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Still More Genetic Variability in Natural Populations

(heat-sensitive allozymes/gel electrophoresis/neutral and selection hypotheses)

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ABSTRACT **Heat-denaturation studies of xanthine dehydrogenase have revealed many more additional alleles at the locus controlling this enzyme than are revealed by electrophoretic studies. In natural populations of species in the virilis group of the genus *Drosophila*, heat-denaturation studies of flies from the same locality revealed 1.74 times as many alleles as did electrophoretic studies. Similarly, studies of several species over their geographic range also revealed 1.74 times as many alleles. In addition, for the nine species studied, electrophoretic analysis had revealed only 11 alleles within the group, whereas heat-denaturation studies revealed a total of 32 alleles. These findings are discussed in the light of the continuing controversy over Darwinian and non-Darwinian theories of evolution.**

Since the advent of electrophoretic techniques for the analysis of genetic variation in natural populations (1–3), numerous studies have confirmed and extended the original findings that a large proportion of the genome is subject to variation (4). The average insect may be heterozygous for as many as 20% of its genes, though for humans this figure may be nearer 5%. The extent to which this value is underestimated is not precisely known, but some have suggested that electrophoretic techniques overlook from $^2/_9$ to $^3/_4$ of the genetic variability per locus (5, 6).

It is widely appreciated that techniques for determination of the sequence of amino-acid residues of proteins could precisely define the amount of genetic variability at a structural gene locus. For enzymes, however, as opposed to luxury proteins such as the hemoglobins, sequencing techniques are enormously time consuming and costly. In this report we show that the simple technique of heat denaturation, performed on extracts from single adult *Drosophila*, discloses from two to three times as many alleles at the xanthine dehydrogenase (*XDH*) locus as were estimated previously. Heat denaturation has been used by others to detect differences between allozymes having different electrophoretic mobility (2, 7, 8), and Harris (9) has documented other quantitative differences in allozymes. To our knowledge, however, this is the first extensive study of heat-stability differences among allozymes sharing the same electrophoretic mobility.

MATERIALS AND METHODS

In this study we used 11 members of the virilis group of *Drosophila*. These included material from long-established laboratory cultures as well as freshly collected strains maintained as iso-female lines. Details of these collections will be published elsewhere (10). In this report we give results from 86 lines inbred from the original stocks to obtain strains exhibiting a single electrophoretic mobility for the enzyme xanthine dehydrogenase. This enzyme was chosen for several reasons. It seems to be representative of loci so far encountered in species of the virilis group. It is moderately polymorphic, certain of its alleles are widely shared among the species of the group, and many polymorphisms are common to several species (Table 1).

Electrophoretic analysis was done in 5.5% acrylamide gel. The xanthine dehydrogenase assay of Prakash, Lewontin, and Hubby was followed (11), except that the 100 ml of 0.1 M Tris buffer was modified by addition of 15 ml of 0.05 M hypoxanthine, and the pH was adjusted to 7.5 rather than to 8.5. Under our conditions, xanthine dehydrogenase migrates as a single band about 1 mm wide and the standard form travels about 3.5 cm. We consider two strains to possess alleles with identical mobility only when both the leading and trailing edges of adjacent bands on the same gel coincide. Samples from different strains are placed alternately on a gel, and under these conditions we can detect mobility differences of less than 0.5 mm under standard conditions. Our requirements for identical mobility are stringent, but they are not unrealistic. In selected cases we were able to show that such differences segregated in regular Mendelian fashion, and there is no doubt they reflect true genetic differences.

For the heat-denaturation studies we used a constant-temperature bath (Chicago Surgical and Electrical Co.) filled with light petroleum oil and outfitted with an automatic stirrer. The temperature is maintained at 71.5° ± 0.25°. This temperature was chosen empirically. At that temperature one of the most common alleles is completely inactivated after 15 min of heat treatment.

In preliminary studies extracts of individual flies were used, but occasionally it was difficult to obtain uniform extracts in this way. To avoid this difficulty, and because our lines were highly inbred, we considered it safe for our purposes to pool several flies per sample. Hence, eight flies from a given line were ground in 80 μl of 0.1 M TBE buffer.† The resulting suspension was then centrifuged in a 0.5-ml centrifuge tube at 21,500 × *g* for 3 min. Ten-microliter aliquots of the supernatant were placed into each of four 0.1-ml centrifuge tubes. These four tubes were then treated as follows: (*1*) maintained at 0° in an ice bath (control); (*2*) treated 5 min at 71.5° in the oil bath, then transferred to the ice bath; (*3*) treated 10 min in the oil bath, then transferred to the ice bath; and (*4*) treated 15 min in the oil bath, then transferred to the ice bath. The four samples were then layered in pockets in the gel and subjected to electrophoresis.

RESULTS

Heat treatment revealed clear differences among many of the

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† 0.1 M Tris-borate buffer, pH 8.9, containing 15 mM EDTA.

TABLE 1. *Distribution and relative mobility of 11 electrophoretic alleles of xanthine dehydrogenase in the virilis group*

Species	0.92	0.96	1.00	1.02	1.04	1.06	1.07	1.10	1.12	1.16	1.17
D. virilis		+	+		+						
D. a. americana	+	+	+	+	+						
D. a. texana		+	+								
D. novamexicana		+	+								
D. lummei					+		+		+		
D. littoralis			+			+	+	+			
D. ezoana								+	+		
D. flavomontana							+				
D. montana			+			+	+	+		+	+
D. borealis									+	+	
D. lacicola					+		+	+	+	+	

The *space* separates species belonging to two cytological phylads. The position of *D. lummei* is not known.

inbred lines. Four different categories of heat sensitivities were found. One form of the enzyme was inactivated after a 5-min treatment, two other forms of the enzyme were inactivated after 10 and 15 min, respectively, and the last form was still active after the 15-min treatment. The differences in heat sensitivity were repeatable and showed no intraline variability, even when 16 individuals were examined separately from each given strain. This observation confirms our expectation that inbreeding had rendered each line homozygous at the xanthine dehydrogenase locus.

For three species we had material that permitted us to assess variability for heat-sensitive alleles within local populations, although the number of samples varied from locality to locality (Table 2). The population of *D. montana* showed no change in variation. The populations of *D. a. americana* showed increases of 1.3- and 1.5-fold, and the population of *D. borealis* showed a 3-fold increase. Adjusting for the number of samples per locality, this averages to a 1.74-fold increase in the number of alleles per strain when heat-sensitive alleles are considered in addition to the electrophoretic alleles. Obviously our sample sizes are very small. Also, our method of isolating inbred lines by no means assured that all alleles of the original strains were detected. Hence, this increase, remarkable enough in itself, must be regarded as an underestimate of the variability in the samples we studied.

We also made a random sampling of alleles from strains of all available species in the group. These results are shown in Table 3. The sample sizes vary here also, but by adjusting for sample size and averaging over all strains, a 1.74-fold increase in the number of alleles per strain is seen here also.

When the alleles for the entire group are considered, a total of 32 temperature-sensitive alleles were discovered from among 11 electrophoretic alleles. This constitutes a 2.9-fold increase in variability for the group as a whole.

It is illuminating to note the manner in which these increases in variability affect previous estimates of genetic similarity between species. This information is given in Table 4. The matrix shows the proportion of alleles in common between species pairs when only electrophoretic alleles are considered (*above diagonal*) and the proportion in common when both electrophoretic and heat-sensitive alleles are considered (*below diagonal*). In comparing *D. virilis* and *D. a. americana*, three out of the five electrophoretic alleles shown by the two forms are shared. However, only four of thirteen temperature-sensitive alleles are in common between them. *D. borealis* and *D. lacicola* share one of five electrophoretic alleles, but they share none of 10 temperature-sensitive alleles. A similar pattern is seen for other comparisons, and generally the amount of genetic similarity between species is lessened when temperature-sensitive alleles are considered. A comparable phenomenon is seen for comparisons between localities (Table 2). For *D. a. americana* three out of four electrophoretic alleles are shared between two localities, but only one of nine heat-sensitive alleles is shared. These two samples were drawn from populations occupying different river systems in Nebraska, but they are separated from each other by only 30 miles, as the crow flies.

DISCUSSION

It is relatively simple to understand why heat-denaturation studies are able to easily reveal hidden variation among electrophoretically identical alleles. For example, the x-ray diffraction analysis of hemoglobin shows only two ionic and four hydrogen bonds holding the two β chains together; other subunits' interactions are few and generally weak (hydrogen bonds and Van der Waal interactions) (12). Thus, single amino-acid substitutions involving any of the amino acids involved in these interactions could change the heat denaturability of this molecule without necessarily changing mobility. Other proteins are known to be stabilized into their quaternary as well as their tertiary structures by a small number of inter- and intramolecular bonds. A large number of proteins are known to be multimers; xanthine dehydrogenase is known to be a multimer (13). We would expect heat denaturation to be a sensitive method of detecting amino-acid substitutions involved in the maintenance of quaternary and tertiary structure. How general is this phenomenon? Only a tentative answer can be offered. Lewontin (personal communication) examined the Esterase-$5^{1.00}$ allele in *D. pseudoobscura* and found three different heat-sensitive alleles. In our laboratory Dr. Rama Singh is finding much hidden variability at the octanol dehydrogenase locus in *D. pseudoobscura* and in various members of the virilis group. Further hidden variability thus promises to be widespread, both among loci and among organisms.

This finding raises interesting questions. Studies of electrophoretic variability to date have revealed two major patterns. One of these shows relatively constant gene frequencies throughout a species range (11). The other tends to reflect one or another of several environmental variables. Often the

TABLE 2. *Variation within localities*

Species	Locality	Iso-female lines examined	Electrophoretic alleles*			
D. a. americana	Woodriver, Nebr.	6	0.96	1.00	1.04	—
	Rockville, Nebr.	7	0.96	1.00	1.04	1.06
D. montana	Craig, Colo.	3		1.16	1.17	
D. borealis	Itaska State Pk., Minn.	5			1.16	

Species	Temperature-sensitive alleles*								
D. a. americana	—	0.96 (15)	1.00 (10)	—	1.00 (15+)	—	1.04 (10)	—	—
	0.96 (10)	0.96 (15)	—	1.00 (15)	—	1.04 (5)	—	1.04 (15)	1.06 (15+)
D. montana	1.16 (15+), 1.17(15)								
D. borealis	1.16 (10), 1.16 (15), 1.16(15+)								

* Alleles are given according to their electrophoretic mobility relative to the standard allele. For temperature-sensitive alleles, *numbers in parentheses* denote the number of min at which complete loss of activity is seen.

variation is clinal in nature and associated with gradients in temperature (14) or moisture (15). From such patterns it has been concluded that this genetic variability is maintained by some kind of selection. One may now wonder how many of these patterns will persist when the loci are examined for heat-sensitive alleles. Our two samples from *D. a. americana* are too small to be of great help in this regard, but they do emphasize the existence of the problem and the seriousness of the question. Separated by only 30 miles, they share only one out of nine temperature-sensitive alleles. The 30 miles may be somewhat misleading, for these are riparian forms and the routes of gene flow would be along the river banks. However, the rivers involved (Loup and Platte) have their confluence about 80 miles downstream from the collection sites so the river distance between the two populations would still be only on the order of 200 miles. If our results are not the result of sampling accident, and they may indeed be so, then the selection hypothesis may be seriously questioned, at least for the maintenance of variation of heat-sensitive alleles.

TABLE 3. *Variation within and between species*

Species	No. of strains	No. of electrophoretic alleles	No. of heat-sensitive alleles	Increase in no. of alleles
	Variation within a species			
D. virilis	10	3	6	2.0
D. a. americana	24	5	11	2.2
D. a. texana	4	2	2	1.0
D. novamexicana	4	2	2	1.0
D. lummei	4	3	3	1.0
D. littoralis	8	4	7	1.75
D. ezoana	4	2	3	1.5
D. montana	13	6	9	1.5
D. flavomontana	1	1	1	1.0
D. borealis	6	2	4	2.0
D. lacicola	8	5	7	1.4
	86		Average	1.74
	Variation between species			
	86	11	32	2.91

A second problem is raised by our data. In spite of the fact that great difference is seen between localities and species, alleles with identical electrophoretic mobilities and heat sensitivities are nonetheless found in populations widely separated geographically and in species quite distant phylogenetically. Within *D. a. americana* the "same" allele is found in populations from Nebraska, Montana, and Vermont. Between species, the "same" allele is found in *D. littoralis* (Finland) and *D. montana* (U.S.), another is shared between *D. ezoana* (Japan) and *D. montana* (U.S.) and so on. When shared alleles are proportionately so few and so widely scattered geographically, what confidence can we have that estimates of genetic similarity derived from electrophoretic studies are at all realistic? In such circumstances convergence becomes a very serious difficulty, and how is that difficulty to be resolved?

Hopefully, the resolutions of these questions will lie somewhere in the middle ground between the chaos intimated by these data and the too comforting patterns painted by electrophoretic studies to date. It is clear that in some systems, at least, genetic variability is far greater than had been suspected. Much additional work will be necessary before specific patterns of variability will be clear enough to allow sound generalization about mechanisms by which such variation is maintained. Existing data do, however, suggest the following as a point of departure. It would seem that for the alleles at the *XDH* locus we detect selection operating at two levels. At one level, selection "sees" on the average those alleles that exhibit changes in electrophoretic mobility of the molecule. Why this should be so we do not know, but in consequence of it electrophoretic alleles at this locus show coherent patterns of geographic distribution and are widely shared among species that

TABLE 4. *Matrix of the proportion of alleles in common between species pairs when electrophoretic alleles are considered (above diagonal) and the proportion in common when both electrophoretic and heat-sensitive alleles are considered (below diagonal)*

	D. virilis	*D. a. americana*	*D. a. texana*	*D. novamexicana*	*D. lummei*	*D. littoralis*	*D. ezoana*	*D. montana*	*D. flavomontana*	*D. borealis*	*D. lacicola*
D. virilis		0.60	0.67	0.67	0.20	0.17	0	0.13	0	0	0.14
D. a. americana	0.31		0.40	0.40	0.14	0.13	0	0.10	0	0	0.11
D. a. texana	0.14	0.18		1.00	0	0.20	0	0.14	0	0	0
D. novamexicana	0	0.08	0.33		0	0.20	0	0.14	0	0	0
D. lummei	0.13	0	0	0		0.17	0.25	0.13	0.33	0.25	0.60
D. littoralis	0.08	0.06	0.13	0	0		0.20	0.67	0.25	0	0.29
D. ezoana	0	0	0	0	0	0		0.14	0	0.33	0.40
D. montana	0.07	0.05	0.10	0	0.09	0.33	0.09		0.17	0.14	0.38
D. flavomontana	0	0	0	0	0.33	0	0	0.11		0	0.20
D. borealis	0	0	0	0	0.17	0	0	0.08	0		0.40
D. lacicola	0.08	0	0	0	0.42	0	0.25	0.14	0.14	0.10	

are closely related genealogically (10, 16). In contrast, temperature-sensitive alleles at this locus tend not to be "seen" by natural selection, and hence they are distributed erratically. Our data may thus support each of the two major hypotheses regarding the mechanisms of maintenance of genetic variability, since both selection and drift seem to be operating simultaneously to determine the patterns of alleles presently seen at the *XDH* locus, in which case the apparent conflict between the selectionist and neutrality hypotheses may be nonexistent. For any given constellation of alleles some fraction may reflect selection; the remainder may be selectively neutral. For most loci these fractions are presently unassessable. The *XDH* locus may be unusual chiefly in that alleles detected by different criteria show substantially different distributions and, hence, probably reflect the operations of different selective regimens. If this is the case, there would no longer be a serious question of whether one mechanism or the other operates generally, since both can be seen to operate always. The relevant question may not be so much which operates as how both operate, either together or in sequence, to generate the specific constellations of alleles seen in particular cases.

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