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Genetic evidence for two species of lugworm (*Arenicola*) in South Wales

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ABSTRACT: Field observations suggest that the common lugworm *Arenicola marina* (L.) has 2 forms on British shores although taxonomists have hitherto mostly recognised it only as a single species showing some morphological variation. Using gel electrophoresis of enzyme systems in homogenised tissue from specimens collected around Swansea (South Wales, UK), we have shown that the 2 forms do not appear to share the same gene pool. The 2 forms are fixed for different alleles at 3 loci out of the 6 which proved to be consistently resolvable and show little similarity in the 2 variable loci at which alleles are shared. Only 4 alleles were found to be common out of 22 investigated. A high value for Nei's Genetic Distance (1.3032) and a low one for Genetic Identity (0.2717) also indicate that they are separate species. An observed heterozygote deficiency is probably due to the mixing of populations as a result of the extended pelagic dispersal phase of larvae and post-larvae.

INTRODUCTION

For many years anglers and bait diggers in Britain have differentiated between 2 forms, varieties or types of the common lugworm *Arenicola marina* (L.), a widely used and very popular winter bait species. The forms are referred to as 'blow lug' (or 'red lug') and 'black lug'. Their earliest mention in the scientific literature appears to be by Gamble & Ashworth (1898) who referred to them as the 'littoral' and 'laminarian' varieties respectively, recognising differences in 'habits and structure'. These differences will be discussed in later publications on morphology and general ecology. Wells (1957), in his paper on variation in this species in which he assigned subspecies status to an Alaskan form, *A. marina glacialis* (Murdoch), expressed serious doubts about this division and considered the littoral and laminarian types of *A. marina* to consist of a single form or variety. An investigation of the impact of intensive bait-digging on lugworm stocks at several locations near Swansea (South Wales, UK), including a study of the possible utilisation of a recently-introduced bait-pump (Cadman 1989), nevertheless led us to the belief that there are clear distinctions in the ecology, morphology and perhaps also physiology of such worms.

It was therefore decided to attempt to resolve these differences by means of horizontal starch gel electrophoresis, a technique that was not available to previous workers. The potential of this technique as a taxonomic tool has been discussed at length by many authors (e.g. Avise 1975, Thorpe 1979, Ward 1989); in combination with morphological and ecological data, electrophoresis has become an accepted way of solving taxonomic problems such as the present one. The method has been used by investigators of both vertebrates (e.g. Jamieson 1974 on various fish stocks, Smith & Robertson 1981 on sprats, Avise & Smith 1974 on sunfish, and Comparini & Rodino 1980 on the Atlantic eel) and invertebrate phyla (e.g. Manwell & Baker 1963 on holothurians, and Shaw et al. 1987 on anthozoans). Shahid (1982) investigated a uniform population of *Arenicola marina* and a number of studies have been carried out on other polychaete genera (e.g. by Mustaquim 1988) and notably by Grassle & Grassle (1976) who used this method to differentiate between members of the *Capitella* sibling species complex which, although they have very different ecological features, show few or no morphological differences – a situation similar to that being examined here. We therefore investigated a number of enzyme systems in order to determine whether there are, in fact, more than one species of this very widely distributed and much studied animal.

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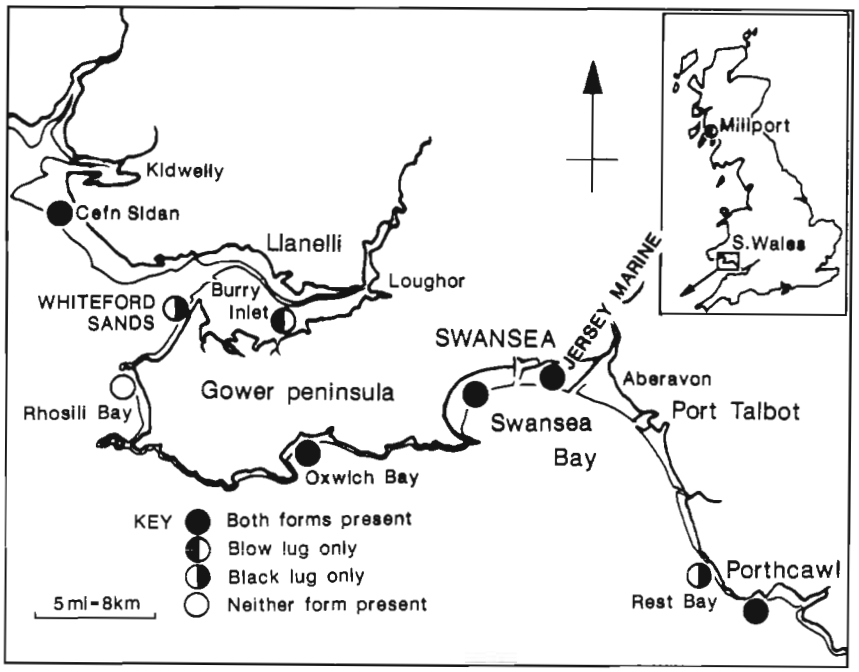

Fig. 1. Location of sampling sites around Swansea and of Millport (Isle of Cumbrae). Worms for the work reported here were taken mainly from Whiteford Sands and Jersey Marine

MATERIALS AND METHODS

Sample collection and preparation. Our main sampling site was near Jersey Marine, at the eastern end of Swansea Bay (Fig. 1); this was the first site which we found to contain sympatric populations of both blow and black lug. Blow lug were also obtained from Kames Bay at Millport, Isle of Cumbrae (in the Clyde Estuary, Scotland) where it is the only form of lugworm present, and black lug were collected at Whiteford Sands on the Gower Peninsula (west of Swansea) where, again, only that form is present.

Blow lug were taken by the traditional method of digging, inserting a garden fork parallel to the axis joining the feeding depression and wormcast (surface markings which are usually obvious) to a depth of 20 to 30 cm. Black lug (Fig. 2) cannot be collected in this way because (1) it is impossible to find the 'set' (as diggers call the surface markings) since there is only very rarely a feeding depression; and (2) the worms appear to lie, head downwards, in an almost vertical shaft with little or no horizontal gallery, to a depth of 50 to 100 cm. When the surface is wet, attempts to dig out the worms fail because the hole fills with a slurry of sand and water. These worms were therefore collected with the 'Alvey Bait Pump', a recent import from Australia resembling an old-fashioned gardening syringe without its jet, which uses a combination of suction and the removal of a core of sediment to extract the worm. Worms which became damaged or broken in this process were still useful for electrophoresis, as only the posterior achaetous end or 'tail' region is required. Worms were collected from sites other than Jersey Marine and gels prepared from their tissues to confirm that any differences in mobility of enzyme systems between the 2 forms, during electrophoresis, are consistent.

The 'tail' of each worm was excised and split longitudinally in order to remove as much of the gut contents, mucus and coelomic fluid as possible, since these materials were found to cause streaking on the gel. Approximately 1 cm^2 of body-wall tissue from the prepared 'tail' was then finely chopped and placed in a small glass tube with 0.2 ml of homogenising buffer (Tris 0.02 M, ETDA [ethylenediaminetetraacetate] 0.001 M, adjusted to pH 7.0; 0.05 g PMSF [phenylmethylsulphanyl fluoride] and 0.05 g NADP [nicotinamide adenine dinucleotide phosphate]) were added to 100 ml of this solution to retard breakdown of the proteins, with 0.05 g bromophenol blue to act as an indicator of the front); finally, a pinch of sterile sand was added to aid in maceration. The tubes and the residue of the samples were then stored at −70 °C until required.

Electrophoresis. The samples were macerated using an electrically-driven rotating glass rod of a size to match the storage tube and then separated in a refrigerated centrifuge at 2790 × *g* for 25 min. A wick of Whatman No. 3 paper was soaked in the supernatant of each sample, blotted dry and placed on a gel prepared using a 12.5 % suspension of hydrolysed starch (Connaught Laboratories; following Pasteur et al. 1988) in a Tris-citrate-EDTA buffer at pH 6.0. Standard electrophoretic techniques, as described by Harris & Hop-

Fig. 2. *Arenicola marina*. Black lug form. Scale bar = 2 cm

kinson (1976), were used, applying 220 V from a regulated power supply (at 44 V cm^{-1}) for ca 4 h and buffering with a continuous Tris-citrate-EDTA system at pH 6.0 to 5.4, again after Pasteur et al. (1988). Samples of blow and black lug were run on the same gel to ensure valid comparisons.

Twelve enzyme systems were initially surveyed, of which 6 were consistently resolvable and could be interpreted accurately for all or most of the individuals. The 6 were: superoxide dismutase (SOD, EC 1.15.1.1), 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), esterase D (ESTD, EC 3.1.1.1), phosphoglucose isomerase (PGI, EC 5.3.1.9); leucine amino-peptidase (LAP, EC 3.4.11.1/2); α-glycerophosphate dehydrogenase (αGPDH, EC 1.1.1.8). Phosphoglucomutase (PGM, EC 2.7.5.1) was scorable only for blow lug so the results for this system have not been included. The stains used were modified from those described by Shaw & Prasad (1970).

To indicate any deviation from Hardy-Weinberg expectations, a χ^2 test was carried out, modified following Levene (1949) and Li (1955) because of the comparatively small sample sizes. This correction also allows for the pooling of the rarer alleles. The test was conducted for black lug at the *6-pgdh* and *Lap* loci and for blow lug at the *α-gpdh* locus. Deviations from Hardy-Weinberg expectations were not calculated for those loci in which the expected frequency of the rarest allele was below 2. The χ^2 test of Nass (1959) was used to determine whether the frequencies of alleles shared between the 2 forms were significantly different.

RESULTS

Staining for 6 protein systems gave 6 scorable genetic loci. Allele frequencies, observed and expected heterozygosity values and deviations from Hardy-Weinberg expectations are listed in Table 1. Genetic distance (D) and genetic identity (I) (Nei 1972) were calculated for all 6 systems. These results are summarized in Table 2.

6PGDH. One locus with 4 alleles was scored for 6PGDH, producing 1- and 3-banded phenotypes. There were no alleles in common between the 2 forms of *Arenicola marina*. The black lug population from Whiteford shares a single common allele with the Jersey Marine black lug population and appears to be monomorphic ($p > 0.99$) but, given the high frequency of this allele and the small size of the Whiteford sample, this is not suprising. The Millport blow lug population has the same alleles as the Jersey Marine population but again, due to the low sample size, no attempt has been made to explain the slightly different frequencies of these alleles between the 2 populations.

ESTD. One locus with 4 alleles was scored producing 1- and 3-banded phenotypes. There are no alleles in common; although the *Est-D* 3/3 homozygote band in blow lug overlaps the *Est-D* 2/2 band in black lug, there is no doubt that these are different alleles because of the obvious difference in mobility. As with *6-pgdh*, very similar allele frequencies were observed between the black and blow lug pairs from the 3 sites.

αGPDH. One locus with 3 alleles was scored; black lug is monomorphic with only a single band, whereas blow lug is polymorphic with 1- and 3-banded phenotypes. Again, no alleles are shared between the 2 forms. The blow lug population at this locus does not deviate significantly from Hardy-Weinberg expectations ($p > 0.5$).

SOD. One locus with a single allele was scored. Both forms are monomorphic ($p > 0.99$), fixed for an allele of the same electrophoretic mobility.

PGI. One locus with 5 alleles was scored for PGI, producing 1- and 3-banded phenotypes. Only two of the alleles (Pgi^1 and Pgi^3) are common to both forms and the frequencies are very different, giving a χ^2 value (Nass 1959) of 123.1 ($p < 0.0001$, 2 df). Black lug has 3

Table 1. *Arenicola marina*. Allele frequencies, observed and expected heterozygote frequencies and probabilities of deviation from Hardy-Weinberg expectations in samples from Great Britain. Most of the sampling was carried out at Jersey Marine, Swansea. Millport and Whiteford samples were examined for only 2 loci, *6-pgdh* and *Est-D*. Alleles are listed in ascending order of mobility. N: no. of individuals in each sample; He: expected frequency of heterozygotes; Ho: observed frequency of heterozygotes; p: values indicate whether the population deviates significantly from Hardy-Weinburg expectations

Locus	Allele	Jersey Marine				Whiteford		Millport	
		Black lug	N	Blow lug	N	Black lug	N	Blow lug	N
6-pgdh	4	0.94	78	0.00	89	1.00	14	0.00	7
	3	0.06		0.00		0.00		0.00	
	2	0.00		0.08		0.00		0.14	
	1	0.00		0.92		0.00		0.86	
Est-D	4	0.08	106	0.00	90	0.04	14	0.00	7
	3	0.00		0.10		0.00		0.00	
	2	0.92		0.00		0.96		0.00	
	1	0.00		0.90		0.00		1.00	
α-gpdh	3	0.00	37	0.23	35	–		–	
	2	0.00		0.77		–		–	
	1	1.00		0.00		–		–	
He		–		0.358					
Ho		–		0.400	$p > 0.5$				
Pgt	5	0.00	44	0.01	47	–		–	
	4	0.00		0.01		–		–	
	3	0.20		0.96		–		–	
	2	0.01		0.00		–		–	
	1	0.79		0.02		–		–	
He	0.336	0.333							
Ho	0.318	$p > 0.5$							
Sod	1	1.00	24	1.00	26	–		–	
Lap	5	0.33	36	0.00	38	–		–	
	4	0.57		0.00		–		–	
	3	0.10		0.94		–		–	
	2	0.00		0.01		–		–	
	1	0.00		0.05		–		–	
He	0.333	$p < 0.025$							
Ho	0.500								

alleles at this locus (Pgi^1, Pgi^2, Pgi^3) and does not deviate from Hardy-Weinberg expectations ($p > 0.5$). Blow lug has 4 alleles (Pgi^1, Pgi^3, Pgi^4, Pgi^5). Shahid (1982) found 5 alleles at this locus, using what appears to be the blow lug form of *Arenicola marina* from various sites around Northumberland (NE England) but, as with this study, the frequency of the 4 less common alleles was very low with the rarest allele having a frequency between 0.001 and 0.005.

LAP. One locus with 5 alleles was scored for LAP, producing 1- and 2-banded phenotypes. Only one allele (Lap^3) is common to both types and, again, the frequency of this allele is very different in the 2 forms. At this locus, black lug deviate significantly from Hardy-Weinberg expectations ($p < 0.025$).

Table 2 includes mean values for genetic identity (I) and genetic distance (D), calculated from all 6 systems studied.

Table 2. *Arenicola marina*. Summary of genetic variation in black lug and blow lug from Jersey Marine (results from Millport and Whiteford are not included)

	Black lug	Blow lug
Mean no. of worms scored per locus	53.7	54.2
No. of loci examined	6	6
Proportion of polymorphic loci ($p > 0.99$)	0.67	0.83
Average no of alleles per locus	2.0	2.3
Genetic identity (I)	0.2717	
Genetic distance (D)	1.3032	

DISCUSSION

From the results given above, the 'blow' and 'black' forms of *Arenicola marina* do not appear to share the same gene pool. They are fixed for different alleles at 3

genetic loci out of the 6 studied (*6-pgdh, Est-D* and *α-gpdh*) and show little similarity in the 2 variable loci at which there are shared alleles. Further evidence, in the form of a high value for Nei's genetic distance (1.3032) and a low value for genetic identity (0.2717), also suggests that they are indeed separate species. Thorpe (1979, 1983) concluded that genetic identity between most congeneric species falls within the range 0.25 to 0.85; between conspecific populations this values lies above 0.90, although it must be pointed out that the values obtained here may be subject to sampling error, owing to the comparatively low number of loci investigated. Nei (1972) pointed out that the greater the number of loci which are investigated, the more reliable is the result. Nevertheless, the presence of only 4 alleles in common out ot a total of 22 is consistent with our proposal that the 2 forms be considered distinct species rather than subspecies, or varieties as proposed by Gamble & Ashworth (1898).

The heterozygote deficiency for black lug at the *Lap* locus is hard to explain, given the amount of data obtained during this study, but may be due to one or more of the following: mutation, migration, genetic drift, natural selection, presence of null alleles or the Wahlund effect, according to Ferguson (1980). It is impossible to determine which is the case here without considerable further work, but a possible cause seems to be the Wahlund effect, as mating choice can be ruled out; given the spawning behaviour of *Arenicola marina*, association of gametes is a random process. A number of workers (Benham 1893, Ashworth 1904, Farke & Berguis 1979) have shown, by the presence of post-larvae or 'Benham larvae' in the plankton from March to June, that the blow form of *A. marina* has a pelagic or dispersal phase which may last several weeks. Blake (1979) pointed out that the Benham larvae may well be an important source of repopulation of beaches 'dug out' by bait collectors and that such recruitment would result in interpopulation mixing. Shahid (1982) found significantly different allele frequencies (at the *Pgi* locus) between one population and the other 4 which he was studying on the northeast coast of Britain, which gives some credence to the idea that discrete populations of blow lug may exist. Both Wells (1957) and Duncan (1959) suggested that there is a strong possibility of discrete races or varieties arising from populations separated by physical discontinuities: Duncan showed great variation in spawning periods, which may indicate genetic differentiation in *A. marina*, and Wells also found considerable morphological variation amongst and between populations. Around the Gower peninsula, there are several beaches which have populations of both blow and black lug, beaches supporting one or the other alone, and also locations which appear unsuitable for either type (Fig. 1). It is possible that the population of black lug at Jersey Marine is a mixture derived from several other South Wales populations, thus giving rise to the observed heterozygote deficiency. This appears to be a common problem when examining animals with a pelagic or other dispersal phase; Tracey et al. (1975) explained in this way the homozygote excess which they reported in the mussel *Mytilus californianus*, suggesting that such an excess (i.e. heterozygote deficiency) occurs partly because very few, if any, young animals settle in the area in which they were spawned.

At present, little or nothing is known of reproduction in black lug, but it is likely that this form uses the same basic strategy reported for blow lug (as undifferentiated *Arenicola marina*) in the literature. The fact that heterozygote deficiency was found for black lug would suggest that their post-larvae also spend a significant amount of time in the plankton. Juvenile black lug and blow lug were both found at Jersey Marine (although only the adults were sampled for electrophoresis), but it is impossible at present to determine whether they were spawned on that beach or were recruited from another location.

We anticipate that morphological and ecological studies, still in progress, will confirm that the 'black' form is a separate species. We will propose a formal taxonomic revision of this lugworm in the near future.

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