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Rapid Mutations in Mice?

Fitch and Atchley (*1*) analyzed genetic variation among inbred mouse strains and concluded that an extraordinarily increased mutation rate occurred. They hypothesized that early in the derivation of the lines there was selection for heterozygosity or increased mutation and concluded that classical population genetic theory cannot explain their data. It may be more simply and realistically concluded that inbred mice do not have extraordinarily high mutation rates for the reasons (i) that a biased sample of loci was used to establish the model for high rates, (ii) that an unbalanced comparison of inbred strains and natural populations was employed, and (iii) that appropriate consideration was not given to mutation rates determined by direct observation.

Fitch and Atchley's model (*1*) is based on the assumption that the loci they compared among inbreds represent a random sample of the mouse genome. Thus, the loci that they did not consider are presumed to vary in frequency and pattern in accordance with loci for which they present data. By their own calculations 300 or more invariant loci among the strains examined would invalidate their model. Several direct analyses of mouse strains by two-dimensional (2-D) electrophoresis indicate that the number of nonvariable proteins among inbreds may easily exceed 300 (*2*). The loci encoding these proteins were not included in the analysis (*1*). Furthermore, the data used by Fitch and Atchley are from a list of loci polymorphic among inbred strains (*3*); it does not contain monomorphic loci. Use of this list (*3*) to compare variation among strains will therefore show a high proportion of loci contributing to interstrain differences, but the proportion will be uninformative in terms of the mouse genome.

The most recent list of mouse loci (*4*), contains 47 reserved gene symbols indicating genetic variation known in man or other organisms but not yet documented in inbred mouse strains. Because there is generally great interest in mouse models it is unlikely that mouse strains have not been examined for a great many more specific loci than those reported to vary among the strains. Understandably, there has simply not been much incentive to maintain a formal documented list of monomorphic loci among inbred mice.

Another basis for the model of Fitch and Atchley is the statement that inbred strains are more variable than wild populations; however, they do not restrict their analysis of variation among inbreds to the same loci that have been studied in wild mice. The bias introduced into the analysis by comparing variabilities between different sets of loci is exemplified by the *H-2* complex, which was included in Fitch and Atchley's data for inbred strain but was not included in the studies cited by Fitch and Atchley for wild mice. The ten strains analyzed by Fitch and Atchley display five different *H-2* haplotypes, and divergence appears among the related C57BL/6, C57BR, and C58 strains as well as between the closely related DBA/1 and DBA/2 strains. This is a greater amount of variability than shown by typical allozymic loci. However, the same *H-2* loci have recently been typed in wild mice (*5*), and these mice also show an extremely high level of polymorphism. The average frequency of a given haplotype within a population was only 0.025 and more than 90 percent of the animals tested were heterozygous at *H-2*.

Similarly, restricting the comparison to loci other than *H-2*, and for which there are corresponding data from wild or ancestral populations and inbreds (Table 1), shows no evidence for an increase in the number of alleles, heterozygosity, or percent polymorphism in the inbreds (*3*, *6*). Thus, the inbreds contain homozygous subsets of the same alleles found in nature, just as expected from ordinary sampling and inbreeding. Small samples from nature with subsequent inbreeding sufficiently explain the high level of apparent parallel (or back) substitution observed by Fitch and Atchley in their phylogenetic analyses of the data (51.7 percent or 71 out of 145 substitutions).

Electrophoretic techniques by which the biochemical variants of inbred mouse strains are recognized have been used for the direct measurement of mutation rates in strains C57BL/6J and DBA/2J

Table 1. Allelic variants at polymorphic loci in both inbred strains and wild populations of *Mus musculus*. Inbred strain data are from Staats (*3*). Data for wild populations are given in (*6*). Total number of alleles in inbred strains, 33; total number of alleles in wild populations, 52; ND, no data available.

Protein locus	Inbred strains analyzed by Fitch and Atchley										Wild mice
	A/HE	AKR	BALB/c	CBA	C3H	C57BL	C57BR	C58	DBA/1	DBA/2	
Akp-1	b	b	b	b	b	a	a	a	a	a	a, b
Es-1	b	b	b	b	b	a	a	b	b	b	a, b, c
Es-2	b	b	b	b	b	b	b	b	b	b	a, b, c, d
Es-3	c	c	a	c	c	a	a	a	c	c	a, b, c, d
Es-5	b	b	b	b	b	b	b	b	b	b	a, b
Es-6	a	a	a	a	a	a	a	a	ND	a	a, b
Gpi-1	a	a	a	ND	b	b	ND	a	ND	a	a, b
Got-1	a	a	a	a	a	a	a	a	ND	a	a, b
Got-2	a	a	a	a	a	a	a	a	ND	a	a, b
Gpt-1	a	a	a	a	a	a	a	a	a	a	a, b, c
Gr-1	a	a	a	a	a	a	a	ND	ND	a	a, b
Hbb	d	d	d	d	d	s	s	s	d	d	s, d, p
Idh-1	a	b	a	b	a	a	b	a	b	b	a, b, c
Ldr-1	a	a	a	a	a	a	a	a	a	a	a, b
Mod-1	a	b	a	b	a	b	b	b	a	a	a, b
Mpi-1	b	b	b	b	ND	a	b	ND	ND	b	a, b
Np-1	a	a	a	a	a	a	ND	ND	a	a	a, b
Pgd-1	a	a	a	a	a	a	a	a	a	a	a, b
Pgm-1	a	a	a	a	b	a	a	a	b	b	a, b
Pgm-2	a	a	a	a	a	a	a	a	ND	a	a, b
Pre-2	b	a	a	a	b	b	a	ND	ND	a	a, b
Tsf	b	b	b	a	b	b	b	b	b	b	a, b

(*7*). In analysis for mutations, mice of the different strains are mated, offspring obtained, tissues removed surgically, and electrophoresis performed on the tissue homogenates. The methodology permits mutations arising in the parental strains to be recognized in the F_1 animals and confirmed by subsequent breeding. Newly arisen mutations are distinguished from preexisting mutations in the process.

Two categories of loci are represented in the electrophoretic analysis (*7*); loci that are homozygous for the same apparent allelic form in both strains and loci that show electrophoretic differences between the strains. In the former category are *Pgm-2*, *Pgd*, *Pep-7*, *Ldh-1*, *Ldh-2*, *Es-2*, *Mpi*, *Trf*, *Pep-2*, *Npi-1*, *Sod-1*, *Pep-1*, and *Gpd-X*; and in the latter, *Idh-1*, *Pep-3*, *Car-2*, *Gpd-1*, *Pgm-1*, *Ggc*, *Hba*, *Hbb*, *Gpi-1*, *Es-1*, *Mod-1*, *Pgm-3*, *Sep-1*, *Acy-1*, *Es-3*, and *Pre-1*. Many of these loci are among those analyzed by Fitch and Atchley.

To date, 40 induced and 9 spontaneous mutations have been found, and 45 of the total mutations have been compared with the available electrophoretic markers found in the common inbred strains (*7*). None of the 45 characterized mutations involves a switch to an alternative form characteristic of any alleles found among inbreds, in contrast to the prediction by Fitch and Atchley (*1*).

Approximately one-half of the induced and spontaneous mutations are nulls (*7*). Null mutations are most efficiently distinguished at loci for which allelic differences exist in the two parental strains. F_1 animals bearing null mutations have one of the two parental bands missing. The appearance of single-band null mutants thus may superficially resemble the effects of a mutation to the alternative parental allele. However, for every identified mutation of this type, a homozygous null phenotype has been isolated by intercrossing presumptive null heterozygotes. Therefore, there is no evidence for mutation in one parental strain resulting in the allelic form characteristic of the other strain, as predicted by the Fitch and Atchley model.

Eight of the nine spontaneous mutations found during electrophoretic analysis (*7*) were naturally preexisting low-frequency variants. One mutant was a newly arisen spontaneous event found among nearly 1 million loci tested for such events. In addition, one case of contamination was detected in which C57L mice were mixed with DBA/2 mice before shipment from the supplier. The mice in this contaminated lot were all nearly identical in coat color, but some animals showed numerous electrophoretic differences. Because a large number of loci were examined in the analysis, it was possible to identify the contaminating mice by their particular phenotype, as distinguished electrophoretically. It is impossible to exclude the possibility that contamination accounts for some of the pattern of variation found among inbreds.

Independent evidence for mutation rates of around 1×10^{-6} was provided by a general morphological (visible) analysis conducted by Schlager and Dickie (*8*). They effectively tested 1.3 to 6.9 million allele generations at each of 40 loci. Additionally, Russell and Kelly (*9*) reported a total of 30 spontaneous mutations in about 4,070,000 loci examined in mice, a spontaneous frequency of about 7.4×10^{-6}, on the basis of seven specific morphological loci. This frequency includes clusters of mutations originating from single events and thus the mutation rate determining the frequency must be somewhat lower, that is approaching 1×10^{-6}. Similar frequencies have been reported from laboratories in England (*10*) and Germany (*11*).

These data provide no evidence for an increased mutation rate in inbred mice.

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We would like to draw attention to two possible sources of error that may have led Fitch and Atchley (*1*) to an erroneously high estimate of the mutation rate in inbred strains of mice.

First, at least one of the differences between the DBA/1 and DBA/2 strains is not likely to have arisen by mutation and probably results from residual heterozygosity. The $H\text{-}2^d$ haplotype of DBA/2 and the $H\text{-}2^q$ haplotype of DBA/1 differ at practically every locus in the *H-2* region, a difference much greater than that seen in any newly arisen *H-2* mutations so far discovered in laboratory strains or crosses. If the *H-2* difference results from initial heterozygosity, the coefficient of kinship must be less than 1.0.

In considering how residual heterozygosity could still be present in 1929 in a strain said to have been inbred since 1909, we have speculated that what was called inbreeding in the early days of mouse genetics may have meant little more than maintaining a closed (and probably small) colony, and that later, when brother-sister mating became the usual practice, it was not common to control subline formation. Writing in 1916, Little and Tyzzer (*2*) say of the dilute brown stock (DBA), "All the present animals are direct descendants of a single pair of closely related, homozygous, dilute brown (silver fawn) mice obtained in the spring of 1909. From the start the stock has been kept free from any out-cross and has therefore an unbroken stretch of more than twenty generations of inbreeding." Nowhere is there a mention of brother-sister matings. The strain went through a bottleneck in 1921, when Little's colony at Cold Spring Harbor was wiped out by mouse typhoid, and the only living DBA's were a trio in an unpedigreed colony that Tyzzer had maintained at Harvard University since 1918. These were sent to L. C. Strong, who obtained offspring from them and provided some to Little (*3*). In 1929, both Little and Strong came to the newly founded Jackson Laboratory bringing their DBA strains with them. The history of the strain at this time becomes quite cloudy, but the two present substrains are said to have originated in 1929–1930, when

"some crosses were made between sublines and several new sublines [were] established" (*4*). If the trio rescued by Strong in 1921 were not highly inbred in the modern sense, and if the sublines that were crossed in 1929 originated not long thereafter, some residual gene differences may well have been transmitted to the two new sublines. The two DBA substrains are undoubtedly closely related, but we believe that the coefficient of kinship used by Fitch and Atchley is too high. A lower coefficient of kinship would lower the intercept of the curve in their figure 3 and result in a lower fixation rate.

Second, and more important, we believe that Fitch and Atchley have underestimated the extent to which unreported unvarying loci may be a source of bias. The loci listed by Staats that form the basis for their calculations cannot be taken as a random sample of loci that might or might not show variation. By definition, these were loci for which variants had been found. Numerous antigens and proteins are known, but their genes do not acquire allelic designations until a variant is discovered. The proportion of variant loci to the total is probably quite low. Elliott (*5*), for example, used two-dimensional electrophoresis to look for protein variants between BALB/c and C57BL/6 strains and found eight variants out of about 250 protein spots revealed by this method. These were proteins of unknown function. Among genes for proteins of known function for which no variants have been found, it is more difficult to determine the number examined. However, the chromosome location of some of these has been found by use of somatic cell hybrids. Twenty-one such loci were listed by Davisson and Roderick (*6*), and there were undoubtedly many more whose chromosome location had not yet been found and which Davisson and Roderick, therefore, did not list. The proportion of loci unreported, that is, not listed by Staats, may very well be high enough so that, if taken into account, the rate of mutation necessary to produce the observed degree of divergence would be close to normal. If so, it is not necessary to account for the preponderance of loci with only two alleles, since the degree of divergence can be explained largely by fixation of alleles present in the original population from which the strains were derived.

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Our original research article (*1*) had two primary aims: (i) to test various methods of reconstructing ancestral relationships with data from taxa whose ancestral relationships were largely known rather than inferred; and (ii) to infer something about the ancestral population structure and divergence of these taxa. Accordingly, 97 loci for ten inbred strains of mice were studied with respect to the first aim and the data proved so robust that five reconstructive techniques all obtained the same (correct) answer.

It is with respect to the second aim that our work has generated the greater interest, for we observed that the average degree of divergence for these loci among major stocks (approximately 45 percent) was greater than could readily be explained by a combination of the customary genetic mechanisms and the then known facts about these strains. It seemed to us that science is not well served when disconcerting data are explained away by plausible but unproved or unprovable folklore remedies, such as bias and contamination. Both of these processes often apply and probably are even present in our inbred mice data; however, it would require considerable special pleading to explain all of our data by bias and contamination. That left such standard genetic processes as mutation rates and the segregation of residual heterozygosity to account for our observation of 45 percent divergence. But we then had to choose between a mutation rate that was abnormally high and an ancestral heterozygosity that seemed too low to permit observing the large divergence. We showed how selection could double the ancestral heterozygosity of 9 percent, but a fivefold increase in genetic divergence is not an anticipated result of this standard genetic process.

Since the known facts appeared opposed to those standard genetic mechanisms and we knew of no other alternatives, we presented the results to the scientific community saying that "we are less concerned that one of the alternatives be proved correct than we are that an explanation for the documented divergence be sought that does not rely on the too facile explanation of contamination." This brings us to the comments of Johnson *et al.* and Green *et al.*

Johnson *et al.* state that we "concluded that an extraordinarily increased mutation rate occurred," and they assert that a "biased sample of loci was used" and "appropriate consideration was not given to mutation rates determined by direct observation." The facts show that these assertions of Johnson *et al.* are either wrong or irrelevant.

Never did we conclude there was an extraordinarily increased mutation rate. At one point, we stated, "because evidence appears to be against both bias and contamination explaining these data, the fixation rate may be equal to the mutation rate and, thus, there may be a high mutation rate in these strains (but see below)." That is as close as we ever came to espousing a high mutation rate. The "below" reference referred to an entire section labeled "*Evidence against a high mutation rate.*" Therein we cited the work of Johnson *et al.* (*2*) and clearly pointed out that their directly observed "mutation rate is two orders of magnitude below our fixation rate." To describe our observations, we deliberately used the term "fixation rate," not "mutation rate," since fixation is a term divorced from any implication of genetic mechanism to account for the large divergence.

Lest anyone doubt that we preferred the mutation rate data of Johnson *et al.* (*2*) over our estimated fixation rate, two paragraphs later we said, "Surely if mutations are occurring at a rate greater than 10^{-3} per locus per generation, many new mutations should have been observed in the generation of their initial occurrence." Johnson *et al.* (*2*) observed no new mutations in their study of inbred mouse strains that we cited.

The conversion hypothesis was considered because of the observation that there were only 2.01 alleles per locus, clearly inconsistent with a simple mutation mechanism. Recognizing that the experimental design of Johnson *et al.* (*2*) was particularly appropriate for providing evidence against the conversion hypothesis, we stated that "we know of no evidence that the mutation rate is increased in inbred mouse strains."

Later, when comparing the gene conversion hypothesis to the residual heterozygosity hypothesis, we returned to the data of Johnson *et al.* (*2*) to evaluate the hypothesis and stated, "In view of the low mutation rates observed in a study of inbred mice, this [mutation hypothesis] is probably the weaker of the two hypotheses." In the absence of a

definitive explanation for the observed divergence, we were reluctant to deny flatly any possible explanation.

Thus, we clearly did not conclude that there was "an extraordinarily increased mutation rate," nor did we fail to address the available refuting evidence.

In their comment, Johnson *et al.* assert that the data are biased. They contend the heterozygosity in these 97 loci is much larger than the average heterozygosity over all loci. They give several reasons, including (i) that our data source excluded monomorphic loci; (ii) that the *H-2* locus is atypically polymorphic; and (iii) that 2-D gel electrophoresis shows much less polymorphism. We accept all of these facts. However, these facts are irrelevant with respect to explaining the great divergence observed. At most, these statements imply that the 45 percent divergence we observed may be atypical of the genome in general.

The average locus in the genome may be less polymorphic than the ones in our data set, as 2-D gels suggest, but the divergence in our 97 loci must be explained in terms of their own ancestral heterozygosity. Our estimate was obtained from these 97 loci (our original figure 3) and therefore was the most appropriate estimate of this heterozygosity available despite a large confidence interval. We did not obtain significantly different results when we separated the data into protein and immunological loci, just larger standard errors. Thus, agreement with other values in the literature for similar protein sets was supportive of our conclusions. If one were to assume that the ancestral heterozygosity of our loci was 2 percent, as Racine and Langley (*3*) found using 2-D gels with a single sample of 25 wild mice, then the 45 percent divergence among our major stocks is not five times, but more than an order of magnitude greater than the ancestral heterozygosity, and therefore the divergence is even more difficult to explain by selection for heterozygosity.

Johnson *et al.*, sometimes with additional data, mostly cover ground that we covered. The only explanation they proffer for our observations is that "small samples from nature with subsequent inbreeding sufficiently explain the high level of apparent parallel (or back) substitution observed by Fitch and Atchley." Yet their comments do not indicate that they support our preferred hypothesis.

Green *et al.* make more relevant suggestions, although they too assert that we espoused a high mutation rate. They provide additional historical information about the DBA stocks. But Green *et al.* "speculate" that inbreeding "may" not have meant systematic brother × sister mating before the 1920's. This is a plausible explanation for the DBA results. It is even more difficult to refute or prove than the colony-contaminating mouse. As such, it is more likely to dissuade one from seeking a simple, testable explanation of the divergence on the basis of determinable facts.

Green *et al.* suggest that our value for the coefficient of kinship may be too high for the DBA stocks. If inbreeding before 1921 were not obligatory brother × sister mating, what would be an appropriate c_{ij}, one minus the coefficient of kinship, for the DBA's in 1929? What would be the effect of assuming they were not like identical twins ($c_{ij} = 0.0$), but rather had a value $c_{ij} = 0.25$? Reanalysis of our data shows that, for $c_{ij} = 0.25$ for the DBA's, the least-squares regression of d_{ij}, the fraction of loci different onto c_{ij} is

$$d_{ij} = 0.148(\pm 0.040) + 0.098(\pm 0.059)c_{ij}$$

for which the estimated ancestral heterozygosity is 0.11 (rather than 0.09) and the fixation rate is 1.3×10^{-3} (rather than 1.4×10^{-3}). Thus, a major change in the coefficient of kinship for the DBA strains produces only a minor effect on the estimates of ancestral heterozygosity or rate of fixation.

Green *et al.* cite divergence at the *H-2* locus in the DBA stocks as a basis for believing that our coefficient of kinship was too great. But varying c_{ij} has an insignificant effect on estimates of the two genetic parameters. However, their suggestion of a potential *H-2* locus effect stimulated us to consider the consequences of linkage to the *H-2* locus.

The *H-2* region has a locus *t* (for tailless) at which all known homozygotes are lethal. Heterozygosity at that locus is therefore assured. Moreover, the *t* locus suppresses crossing over in that region. Thus, there might be a hitchhiking effect on loci closely linked to the *t* locus. In our data, there are six closely linked loci in this region: *H*-2, *Qa*-2, *Qa*-3, *Qed*-1, *Ce*-2, and *Tla*. Four of these loci are different in the DBA/1 and the DBA/2 strains. To remove the potential effects of such linkage, we recalculated divergence between DBA/1 and DBA/2, excluding these six sites, and reevaluated the regression line in figure 3. The estimate of percent divergence between DBA/1 and DBA/2 is then based on 86 loci and is 0.08. The ancestral heterozygosity is then estimated as 0.17 or 0.19, depending upon whether c_{ij} for the DBA's was 0.0 or 0.25. The rate of fixation in either case remains just below 10^{-3}. Although these values for ancestral heterozygosity and fixation rate lie within the 95 percent confidence limits of our earlier estimate, they are perhaps more accurate, as they corroborate an independently derived estimate of ancestral heterozygosity made by another method described below.

Just before publication of our research article, Bishop *et al.* (*4*) reported that all six of the strains they tested that were among our ten possessed Y chromosome (male)–specific DNA sequences that are diagnostic of *Mus musculus musculus*. This should be considered in the context of the observation by Ferris *et al.* (*5*) that the mitochondria (female-derived) of our strains all came from *M. m. domesticus*, probably from an English female mouse. This is strong evidence that the "old" inbred lines, such as those examined in our research article, arose by a cross of a *domesticus* female and a *musculus* male. Such a cross could easily produce average heterozygosity values considerably greater than estimates for the wild-type populations of 0.09 for loci of the kind in our study.

We tested this hypothesis by using the data of Sage (*6*) and considering the expected heterozygosity of a cross between a mouse from Abingdon, England, and a mouse from Bratislava, Czechoslovakia. The result was a heterozygosity of 0.21. This is the value that the estimates for our data (minus the *H-2* region) support. While this is only half the divergence value to be explained, we noted in our original research article how one might double the genetic divergence by inbreeding. Thus, there is now evidence, not just speculation, to support the idea that the ancestral heterozygosity was 0.2 or more and that inbreeding accompanied by selection for heterozygosity can account for the remainder of the documented divergence.

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