bactrianus* and of *M. m. castaneus* on digestion with *Hpa*II and *Hae*III differed (Figure 3), but the differences were infinitesimal; only one fragment change was detected in each case. The mtDNA cleavage patterns from the various subspecies were quite distinct.

Figure 2 shows that *Hind*III and *Pst*I digestions gave identical cleavage patterns for mtDNAs from three Asian subspecies (type B), *Bam*HI for *M. m. bactrianus* and *M. m. casteneus* (type B), and *Hae*II and *Eco*RI for *M. m. casteneus* and *M. m. molossinus* (type C and B). On the other hand, *Hind*II, *Hpa*I *and Hpa*II digestions gave identical cleavage patterns for mtDNAs from *M. m. domesticus* and *M. m. molossinus* (type A) and *Bgl*I for *M. m. domesticus*, *M. m. castaneus* and *M. m. molossinus* (type A). These results suggest that Asian subspecies are


1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
A C C D A C B B A B C A A B B B A B C A A B C C A B B B B A B A A
BamHI EcoRI HindII HindIII HpaI HaeII PstI BglI

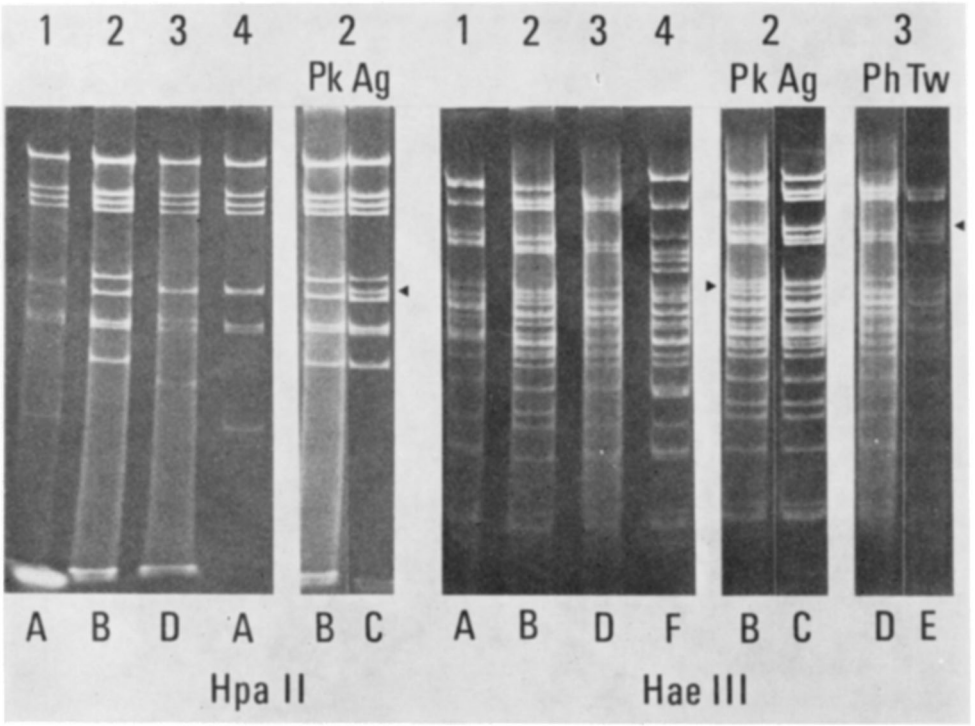


FIGURE 3.—Comparison of mtDNA cleavage patterns from four subspecies of mice and two local races of *M. m. bactrianus* and *M. m. castaneus* after digestion with *Hpa*II and *Hae*III. All digested mtDNAs were electrophoresed in 4% polyacrylamide slab gels. Pk, Af, Ph and Tw represent the mice trapped in Pakistan, Afghanistan, Philippines and Taiwan, respectively. Others are the same as those shown in Figure 2. "◀" indicates the occurrence of additional band.

closely related with one another, although *M. m. molossinus* is slightly different from the other two. The cleavage patterns with *Hind*II, *Hpa*I and *Hpa*II of *M. m. molossinus* are identical to those of *M. m. domesticus*, while those of the other Asian mice are not (Table 1).

Since at least one of the restriction enzymes gave cleavage patterns that were common to two or more subspecies (*e.g.*, *M. m. bactrianus*, *M. m. castaneus* and *M. m. urbanus* with *Bam*HI, *M. m. domesticus* and *M. m. molossinus* with *Hind*II, *Hpa*I and *Hpa*II), we suggest that the mtDNAs from the five subspecies have identical molecular weight. The accuracy of determining the sizes of the fragments does not preclude the existence of small deletion/insertion or small fragments invisible in the gels. However, this may not bring about serious errors in the estimates of total molecular weights or nucleotide sequence divergence

FIGURE 2.—Comparison of mtDNA cleavage patterns from four subspecies of mice. (1) *M. m. domesticus*, (2) *M. m. bactrianus*, (3) *M. m. castaneus*, (4) *M. m. molossinus*. The mtDNAs after digestion with a restriction enzyme were electrophoresed in 1% agarose slab gels. Letters under the photographs refer to the type of cleavage patterns observed.

(GOTOH *et al.* 1979). The molecular weight of total mtDNA genome was estimated to be $(10.6 \pm 0.25) \times 10^6$ [(16,000 ± 380) base pairs] with gel electrophoresis, and $(10.7 \pm 0.54) \times 10^6$ [(16,200 ± 810) base pairs] by electron microscopy using *E. coli* plasmid pBR322 (2.88×10^6) [4362 base pairs] as a molecular weight standard (SUTCLIFFE 1979). The molecular weight of each restriction fragment of these mtDNAs was estimated, as described in MATERIALS AND METHODS and is summarized in Table 2. Most molecular weights of some of the large fragments were estimated by gel electrophoresis, but molecular weights of some of the larger fragments were estimated by determining the relative length of the fragment to that of whole mtDNA molecule using electron microscopy (Table 2).

Cleavage maps of the mtDNAs were constructed by partial digestion and by double digestion techniques. *Hind*II maps were derived from *Hpa*I maps as described by MOORE *et al.* (1977). The cleavage maps are shown in Figure 4. The map of *M. m. domesticus* was identical to those described by PARKER and WATSON (1977) and MOORE *et al.* (1977).

Comparing these maps, we found that, for a particular endonuclease, some cleavage sites were common to all four and others absent in one or more of the maps. The numbers of common and different sites are: 1 and 3 for *Eco*RI, 1 and 1 for *Pst*I, 2 and 4 for *Bam*HI, 2 and 5 for *Hind*II, 2 and 1 for *Hind*III, 2 and 2 for *Hpa*I, 1 and 3 for *Hae*II and 3 and 1 for *Bgl*I, respectively. While the variable sites appear to be scattered over the mtDNA molecules, common sites seem to be clustered on the molecules at 20–30% and 65–90% genome units (Figure 4). The latter clustered region includes the mitochondrial rRNA genes (BATTEY and CLAYTON 1978).

Quantitative estimates of nucleotide sequence divergence between mtDNAs of these five subspecies of *Mus musculus* were obtained by the method described by GOTOH *et al.* (1979). This method, although similar to those described by NEI and LI (1979) and KAPLAN and LANGLEY (1979), is preferred because it takes into account redundant sequences that are recognized by some restriction enzymes used in this study.

The divergences between two races of *M. m. bactrianus* and of *M. m. castaneus* (intrasubspecific divergences) were estimated from their cleavage maps (Figure 4, Table 3) and patterns (Figure 3). The numbers of common and different fragments in the patterns between the races of *M. m. bactrianus* are 10 and 1 for *Hpa*II and 23 and 1 for *Hae*III. These numbers in *M. m. castaneus* are 9 and 0 for *Hpa*II, and 22 and 1 for *Hae*III. Since restriction maps of these four-base enzymes have not been constructed, the numbers of common and different sites were inferred from those of the fragments (see MATERIALS AND METHODS). In *M. m. bactrianus*, the nucleotide sequence divergence between the two races was estimated to be less than 0.4% and, in *M. m. castaneus*, less than 0.3%. In both subspecies, the values for time of divergence of these races were calculated to be $0.1–0.2 \times 10^6$ years. These values are comparable to those NEI (1975) obtained for European subspecies through protein polymorphisms.

TABLE 2

Molecular weights of mtDNA restriction fragments from the mouse subspecies examined

Enzyme	Molecular weights ($\times 10^6$)			
	M. m. domesticus	*M. m. bactrianus*	*M. m. castaneus*	*M. m. molossinus*
*Bam*HI	5.2	3.6	3.6	10‡
	4.8	3.4(d)†	3.4(d)†	0.7
	0.4	0.7	0.7	—
	0.2	—	—	—
Total	10.6	11.1	11.1	10.7
*Eco*RI	9.3	8.3	6.4	6.4
	1.3	2.9	2.8	2.8
	0.1	—	1.3	1.3
Total	10.7	11.2	10.5	10.5
*Hind*II	5.9	3.6	5.0	5.9
	2.1	2.7	3.2	2.1
	1.5	2.1	1.5	1.5
	1.0	1.5	0.5	1.0
	0.2	0.5	0.2	0.2
	—	0.2	—	—
Total	10.7	10.6	10.4	10.7
*Hind*III	8.9	9.6	9.6	9.6‡
	1.3	0.5	0.5	0.5
	0.5	—	—	—
Total	10.7	10.1	10.1	10.1
*Hpa*I	7.1	6.4	8.7	7.1
	2.1	2.1	1.5	2.1
	1.5	1.5	0.5	1.5
	—	0.5	—	—
Total	10.7	10.5	10.7	10.7
*Hae*II	10.7‡	7.0	7.2	7.1
	—	1.6	2.6	2.6
	—	1.2	1.2	1.2
	—	1.0	—	—
Total	10.7	10.8	11.0	11.0
*Pst*I	8.1	10.6	10.6	10.6‡
	2.6	—	—	—
Total	10.7	10.6	10.6	10.6
*Bgl*I	7.0	6.8	7.0	7.0
	1.9	3.5	1.9	1.9
	1.5	—	1.5	1.5
Total	10.4	10.3	10.4	10.4

† Doublet.
‡ Based on electron microscopic study.

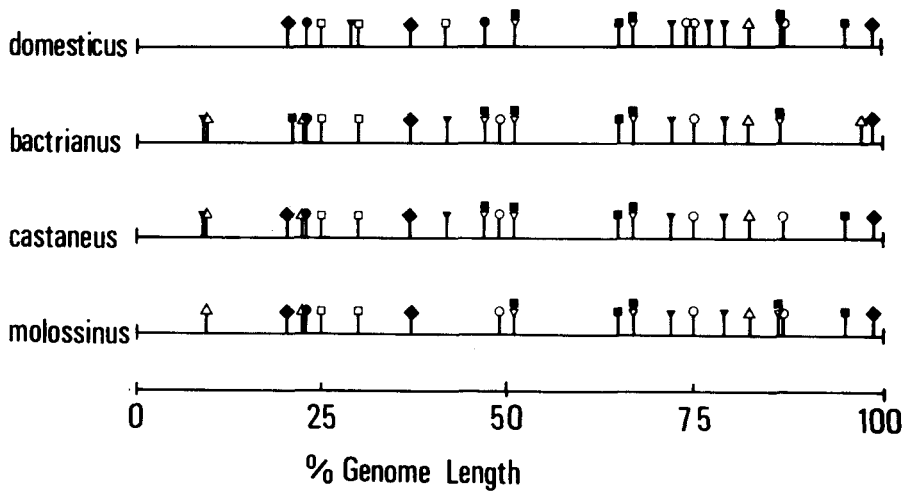


FIGURE 4.—Cleavage maps of mtDNAs from four subspecies of mice. Cleavage sites for individual restriction enzymes are identified by vertical lines topped with the following symbols: ▼ *Bam*HI, ○ *Eco*RI, ■ *Hind*II, □ *Hind*III, ▽ *Hpa*I, △ *Hae*II, ◆ *Bgl*I and ● *Pst*I. The linear map is arranged by assuming that the origin of DNA replication is at position 0. Length is given in percent of total genome.

TABLE 3

Numbers of cleavage sites that are common (c_i) or different (d_i) between pairs of Mus musculus *subspecies*

	M. m. domesticus vs.					
	M. m. bactrianus		*M. m. castaneus*		*M. m. molossinus*	
Enzyme	c_i	d_i	c_i	d_i	c_i	d_i
*Bam*HI	2	4	2	4	2	2
*Eco*RI	1	3	2	2	2	2
*Hind*II	4	3	4	2	5	0
*Hind*III	2	1	2	1	2	1
*Hpa*I	3	1	2	2	3	0
*Hae*II	1	3	1	2	1	2
*Pst*I	1	1	1	1	1	1
*Bgl*I	2	1	3	0	3	0

	M. m. bactrianus vs.				*M. m. castaneus* vs.	
	M. m. castaneus		*M. m. molossinus*		*M. m. molossinus*	
Enzyme	c_i	d_i	c_i	d_i	c_i	d_i
*Bam*HI	4	0	2	2	2	2
*Eco*RI	2	1	2	1	3	0
*Hind*II	4	3	4	3	4	2
*Hind*III	2	0	2	0	2	0
*Hpa*I	3	1	3	1	2	2
*Hae*II	3	1	3	1	3	1
*Pst*I	1	0	1	0	1	0
*Bgl*I	2	1	2	1	3	0

The nucleotide divergences between the European subspecies *M. m. domesticus* and two Asian subspecies, *M. m. bactrianus* and *M. m. castaneus*, were 5.7 to 7.1%, while that between the European subspecies and the other Asian subspecies *M. m. molossinus* was 3.2% (Table 4). The latter value is comparable to those obtained in comparisons between Asian subspecies (about 3%). These results show thatt he European subspecies *M. m. domesticus* is distantly related to the Asian subspecies *M. m. bactrianus* and *M. m. castaneus*. On the other hand, another subspecies, *M. m. molossinus*, is equidistantly related to the European and the other Asian subspecies, suggesting that *M. m. molossinus* occupies a unique phylogenic position in Asian subspecies.

Most of the *Mus musculus* subspecies studied have unique sets of cleavage patterns. However, some of the patterns, such as those of *M. m. urbanus*, were indistinguishable form those of *M. m. castaneus*. This suggests close relationship between *M. m. urbanus* and *M. m. castaneus*. Finally, this technique was applied to four groups of mice whose subspecies designation was not completely identified. As shown in Table 1, the cleavage patterns of mouse trapped in Borneo (*M. m.* subsp. A) were the same as that of *M. m. bactrianus*. On the other hand, the patterns of another Borneo mouse (*M. m.* subsp. B) were similar to those of *M. m. castaneus*, although the *Eco*RI cleavage pattern of this mouse differed from those of other mice (data not shown). Judging from the facts that the two mice were trapped in the locality where *M. m. castaneus* is common and that their morphologies were similar to *M. m. castaneus* but not to *M. m. bactrianus*, the former may be the hybrid between *M. m. bactrianus* and another subspecies, perhaps *M. m. castaneus*, while the latter is perhaps a variant of *M. m. castaneus*.

The patterns of mice originally trapped at Mauritius and the Seychelles were identical to each other (Table 1) and morphologically similar to *M. m. domesticus*. The cleavage patterns of their mtDNAs, except for the *Hind*III patterns,

TABLE 4

Sequence divergence of mtDNA and time of divergence between subspecies of Mus musculus *and their local races**

	Upper right half: Sequence divergence (%) P_0† (P_l–P_h)‡			
	M. m. domesticus	*M. m. bactrianus*	*M. m. castaneus*	*M. m. molossinus*
M. m. domesticus	—	7.1(5.8–9.6)	5.8(4.5–8.0)	3.2(2.5–5.0)
M.m. bactrianus	2.6(2.1–3.6)	—	2.6(2.0–4.2)	3.6(2.8–5.4)
M. m. castaneus	2.1(1.6–2.9)	1.0(0.7–1.5)	—	2.8(2.1–4.3)
M.m. molossinus	1.1(0.9–1.8)	1.3(1.0–1.9)	1.0(0.7–1.5)	—
Lower left half: Time of divergence (million years)§				

* Intrasubspecific heterogeneity was found in *M. m. bactrianus* (<0.4%, $<0.2 \times 10^6$ years) and in *M. m. castaneus* (<0.3%, $<0.1 \times 10^6$ years), but not in other subspecies. The effect of the intrasubspecific heterogeneity was neglected in calculating the intersubspecific heterogeneity (see Text).

† P_0; the most probable value.

‡ ($P_l - P_h$); a confidence interval of P_0 with confidence coefficient of 0.683.

§ Calculated from P_0 and ($P_l - P_h$) (see MATERIALS AND METHODS).

were, as might be expected, similar to those of *M. m. domesticus*. The *Hind*III patterns were identical to those of Asian mice. This result suggests that those mice are variants of *M. m. domesticus*.

DISCUSSION

In the present study, we found both intra- and intersubspecific heterogeneity of mtDNAs from five subspecies of *Mus musculus*. Table 4 shows the occurrence of intrasubspecific heterogeneity in two subspecies, *M. m. bactrianus*, which has 0.4% sequence divergence and *M. m. castaneus*, which has 0.3%, while heterogeneity was not detected in *M. m. domesticus* and *M. m. molossinus*. This intrasubspecific divergence in *Mus musculus* is comparable to that within human races (BROWN 1980), but smaller than that found in the local races of *Peromyscus* and *Geomys* (AVISE *et al.* 1979 a,b). A possible explanation for the reduced diversity observed in *Mus musculus* mtDNA as compared to other animals is as follows. The ancestral subspecies of the mice originally evolved in several isolated localities, probably in western Asia, and a small number of mice that had a uniform mtDNA sequence rapidly dispersed from there over a wide area in the world in association wiht human movements. For the example, *M. m. domesticus* in North America is thought to have been transported from Europe by Puritans and emigrants and *M. m. brevirostris* in South America from Spain (KEELER 1931; SCHWARZ and SCHWARZ 1943).

The number of variant forms of mtDNA in the sample is likely to depend largely on the sample size (NEI and TAJIMA 1981). However, the homogeneity found in *M. m. domesticus* and *M. m. molossinus* cannot be attributed to the proximity of collection localities because the Japanese mice examined here were obtained from four islands that are distantly separated from each other (Figure 1, Table 1). We also found that about forty strains and substrains of laboratory mice reveal no heterogeneous mtDNA, as judged by cleavage patterns with 8 to 10 restriction enzymes. Furthermore, these patterns were identical to those of *M. m. domesticus* trapped in Canada (YONEKAWA *et al.* 1980; unpublished results). Since at least one strain of inbred mice, SK/Cam, had its origin independent of the rest (Skokholm Island off the coast of Wales), *M. m. domesticus* appears quite homogeneous. These results suggest that the intrasubspecific heterogeneity of these mice is very small, although it can possibly be detected by increasing the sample size and using more restriction enzymes. In fact, the wild mice trapped in the Seychelles and Mauritius (Figure 1) show cleavage patterns similar to, but clearly different from those of *M. m. domesticus* (Table 1). Their morphology, biochemical markers, chromosome C-band patterns (MORIWAKI, unpublished) and the fact that these islands had long been governed by Europeans all strongly suggest that these mice are variants of *M. m. domesticus* or intermediates of subspecies differentiation.

Since *Mus musculus* subspecies have less divergent mtDNA populations than *Rattus*, *Geomys* and *Peromyscus* (HAYASHI *et al.* 1979; AVISE *et al.* 1979a, b), the restriction enzyme analysis of mtDNA facilitates the taxonomic reconstitution of the species. For example, though *M. m. urbanus* was considered an inde-

pendent subspecies in *Mus musculus* by SCHWARZ and SCHWARZ (1943), MARSHALL (1977) reported that the subspecies is the same as *M. m. castaneus*. Our finding that the cleavage patterns of *M. m. urbanus* mtDNA were identical to those of *M. m. castaneus* supports MARSHALL's view. Similarly, some Japanese taxonomist thought that there were at least four subspecies of *Mus musculus* in Japan, *M. m. yesonis* in Hokkaido, *M. m. orii* in Yaku-Island, *M. m. yonakuni* in Yonaguni-Island and *M. m. molossinus* in various localities in Japan (KURODA 1940). Our results, however, clearly show that the cleavage patterns of mtDNA from the mice trapped in Yonaguni-Island are identical to that of *M. m. molossinus* trapped in Mishima, Hakozaki and Tsushima (Figure 1, Table 1). Thus, it is probably more accurate to regard *M. m. yonakuni* not as a subspecies, but as one of the local races of *M. m. molossinus*. This will be discussed in greater detail elsewhere.

Though there have been a few reports on genetic distances between the various subspecies of *Mus musculus*, especially Asian subspecies (SELANDER and YANG 1964; SELANDER, HUNT and YANG 1969; MORIWAKI *et al.* 1979; BRITTON and THALER 1979; MINEZAWA, MORIWAKI and KONDO 1979, 1981), our results show that the two Asian subspecies *M. m. bactrianus* and *M. m. castaneus* are genetically distant from the European subspecies *M. m. domesticus*. The sequence divergence and time of divergence between them were estimated to be 5.8–7.1% and 2.1–2.5 $\times$ 10^6 years, respectively. However, the values for genetic distances between *M. m. molossinus* and the other two Asian subspecies are comparable to those between *M. m. molossinus* and the European subspecies (about 3%, 1 $\times$ 10^6 years; Table 3). Our estimation between *M. m. molossinus* and *M. m. domesticus* agrees well with values obtained from protein polymorphism (MORIWAKI *et al.* 1979; MINEZAWA, MORIWAKI and KONDO 1981).

These results shed light on the evolution of *M. m. molossinus*. Two opposite hypotheses on the evolution of this group have been proposed by SCHWARZ and SCHWARZ (1943) and by MARSHALL (1977). SCHWARZ and SCHWARZ proposed that *M. m. molossinus* originally developed in Far East Asia and formed a unique group within the species, as judged by their morphology and habitat. On the other hand, MARSHALL argued that *M. m. molossinus* belonged to the "*castaneus*" group, as judged by the characteristic morphology of their zygomatic plate. If MARSHALL's hypothesis were correct, the value for genetic distance of mtDNAs between *M. m. molossinus* and *M. m. castaneus* should be smaller than that between *M. m. molossinus* and *M. m. bactrianus* or *M. m. domesticus*. As shown in Table 4, however, this assumption is not supported by our results. On the other hand, if SCHWARZ and SCHWARZ were correct in their hypothesis, we should find many unique cleavage patterns in *M. m. molossinus*. These we did not find. Therefore, it is unlikely that *M. m. molossinus* has diverged greatly from the other subspecies. Comparing the mtDNA cleavage maps of *M. m. molossinus* and the other three subspecies, we found that most cleavage sites on *M. m. molossinus* mtDNA are common sites found in both the Asian subspecies and the European subspecies (Figure 4).

Based on the most probable values (P_0) in the genetic distance matrix (Table 4), we constructed phylogenetic diagrams by two different methods, the un-

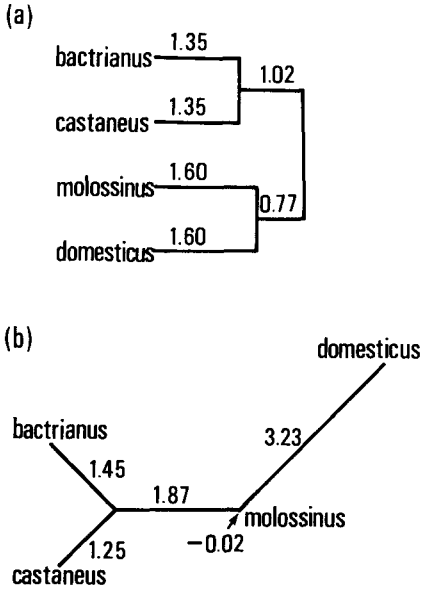


FIGURE 5.—Phylogenetic diagrams of four subspecies of *Mus musculus*. (a) Unweighted pair-group clustering method and (b) modified Farris method. The length of each branch is proportional to the sequence divergence of value (%) shown beside.

weighted pair-group clustering method (SOKAL and MICHENER 1958) and the modified Farris method (TATENO and NEI, unpublished) (Figure 5). The negative value in the *M. m. molossinus* branch obtained by the modified Farris may be regarded as 0, within the statistical ambiguity intrinsic to each P_0 value. These two diagrams show that *M. m. molossinus* is distantly related to other Asian subspecies. This, together with the results described above, suggests that *M. m. molosinus* occupies a unique position among the Asian subspecies. Unfortunately, we have no additional evidence to confirm this suggestion. However, of interest is the fact that the genetic distance between *M. m. domesticus* and *M. m. molossinus*, the latter coming from distantly separated localities, is smaller than that between *M. m. domesticus* and *M. m. bactrianus* or *M. m. castaneus*.

In conclusion, the mtDNA endonuclease cleavage patterns provide support for many of the subspecies that have been described within the species *Mus musculus*. The endonucleases used here are insufficient in number to determine the phylogenetic relationships within a subspecies. Population structure within local races of a subspecies will be refined by increasing the use of four-base restriction enzymes.

We thank Y. TATENO of The Physical and Chemical Research Institute for constructing phylogenetic diagrams, K. KONDO, H. NISHIMURA and M. HARADA for supplying wild mice or mouse strains and Y. NAKAMURA of The Institute of Medical Science, Tokyo University for providing the *E. coli* plasmid pBR322. We are also grateful to T. SHIROISHI of The National Institute of Genetics for helpful discussions and S. KUWABARA of Saitama Cancer Center and H. HIRAI of Kumamoto University for excellent technical assistance.

LITERATURE CITED

AVISE, J. C., R. A. LANSMAN and R. O. SHADE, 1979a The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus *Peromiscus*. Genetics **92**: 279–285.

AVISE, J. C., C. GIBLIN-DAVIDSON, J. LAERM, J. C. PATTON and R. A. LANSMAN, 1979b. Mitochondrial DNA clones and matriarchal phylogeny within and among geographical populations of the pocket gopher, *Geomys pinetis*. Proc. Natl. Acad. Sci. U.S. **76**: 6695–6698.

BATTEY, J. and D. A. CLAYTON, 1978 The transcription map of mouse mitochondrial DNA. Cell **14**: 143–156.

BERRY, R. J., 1980 The great mouse festival. Nature **283**: 15–16.

BRITTON, J. and L. THALER, 1979 Evidence for the presence of two sympatric species of mice (Genus *Mus* L.) in southern France based on biochemical genetics. Biochem. Genetics **16**: 213–225.

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

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

GOTOH, O., J.-I. HAYASHI, H. YONEKAWA and Y. TAGASHIRA, 1979 An improved method for estimating sequence divergence between related DNAs from changes in restriction endonuclease cleavage sites. J. Mol. Evol. **14**: 301–310.

HAYASHI, J.-I., H. YONEKAWA, O. GOTOH, Y. TAGASHIRA, K. MORIWAKI and T. H. YOSHIDA, 1979 Evolutionary aspects of variant types of rat mitochondrial DNAs. Biochim. Biophys. Acta **264**: 202–211.

HAYWARD, G. S. and M. G. SMITH, 1972 The chromosome of bacteriophage T_5. I. Analysis of the single stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. **63**: 383–395.

KAPLAN, N. and C. H. LANGLEY, 1979 A new estimate of sequence divergence of mitochondrial DNA using restriction endonuclease mappings. J. Mol. Evol. **13**: 295–304.

KEELER, C. D., 1931 *The Laboratory Mouse: Its Origin, Heredity and Culture*. Harvard University Press, Cambridge, Mass.

KLEIN, J., 1975 *Biology of the Mouse Histocompatibility-2 Complex*. Springer-Verlag, New York.

KOHNE, D. F., 1970 Evolution of higher organism DNA. Quart. Rev. Biophys. **3**: 327–375.

KURODA, N., 1940 *A Monograph of Japanese Mammals* (in Japanese). Sanseido, Tokyo.

MARSHALL, JR., J. T., 1977 A synopsis of Asian species of *Mus* (Rodentia, Muridae). Bull. Am. Museum Natl. Hist. **153**: 172–220.

MINEZAWA, M., K. MORIWAKI and K. KONDO, 1979 Geographical distribution of Hbb^p allele the Japanese wild mice, *Mus musculus molossinus*. Japan. J. Genetics **54**: 165–173.

MINEZAWA, M., K. MORIWAKI and K. KONDO, 1981 Geographical survey of protein variations in wild populations of Japanese house mouse, *Mus musculus molossinus*. Japan. J. Genetics **56**: 27–39.

MORIWAKI, K., T. SHIROISHI, M. MINEZAWA, T. AOTSUKA and K. KONDO, 1979 Frequency distribution of histocompatibility-2 antigenic specificities in the Japanese wild mouse genetically remote from the European subspecies. J. Immunogenetics **6**: 97–113.

MORSE III, H. C., 1978 *Origins of Inbred Mice*. Academic Press, New York.

MOORE, K. H., P. H. JOHNSON, S. E. M. CANDER and L. I. GROSSMAN, 1977 A restriction endonuclease cleavage map of mouse mitochondrial DNA. Nucleic Acids Res. **4**: 1273–1289.

NEI, M., 1975 Molecular population genetics and evolution. pp. 175–209. In: *Frontiers of Biology*. Vol. 40, North Holland, Amsterdam.

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

NEI, M. and F. TAJIMA, 1981 DNA polymorphism detected by restriction endonucleases. Genetics **98**: 145–163.

PARKER, R. C. and R. M. WATSON, 1977 Restriction endonuclease cleavage maps of rat and mouse mitochondrial DNAs. Nucleic Acids Res. **4**: 1291–1299.

PHILLIPS, W. W. A., 1935 Genus *Mus*. pp. 275–278. In: *Manual of the Mammals of Ceylon*. Dulau Co., Ltd, London.

SCHWARZ, E. and H. K. SCHWARZ, 1943 The wild and commensal stocks of house mouse, *Mus musculus* Linnaeus. J. Mammal. **24**: 59–72.

SELANDER, R. K. and S. Y. YANG, 1969 Protein polymorphism and genetic heterozygosity in a wild population of the house mouse (*Mus musculus*). Genetics **63**: 653–667.

SELANDER, R. K., W. G. HUNT and S. Y. YANG, 1969 Protein polymorphism and genetic heterogeneity in two European subspecies of house mouse. Evolution **23**: 379–390.

SHAH, D. M. and C. H. LANGLEY, 1979 Inter- and intraspecific variation in restriction maps of *Drosophila* mitochondrial DNAs. Nature **281**: 696–697.

STAATS, J., 1976 Standardized nomenclature for inbred strains of mice: Sixth listing. Cancer Res. **36**: 4333–4377.

SOKAL, R. R. and C. D. MICHENER, 1958 A statistical method for evaluating systematic relationship. Univ. Kansas Sci. Bull. **38**: 1409–1438.

SUTCLIFFE, J. G., 1979 Complete nucleotide sequence of the *Escherichia coli* plasmid pBR 322. Cold Spring Harbor Symp. Quant. Biol. **43**: 77–90.

UPHOLT, W. B. and I. B. DAVID, 1977 Mapping of mitochondrial DNA of individual sheep and goats: Rapid evolution in the D-loop region. Cell **11**: 571–583.

YONEKAWA, H., O. GOTOH, J. MOTOHASHI, J.-I. HAYASHI, and Y. TAGASHIRA, 1978 Positioning of the A·T-rich regions in rat mitochondrial DNA by electron microscopy and analysis of the hysteresis of denaturation. Biochim. Biophys. Acta **251**: 510–519.

YONEKAWA, H., K. MORIWAKI, O. GOTOH, J. WATANABE, J.-I. HAYASHI, N. MIYASHITA, M. L. PETRAS and Y. TAGASHIRA, 1980 Relationship between laboratory mice and the subspecies *Mus musculus domesticus* based on restriction endonuclease cleavage patterns of mitochondrial DNA. Japan. J. Genetics **55**: 289–296.

Corresponding editor: M. NEI