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Protein variation in the plaice, *Pleuronectes platessa* L.

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SUMMARY

Thirty-nine enzyme loci and seven non-enzymic protein loci have been screened for electrophoretically detectable variation in a population of *Pleuronectes platessa*, a marine flatfish. The mean heterozygosity per individual is $0{\cdot}102 \pm 0{\cdot}026$, or $0{\cdot}118 \pm 0{\cdot}030$ if the non-enzymic proteins are excluded. The distributions of allele frequencies and single locus heterozygosities are given, and the results discussed with reference to current theories concerning the nature of protein variation.

1. INTRODUCTION

Electrophoretic techniques have been widely used to study the population genetics of a wide range of animal species. Avise (1974) and Powell (1975) have written useful reviews of such work. We are interested in the structure and dynamics of the gene pool of the plaice, a commercially important marine flatfish. This paper describes the extent of protein variation in this species, and the results are discussed in the light of current theories concerning the nature of protein variation. Other papers will discuss the evidence which bears upon the possible adaptive significance of such variation (Beardmore & Ward, 1977). Genetic homologies with closely related flatfish species are also being measured (Ward & Galleguillos, 1977).

The plaice, *Pleuronectes platessa* Lin., is a member of the family Pleuronectidae, order Heterosomata. It lives on the sandy parts of the continental shelf of north-western Europe and Iceland. Although spawning may occur throughout this area, a number of distinct spawning grounds are known between which migration is limited. Most of the present study centres on the Bristol Channel population, which has been shown by tagging experiments to be reasonably distinct from neighbouring populations, although migration in and out does occur (Macer, 1972). The spawning ground for this population is located off the north coast of Cornwall (Simpson, 1959). Wimpenny (1953) has given a general review of the biology of the species.

2. MATERIALS AND METHODS

(i) *General*

Adult fish (2+ years old) were trawled from the R.V. *Ocean Crest* on single day trips from Swansea to Carmarthen Bay (South Wales). They were kept

refrigerated until the following day when tissues were dissected out, these and the carcasses then being stored at −20 °C until required. Stability of muscle enzymes proved to be excellent, most systems still being easy to type after 2 years of storage. Thus although the enzyme adenosine deaminase was not initially assayed, once screening for this enzyme had commenced the availability of stored samples permitted all fish to be unambiguously typed. Liver-specific enzymes posed greater problems and stability at −20 °C only proved satisfactory for up to 2 weeks. Later, facilities for storage at −76 °C became available and this effected an improvement in storage times.

0-group fish (those in their first year of life) were collected from beaches off Swansea and Pendine (South Wales) by pushnetting (mesh size 8 mm) and were either kept alive in tanks in the laboratory or stored frozen until typed. Stability of muscle enzymes was again excellent, but some liver enzymes rapidly deteriorated. The data in the present paper do not distinguish between 0-group and older fish, but data from runs where there has been enzyme degradation have been discarded.

(ii) *Sample preparation*

Muscle enzymes: small pieces of skeletal muscle placed in 0·5 cm diameter wells sunk into perspex blocks were moistened with distilled water and ground manually using a glass rod. The crude homogenate was absorbed directly onto squares of Whatman No. 1 filter paper.

Liver enzymes: the entire livers from 0-group fish were placed in the perspex blocks, a drop of distilled water added and the tissue homogenized using an electrically driven glass rod. The homogenate was absorbed directly on to pieces of filter paper. Segments were cut from the livers of older fish, homogenized in 0·2 ml distilled water, and centrifuged at 5000 *g* for 30 min. The supernatant beneath the surface fat layer was used as the enzyme source.

Eye enzymes: Intact eyes together with a few drops of water and a little acid-washed sand were ground using a pestle and mortar. The homogenate was centrifuged at 5000 *g* for 30 min and the supernatant absorbed onto the filter paper squares.

(iii) *Electrophoresis*

Horizontal starch-gel electrophoresis was carried out using 12·5% starch gels (Connaught starch). Three buffer systems were utilized:

(I) *Discontinuous tris-citrate* (Poulik). Electrode: 0·30 M borate, pH 8·2 (18·55 g boric acid, 2·40 g sodium hydroxide/l.). Gel: 0·076 M tris, 0·005 M citric acid, pH 8·7 (9·21 g tris, 1·05 g citric acid/l.). Potential: 300 V for 3 h.

(II) *Continuous tris-citrate*. Electrode: 0·25 M tris, 0·057 M citric acid, pH 8·0 (30·29 g tris, 11·98 g citric acid/l.). Gel: dilute electrode buffer 1:25. Potential: 200 V for 4 h. For G3PDH staining, 0·02 ml mercaptoethanol and 10 mg NAD were added to the 220 ml starch gel (Wright, Siciliano & Baptist, 1972).

(III) *Histidine/citrate*. Electrode: 0·41 M trisodium citrate, pH 7·0 (120·58 g trisodium citrate/l., adjust pH with 0·5 M citric acid). Gel: 0·005 M histidine HCl,

pH 7·0 (0·96 g l-histidine hydrochloride/l., adjust pH with 0·1 M sodium hydroxide). Potential: 150 V for 4 h.

Gels were 6 mm deep and were sliced twice to provide three slices, each of which could be stained for a different enzyme. Twenty samples per gel were run and runs were carried out at 4 °C. A summary of the enzymes screened, together with the relevant buffer schemes, is provided in Table 1. Most enzyme stains were modified from those listed in Shaw & Prasad (1970). References for additional stains are as follows: esterase-D, Hopkinson *et al.* (1973); adenosine deaminase, Spencer, Hopkinson & Harris (1968). The substrate used for esterases 1–4 was α-naphthyl acetate, the dipeptide used for aminopeptidase was phenylalanyl leucine. Samples showing rare alleles for any systems were collected together and run against one another, thus allowing unambiguous determination of alleles.

3. RESULTS

(i) *Proteins scored*

The proteins assayed are listed in Table 1, and brief comments on each of them follow. The tissues listed are those in which each protein was routinely scored, but this does not necessarily imply that the loci involved are inactive in other tissues.

Alcohol dehydrogenase (liver). Homozygotes with one strong band and a weak faster satellite band. Heterozygotes with three major bands.

Octanol dehydrogenase (liver). Homozygotes single banded, heterozygotes three banded.

α-Glycerophosphate dehydrogenase. Two loci scored, *αGpdh-1* from muscle tissue and *αGpdh-2* from liver tissue. Multiple *αGpdh* loci have been recorded from several species of teleost (e.g. Engel, Schmidtke & Wolf, 1971; Clayton, Franzin & Tretiak, 1973). For both systems homozygotes have one major band and weak faster satellite zones, while heterozygotes have three major bands. Purdom, Thompson & Dando (1976) term this enzyme glycerol-3-phosphate dehydrogenase (G3PDH) in their studies on the plaice; their *G3PDH* locus corresponds to our *αGpdh-1* locus.

Sorbitol dehydrogenase (liver). Homozygotes with one major band together with several faster but weaker satellite bands. Heterozygotes have the five major bands expected of a tetrameric molecule, with the heterotetramers staining most strongly, but there is variation in the relative intensities of these bands from individual to individual.

Lactate dehydrogenase. A single monomorphic locus, *Ldh-1*, is active in muscle tissue, and specifies a slowly migrating isozyme. This is the A_4 isozyme (Markert & Faulhaber, 1965). Storage at −20 °C leads to the formation of faster satellite bands. Electrophoresis of eye tissue yields a multiplicity of bands, but the presence of a weakly polymorphic locus enables a reasonable interpretation of the isozyme patterns to be made (Fig. 1). The *Ldh-4* locus probably codes for the C subunit giving the rapidly migrating C_4 homotetramer (Markert, Shaklee & Whitt, 1975) and *Ldh-2* may well code for the B subunit. The nature of the subunit coded for by

Table 1. *Proteins investigated in* Pleuronectes platessa

Protein	EC no.	Structure	Function	Buffer	No. of loci	No. of loci polymorphic* A	B
Oxidoreductases							
Alcohol dehydrogenase (ADH)	1.1.1.1	Dimer	NG	DTC	1	1	0
Octanol dehydrogenase (ODH)	1.1.1.1	Dimer	NG	HC	1	1	0
α-Glycerophosphate dehydrogenase (αGPDH)	1.1.1.8	Dimer	G	HC	2	2	2
Sorbitol dehydrogenase (SDH)	1.1.1.14	Tetramer	NG	DTC	1	1	1
Lactate dehydrogenase (LDH)	1.1.1.27	Tetramer	G	CTC	4	1	0
Malate dehydrogenase (MDH)	1.1.1.37	Dimer	G	HC	2	1	1
Malic enzyme (ME)	1.1.1.40	Tetramer	G	HC	1	0	0
Isocitrate dehydrogenase (IDH)	1.1.1.42	Dimer	G	HC	2	2	1
6-Phosphogluconate dehydrogenase (6PGDH)	1.1.1.44	Dimer	G	CTC	1	1	0
Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	1.2.1.12	Tetramer	G	CTC†	2	0	0
Superoxide dismutase (SOD)	1.15.1.1	Dimer	NG	HC(CTC)	1	0	0
'Nothing dehydrogenase' (NDH)	—	Monomer	—	HC	1	1	1
Transferases							
Glutamate oxalate transaminase (GOT)	2.6.1.1	Dimer	G	HC(CTC)	2	2	2
Creatine kinase (CK)	2.7.3.2	Dimer	NG	HC	2	0	0
Adenylate kinase (AK)	2.7.4.3	Monomer	NG	HC	1	0	0
Phosphoglucomutase (PGM)	2.7.5.1	Monomer	G	CTC(HC)	2	2	2
Hydrolases							
Esterases 1–4 (EST)	3.1.1.1	Monomer	NG	DTC	4	2	0
Esterase D (EST)	3.1.1.1	Dimer	NG	HC	1	0	0
Alkaline phosphatase (ALP)	3.1.3.1	Dimer	NG	DTC	1	0	0
Acid phosphatase (ACP)	3.1.3.2	Dimer	NG	DTC	1	0	0
Leucine aminopeptidase (LAP)	3.4.1.1	Monomer	NG	DTC	1	1	0
Aminopeptidase (AP)	—	—	NG	DTC	2	0	0
Adenosine deaminase (ADA)	3.5.4.4	Monomer	NG	HC	1	1	1
Isomerases							
Phosphoglucose isomerase (PGI)	5.3.1.9	Dimer	G	CTC	2	2	1
Non-enzymic proteins							
Muscle protein (PROT)	—	—	—	DTC	5	1	0
Haemoglobin (HB)	—	—	—	DTC	2	0	0
				Total	46	22	12

For footnotes see facing page.

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Ldh-3 remains obscure, but we feel that this isozyme is more likely to be a homotetramer than a heterotetramer. However, it is possible that this is a translational modification of the LDH-4 isozyme rather than a distinct enzyme. Lush, Cowey & Knox (1969) studied *Pleuronectes platessa* in a survey of the LDH isozymes of a number of flatfish species, but the absence of genetic variation in their small sample of plaice precluded a firm interpretation of the isozyme patterns. Our interpretation is supported by the LDH patterns of the closely related flatfish *Limanda limanda*, L. (the dab, syn. *Pleuronectes limanda* L.). Some of the major bands in this species differ in their mobility from those of the plaice, but the presumed hybrid zones are shifted in the manner predicted. Analysis of the separate eye tissues may give further useful information, since all four loci may not be active in all eye tissues. Compartmentalization may account for the lack of certain hybrid classes. In the plaice it is clear that asymmetrical heterotetramers between the products of different loci are either not formed or are unstable. This phenomenon is common in many fish species (Markert & Faulhaber, 1965; Markert, Shaklee & Whitt, 1975). However, asymmetrical LDH-2 heterotetramers are formed in individuals heterozygous for *Ldh-2*, all five allozymes being expressed (see Fig. 1).

Malate dehydrogenase. Two loci coding for the supernatant enzyme were detected. Mitochondrial MDH was not observed. Both *Mdh-1* and *Mdh-2* are active in muscle tissue, and the heterodimers between them produce a band of intermediate mobility. Duplicate supernatant *Mdh* loci have been recorded in several species of teleost (Bailey, Cocks & Wilson, 1969; Clayton, Tretiak & Kooyman, 1971; Wheat *et al.* 1971; Wheat, Whitt & Childers, 1972). In individuals heterozygous for either *Mdh-1* or *Mdh-2*, the expected six bands were observed. MDH-2, the more anodally migrating system, corresponding to MDH-A of Purdom *et al.* (1976), is almost undetectable in liver tissue.

Malic enzyme (muscle/liver). Only a single species of this enzyme was detected. Homozygotes have a single band, and no heterozygotes were observed. This enzyme commonly exists as a tetramer in vertebrate species (Nevaldine, Bassel & Hsu, 1974; Hopkinson, Edwards & Harris, 1976) and is so considered here.

Isocitrate dehydrogenase. Two loci were detected: *Idh-1* active in liver tissue and *Idh-2* in muscle tissue. Both give three-banded heterozygotes.

Table 1 footnotes.

Quaternary structures have been assigned primarily from patterns of electrophoretic variants. Where the enzymes are invariant in the plaice, quaternary structures have been assumed by extrapolation from other species. For further details see text. The structure of AP-1 and AP-2 is unknown.

Function: G designates glucose metabolizing enzymes, i.e. those catalysing steps in the glycolytic pathway, the TCA cycle, and closely related reactions. NG designates non-glucose metabolizing enzymes, i.e. all other enzymes.

* Criterion A: frequency of most common allele $\geqslant$ 0 99. Criterion B: Frequency of most common allele $\geqslant$ 0·95.

† Modified buffer system, see text.

6-Phosphogluconate dehydrogenase (muscle/liver). Homozygotes are single banded, heterozygotes triple.

Glyceraldehyde-3-phosphate dehydrogenase. Two loci, both monomorphic. *G3pdh-1* is found in muscle; *G3pdh-2*, found in eye tissue, codes for an isozyme migrating

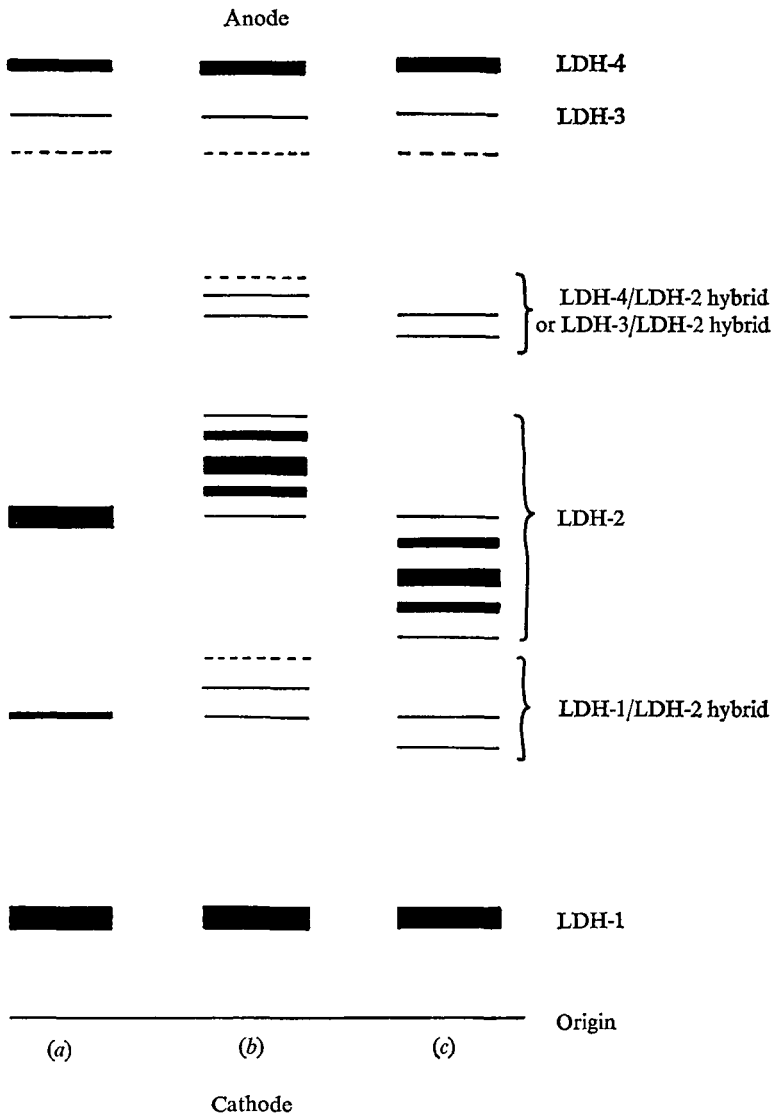

Fig. 1. LDH patterns in the eye of the plaice, and proposed interpretation. *Ldh-2* genotypes: (*a*) *Ldh-2*^{100}/*Ldh-2*^{100}, (*b*) *Ldh-2*^{120}/*Ldh-2*^{100}, (*c*) *Ldh-2*^{100}/*Ldh-2*^{75}.

rapidly towards the anode. Probably a tetramer, as it is in the fish genus *Xiphophorus* (Wright *et al.* 1972) and in man (Hopkinson *et al.* 1976).

Superoxide dismutase (liver). Formerly known as tetrazolium oxidase or indophenol oxidase. It appears on gels stained for ADH, ODH, SDH, αGPDH and

MDH. Homozygotes are single banded, heterozygotes triple banded. This is the supernatant enzyme.

'*Nothing dehydrogenase*' (liver). This enzyme appears on gels stained for phosphoglucomutase and malic enzyme, and is assumed to be an NADP-dependent dehydrogenase. The nature of its substrate remains obscure. Homozygotes are single banded, heterozygotes double.

Glutamate oxalate transaminase (muscle/liver). Also known as aspartate aminotransferase. Two systems appear, of which the more anodally migrating, GOT-2, is assumed to represent the supernatant form (Selander *et al.* 1971; Avise & Selander, 1972; Harris & Hopkinson, 1976). GOT-1 is then the mitochondrial form. Both loci give single-banded homozygotes and triple-banded heterozygotes. Plaice GOT-2 shows a high degree of polymorphism, but resolution is frequently poor and it is unsuitable for routine screening. Genotypes at this locus were only scored from those runs giving reasonable resolution.

Creatine kinase. Electrophoresis of brain or eye tissue produces three bands of CK activity. These are assumed to represent the products of two monomorphic loci, the isozyme with intermediate mobility being the heterodimer. Hybridization of the products of different CK loci has been reported from salmonid fishes and higher vertebrates, including man (Eppenberger, Scholl & Ursprung, 1971; Harris & Hopkinson, 1976). The *Ck-1* locus codes for the slowest and most intense isozyme, *CK-2* for the fastest. Only *Ck-1* appears to be active in muscle or liver tissue, and activity in these tissues is low.

Adenylate kinase (muscle/eye/brain). Homozygotes are single banded and no heterozygotes were observed. As expected, AK appears on gels stained for CK when using the stain of Shaw & Prasad (1970). This enzyme is a monomer in man (Hopkinson *et al.* 1976) and is assumed to be monomer in the plaice.

Phosphoglucomutase. Two loci scored. *Pgm-1* is active in muscle and liver tissue, and corresponds to the *PGM* locus of Purdom *et al.* (1976). Homozygotes are single banded, heterozygotes double. Sometimes a faster satellite zone is seen, particularly with the histidine/citrate buffer system. PGM-2 is only scorable in young 0-group fish up to 50 mm in length, and is not observed in muscle samples from larger fish. Homozygotes are single banded, heterozygotes double.

Esterases. Liver esterases gave complex patterns and could not be scored with any certainty. EST-1 (eye): a slow system with single-banded homozygotes; no heterozygotes were observed; most vertebrate esterases active toward α-naphthyl acetate behave as monomers, and this enzyme is assumed to be monomeric. EST-2 (muscle): homozygotes are single banded, heterozygotes double. EST-3 (muscle): an enzyme with a higher anodal mobility than EST-2; Homozygotes have one strong band together, with a weaker faster band; heterozygotes have an additional slower band. EST-4 (muscle): the most anodal system; homozygotes have two bands, heterozygotes four, the extra two bands of the heterozygote overlapping those of the common homozygote. EST-D (muscle): a fast system, specific to methyl umbelliferyl acetate; homozygotes are single banded, heterozygotes triple; this enzyme is termed EST-D in order to stress its probable

homology with EST-D of man, which is also dimeric and reacts specifically with umbelliferyl esters. (Hopkinson *et al.* 1973.)

Alkaline phosphatase (liver). Homozygotes are single banded in fresh material, but additional slower bands form during storage. Heterozygotes are three banded.

Acid phosphatase (liver). A monomorphic single-banded system, no heterozygotes were observed. Most vertebrate acid phosphatases, with the exception of the monomeric human red blood cell acid phosphatase (Hopkinson *et al.* 1976), behave as dimers, and the plaice enzyme is here classified as a dimer.

Leucine aminopeptidase (muscle). A rapidly migrating system. Homozygotes are single banded, heterozygotes double. Other LAP systems are too unreliable or too diffuse to score.

Aminopeptidase (muscle). Two loci were scored, both of which were invariant. Aminopeptidases are a somewhat heterogeneous group of enzymes showing variable quaternary structure (Hopkinson *et al.* 1976), and this precludes the immediate designation of quaternary structure in the absence of heterozygote patterns.

Adenosine deaminase (muscle/liver). Single-banded homozygotes, double-banded heterozygotes.

Phosphoglucose isomerase. Also termed glucose phosphate isomerase or phosphohexose isomerase. Specified by two loci in muscle tissue, the products of which hybridize to give a heterodimer of intermediate mobility. This pattern is common in fish (Avise & Kitto, 1973). The locus coding for the slowest isozyme is here designated *Pgi-1*, and corresponds with that termed *GP1-B* by Purdom *et al.* (1976). Individuals heterozygous at a single locus show the expected six bands of activity from muscle tissue. Only *Pgi-2* is active in liver tissue.

Muscle protein. Five zones scored as the products of separate loci, other proteins were too faint and/or diffuse to score. Only the *Prot-3* locus is polymorphic. Homozygotes are single banded, heterozygotes double. This system runs just behind the major muscle protein, coded for by *Prot-4*.

Haemoglobin. Two major bands, flanked either side by weaker bands. The two major bands are invariant. There is some variation in the weaker bands but this may result from non-genetic causes. Scored as the products of two monomorphic loci.

Allele frequencies for these loci are given in Tables 2 and 3. The superscript 100 designates the most common allele at that locus and other alleles are given numbers that describe their relative mobility to that allele.

(ii) *Breeding data*

An extensive series of breeding tests utilizing the five loci *Mdh-2*, *Pgm-1*, *Pgi-1*, *Pgi-2* and *αGpdh-1* were carried out by Purdom *et al.* (1976). These loci, active in muscle tissue, can be typed in premetamorphosed fish. We present here a much smaller amount of data using the first four of these loci, but this work was carried out in the early stages of this project and the formal genetics of most systems has still not been proven. Progeny ratios (Table 4) are not significantly different from

Table 2. *Allele frequencies for the polymorphic loci*

Locus	Tissue	*n*	Allele designations and frequency					Heterozygosity	
			258	166	100	10	—	Obs.	Exp.
Adh	Liver	468	0·001	0·003	0·983	0·013	—	0·034	0·034
			129	125	119	100	75		
Odh	Liver	573	0·004	0·005	0·017	0·971	0·002	0·056	0·055
			135(7)	113(6)	100(5)	73(4)	65(3)		
αGpdh-1	Muscle	2275	0·055	0·002	0·872	0·066	0·001	0·234	0·232
			58(2)	50(1)	—	—	—		
			0·001	0·003	—	—	—		
			143	131	115	100	79		
αGpdh-2	Liver	294	0·002	0·012	0·352	0·599	0·036	0·466	0·518
			172	137	100	—	—		
Sdh	Liver	320	0·003	0·342	0·655	—	—	0·431	0·454
			120	100	75	—	—		
Ldh-2	Eye	418	0·002	0·988	0·010	—	—	0·024	0·024
			126(3)	100(2)	77(1)	—	—		
Mdh-2	Muscle	2275	0·005	0·876	0·118	—	—	0·216	0·219
			131	118	100	81	74		
1dh-1	Liver	949	0·002	0·102	0·512	0·374	0·009	0·573	0·585
			100	88	—	—	—		
1dh-2	Muscle	742	0·987	0·013	—	—	—	0·026	0·026
			117	100	86	—	—		
6Pgdh	Mus./liv.	2114	0·021	0·976	0·004	—	—	0·047	0·049
			111	100	85	—	—		
Ndh	Liver	210	0·017	0·938	0·045	—	—	0·114	0·118
			100	79	—	—	—		
Got-1	Mus./liv.	591	0·984	0·016	—	—	—	0·032	0·031
			116	100	84	66	—		
*Got-2**	Mus./liv.	219	0·167	0·463	0·358	0·011	—	0·630	0·627
			228(8)	186(7)	171(6)	148(5)	140(4)		
Pgm-1	Mus./liv.	2273	0·012	0·006	0·002	0·002	0·360	0·483	0·499
			100(3)	66(2)	59(1)	—	—		
			0·611	0·005	0·002	—	—		
			118	110	100	93	—		
Pgm-2	Muscle	56	0·036	0·259	0·652	0·045	—	0·482	0·489
			105	100	95	—	—		
Est-3	Muscle	1039	0·001	0·988	0·012	—	—	0·024	0·026
			103	100	97	—	—		
Est-4	Muscle	953	0·005	0·971	0·024	—	—	0·058	0·057
			100	95	—	—	—		
Lap	Muscle	58	0·974	0·026	—	—	—	0·052	0·051
			117(6)	108(5)	100(4)	96(3)	91(2)		
Ada	Mus./liv.	2272	0·002	0·110	0·740	0·013	0·128	0·418	0·424
			80(1)	—	—	—	—		
			0·007	—	—	—	—		
			500	450	287	250	237		
Pgi-1	Muscle	2270	0·003	0·001	0·002	0·002	0·001	0·058	0·057
			162	100	50	13	−25		
			0·001	0·971	0·002	0·018	0·001		
			109(5)	104(4)	100(3)	90(2)	83(1)		
Pgi-2	Mus./liv.	2275	0·005	0·038	0·943	0·013	0·001	0·110	0·109
			100	94	—	—	—		
Prot-3	Muscle	67	0·970	0·030	—	—	—	0·060	0·058

The allele designations given in parentheses for the five loci *αGpdh-1*, *Mdh-2*, *Pgm-1*, *Ada* and *Pgi-2* are those used in Beardmore & Ward (1977).

* Allele frequencies should be regarded as provisional. See text.

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expected, although sample sizes were small, and no examples of the anomalous individuals observed by Purdom *et al.* were recorded.

Purdom *et al.* also studied the linkage relationships between *Pgi-1*, *Pgi-2*, *Pgm-1* and *αGpdh-1*, although that between the pair *Pgi-2* and *αGpdh-1* was not tested. However, their data, as presented, make it impossible to test for linkage. Progeny from single crosses have been assigned to complementary classes, but it appears that complementary classes from different double heterozygotes have been pooled. This will have the effect of obscuring any linkage relationships, since

Table 3. *Allele frequencies at monomorphic loci*

Locus	Tissue	*n*	Allele frequency			Observed heterozygosity
			100	Rare alleles		
				126	72	
Mdh-1	Mus./liv.	2202	0·997	0·002	0·001	0·006
				195	37	
Sod	Liver	1058	0·995	0·005	0·001	0·010
				103	97	
Est-2	Muscle	1463	0·997	0·002	0·001	0·007
				81	—	
Est-D	Muscle	241	0·994	0·006	—	0·012
				89	—	
Alp	Liver	334	0·997	0·003	—	0·006

No genetic variability was detected for the following loci (tissues and sample sizes given): *Ldh-1*, muscle/eye, 654; *Ldh-3*, eye, 401; *Ldh-4*, eye, 58: *Me*, muscle/liver, 156; *G3pdh-1*, muscle, 39; *G3pdh-2*, eye, 353; *Ck-1*, eye/brain, 55; *Ck-2*, eye/brain, 55; *Ak*, muscle/eye/brain, 70; *Est-1*, eye, 112; *Acp*, liver, 52; *Ap-1*, muscle, 412; *Ap-2*, muscle, 237; *Prot-1*, muscle, 40; *Prot-2*, muscle, 52; *Prot-4*, muscle, 79; *Prot-5*, muscle, 81; *Hb-1*, red blood cells, 156; Hb-*2*, red blood cells, 156.

Table 4. *Segregation in crosses involving the* Mdh-2, Pgm-1, Pgi-1 *and* Pgi-2 *loci*

Locus	Cross	Parental genotypes		Progeny genotypes			
		Female	Male				
Mdh-2	10 × 2	100/77	100/77	8 100/100	12 100/77	8 77/77	—
	4 × 2	100/100	100/77	12 100/100	10 100/77	—	—
Pgm-1	10 × 2, 4 × 2	100/100	228/140	16 140/100	23 228/100	—	—
	7 × 14	140/100	228/100	2 100/100	4 228/100	1 140/100	1 228/140
	13 × 15	140/100	140/100	3 140/140	9 140/100	6 100/100	—
Pgi-1	10 × 2, 4 × 2	100/100	500/100	33 100/100	27 500/100	—	—
	4 × 12	100/100	287/100	3 100/100	5 287/100	—	—
Pgi-2	4 × 2, 4 × 12	104/100	100/100	20 100/100	11 104/100	—	—

Crosses are carried out by artificial mixing of eggs and sperm, thus eggs from one female can be crossed separately with sperm from a number of males and vice versa.

the linkage phases of the double heterozygotes are unknown. The very small amount of data we have shows the pairs of loci *Mdh-2/Pgm-1 Mdh-2/Pgi-1* and *Pgi-1/Pgm-1* to be either not linked or only loosely linked (Table 5).

Table 5. *Pairwise comparisons for possible linkage between the* Mdh-2, Pgm-1 *and* Pgi-1 *loci*

Loci	Cross	No. of the progeny in the complementary classes	
Mdh-2 and *Pgm-1*	4 × 2	13	11
Mdh-2 and *Pgi-1*	4 × 2	14	10
Pgm-1 and *Pgi-1*	4 × 2 10 × 2	16	11

Genotypes of parents are given in Table 6. The complementary classes can be described as the +/+, −/− progeny versus the +/−, −/+ progeny, or +/−, −/+ versus +/+, −/−. Crosses 4 × 2 and 10 × 2 can justifiably be summed since the double heterozygote is the male (2), and the two females are both double homozygotes.

Table 6. *Levels of protein variation in the Bristol Channel plaice population*

	n	Prop. of loci polymorphic*		Mean heterozygosity ± S.E.
		Criterion A	Criterion B	
Enzymes	39	0·564	0·308	0·118 ± 0·030
Other proteins	7	0·143	0·000	0·009 ± 0·009
All proteins	46	0·478	0·261	0·102 ± 0·026

* Frequency of most common allele ⩽ 0·99 (criterion A), or ⩽ 0·95 (criterion B).

(iii) *Genic heterozygosity*

The proportion of loci polymorphic in the Bristol Channel population and the average heterozygosity per fish are given in Table 6. In the young fish (0 group) direct observation can lead to the incorrect assignment of sex, and much of the work on the polymorphic loci has concentrated on these small fish (Beardmore & Ward, 1977). An analysis of adult individuals failed to reveal any significant differences in genotype or allele proportions between the two sexes for any polymorphic locus. Between 200 and 300 individuals of each sex were typed for *αGpdh-1*, *Mdh-2*, *1dh-1*, *6Pgdh*, *Got-1*, *Pgm-1*, *Est-3*, *Pgi-1 and Pgi-2*, between 100 and 200 for *Adh*, *Odh*, *Sdh*, *αGpdh-2*, *Ldh-2*, *1dh-2*, *Got-2* and *Est-4* and less than 100 of each sex for *Ndh*, *Lap* and *Prot-3*. The *Pgm-2* locus, only scored from very young individuals, was not classified with respect to sex. A sample of 520 individuals, of varying age (2–5 years), gave a sex ratio of 0·89 (♂:♀).

Table 7 relates enzyme heterozygosity to function (glucose metabolizing versus non-glucose metabolizing) and to quaternary structure (monomeric, dimeric and tetrameric classes).

Fig. 2 gives a plot of single locus heterozygosity against numbers of loci. Exactly the same histogram is obtained whether one considers the Bristol Channel

population alone or combines it with the Irish Sea data (following section). Fig. 3 plots numbers of alleles against allele frequency. Here the Bristol Channel and Irish Sea data are combined, and allele frequencies calculated allowing for any

Table 7. *The relationship between enzyme heterozygosity, function and quaternary structure (data from table 2 and table 3)*

		Quaternary structure		
	Overall	Monomer	Dimer	Tetramer
Glucose enzymes				
$\bar{x}$	0·169	0·483	0·218	0·003
n	20	2	11	7
S.E.	0·050	0·001	0·070	0·003
Non-glucose enzymes				
$\bar{x}$	0·062	0·079	0·015	0·431
n	18	7	8	1
S.E.	0·031	0·057	0·007	—
All enzymes				
$\bar{x}$	0·118	0·164	0·132	0·057
n	39	10	19	8
S.E.	0·030	0·066	0·202	0·054

The substrate and function of the monomeric enzyme NDH is unknown. The quaternary structures of the two non-glucose metabolizing enzymes AP-1 and AP-2 are unknown.

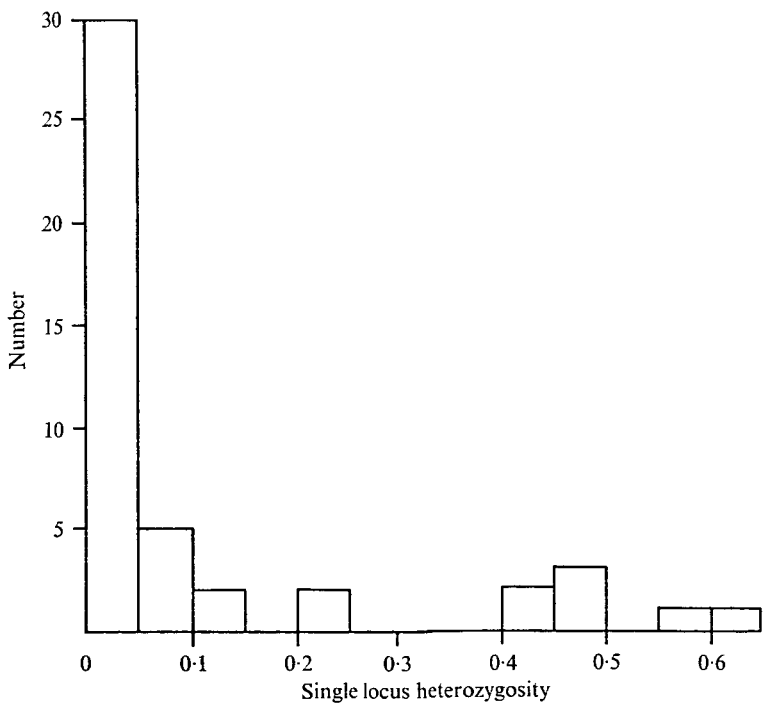

Fig. 2. Distribution of average heterozygosity per locus for 46 loci.

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differences in sample size. The major effect of subtracting the Irish Sea data is to decrease by four the numbers of alleles with a frequency less than 0·01.

(iv) *Patterns of genetic variation*

Most of the polymorphic loci and many of the monomorphic loci have also been assayed in samples from a North-East Irish Sea population trawled off the North Wales coast (Table 8). There are no significant differences between the two populations at these loci, although they do show differences in some meristic characters. (Ward, unpublished). Purdom *et al.* (1976) found no significant heterogeneity at the five loci *αGpdh-1*, *Pgi-1*, *Pgi-2*, *Pgm-1* and *Mdh-2* in samples from different

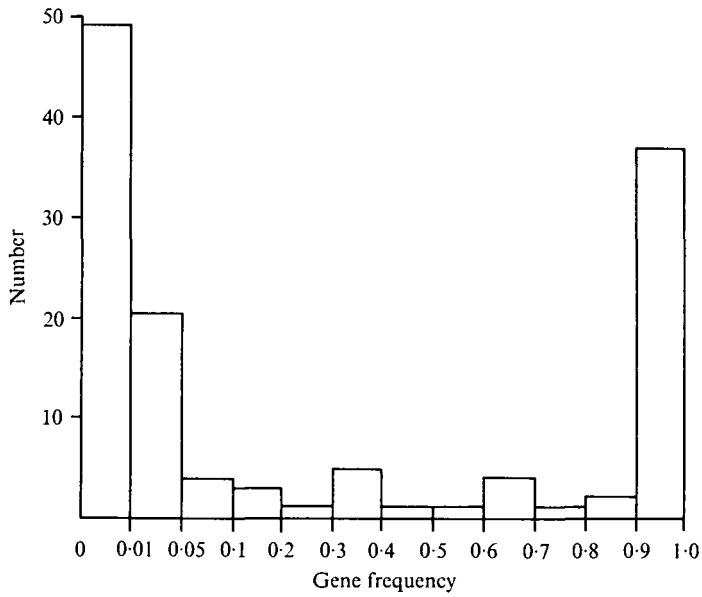

Fig. 3. Distribution of all alleles at 46 loci in the Bristol Channel and N.E. Irish Sea populations by frequency of individual genes.

North Sea fishing grounds, and although their data for these five loci (tables 9 and 10) show broad agreement with ours, there are some interesting differences. In the Bristol Channel and N.E. Irish Sea populations *Pgi-2* is more heterozygous than *Pgi-1*, but in the North Sea this situation is reversed. Perhaps the most striking difference is at the *αGpdh-1* locus. Here the Bristol Channel and N.E. Irish Sea populations, which are in geographic terms essentially contiguous, show double the observed heterozygosity of the North Sea populations. This difference is statistically highly significant ($\chi^2_1 = 14{\cdot}75$, $P < 0{\cdot}001$). It is worth noting that the distribution of genotypes at *αGpdh-1* in the North Sea does not conform with Hardy-Weinberg expectations (Purdom *et al.* 1976). These apparent interpopulation differences need further investigation. The distributions of genotypic and allelic frequencies at *Mdh-2* and *Pgm-1* are similar in all three populations.

Table 8. *Allele frequencies in an Irish Sea population*

Locus	*n*	Allele frequencies					Heterozygosities: Observed	Heterozygosities: Expected
		119	100	75	—	—		
Odh	68	0·015	0·978	0·007	—	—	0·044	0·043
		148	135	113	100	73		
αGpdh-1	143	0·004	0·073	0·004	0·867	0·046	0·238	0·241
		65	58	—	—	—		
		0·004	0·004	—	—	—		
		131	115	100	79	—		
αGpdh-2	144	0·007	0·357	0·608	0·028	—	0·517	0·502
		137	100	65	—	—		
Sdh	54	0·287	0·704	0·009	—	—	0·370	0·422
		100	75	—	—	—		
Ldh-2	135	0·993	0·007	—	—	—	0·015	0·014
		126	100	77	—	—		
Mdh-2	144	0·007	0·896	0·097	—	—	0·208	0·188
		118	100	81	74	—		
1dh-1	144	0·087	0·517	0·389	0·007	—	0·590	0·574
		100	88	—	—	—		
1dh-2	119	0·979	0·021	—	—	—	0·042	0·041
		117	100	—	—	—		
6Pgdh	144	0·024	0·976	—	—	—	0·049	0·047
		195	100	—	—	—		
Sod	120	0·008	0·992	—	—	—	0·017	0·016
		100	85	—	—	—		
Ndh	73	0·945	0·055	—	—	—	0·110	0·104
		100	79	62	—	—		
Got-1	120	0·971	0·025	0·004	—	—	0·058	0·057
		228	222	186	140	—		
Pgm-1	144	0·024	0·003	0·014	0·333	—	0·507	0·522
		100	66	59	—	—		
		0·615	0·003	0·007	—	—		
		103	100	—	—	—		
Est-2	80	0·006	0·994	—	—	—	0·013	0·012
		100	95	—	—	—		
Est-3	46	0·989	0·011	—	—	—	0·022	0·022
		100	97	—	—	—		
Est-4	64	0·984	0·016	—	—	—	0·031	0·031
		117	108	100	96	—		
Ada	144	0·003	0·125	0·733	0·017	—	0·410	0·434
		91	80	—	—	—		
		0·115	0·007	—	—	—		
		500	287	100	50	—		
Pgi-1	144	0·003	0·003	0·965	0·003	—	0·069	0·068
		13	—	—	—	—		
		0·024	—	—	—	—		
		109	104	100	90	—		
Pgi-2	143	0·014	0·045	0·927	0·014	—	0·147	0·138
		100	94	—	—	—		
Prot-3	100	0·985	0·015	—	—	—	0·030	0·030

The following loci were also screened but found to be invariant. Sample sizes are given in parentheses. *Adh* (60); *Ldh-1, -3, -4* (142, 133, 133); *Mdh-1* (144); *G3pdh-1, -2* (75, 121); *Ak* (77); *Alp* (20); *Acp* (20); *Ap-1, -2* (144, 57); *Prot-1, -2, -4, -5* (23, 76, 100, 100).

Table 9. *Allele frequencies at five enzyme loci from North Sea populations*

Enzyme locus and sample size				
αGpdh-1, 187	*Mdh-2*, 108	*Pgm-1*, 202	*Pgi-1*, 202	*Pgi-2*, 201
0·040 (1; 135)	0·866 (2; 100)	0·015 (1; 228)	0·007 (2; 500–162?)	0·037 (1; 104)
0·920 (2; 100)	0·134 (3; 77)	0·012 (2; 186, 171, 148)	0·050 (4; 500–162?)	0·950 (2; 100)
0·032 (3; 73)	—	0·374 (3; 140)	0·921 (6; 100)	0·012 (3; 90)
0·008 (4; 65, 58, 50)	—	0·592 (4; 100) 0·007 (5; 66, 59)	0·022 (7; 13)	

Data calculated from Purdom *et al.* (1976). Figures in parentheses give the allele notation of Purdom *et al.* followed by our notation.

Table 10. *Comparison of heterozygosity at five loci in three plaice populations*

	Bristol Channel	N.E. Irish Sea	N. Sea
αGpdh-1	0·234 ± 0·009	0·238 ± 0·036	0·112 ± 0·023 (0·151)
Mdh-2	0·216 ± 0·009	0·208 ± 0·034	0·231 ± 0·041 (0·232)
Pgm-1	0·483 ± 0·011	0·507 ± 0·042	0·540 ± 0·035 (0·509)
Pgi-1	0·058 ± 0·005	0·069 ± 0·021	0·149 ± 0·025 (0·149)
Pgi-2	0·110 ± 0·007	0·147 ± 0·030	0·094 ± 0·021 (0·096)

Standard errors are given. Observed and expected (in parentheses) heterozygosities for the North Sea population are calculated from Purdom *et al.* (1976).

4. DISCUSSION

The Bristol Channel population of plaice has been screened at a total of 46 loci, making it the most comprehensive investigation of protein variation in any fish species to date. The mean heterozygosity per individual, 0·102 ± 0·026 (0·118 ± 0·030 if non-enzymic proteins are excluded), is very nearly twice the mean average heterozygosity of a range of fish species (0·058 ± 0·006, Powell, 1975) and is twice that of vertebrate species as a whole (0·050 ± 0·004, Powell, 1975). It is perhaps profitless to speculate upon the causes of this high variability; the particular sample of loci scored may not have been representative of the gene pool as a whole; but it should be noted that this comparatively high value does not accord with the predictions of Ayala and Valentine (Ayala *et al.* 1975; Valentine, 1976). They have proposed that the genetic variability of marine species is related to trophic resource variability in such a way that genetic variability is expected to be highest where trophic resources are the least seasonal (i.e. in tropical waters), and that with increasing latitude genetic variability should decrease. Most of the evidence in favour of this hypothesis comes from surveys of marine invertebrates, but it does predict that the plaice should show average variability rather than the high levels observed. The plaice certainly perceives its trophic resources to be highly seasonal: feeding occurs during the spring, summer and autumn months

and almost ceases during the winter. Soulé (1976) has shown that protein heterozygosity is related to population size, although apparently not to the extent predicted by the neutralist hypothesis. In 1953 it was estimated that 3×10^6 female plaice spawned in the Irish Sea (Simpson, 1959). The population has shrunk since then, but the effective population size of the Bristol Channel may be of the order of 10^6. This is, of course, a very rough approximation and will be significantly affected by the extent of migration into and out of this area, but the high variability of the plaice may at least partly reflect its undoubtedly large population size.

The observed patterns of protein variation are, in broad terms, similar to those for other organisms where large numbers of loci have been studied. The distribution of single locus heterozygosity against frequency (Fig. 2) gives the typical L-shaped distribution with a small peak around $h = 0{\cdot}5$. This is also sometimes termed a reverse J distribution. Ayala *et al.* (1974) expected that the neutral model (Kimura & Ohta, 1971) for the maintenance of electrophoretically detectable protein variation should give an approximately normal distribution of heterozygosities around the mean value. Significant deviations from such a distribution may then indicate the action of balancing selection. However, more recent simulations of neutral models have shown that a reverse J distribution *is* expected (Nei, Fuerst & Chakraborty, 1976), and it therefore appears that the form of the distribution of single locus heterozygosity does not yet allow discrimination between the neutralist and selectionist models.

Plotting numbers of alleles against allele frequency (Fig. 3) shows that the largest allele class is composed of rare alleles. Ohta (1976) analysed data from *Drosophila willistoni* and man and demonstrated that in these species there is a significant excess of alleles with very low frequencies over the numbers expected from strict neutral theory. The allele distribution in plaice is similar to that of *Drosophila* and man and an abundance of rare alleles is again observed. Furthermore, some, possibly many, rare alleles must have been missed, particularly for those loci where sample sizes were not large. It is difficult to imagine that such rare alleles are effective components of a balanced polymorphism, and it may be that, as Ohta (1976) suggests, these low-frequency alleles are maintained in populations by a mutation-selection balance (where selective coefficients are similar to mutation rates).

The figures given by Powell (1975) lead to the conclusion that in most groups of animals, although apparently not in mammals, non-enzymic proteins are on average less variable than the enzymes commonly studied. This conclusion is reinforced by our data.

Correlations between enzyme properties and enzyme heterozygosity have been sought by a number of workers. Perhaps the most widely accepted view is that of Gillespie & Kojima (1968; Kojima, Gillespie & Tobari, 1970), who showed that the non-glucose metabolizing enzymes of *Drosophila* are genetically more variable than those metabolizing glucose. They proposed that the non-glucose enzymes, being less specific with respect to substrate, are subject to less rigid structural constraints than the glucose metabolizing enzymes. However, this relationship

may not hold for vertebrate species. Here the difference in mean heterozygosity between the two groups of enzymes is very small (Ward, 1977). It can be seen that in the plaice the glucose metabolizing enzymes are considerably *more* variable than the non-glucose enzymes, although in fact the difference in mean heterozygosity is not statistically significant ($P \simeq 0{\cdot}085$).

It has been proposed that heterozygosity is related to subunit number (Ward, 1977). In general, monomeric enzymes are more variable than dimeric enzymes, and dimeric enzymes are more variable than tetrameric forms. Zouros (1976) also found monomeric enzymes to be more variable than multimeric species. This may result from the possession by multimeric enzymes of critical amino acid sites required for subunit binding, thus subjecting multimeric enzymes to more severe structural constraints than the monomeric forms. Data from the plaice support this relationship, although the errors attached to the mean heterozygosities of the different structural classes render the differences statistically non-significant.

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