

# Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice

**ABSTRACT:** As judged by restriction analysis, mitochondrial DNA shows strictly maternal inheritance during 6-8 generations of backcrossing in both directions between *Mus domesticus* and *Mus spretus*. The average number of paternal mitochondrial genomes contributed to the next generation is estimated to be no more than one per thousand maternal mitochondrial genomes contributed. Despite the estimated accumulation of over 2000 mutational differences between *M. spretus* and *M. domesticus* mtDNAs since their divergence from a common ancestor, each of these mitochondrial DNAs, whether on a *M. spretus* or a *M. domesticus* nuclear background, allows mice to develop with seemingly normal viability and fertility.

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THE INHERITANCE of mitochondrial DNA (mtDNA) is predominantly maternal in all multicellular animals tested. These include three genera of mammals (*Equus*<sup>24</sup>, *Homo*<sup>17</sup>, and *Rattus*<sup>5,14,21</sup>), one genus of frogs (*Xenopus*<sup>9</sup>), and two genera of insects (*Drosophila*<sup>31</sup> and *Heliothis*<sup>25</sup>). The present study, which extends this generalization to mice (*Mus*), is notable because it spans eight generations and establishes that mtDNA inheritance can be at least 99.9 percent maternal. Previous studies of mtDNA inheritance in vertebrates spanned from one to three generations. Thus, they could not exclude paternal contributions as small as 0.1 percent per generation.

The species chosen for our investigation, *Mus spretus* and *M. domesticus*, are the most distantly related pair of mouse species known to be capable of producing fertile female hybrids<sup>20</sup>. The mtDNAs of these two species have been shown by restriction analysis to differ by more than 2000 mutations<sup>13</sup>. The present investigation of mitochondrial transmission during extensive backcrossing bears on attempts to understand the abilities of mtDNA to coevolve with nuclear DNA<sup>6</sup> and to invade both wild populations and laboratory strains of mice<sup>10-13,23,36</sup>. The results also are relevant to the use of backcrossing as a technique for saving species on the brink of extinction<sup>34</sup>.

## Materials and Methods

The inbred strain, C57BL/6J, of *M. domesticus* was purchased from the Jackson Laboratory, Bar Harbor, Maine in May 1982. One male and two females from a randomly bred strain of *M. spretus* were obtained in July 1981 from Dr. Richard Sage, University of California, Berkeley, who collected the original stock 7 km northwest of Azrou, Morocco in 1978.

Reciprocal crosses between these two parental strains were performed as follows: 1) *domesticus* female × *spretus* male; 2) *spretus* female × *domesticus* male. The F<sub>1</sub> females from the *domesticus* × *spretus* cross were then backcrossed to *spretus* males; the progeny of this cross are referred to as the BC<sub>1</sub> generation. Backcrossing of the female descendants to *spretus* males was continued for a total of five generations. Likewise, F<sub>1</sub> females from the *spretus* × *domesticus* cross were backcrossed to *domesticus* males; backcrossing was continued for seven generations (see Cover). Fertile female offspring were obtained in every generation and in both directions. By contrast, F<sub>1</sub> male hybrids were sterile. In backcross generations, some fertile males were observed, the frequency after the BC<sub>3</sub> generation being about as high as in the parental strains.

Guénet and Bonhomme<sup>20</sup> were unable to

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obtain live offspring without surgical procedures from the cross *Mus spretus* × *M. domesticus*. The present work shows that it is indeed possible to obtain progeny from this cross providing the *spretus* females have borne previous litters.

A model of mtDNA inheritance during

extensive backcrossing appears in Figure 1. It assumes that the ratio of the number of mtDNA molecules contributed by the sperm to that contributed by the egg is 0.0005 per generation<sup>22,27</sup>. There are two ways of testing this model, depending on which of two alternative assumptions is made about the effective

size of the mtDNA population in the germ line.

If the effective size is large (e.g.,  $10^3$ – $10^5$  molecules per cell), the variance in the proportion of paternal mtDNAs among backcross individuals will be small. In this case, the magnitude of the paternal contribution can be estimated by examining a small number of progeny after many generations of backcrossing. In recognition of the fact that the number of mtDNA molecules in an egg is indeed large<sup>22,27</sup>, the test we have conducted pertains to this case.

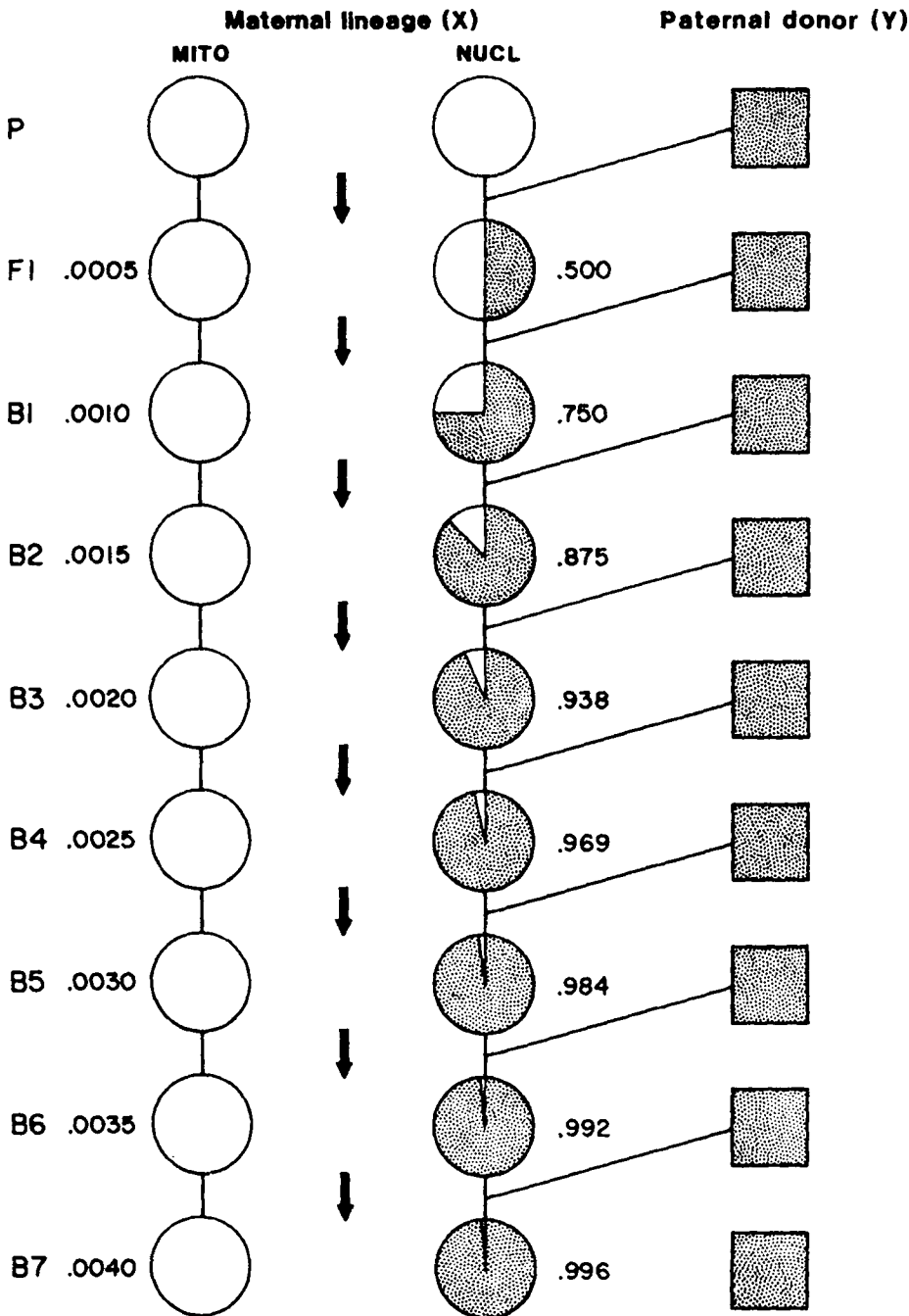
If the effective population is small (e.g., 1 to 10 molecules per cell), however, genetic drift will be an important factor. In this case, the variance among backcross individuals (in the proportion of paternally derived mtDNAs) will be large. Accordingly, a large number of individuals would need to be tested in order to detect paternally derived mtDNA; moreover, the number of backcross generations would not need to be large.

MtDNA was purified from the livers and kidneys of two specimens from each parental strain and three from each of the two final backcross progeny by isopycnic centrifugation in a solution containing caesium chloride and propidium diiodide<sup>4</sup>. The purified mtDNA was digested with three restriction enzymes, *HincII*, *AvaiI*, and *HindIII*, under the conditions recommended by the supplier (New England Biolabs, Beverly, MA). The fragments were then labeled by filling the 3' ends with ( $\alpha^{32}P$ ) dNTP, in the presence of the large fragment of DNA polymerase I (Klenow), and separated electrophoretically in 1.2 percent horizontal agarose gels. Fragments were visualized by autoradiography of dried gels at room temperature. A series of exposures allowed detection of minor components. Fragment sizes were determined by comparing them to the sizes of the mouse mtDNA fragments of known sequence<sup>1,13</sup> and to phage PM2 fragments generated by digestion with *HindIII*<sup>29</sup>.

## Results

### Parental MtDNA

The fragment patterns obtained with *HincII* are shown in Figure 2 for the parental strains in lanes 1 and 5. The *domesticus* pattern (lane 5) matches that expected for the old inbred type of mtDNA<sup>10,13</sup>, which has five *HincII* sites at nucleotide positions 364, 1858, 5123, 5452, and 7718<sup>1,13</sup>. Cleavage at these sites yields five fragments whose sizes in kilobases are 8.94, 3.27, 2.27, 1.49, and 0.33 (not seen in this gel). The seven visible fragments in the *spretus* pattern (lane 1) can



**FIGURE 1** Scheme illustrating one model of predominantly maternal inheritance of mtDNA during repeated backcrossing of a lineage derived from a female of species X to male(s) from species Y. P, parental generation; F<sub>1</sub>, first filial generation; B<sub>1</sub>–B<sub>7</sub> backcross generations (referred to as BC<sub>1</sub>–BC<sub>7</sub> in the text). Empty and solid symbols refer to genes from species X and Y, respectively. The numbers refer to the accumulated fraction of genes expected to be contributed by species Y to the descendants of X, assuming that there is no selection for or against paternal mtDNA or nuclear DNA and that the ratio of the numbers of mtDNA molecules contributed by the sperm to that contributed by the egg is 0.0005.

be explained by supposing that the Azrou type of *spretus* mtDNA contains all five of the *domesticus* *HincII* sites and four additional sites at positions 5754, 8637, 12658, and 15606. The old-inbred *domesticus* sequence has semisites at those four positions, which could mutate to sites by a total of four base substitutions (see also Ferris et al.<sup>13</sup>). Cleavage at these nine sites is expected to yield nine fragments, seven of which are visible on this gel, having the following sizes in kilobases: 4.02, 3.27, 2.95, 1.96, 1.49, 1.05, 0.92, 0.33 (not visible), and 0.30 (not visible). (The Azrou pattern differs slightly from that reported for a *spretus* mtDNA from Cadiz, Spain; the difference can be accounted for by a site gain at position 6222 in Cadiz mtDNA<sup>13</sup>.)

### Backcross MtDNA

Lanes 2-4 in Figure 2 show the fragment pattern obtained after backcrossing the female descendants of the *spretus* female × *domesticus* male cross to *domesticus* males for seven generations as illustrated in Figure 1. The pattern is clearly *spretus*-like; there is no hint of the presence of paternally contributed *domesticus* mtDNA in these lanes. Likewise, lanes 6 and 7 show the mtDNA fragments obtained after backcrossing the

female descendants of the *domesticus* female × *spretus* male cross to *spretus* males for five generations. They are clearly *domesticus*-like; there is no hint of the presence of a paternally contributed *spretus* mtDNA. Similar results were obtained when the enzymes *AvaI* and *HindIII* were used.

### Calculations

Reconstruction experiments conducted with *spretus* and *domesticus* mtDNA by Russell Higuchi and Christopher Martin at Berkeley indicate that under the conditions used, a minor component of 0.5 percent would have been detected in lanes 3 and 4, which have the most DNA. Similarly, we expect to have detected a 1 percent minor component in lanes 2 and 7 and a 2 percent minor component in lane 6, which has the least amount of DNA.

From these values, an upper limit can be calculated for the average proportion of mtDNA molecules inherited from the sperm per generation ( $s$ ), using equation 1

$$s = p/n \quad (1)$$

where  $p$  is the accumulated proportion of paternal mtDNA molecules at the  $n^{\text{th}}$  generation of backcrossing. This simplification of the equation given by Lansman et al.<sup>25</sup> is satisfactory only when  $s$ ,  $p$ , and  $n$  are small. Taking the results shown in lanes 3 and 4 of Figure 2 as giving the best chance to detect a paternal contribution by *domesticus*, we estimate, from  $P < 0.005$  and  $n = 8$ , that  $s < 0.0006$ . Likewise, from the results shown in lane 7, we estimate that the paternal contribution ( $s$ ) from *spretus* is probably no greater than 0.0017.

### Discussion

#### High ratio of egg to sperm MtDNA

The estimate from backcrossing experiments that at least 99.8 percent to 99.9 percent of the mtDNA molecules in a mouse are inherited from the mother can be considered in relation to the microscopical evidence that in mice, as in many other vertebrates, the midpiece of the sperm contains mitochondria and enters the egg<sup>30,33,35</sup>. The mouse sperm delivers about 50 molecules of paternal mtDNA<sup>22</sup> into the egg, which contains about  $10^5$  copies of maternal mtDNA<sup>22,27</sup>. From this, one calculates that the percentage of maternally derived mtDNA molecules in a fertilized mouse egg is about 99.9 percent. This estimate based on studies of sperm and eggs agrees with that based on our backcrossing experiments. The backcrossing experi-

ments, therefore, do not rule out the possibility of a paternal contribution to mtDNA inheritance.

### Coevolution of MtDNA and nuclear DNA

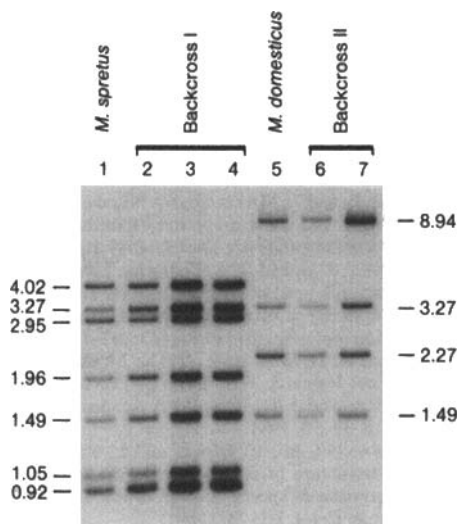
Our experimental results do render it unlikely that paternal mtDNA has a replicative advantage over maternal mtDNA in backcross mice. The possibility of such a replicative advantage merits discussion in the light of the evidence that mtDNA coevolves with nuclear genes.

Empirical evidence for coevolution comes from both functional tests and evolutionary rate comparisons. We refer to coevolution between cytochrome *c*, encoded by nuclear DNA, and cytochrome oxidase II, encoded by mtDNA<sup>6</sup>, and to that between nuclearly and mitochondrially encoded components of the translation apparatus<sup>6</sup>. Since the evidence for such coevolution comes from the comparison of rodents with other mammals, the time scale on which this phenomenon became evident is about 80 million years.

On theoretical grounds, such coevolution also is expected to occur in both the replication and transcription machinery. It is notable that 1) the proteins required for replication and transcription of mtDNA are encoded mainly or exclusively in the nucleus<sup>7,8</sup>, and 2) mtDNA evolves unusually fast, especially in the noncoding regions that contain the origins of replication and transcription<sup>1,7,8,12,13,19</sup>. This leads one to expect that those nuclearly encoded amino acid sequences that must recognize the mtDNA sequences might coevolve with them rapidly.

Conceivably, the ability of the *domesticus* DNA and RNA polymerases to recognize *spretus* mtDNA differs from their ability to recognize *domesticus* mtDNA and vice versa. Such a difference could affect the relative rates of replication of these two types of mtDNA on a given nuclear background. A possible outcome would be a higher rate of replication for the homologous mtDNA than for the heterologous mtDNA. In the case of extensively backcrossed mice, like those we have dealt with, the nuclear background is estimated to be predominantly of the paternal type (98.4 percent to 99.6 percent; see Figure 1) and thus could be conducive to faster replication of the paternal mtDNA (which is homologous) than of the maternal mtDNA (which is heterologous). The results of our experiments, however, suggest that if such a replicative advantage exists for the paternal mtDNA, it must be small.

The ability of mtDNA from one species to function on the nuclear background of another species has already been shown for four



**FIGURE 2** Electrophoretic separation of fragments made by digesting seven samples of mouse mtDNA with *HincII*. The sizes of the fragments, given in kilobases, were determined with reference to the known sizes of fragments from the mouse mtDNA of known sequence<sup>1</sup> and from the phage PM2 DNA digested with *HindIII*<sup>29</sup>. Lane 1, *spretus*; lanes 2-4, *spretus* maternal lineage after eight generations of crossing to *domesticus* males; lane 5, *domesticus* (C57BL/6J); lanes 6 and 7, *domesticus* maternal lineage after six generations of crossing to *spretus* males.

closely related pairs of mouse species: *domesticus* and *molossinus*<sup>10</sup>, *domesticus* and *musculus*<sup>2,12</sup>, *castaneus* and *molossinus*<sup>37</sup>, and *castaneus* and *domesticus* (Gyllensten and Wilson, unpub. data). In these cases, the extent of divergence between the mtDNAs compared is less than 5 percent<sup>13</sup>. The case of *spretus* and *domesticus* is more remarkable because the estimated divergence is about 13 percent<sup>13</sup>, which corresponds to over 2000 mutational differences. The nuclear genomes of these two species also are divergent as regards both repetitive sequences<sup>3</sup> and genes coding for proteins. There are, on average, 0.3 electrophoretically detectable substitutions per polypeptide compared, according to Sage<sup>32</sup>. Hence, many of the 200 or so nuclear encoded proteins that function in the mitochondria have probably accumulated one or more amino acid substitutions since the divergence of *spretus* and *domesticus*. Yet, each of those mtDNAs, whether on a *spretus* or a *domesticus* nuclear background, supports the development of extensively backcrossed mice with seemingly normal viability and fertility (D. Wharton, ms. in prep.). The implication is that these mutational differences are physiologically inconsequential or nearly so—an inference consistent with the hypothesis that most of molecular evolution could be due to the spread of neutral mutations. During the 3–6 million years that probably have elapsed since these two species had a common ancestor<sup>13</sup>, there seems to have been virtually no coevolution between nuclear and mtDNA in either species. (By contrast, in the case of two species of moths (*Heliothis*) that have been backcrossed for 91 generations, the male progeny are consistently sterile, a result that could indicate that coevolution has resulted in an incompatibility between the mtDNA of one species and the nuclear background of the other species<sup>26</sup>.)

#### Potential for restoration of species by backcrossing

In terms of mtDNA divergence, *spretus* and *domesticus* are among the most distantly related pairs of mammalian species known to produce fertile hybrids in either direction. The only such species known to exceed the *spretus*-*domesticus* divergence are certain pairs of macaque species whose mtDNAs diverge by 15 percent<sup>15</sup>. Indeed some pairs of species that are less divergent at the molecular level are incapable of producing fertile hybrids, e.g., the horse and donkey<sup>18</sup>, whose mtDNAs have diverged by about 6 percent<sup>16</sup>, or the gibbon and siamang<sup>28</sup>, whose mtDNAs also differ by 6 percent (Ferris,

Prager, and Wilson, unpub. data); the limiting factor in these cases is probably karyotypic divergence<sup>28</sup>. The fact that such divergent mtDNAs as those of *spretus* and *domesticus* can support normal development on the predominantly heterologous nuclear background implies that the use of backcrossing to rescue species on the brink of extinction will not necessarily be limited by mtDNA.

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