Analysis of distributions of single-locus heterozygosity as a test of neutral theory

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Summary

Three tests of neutral theory were carried out using a large dataset of vertebrate allozyme studies. The first test considered the relationship between the mean and variance of single locus heterozygosity across a sample of enzymes and non-enzymatic proteins. The second test compared the distributions of heterozygosity between paired proteins in balanced datasets in which each protein is scored for the same sample of species. The third test compared the observed distribution of single locus heterozygosity with theoretical distributions predicted by neutral theory. The results show an excellent quantitative fit with the predictions of neutral theory, though some small deviations from neutrality were observed which are consistent with the action of natural selection.

1. Introduction

Since Kimura first published his neutral theory of molecular evolution (Kimura, 1968*a*, *b*), emphasizing the effect of neutral mutation and genetic drift in an effort to account for protein polymorphism revealed in populations by electrophoretic techniques, there have been many approaches to testing predictions from neutral theory. These have ranged from a broadly neutralist (Chakraborty *et al.* 1978, 1980; Fuerst *et al.* 1977; Gojobori, 1982; Nei & Graur, 1984; Nei *et al.* 1976; Watterson, 1978; Yamazaki & Maruyama, 1972, 1974) to a broadly selectionist perspective (Ayala & Gilpin, 1973*a*, *b*, 1974; Nevo *et al.* 1984; Nevo & Beiles, 1988). The two parameters of equilibrium neutral theory which determine the levels of polymorphism and heterozygosity in natural populations are effective population size, N, and neutral mutation rate, u (Kimura & Crow, 1964; Ohta & Kimura, 1973). These parameters are very difficult to estimate in natural populations, but their theoretical manipulation is recognized as being less complex than the many parameters and variables of selection theory.

In this paper, the distributions of single locus heterozygosity (H) across vertebrate species for each of a number of different allozyme proteins are used to test a number of predictions of neutral theory. By strictly neutral theory, a plot of the variance of H against the mean of H should fall on a curve of increasing slope intersecting the origin, even if the data for those proteins differing in H are contributed by different species with different N (Gojobori, 1982). Within the context of equilibrium neutral theory, deviations above this curve could be caused by differences in the distribution of N or u between proteins across species. By contrast deviations below the curve could occur in the version of neutral theory incorporating slightly deleterious mutations (Ohta, 1973, 1976; Gojobori, 1982) or if heterozygosity is below the equilibrium expectation as a result of past bottlenecks in populations size (Gojobori, 1982). The relationship between the mean and variance of H has been considered by a number of authors (Fuerst *et al.* 1977; Gojobori, 1982; Nevo, 1983; Solé-Cava & Thorpe, 1991). The present study extends these analyses by carrying out the test using a set of vertebrate allozyme data considerably larger than that used in previous studies.

A consequence of this theoretical relationship between the mean and variance of H is that proteins with the same mean H are expected to have the same variance of H. Neutral theory is further tested in the present study by comparing the distributions of H within pairs of proteins in which each member of the pair has approximately the same mean heterozygosity. The comparison is carried out using a balanced dataset in which the same set of species is used for

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Table 1. *Proteins used for analysis with mean* ($\hat{H}$) *and variance* (S_H^2) *of heterozygosity, and number of species scored. Alternative names and abbreviations appear in parentheses.*

Protein abbreviations	Protein name	EC no.	$\hat{H}$	S_H^2	No. of species
ADH	Alcohol dehydrogenase	1.1.1.1	0·071	0·022	290
MDH	Malate dehydrogenase	1.1.1.37	0·032	0·010	719
α-GPDH	α-glycerophosphate dehydrogenase	1.1.1.8	0·069	0·019	531
IDH	Isocitrate dehydrogenase (NADP)	1.1.1.42	0·071	0·022	603
SDH	Sorbitol dehydrogenase	1.1.1.14	0·085	0·026	307
(*IdDH*)	(*Iditol dehydrogenase*)				
6PGDH	6-Phosphogluconate dehydrogenase	1.1.1.44	0·113	0·029	566
LDH	Lactate dehydrogenase	1.1.1.27	0·036	0·011	725
ME	Malic enzyme	1.1.1.4	0·092	0·031	339
G6PDH	Glucose-6-phosphate dehydrogenase	1.1.1.49	0·059	0·020	185
SOD	Superoxide dismutase	1.15.1.1	0·038	0·013	582
(*IPO*)	(*Indophenol oxidase*)				
(*TO*)	(*Tetrazolium oxidase*)				
AAT	Aspartate aminotransferase	2.6.1.1	0·071	0·022	641
(*GOT*)	(*Glutamate oxalate transaminase*)				
PGM	Phosphoglucomutase	5.4.2.2	0·109	0·030	688
EST	Esterase (excluding EST-D)	*******	0·160	0·048	478
	(*including carbonic anhydrase, CA*)				
PGI	Phosphoglucose isomerase	5.3.1.9	0·097	0·027	599
(*GPI*)	(*Glucosephosphate isomerase*)				
XDH	Xanthine dehydrogenase	1.2.1.37	0·062	0·022	165
GDH	Glutamate dehydrogenase	1.4.1.2/3	0·029	0·011	198
PEP	Peptidases (*excluding LAP*)	*******	0·096	0·028	410
FUM	Fumarase	4.2.1.2	0·058	0·015	182
LAP	Leucine aminopeptidase	3.4.11.1/2	0·048	0·015	239
TF	Transferrin	*******	0·172	0·047	163
PROT	General protein	*******	0·029	0·010	394
HB	Haemoglobin	*******	0·055	0·020	198
ALB	Albumin	*******	0·108	0·036	237
AK	Adenylate kinase	2.7.4.3	0·026	0·008	173
CK	Creatine kinase	2.7.3.2	0·020	0·006	257
ADA	Adenosine deaminase	3.5.4.4	0·169	0·046	167
MPI	Mannose phosphate isomerase	5.3.1.8	0·141	0·038	294
ACP	Acid phosphatase	3.1.3.2	0·055	0·016	168
(*MUP*)	(*Methyl umbelliferyl phosphatase*)				
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	0·018	0·007	128
(*TPDH*)	(*Triosephosphate dehydrogenase*)				
ACO	Aconitase	4.2.1.3	0·098	0·027	112

each of two proteins. This ensures that the distribution of N will be the same for each protein and thus be eliminated as a potential cause of differences in the distribution of H.

The predictions of neutral theory will also be tested by comparing the observed distribution of H with the neutral expectation. The analyses again extend the results of earlier studies (Nei *et al.* 1976; Fuerst *et al.* 1977) by using a substantially larger allozyme dataset.

It is concluded that although some slight deviations from neutrality are observed, consistent with the action of natural selection, the results show a very close quantitative fit with the predictions of neutral theory. If the overall patterns of polymorphism and heterozygosity in natural populations are caused by selection so strong that it dominates mutation and drift, it must be of a form which mimics the neutral model.

2. Methods of analysis

The data for the present analyses have been collated from the large amount of electrophoretic literature available for vertebrates. The criteria used for acceptance of data are that an electrophoretic study is of natural populations and that 15 or more protein loci have been screened in at least 15 individuals per locus. A full bibliography of the sources of data can be obtained on request by E-mail from DOFS (BASKIBIN@UK.AC.SWAN.VAX).

Heterozygosity at a locus in each species was calculated as the Hardy–Weinberg expectation:

$$H = 1 - \sum_{k=1}^{K} x_k^2$$

where x_k is the frequency of the kth of K alleles. Studies in which allele frequencies were assayed for
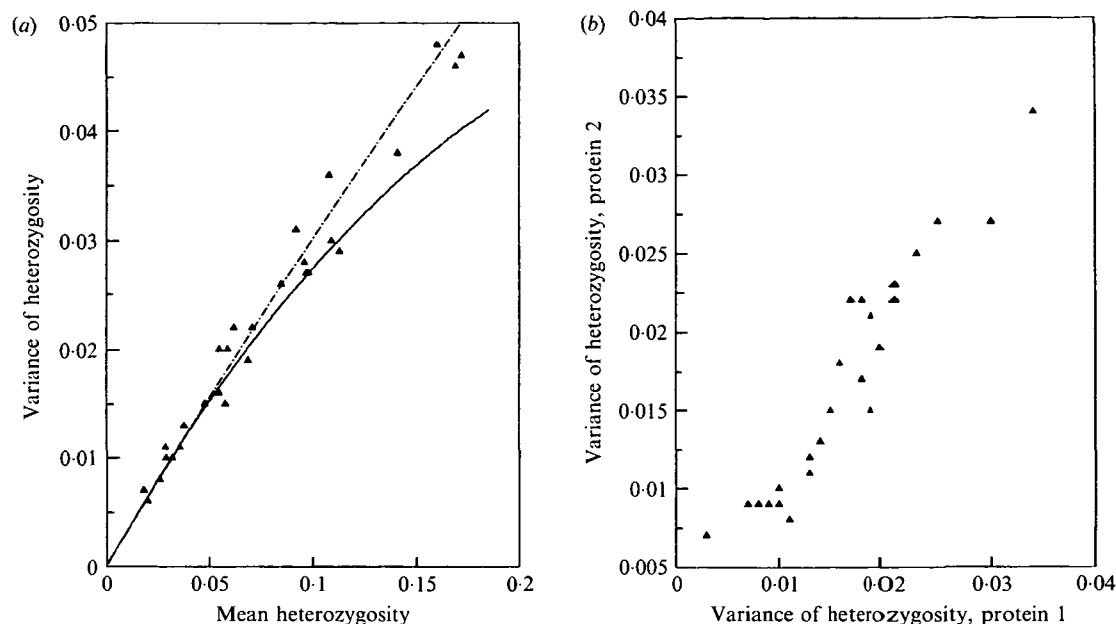
Fig. 1. (a) Relationships between mean and variance of heterozygosity for 30 proteins used in the analysis, data from Table 1. The solid line represents the theoretical prediction from the infinite allele model with constant mutation rate among loci, the dashed line the prediction from the same model but with varying mutation rate among loci. (b) Relationship between variance of first protein (protein 1) and variance of second protein (protein 2), data from Table 2. Proteins within each pair were assigned as protein 1 or protein 2 at random.

several populations within a species (intraspecies studies) had their allele frequencies averaged arithmetically over populations to provide species average values from which expected heterozygosities were calculated. If a species had been scored at more than one locus for a particular protein, one locus was chosen at random to provide a heterozygosity value. Thirty proteins were selected for analysis. These are given in Table 1 with mean and variance of heterozygosity and number of species in which they were scored across the whole dataset. These proteins were chosen because they contributed to the protein pairs analysed, as explained below.

Pairs of proteins having approximately the same mean heterozygosity in an initial screening of the whole dataset were selected, and then reanalysed using only those species which had been scored for both members of that protein pair, that is using a balanced dataset with no missing values. The advantage of ensuring that the same set of species provided data for the two proteins in the comparison is that the effect of species level N on the distribution of H should be the same for both proteins. Only protein pairs that had been scored in 100 or more vertebrate species and whose two proteins had mean heterozygosity within an arbitrary limit of ± 0.005 of each other were used for analysis.

Two methods were used to test for differences between protein distributions. The first method employed the non-parametric Kruskal–Wallis analysis of variance. The second method employed the χ^2 contingency test of Nass (1959) which can be applied

when some expected values are less than unity. For the latter test, the distributions were divided up into frequency classes of 0.05 heterozygosity units in width, apart from monomorphic loci which were treated as a separate class. The numbers of observations in each class were counted for each protein to give a $2 \times M$ table, where M is the number of classes.

The same frequency classes were used to compare the protein distributions with expectations derived from neutral theory (Fuerst *et al.* 1977). The Nass χ^2 test was used to compare the observed and expected numbers of loci in each class.

3. Results

The relationship between the mean and variance of H for the 30 proteins given in Table 1 is shown in Fig. 1. There is a strong correlation between the two variables ($r = 0.985$, $P < 0.001$). However, at high heterozygosity the variance exceeds the theoretical prediction for the infinite allele model with constant N (solid line).

Of the 25 protein pairs with mean heterozygosity within ± 0.005 of each other, all also had closely similar variance values (Table 2). The high correlation of $r = 0.95$ (Fig. 1) again suggests a strong quantitative agreement with predictions of neutral theory. The results of comparing the distributions of the two proteins within each pair using the Kruskal–Wallis and Nass χ^2 tests are shown in Table 2. For the latter test, of 25 protein pairs, 10 (40%) were significant at the 5% or higher level. On applying the sequential

Table 2. *Results of comparison of distribution of heterozygosity for protein pairs with very similar mean heterozygosity ($\hat{H}$) and variance (S_H^2) over the whole vertebrate dataset. Results given are for the Nass χ^2-test and for the Kruskal–Wallis test (N = number of species)*

Pair	Proteins	$\hat{H}$	S_H^2	N	Sig[1]	Sig[2]
1	LDH	0·015	0·003	126	$P < 0.05$	NS
	G3PDH	0·018	0·007			
2	MDH	0·024	0·009	165	$P < 0.05$	NS
	AK	0·022	0·007			
3	LDH	0·027	0·011	257	NS	NS
	CK	0·024	0·008			
4	MDH	0·030	0·008	252	$P < 0.10$	$P < 0.05$
	CK	0·026	0·009			
5	MDH	0·031	0·009	385	$P < 0.01$	NS
	GEN.PROT.	0·026	0·009			
6	LDH	0·032	0·009	392	$P < 0.01$	$P < 0.1$
	GEN.PROT.	0·027	0·010			
7	MDH	0·032	0·010	710	$P < 0.10$	NS
	LDH	0·034	0·010			
8	MDH	0·036	0·012	194	$P < 0.05$	$P < 0.05$
	GDH	0·034	0·013			
9	LDH	0·035	0·011	576	$P < 0.05$	NS
	SOD	0·038	0·013			
10	LDH	0·041	0·013	237	NS	NS
	LAP	0·045	0·014			
11	αGPDH	0·048	0·015	140	NS	NS
	XDH	0·047	0·015			
12	AAT	0·051	0·016	182	NS	NS
	HB	0·050	0·018			
13	αGPDH	0·054	0·013	160	NS	NS
	FUM	0·051	0·014			
14	AAT	0·057	0·020	138	NS	NS
	ACP	0·052	0·019			
15	PGI	0·063	0·017	138	$P < 0.001$	$P < 0.01$
	ACP	0·058	0·018			
16	αGPDH	0·060	0·015	255	NS	NS
	ADH	0·065	0·019			
17	αGPDH	0·066	0·018	449	NS	NS
	IDH	0·071	0·022			
18	IDH	0·063	0·019	249	NS	NS
	ADH	0·068	0·021			
19	αGPDH	0·064	0·017	490	$P < 0.05$	NS
	AAT	0·069	0·022			
20	IDH	0·069	0·021	531	$P < 0.10$	NS
	AAT	0·068	0·022			
21	SDH	0·074	0·021	162	NS	NS
	ADH	0·070	0·023			
22	SDH	0·074	0·023	101	$P < 0.10$	NS
	ALB	0·079	0·025			
23	PGM	0·090	0·025	109	$P < 0.01$	NS
	ACO	0·089	0·027			
24	PGI	0·098	0·027	372	$P < 0.01$	NS
	PEP	0·102	0·030			
25	ALB	0·101	0·034	119	NS	NS
	PEP	0·105	0·034			

[1] Results of the Nass χ^2-test in comparing the distributions.
[2] Results of the Kruskal–Wallis oneway ANOVA in comparing the distributions.

Bonferroni procedure for table-wide significance (Rice, 1989), only the result for PGI–ACP remained significant. However, as quite low probability values were observed in many pairs, an overall test of significance was considered appropriate and the combining probabilities procedure adopted (Sokal & Rohlf, 1981, pp. 779–782). This test requires that the probabilities to be combined arise from independent tests. In order to accommodate this requirement, some protein pairs were removed from the analysis. For example, the three protein pairs, MDH–General-Protein, LDH–General-Protein and MDH–LDH are
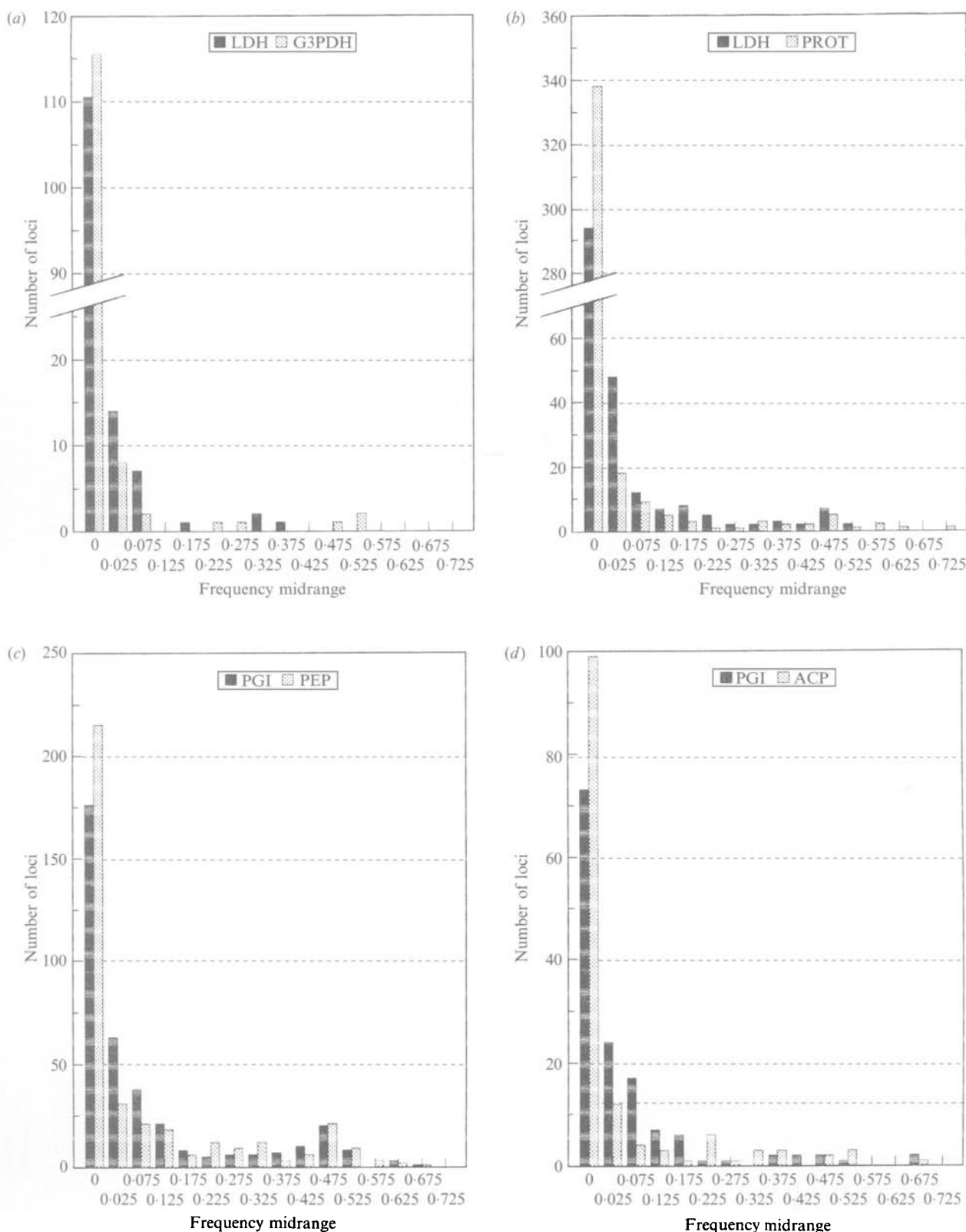
Fig. 2. Distribution of single locus heterozygosity in (*a*) LDH and G3PDH, data from 126 species; (*b*) LDH and General-Protein, data from 392 species, (*c*) PGI and PEP, data from 372 species, and (*d*) PGI and ACP, data from 138 species.

non-independent, but any two of these pairs would be independent in the sense that knowledge of the probability value for one of the two pairs would not provide information on the probability value for the

second pair. One pair in such triplets was removed at random. The results of the test, applied over the remaining 22 protein pairs, provides evidence of a significant tendency towards differences in the dis-
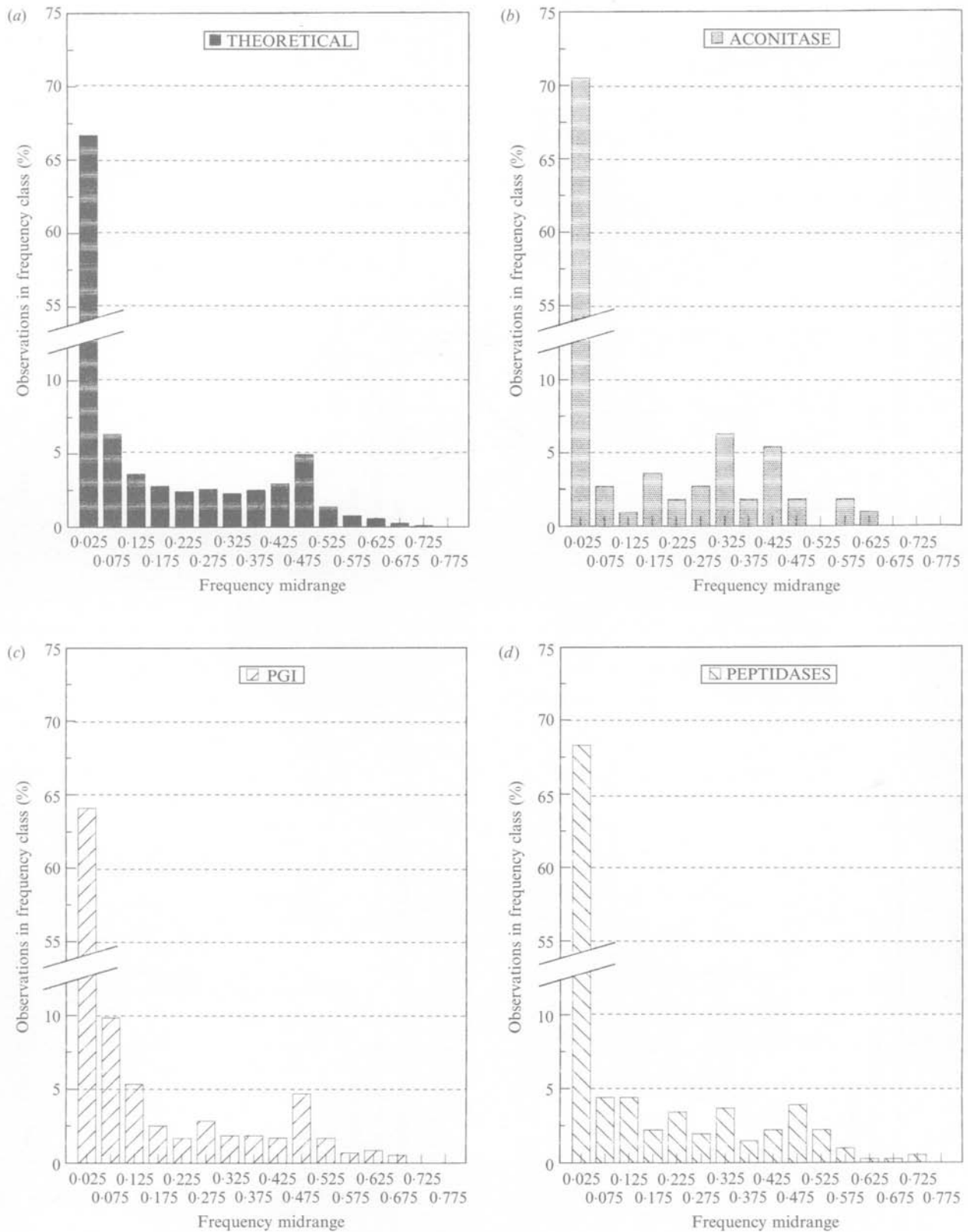
Fig. 3. Comparison of the distributions of single-locus heterozygosity in PGI, Peptidases and Aconitase with a theoretical distribution for $H = 0.10$ from Fuerst *et al.* (1977).

tribution of H between proteins within pairs ($\chi^2 = 108.414$, $P < 0.001$).

The Kruskal–Wallis tests performed on the 25 protein pairs gave significant results for only four pairs (16%) MDH–CK, LDH–General-Protein, MDH–GDH and PGI–ACP. On applying the sequential Bonferroni procedure no protein pairs remained significant. However, the combining probabilities test performed on 22 protein pairs proved to be significant ($\chi^2 = 75.802$, $P < 0.01$), in agreement with the results

obtained from the Nass χ^2 test. Taking the results of these two tests together, there is evidence of an overall tendency for the two proteins within the pairs tested to differ significantly in their distributions of single-locus heterozygosity, even though quantitatively the variances are quite similar.

On examining histograms for individual protein pairs with significantly different distributions under the Nass χ^2 test, a trend can be seen (examples in Fig. 2). In each case, one protein in the pair has been scored both for a greater number of both monomorphic loci and highly heterozygous loci ($\sim H > 0.45$) in the subset of species making up the comparison. The other protein in the comparison, with fewer monomorphic loci, has a greater number of loci with low heterozygosity ($\sim 0.001 < H < 0.15$).

The distributions observed are of the same general type as those observed by earlier workers (Fuerst *et al.* 1977; Nei *et al.* 1976; Stewart, 1976, 1977). Fuerst *et al.* (1977) presented theoretical distributions for single-locus heterozygosity for a range of mean expected heterozygosities, using computer simulated allele frequencies generated using the infinite allele model (Kimura & Crow, 1964). Over the whole vertebrate dataset used in this analysis, three proteins (ACP, $\hat{H} = 0.055$ over 168 species; HB, $\hat{H} = 0.055$ over 198 species; LAP, $\hat{H} = 0.048$ over 239 species) had mean heterozygosities within 0.005 of the $H = 0.05$ distribution and three proteins (PEP, $\hat{H} = 0.097$ over 409 species; PGI, $\hat{H} = 0.098$ over 598 species; ACO, $\hat{H} = 0.098$ over 112 species) were within 0.005 of the $\hat{H} = 0.10$ distribution. The distributions for the latter three proteins are given in Fig. 3. If these distributions are compared with the theoretical distribution for proteins with a mean heterozygosity of 0.05 and 0.10 respectively, using the Nass χ^2, none of the six proteins is found to be significantly different from the theoretical distribution. Thus it would seem that the series of theoretical distributions from Fuerst *et al.* (1977) are an accurate prediction of empirical behaviour.

Another aspect of the theoretical distributions described by Fuerst *et al.* (1977) is that they display a peak in the frequency class, $H \sim 0.045$–0.050. The datasets, for both individual proteins and protein pairs, generally corroborate this prediction (see, for example, Figs 2–3). The peak is more noticeable in the distributions of proteins with relatively higher mean heterozygosity.

4. Discussion

The observation of a strong positive correlation between the mean and variance of H is in agreement with the predictions of neutral theory. In contrast to the results of previous studies (Gojobori, 1982; Nevo, 1983), slight deviations from prediction were observed in the direction of greater than expected variance of H. Within the context of neutral theory, this could be

explained by differences between proteins and taxa in the variance of neutral mutation rate or effective population size, but not by the presence of slightly deleterious mutations (with selection coefficients of the order $1/N$) nor by population bottlenecks (Gojobori, 1982). These deviations could equally well be explained by some form of strong selection (with selection coefficients much greater than $1/N$).

The use of balanced datasets to compare the distribution of H provides the means of controlling for variation in the distribution of N between proteins. The comparison, in this circumstance, of proteins with the same mean heterozygosity provides an additional approximate control for mean neutral mutation rate. These controls permit few if any degrees of freedom for neutral theory to explain differences in the variance of heterozygosity between proteins. Thus the observation that proteins with the same mean H have closely similar variance values provides good support for neutral theory.

Evidence was obtained of residual differences in the distribution of H between paired proteins with the same mean and variance of H. Neutral theory can accommodate these observations by assuming that they arise from variation between proteins in the distribution of neutral mutation rate, which might in turn be related to variation in functional constraint caused by rare positive directional selection. Quaternary structure and subunit size, two factors which might influence the level of constraint, have been shown to be related to variation in protein heterozygosity (Koehn & Eanes, 1978; Nei *et al.* 1978; Ward 1978; Ward *et al.* 1992).

The reverse J shaped distribution of single locus heterozygosity, predicted by neutral theory, was observed in this study and no significant differences were observed between empirical and theoretical distributions for any of the proteins tested. Some models of strong selection give rise to the same distribution as neutral theory. An example of such a model in the SAS–CFF model, in which polymorphisms arise and are maintained by fluctuating selection in a random environment (Gillespie, 1991). The distribution of allele frequencies in selection models is in general very much dependent on the details of the model (Gillespie, 1991). Thus the empirical results obtained in this study narrow down the field of selection models, eliminating all but those making predictions identical or nearly identical to those of natural theory.

In conclusion, the high correlation between the mean and variance of heterozygosity, the close similarity between protein distributions in balanced datasets, and the close agreement between empirical and theoretical distributions of heterozygosity provide good quantitative support for neutral theory. Although some models of selection might make similar predictions neutral theory has fewer parameters, and for this reason might be preferred.

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