Biochemical systematics of certain polychaetes with special reference to *Polydora ciliata* (Johnston)

by

Javed Mustaquim

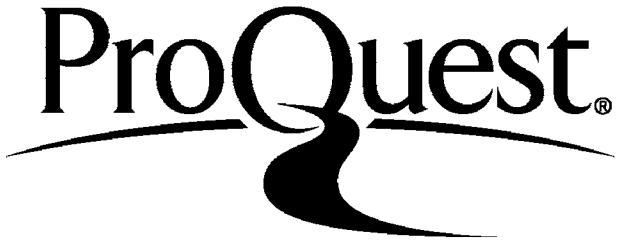
A thesis submitted to London University

for the degree of

Ph.D.

Bedford College, London

April, 1982



ABSTRACT

Intraspecific variation in Polydora ciliata (Johnston) was assessed on the basis of evidence from morphology and population genetics. Morphological characters were studied by scanning electron microscopy and differences were observed between populations of P. ciliata with respect to the setae of the enlarged 5th segment and in the length of caruncle.

Gene frequencies were determined for all the nine populations of P. ciliata by the use of polyacrylamide slab gel electrophoresis. Seven enzyme systems comprising 13 loci were investigated from individual worms. The enzyme systems were: 1. acid phosphatase, 2. esterase, 3. glucose-6-phosphate dehydrogenase, 4. malate dehydrogenase, 5. malic enzyme, 6. phosphoglucose isomerase, and 7. xanthine dehydrogenase. The mean genetic identity and genetic distance were calculated using Nei's index. The genetic identity calculations revealed a high degree of similarity between populations of P. ciliata which bore in hard substrates. The 'boring' and 'non-boring' forms of P. ciliata were significantly different from each other with respect to phosphoglucose isomerase, esterase and acid phosphatase.

Two other species of Polydora, P. ligni Webster and P. limicola (Annenkova) were also studied morphologically and electrophoretically in order to assess interspecific variation. P. ligni was found very similar genetically to the 'non-boring'

form of P. ciliata.

Other polychaetes which were studied electrophoretically include; 1. Arenicola marina, 2. Nereis diversicolor, 3. N. virens and N. fucata. Inter and intraspecific variation was observed but due to small sample sizes gene frequencies were not determined.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Prof. R. P. Dales for his supervision, critical discussion and constant encouragement throughout this study. With out his help this research project could not have been completed.

I am also indebted to the following people for their help in collecting specimens: Dr P. E. Gibbs of the Marine Laboratory, Plymouth; Dr M. Kent, while working at the Marine Laboratory, Plymouth; Mr M. Kendall of the Wellcome Marine Laboratory, Robin Hood's Bay; Mrs. Jan Thorne, Miss Elizabeth Storer, Mr K. Jennings, Mr D. Herbert, Mr A. Brimble and Dr Lynda Warren of Bedford College, London. I am also grateful to Dr J. R. Lewis of the Wellcome Marine Laboratory for obtaining permission from the Port authority and arranging my trip to Tees estuary (Seal sand). The personal delivery of living worms from St. Andrews by Dr C. D. Todd of the Gatty Marine Laboratory is especially appreciated.

My thanks are due to Miss Mandy Havard of University College, Cardiff; Dr P. R. Garwood of the Dove Marine Laboratory, Cullercoats and Dr S. A. Siddiqui of the Oceanography Department, Liverpool University for their hospitality during my visit and help in collecting specimens.

I would like to thank Dr P. R. Dando of the Marine Laboratory, Plymouth and Mr H. Jamil of the Biochemistry

Department, Bedford College for useful discussions on electrophoretic problems. The general help extended by my colleague Mr G. Arason is gratefully acknowledged.

My thanks are also due to Mr D. J. S. Field, Chief Technician and his staff for technical assistance, especially Mr Z. Podhorodecki for taking gel photographs. I am grateful to Mr S. Davies for assistance with scanning electron microscopy.

My unending gratitude is herein extended to my wife for her untiring support throughout this study.

TABLE OF CONTENTS

LIST OF TABLES

Table 10. Summary of gene and genotype distributions and heterozygosities for the EST-2 locus for different populations of Polydora.

Table 11. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of EST-2 locus among eleven populations of Polydora.

Table 12. Summary of gene and genotype distributions and heterozygosities for the EST-3 locus for four populations of Polydora which were polymorphic.

Table 13. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of EST-3 locus among eleven populations of Polydora.

Table 14. Estimates of mean genetic identity (below diagonal) and genetic distance (above diagonal) of 13 loci among eleven populations of Polydora.

Table 15. Estimates of mean genetic identity (below diagonal) and genetic distance (above diagonal) of pooled data of 'boring' and 'non-boring' forms of P. ciliata, P. ligni and P. limicola for 13 loci.

Table 16. Average heterozygosity ($\bar{H}$) per locus in different populations of Polydora.

Table 17. Average genetic similarity, $\bar{I}$ and genetic distance, $\bar{D}$ between taxa of various levels of evolutionary divergence in the Drosophila willistoni group (From Ayala et al. 1974).

LIST OF FIGURES

in different populations of Polydora.

Figure 13. Histograms showing distribution of caruncle length with respect to segment number in different populations of Polydora.

Figure 14. Scanning electron micrograph of the anterior region (dorsal view) of P. ciliata, from Dumpton, showing caruncle.

Figure 15. P. ligni; scanning electron micrograph of a caruncle bearing nuchal antenna.

Figure 16. P. ciliata (from Whitstable); scanning electron micrograph of a caruncle bearing nuchal antenna.

Figure 17. P. ligni; scanning electron micrograph of enlarged nuchal antenna.

Figure 18. Population range diagram showing variation in the number of heavy spines in the 5th enlarged segment of Polydora species.

Figure 19. Scanning electron micrograph of Polydora showing arrangement of various types of setae on the 5th enlarged segment.

Figure 20. P. ligni; scanning electron micrograph of the 5th enlarged segment; arrow indicates an extra ordinary heavy spine.

Figure 21. P. limicola; scanning electron micrograph of the 'posterio-ventral' bundle of setae on the 5th enlarged segment.

Figure 22. P. ciliata (from Barry); scanning electron micrograph of a heavy spine of the 5th enlarged segment.

Figure 23. P. ligni; scanning electron micrograph of a heavy spine of the 5th enlarged segment.

Figure 24. Average number of hooded hooks per segment in different populations of Polydora species with standard error of the mean.

Figure 25. Diagram showing various criteria used to distinguish different loci on zymogram.

Figure 26. Diagram showing position of bands of glucose-6-phosphate dehydrogenase (G6PD) and malate dehydrogenase(MDH) found in eleven populations of Polydora.

Figure 27. Diagram showing position of bands of malic enzyme (ME) and xanthine dehydrogenase (XDH) found in eleven populations of Polydora.

Figure 28. Photograph of malate dehydrogenase zymogram of P. ciliata, P. ligni and P. limicola.

Figure 29. Photograph of xanthine dehydrogenase zymogram of P. ciliata, P. ligni and P. limicola.

Figure 30. Diagram of acid phosphatase zymogram showing two types of patterns found in Polydora species.

Figure 31. Diagram of phosphoglucose isomerase zymogram showing three different types of patterns found in Polydora species.

Figure 32. Photograph of acid phosphatase zymogram showing two

types of patterns found in Polydora species.

Figure 33. Photograph of phosphoglucose isomerase zymogram of P. ligni, P. limicola and P. ciliata.

Figure 34. Diagram of esterase zymogram showing two types of patterns found in Polydora species.

Figure 35. Photograph of esterase zymogram of P. ciliata from Dumpton ('boring' form).

Figure 36. Diagram showing position of bands of lactate dehydrogenase (LDH) and peroxidase (POX) in Polydora species.

Figure 37. Diagram showing position of bands of isocitrate dehydrogenase (IDH) and tetrazolium oxidase (TO) in Polydora species.

Figure 38. Phenogram showing relationships of four groups of Polydora generated according to UPGMA method of cluster analysis and based on the data in Table 15.

Figure 39. Camera lucida sketch of the heavy spines of the 5th enlarged segment in a specimen of P. ciliata from Robin Hood's Bay which demonstrate the diversity of shape of the heavy spines (from Kendall, 1980).

Figure 40. Map showing locations of Arenicola marina populations sampled.

Figure 41. Map showing locations of Nereis diversicolor, N. fucata and N. virens populations sampled.

Figure 42. Diagram of phosphoglucose isomerase (PGI) and

LIST OF ABBREVIATIONS

(used in Tables)

ACP.	acid phosphatase
ADH.	alcohol dehydrogenase
AK.	adenylate kinase
ALD.	aldolase
α-ALDH.	α-alanine dehydrogenase
ALP.	alkaline phosphatase
AMY.	amylase
AO.	aldehyde oxidase
ARK.	arginine kinase
CA.	carbonic anhydrase
CAT.	catalase
CER.	ceruloplasmin
CPK.	creatine phosphokinase
DO.	dianisidine oxidase
EN.	enolase
EST.	esterase
FBA.	Fructose-biphosphate aldolase (=ALD)
FK.	fructokinase
FUM.	fumarase
β-GA.	β-galactosidase
GAPD.	glyceraldehyde-phosphate dehydrogenase
GDH.	glutamate dehydrogenase
GK.	glucokinase
β-GLU.	β-glutamate-oxaloacetate transaminase (=β-GOT)
GOT.	glutamate oxalate transaminase

GOXT.	glutamate-oxaloacetate transaminase (=GOT)
G3PD.	glyceraldehyde-3-phosphate dehydrogenase (=GAPDH)
G6PD.	glucose-6-phosphate dehydrogenase (=G6PDH)
α-GPD.	α-glycerophosphate dehydrogenase(=GPD)
GPI.	glucosephosphate isomerase
GPT.	glutamate-pyruvate transaminase
HAEM.	haemoglobin
β-HDH.	β-hydroxybutyrate dehydrogenase (=β-HBDH)
H_e	heterozygosity (expected)
HEXD.	hexanol dehydrogenase (=HEXDH)
HK.	hexokinase
H_o	heterozygosity (observed)
IDH.	isocitrate dehydrogenase (=ICD)
LAP.	leucine aminopeptidase
LDH.	lactate dehydrogenase
M.	monomorphic
MDH.	malate dehydrogenase
ME.	malic enzyme
MPI.	mannosephosphate isomerase
MYOG.	myoglobin
NDH.	'nothing' dehydrogenase
NK.	'nothing' kinase
NP.	nucleoside phosphorylase
ODH.	octanol dehydrogenase
P.	polymorphic
PEP.	peptidase
PGAM.	phosphoglyceromutase
6PGD.	6-phosphogluconate dehydrogenase (=PGD)

PGI.	phosphoglucose isomerase (=GPI)
PGK.	phosphoglycerate kinase
PGM.	phosphoglucomutase
PHI.	phosphohexose isomerase(=GPI)
PK.	pyruvate kinase
POX.	peroxidase
PROT.	protein
SDA.	superoxide dismutase (=SOD)
SDH.	sorbitol dehydrogenase
TO.	tetrazolium oxidase (=SOD)
TPD.	triose-phosphate dehydrogenase (=GAPDH)
TPI.	triose-phosphate isomerase
XDH.	xanthine dehydrogenase

Synonyms used in the literatures are noted on the right.

Abbreviations used in this thesis are on the left hand side.

INTRODUCTION

The morphological variation in natural populations has attracted the attention of biologists for centuries. It has been the basis for classification of organisms and it is still the most practical method of classifying living and preserved organisms. Taxonomy has a long history and it goes back to ancient Greece but it was about the middle of the eighteenth century, when taxonomy became a distinct discipline. In the middle eighteenth century taxonomic studies were confined to studies of the local faunas and it was thought that the species were fixed and uniform. It was believed that every species had an invariant generalized or idealized pattern shared by all members of the species. Variation within species was regarded as accidental and thought to have no taxonomic significance. It was during this period that Linnaeus published his work 'Systema Naturae' (1758) and introduced the binominal system of nomenclature. He accepted the idea of the fixity of species and shared the view that all individual members of a particular species were descendants of the animals created in a form which had remained virtually unchanged to the present day. However, there were some naturalists during the eighteenth century who began to doubt that species were unchanging. Buffon, for instance, advocated the idea of the fixity of species, nevertheless favoured the idea of transformation.

Lamarck was the first naturalist to discard the concept of fixed species. He was also the first to state that complex

organisms have evolved from similar ones. He regarded the simplest organisms known to him as the original organisms calling them 'infusores'. Although the root of evolutionary theory goes back to the eighteenth century it was not generally accepted until the end of nineteenth century, mainly due to the work of Charles Darwin (1809 - 1882). Darwin conceived evolution as a consequence of natural selection. The importance of Darwin's contribution is reflected in the immediate acceptance of his theory by many biologists and in the vast amount of research on evolutionary problems that took place during the second half of the nineteenth century.

The typological concept of the species which was already shaky in the early nineteenth century was replaced by a dynamic species concept. Interest reverted towards the fauna of local areas and the study of variation within populations. Species were no longer considered as fixed and uniform, but rather as polytypic, consisting of many sub-species and local populations, each differing from the others and each showing considerable variability within itself. The difficulties of the theory of natural selection which Darwin clearly recognized and set forth in the final edition of 'origin of species' (1872) were not being cleared up until biologists rediscovered Mendel's laws of inheritance in 1900. As long as no coherent theory of heredity existed, the basis of natural selection could not be understood.

The rediscovery of Mendel's work led to the rise of the study of genetics and in the first two decades of twentieth century Mendelian genetics was largely responsible for a temporary decline in Darwin's reputation among biologists. Early Mendelians (such as de Vries and Bateson) thought that the new species were produced in a single step by a large mutation and even disregarded the environment as a selective agent. The restoration of Darwinian natural selection as the principal guiding factor in evolution began with the development of population genetics in the 1920s, based upon the work of Fisher and others. Fisher in his book 'The genetical theory of natural selection' (1930) described that even a very small selective advantage of a new gene or gene combination could cause in time a genetic transformation of a population.

The taxonomic work of the twentieth century, especially since about 1940, is referred to as the 'new systematics' (Huxley, 1940) or 'biosystematics' and everything which preceded it as 'classical taxonomy' (Cronquist, 1964). In biosystematics the purely morphological species concept has been replaced by a concept which takes other biological factors into consideration. There is no doubt that the conclusion deduced from purely morphological data are of extreme importance but it is equally certain that morphology alone does not cover the whole story. It does not represent adequately the relationship between organisms. Many kinds of individuals were found that were clearly conspecific, inspite

of striking differences in structure due to sexual dimorphism, age-differences, polymorphism or other forms of individual variation. On the other hand many instances are known where morphologically identical populations live sympatrically and do not interbreed (sibling species). Situations like these have led to revision of the species concept. It is due to this reason that in biosystematics other biological aspects such as the ecology, geography, genetics and physiology of the species are included. The population, represented by an adequate sample, has become the basic unit rather than the individual.

The impact of this change has been enormous. Populations are variable and consequently the description, measurement and evaluation of variation became one of the major problems and tasks of the taxonomists. The methods of population analysis and statistics became a standard part of the process of taxonomy. In spite of all practical difficulties, reproductive isolation has proved to be the soundest theoretical criterion of a species. Mayr et al. (1953) defined species as "groups of actually (or potentially) interbreeding natural populations which are reproductively isolated from other such groups". The term 'population' can be used in different ways and has different meanings even in scientific language. For example, an ecologist may speak of the rodent population in a meadow. This might include mice, pocket gophers and individuals of some others species. A demographer, on the other hand, may speak of the 'English population' referring

to all men, women and children in England. When a taxonomist speaks of population he refers a 'reproductive community' or 'Mendelian population'. A Mendelian population can be defined as a "community of individuals of a sexually reproducing species within which mating takes place" (Dobzhansky et al., 1977). The individuals in a Mendelian population interbreed among themselves, exchange genes freely and randomly, and are said to share a common gene pool. A gene pool of a population is "the sum total of the genotypes of all individuals in a reproductive community or Mendelian population" (Dobzhansky et al., 1977). It is this Mendelian population that has become the basic unit of systematics and it is this population in which evolutionary changes occur, not in individuals. The genetic constitution of individuals remains the same throughout life, but the genetic constitution of a population may change from generation to generation due to migration, genetic mutation, drift and natural selection.

Two models or theories of genetic structure of populations have been proposed, the 'classical' and the 'balance' theory. According to the classical theory of population genetics, most individuals within a species would be homozygous for the wild type allele at most gene loci; at a very small proportion of its loci the individual would be heterozygous for a wild and mutant allele. Under this classical model mutations are thought to be constantly introduced into the population, but most of these are assumed to be deleterious and subject to

removal by natural selection. Rarely, a beneficial mutant allele might arise, conferring higher fitness upon its carriers than the preexisting wild type allele. This beneficial allele would gradually increase in frequency by natural selection to become the new wild type allele, while the former less beneficial wild type allele would be eliminated.

On the other hand according to the balance theory of population genetics, there is generally no single wild type or 'normal' allele. Most individuals would be heterozygous at a large proportion of their gene loci. The balance model derives its name from the fact that multiple alleles are thought to be maintained by balancing selection.

The balance model of the genetic structure of populations has now become established and evidence has accumulated over the years showing that genetic polymorphism is wide-spread. The maintenance of a high degree of polymorphism by various forms of balancing selection is, however, not clear.

The degree of genetic variation in a population can be estimated by detecting allelic variants in single genes representing a random sample of the total genome. In Mendelian genetics the presence of a gene is ascertained by studying segregation in mating between individuals. Only genes with allelic variation can be shown to exist. Therefore, it is not possible to obtain a sample of the genome random with respect

to variation, since only variable genes can be studied. Another problem is in Mendelian studies of single genes, individuals must be assigned to discrete classes which are readily distinguishable. Yet most of the genetic variation relevant to evolution affects characters within continuous expression, such as fecundity, viability, longevity, rate of development and size.

Recent development in molecular genetics have made it possible to obtain a random sample of the genome, and to detect allelic variation in individual loci. The genetic information encoded in the nucleotide sequence of the DNA of structural genes is translated into a sequence of amino acids making up a polypeptide chain. Most enzymes and proteins are the products of the individual genes, although some are made up of polypeptides coded by two or more genes (Lewontin, 1974; Dobzhansky et al., 1977). It is possible, to a first approximation, to equate variation in enzymes or proteins with variation in genes. A sample of proteins, random with respect to their genetically determined variation, may be obtained. The genes coding for such proteins represent a random sample of the genome. Variant as well as invariant loci can be surveyed.

New methods in systematics :

There are a number of methods which have been recently introduced into systematics. These methods have been used in

the hope that basic differences in the protein composition of different species of organisms might be revealed. I shall briefly review them so that it can be appreciated why a particular technique was chosen for the present study. Ferguson (1980) has discussed many of the methods and some of descriptions which follow are derieved from his book.

Serology : The 'systematic serology' or 'immunological taxonomy' grew out of medical immunology, from the pioneering work of Nuttall (1901, 1904), who first recognised that antigen-antibody reactions can be used in systematics. Latter it was greatly expanded by Boyden and his colleagues and the reviews of Boyden (1942, 1953, 1958) are valuable.

The basic principle is that the proteins are antigenic and they have distinct sites on their surface called 'antigenic determinant' against which antibodies are produced. The exact nature of these antigenic determinants and their relation to amino acids sequence of the protein is unknown. However, change in the amino acid sequence of a protein alter the nature of the antigenic determinants. If these antibodies are mixed with the original protein in the presence of a suitable electrolyte, an antigen-antibody reaction will take place in which the combining sites of the antibody link on to the antigenic determinant sites of the antigen. If the antigen has several antigenic determinants per molecule (multivalent) then, since antibodies have at least two combining sites, large aggregates of antigen-antibody will form and will precipitate from solution when antigen and antiserum are present in approximately equal amount.

If antibodies to a protein of one species are mixed with the same protein from another species - heterologous reaction - and if the two proteins have antigenic determinants in common, then antigen-antibody reaction will take place. The fewer the determinants in common and the poorer the matching of the combining sites, the weaker the antigen-antibody reaction will be. Based on the magnitude of this, an immunological distance (ID) can be calculated as described by Mainardi (1959) :

$$I.D. = \sqrt{\frac{O_a}{E_a} \cdot \frac{O_b}{E_b}}$$

where O and E stand for the homologous and heterologous titres respectively with the antisera a and b. The titres are the dilution of serum giving the end point, and not the concentration. This I.D. is proportional to the difference in structure between a test protein and the protein used to prepare the heterologous antibodies.

There are some problems in the use of serology, which Boyden (1942, 1958) and Lewontin (1974) have pointed out. 1) The rabbits which are often used in the preparation of antibodies may respond differently to the same antigen. The amount of antibody produced to determinants may vary from rabbit to rabbit and occasionally individual rabbits may fail to form antibodies to one or more determinants. 2) Sometimes it is not the proteins but carbohydrates which are resposible for cross reaction in distant taxa. It is not surprising that two creatures which both produced large amounts of, say, a

polymer of glucuronic acid, could show a strong serological cross reaction which dominates the true cross reaction. 3) It is possible to detect differences from the standard but it is very difficult to detect differences among variants and it is impossible to differentiate heterozygote from homozygote. 4) The antigen-antibody reaction against a single amino acid substitution, differs quantitatively rather than qualitatively, so the clear cut and simple amino acid sequence difference is converted into a continuously varying character.

Salthe (1969) tried to use micro-complement fixation to characterize genetic variation for lactate dehydrogenase in frogs, and although he demonstrated heterogeneity within and between populations, the resolution of the technique was inadequate for classifying genotypes (Lewontin, 1974).

Chromatography : Chromatography was first introduced by the Russian botanist, Tswett in 1906 (cited in Stevens, 1969), to separate plant pigments. He used adsorption chromatography in glass columns, but later Consden et al. (1944) described a new method of chromatography using sheets of filter paper.

Wright (1959) has reviewed these methods, mentioning for example, work on insects, fish, mollusc and echinoderm and the use of tissue fluids, muscle squashes and mucus, which were examined for amino acids, pigments and fluorescent substances.

The chromatographic separations are achieved by the

distribution of components in a mixture between a fixed and a moving phase, referred to as the stationary and the mobile phase respectively. Separation between two substances begins to occur when one is held more strongly by the stationary phase than the other, which tends to move on faster in the mobile phase. In general, a drop of the solution containing the components to be separated is placed near the end of a paper strip and allowed to dry. The strip is then placed so that it dips a few millimeters into a solvent. The solvent then commences to flow along the paper. After some time the strip is withdrawn and rapidly dried. Sometimes, after drying the paper completely, it is turned at right angles and run in a second solvent which performs a further separation and causes the components to be distributed on the paper in two dimensions instead of only one dimension. Since the majority of compounds of biological importance are colourless and not visible on paper, they are developed either by spraying or dipping the chromatogram in a bath of the reagent followed by some appropriate treatment such as allowing the paper to dry in air or heating until the colours appear.

Unlike serology, the result obtained by chromatography is not a similarity index but it is a set of data on the occurrence of individual chemical substances, which are good characters for taxonomic use. On the other hand like serology this method is also inadequate for classifying a genotype and it is difficult to distiguish hetero- and homozygous individuals.

Amino acid sequencing : Perhaps the maximum amount of systematic information is available when the complete amino acid sequence of a protein is known. Usually the Edman degradation process is used for determining amino acid sequence. In this process, amino acids are sequentially removed from the amino-terminus of the polypeptide. The terminal amino group of the polypeptide is coupled with a reagent called phenylisothiocyanate and a phenylthiocarbamyl derivative is formed. Under anhydrous acidic condition, the sulphur of the phenylthiocarbamyl group attacks the carbamyl component of the first peptide bond, resulting in a change of the terminal alpha amino acid as a thiazoline. This cleaved amino acid is separated from the residual polypeptide by extraction with solvent and identified after converting it to a more stable phenylthiohydantion form. The shortened peptide now has a new amino-terminal. By repeating the cycle further amino acids from the amino-terminal can be cleaved, removed and identified. This can establish the sequence of amino acids on a polypeptide. There are various methods by which a cleaved amino acid can be identified including gas-liquid chromatography, thin layer chromatography or mass spectrometry. A free amino acid (after hydrolysis of the thiazolinones or thiohydantoins) can be identified by ion exchange column chromatography in automatic amino acid analysers.

Edman degradation cannot be carried out for more than 60 - 70 cycles (Ferguson, 1980). Therefore, large proteins are broken into small fragments of peptides with enzymes. The

endopeptidase trypsin and chymotrypsin are commonly used for this purpose. Trypsin breaks a polypeptide chain by hydrolising the bonds just after the point where the positively charged lysine and arginine occurs, while chymotrypsin similarly severs the chain after phenylalanine, tyrosine or tryptophan. The peptides resulting from digestion of a polypeptide with these two enzymes will be different but overlapping. From the sequence of these overlapping fragments, the overall sequence can be deduced.

Insulin which consists of 51 amino acids was the first protein to be sequenced by Sanger and Thompson (1953). The procedures to establish the amino acid sequence of proteins are extremely laborious and time consuming. Current technology is not far enough advanced to allow such a procedure to be carried out on hundreds of individuals for scores of proteins.

Electrophoresis : The electrophoretic technique has been given much attention by taxonomists and is being used more widely than any other biochemical methods. In order to appreciate the type of approach that has been used, a brief outline of the physiochemical properties of proteins is appropiate. Proteins are composed of one or more polypeptides and these polypeptides are chains of some 20 essential amino acids. Amino acids are nitrogen-containing molecules with an amino group (NH_2) and a carboxyl group (COOH) and a general formula of :

$$NH_2 — \underset{\displaystyle R}{\underset{|}{CH}} — COOH$$

where R is specific to the amino acid. The simplest amino acid is glycine, in which R is a hydrogen atom. The individual amino acids are linked through an amide or a peptide bond (—CO.NH—) to form a polypeptide chain. The sequence in which amino acids are linked to form a polypeptide chain is known as the 'primary structure'. Each polypeptide has a free amino group at one end and a free carboxyl group at the other end. Most polypeptides are coiled by formation of hydrogen bonds between adjacent amino acids and this coiling of peptides is referred to as 'secondary structure'. Disulphide bonds can bridge two cysteine residues in different parts of the coiled chain and this, and other bonding, results in folding of the molecule and is known as the 'tertiary structure'.

The R group of some amino acids are acidic (negative charge) or basic (positive charge) or neutral. A polypeptide made up of a mixture of differently charged amino acids will have a net negative or positive charge depending on the balance of the charges and the folding of the molecule. If the pH of the environment is lowered (H^+ increased) NH_2 will be positively charged (NH_3^+), while acidic (COO^-) ions will be saturated and become neutral (COOH). The result is that the polypeptide will take a positive charge. The reverse will happen if the pH is raised (H^+ decreased). The pH where negative and positive charges just balance each other, to give a neutral polypeptide, is known as the iso-electric point (pI). The iso-electric points of most proteins in

animals are slightly alkaline, around pH 8 (Lewontin, 1974).

If an allelic change occurs at a locus or gene in such a way that an amino acid is replaced by another amino acid of different charge, the net charge of the protein will be altered, at a given pH. Such changes in charge can be used to separate proteins and thus to identify the products of different alleles.

Electrophoretic methods permit detection of allelic variants in individual genes. Both variant and invariant gene loci can be identified. A random sample of genes with respect to variation is possible. Proteins and enzymes for which the appropriate assay technique exist can be chosen for study without any prior knowledge whether they are variable or not and if variable how variable. This technique allows reasonably rapid examination of large numbers of individuals and many enzymes and proteins.

In general, electrophoresis is the movement of charged particles under the influence of an electric field and the apparatus mainly used consists of a gel slab (starch, agar, acrylamide) whose two ends are in contact with the opposite poles between which a current flows (Figure 1). Usually electrophoresis is carried out in pH 8 - 9 buffers, at which pH most proteins are negatively charged and migrate towards the anodal end of the gel. A protein sample for electrophoresis is introduced into wells at one end of the gel and any charged

molecule will move down along the gel towards the anodal end under the influence of electric current applied. After a high voltage has been applied to the gel for few hours, the various proteins that migrated at different rates will be concentrated at different points along the gel and these can be visualised by staining the gel with a general protein stain. Enzymes generally exist in low concentration and a particular enzyme can only be located by a dye-coupled enzyme substrate reaction. In this reaction the enzyme is used to break-down the specific substrate, and in so doing to bring about the oxidation or reduction of a soluble substance to an insoluble, coloured form. The result is a coloured precipitate which forms at the site of the enzyme activity.

Proteins for electrophoresis must be in solution and can be extracted from various tissues by homogenisation, sonication or by freezing and thawing. The concentration and pH of the extraction mixture is important. Any change in ionic strength or pH can bring about differential extraction of protein. Proteins already in solution forms like milk or blood, can be applied directly.

Review of electrophoretic methods :

Tiselius in 1937 (cited in Brewer, 1970) developed the moving boundary electrophoresis in a free solution and he may be considered as 'the father of electrophoresis' (Brewer, 1970). Subsequent development involved zone-electrophoresis in which

Figure 1. Diagram of a vertical slab gel electrophoresis apparatus.

A. electrode vessel; B. bands of haemoglobin; C. power supply; D. gel slab.

solid media are used to separate various components of proteins in distinct zones or bands. The moving boundary method is technically difficult and requires relatively large samples. It is possible to stabilize solutions to a certain extent by using an electrophoretically immobile solute such as sucrose, but it is more feasible to use a solid medium instead. Detection of the protein zones by refraction in solid media is not possible due to light scattering. Hunter and Markert (1957) introduced histochemical techniques for detection of proteins and enzymes after electrophoresis and these are now used widely. Because of the importance of developing appropiate media for electrophoresis, the important historical features in this technique consist, in large part of a description of the introduction of different media. Only analytical methods will be discussed and not the preparative methods which are usually used for enzyme separation for further analysis.

1) Paper electrophoresis : Filter paper was first used as a supporting medium for zone electrophoresis. Most work has been done on Whatman No. 1 filter paper. The capacity of the wet papers to accomodate the sample is limited, with Whatman No. 1 paper about 3 - 4 µl/cm can be applied, while thicker grades can take up to 8 - 10 µl/cm (Wilkinson, 1970). Whatman chromatography paper No. 100 is also used as it is capable of holding relatively large samples. Paper electrophoresis is of two types, the 'vertical' where paper strips are held vertically and 'horizontal' where it is held

horizontally. In practice, both types give almost identical separation in paper electrophoresis (Smith, 1968).

The resolving power of paper electrophoresis is not very high and the paper is not as homogenous as other media which are described later. Streaking of bands and adsorption make it impossible to obtain a pure white background.

2) Cellulose acetate electrophoresis : Cellulose acetate membrane (CAM) filter electrophoresis was introduced by Kohn (1957). There are many advantages of cellulose acetate membrane ~~filter paper~~ as a supporting medium for electrophoresis. The material is more homogenous and microporous than paper. Adsorption is minimal and it eliminates the 'tailing effect' resulting in sharp and well defined bands. The background is white. Adequate separation of protein bands for analytical purposes can usually be achieved in 1 - 2 hours. The cellulose strip can be cleared by using a suitable swelling agent like glacial acetic acid or by immersion in an appropiate clearing fluid, such as whitmore oil 120, to glass like transparency. The latter method has the advantage that the cleared strips can be returned to their original dry state by washing them with petroleum ether. A cleared strip can be scanned and photographed.

Cellulose acetate is also available in gel form in blocks and strips called 'cellogel'. It is supplied moistened with 50% methanol, which must be removed by blotting .

and soaking in buffer before use. The gel strip can be used in the same electrophoretic apparatus as for cellulose acetate paper.

3) Agar gel electrophoresis : Agar is a polysaccharide prepared from algae and agar gel was first used by Gordon et al. (1949) as an electrophoretic medium. It gels in concentrations as low as 0.75%. This method of electrophoresis is very quick and usually takes only 25 - 30 minutes. It was first used for isozyme studies by Wieme (1959) with lactate and sorbitol dehydrogenases.

Unlike paper and cellulose acetate, agar is transparent and allows direct densitometric measurements after staining. It has the particular advantage that during isozyme separation passive diffusion is less than with paper or cellulose acetate and its resolving power is greater than either paper or cellulose acetate. It has seen widespread use for immunoelectrophoresis, discussed elsewhere.

An excellent account of the technique and application of agar gel electrophoresis has been compiled by Wieme (1965).

4) Starch gel electrophoresis : In 1955 Smithies introduced starch gel as the supporting medium for electrophoresis. This technique has been used more widely than any other method. Powdered starch is hydrolised by heating in buffer or aqueous solution and, upon cooling, it forms a gel. The electrophoretic

separation on paper, cellulose acetate and agar gel are based on a simple electrophoretic effect, the migration of charged particles under the influence of an electric current. The starch gel contributes an additional factor to the separation of proteins. The pore size of the gel is of the same order of magnitude as the diameter of the globular proteins migrating through them. Hence separation in starch gel is not only effected by the electric charge but also by the molecular size. Evidence to support the hypothesis that molecular size influences the mobility in starch gel is provided by comparing the migration rates of proteins of known molecular weight during filter paper and starch gel electrophoresis at the same pH. For example on filter paper haemoglobin, transferrin and β-lipoprotein, whose molecular weights are 67,000; 90,000 and 1,300,000 respectively, all migrate to one position, but on starch gel they are widely separated (Flynn, 1968).

Hunter and Markert (1957) first used starch gel for enzyme studies and suggested the term "zymogram" for the starch gel strip stained by histochemical methods.

Both horizontal and vertical methods can be used in starch gel electrophoresis. The original method was horizontal but Smithies later introduced a vertical gel slab method in 1959 to overcome the distortion in the electrophoretic pattern caused by electro-decantation when protein samples are pippetted into slots preformed in horizontal gels. Resolution

is usually superior in the vertical method and there is more uniform migration from the sample slots (Shaw and Koen, 1968). Besides improved resolution, starch gel has various other advantages over previously described methods. A large sample can be applied and, after electrophoretic run, the gel can be sliced into two or more slices, each of which can be stained for different proteins or enzymes. However, the technique requires more time and skill than paper and cellulose acetate electrophoresis.

5) Acrylamide gel electrophoresis : Raymond and Weintraub (1959) introduced acrylamide gel as an electrophoretic medium and disc electrophoresis in acrylamide gel was described by Ornstein and Davis (1962). In spite of its recent introduction, this medium has already seen considerable use because it has excellent resolving power (Brewer, 1970).

Like starch gel, acrylamide gel also possess a sieving effect. An additional advantage of acrylamide gel is that it can be tailored so that the pore size of the gel can be varied in a known manner. Thus it is as simple to produce a gel of 3 per cent concentration as one of 5, 7.5, 10, 15 or more per cent concentration. This facility enables the gel to be used not only as a routine analytical tool based on a fixed concentration but as a versatile molecular sieve. The acrylamide gels are less fragile and can be handled very easily. A very important advantage is that gels are optically clear, making isozyme bands easier to locate and quantify by

densitometry. It can be used over a wide pH range (Wilkinson, 1970). Complications due to electro-osmotic flow do not normally occur with this medium. The main disadvantage of acrylamide gel is that the acrylamide monomer and the bis-acrylamide are neurotoxic as long as they are unpolymerised. The polymerised gel is, however, not neurotoxic. The gel is used either as a horizontal or vertical slab or in the 'disc' electrophoresis.

5.a) Disc electrophoresis : Disc electrophoresis was developed by Ornstein and Davis in 1962 for the analytical separation of protein mixtures. By this method proteins are concentrated into thin starting zones and then separated by the combined action of electrophoresis and molecular sieving. It is performed in small columns of acrylamide gel consisting of three parts : (1) a large pore 'sample gel' into which the protein sample is introduced, (2) a large pore 'spacer gel' in which the sample is electrophoretically concentrated and (3) a 'small pore gel' in which the sample is separated into various discrete bands or zones.

The gel tubes or columns are held vertically in the apparatus and after the electrophoretic run gels are withdrawn from the tubes and stained.

Acrylamide split gel method for comparing identical amounts of different samples in the same tube has been described by Wright (1974). Clarke (1964) showed that both spacer and

sample gel can be dispensed with and many workers now use only the running gel. This is often advantageous as some protein samples inhibit the gelling of sample gel and thereby create further problems.

5.b) Slab gel electrophoresis : This technique is a modification of the original 'disc' electrophoresis and in this gel slabs are used in place of tubes. Sample and spacer gels may be used but it has been discussed in detail by Raymond (1964) that 'these are unnecessary complications'.

The slab gel is ideal, especially for systematic studies, as it permits comparision of a number of samples side-by-side processed under identical conditions. A large number of samples can be run in a single gel, making the technique easier to carry out in the laboratory. Another important advantage is that the slab permits the application of a two directional technique which is impossible in the tube gel method. The slab gel, like starch gel, can be sliced into two or more slices and each slice can be used for a different enzyme assay.

The acrylamide slab can be used in either vertical or horizontal position. The distortion in the electrophoretic pattern caused by electro decantation can, however, be avoided by the vertical method.

With the combination of various supporting media and buffers of different composition, pH and ionic strength, a wide

range of electrophoretic techniques can be applied. Apart from the nature of the supporting media, electrophoretic technique can be divided into following groups.

a) Continous buffer electrophoresis :

In continuous buffer electrophoresis the buffer present in the supporting medium is the same as the buffer present in the electrode tanks. This system is normally used in cellulose acetate and in agar electrophoresis. It is also used in acrylamide gel electrophoresis where the separation is on molecular size only. In this case gels are prepared with a continously varying pore size from one end of the gel to the other also called 'gradient gel'. In this gradient pore technique the electric charge is used only to move the proteins to their pore limit, and therefore, separation is on the basis of molecular size.

b) Discontinous buffer electrophoresis :

In the early work on electrophoresis in starch gel it was discovered that if a different buffer was used in the electrode tanks from that in the gel, resolution improved considerably. The various components of crude horse-radish peroxidase are resolved much more sharply when tris-citrate buffer is used for gel preparation and the electrode vessels are filled with borate buffer than when either solution is used in a continous system (Wilkinson, 1970). Discontinous buffer systems have been

exploited to a large extent in acrylamide gels where they can be combined with regions of different pore sizes.

c) Isoelectric focusing :

This is an electrophoretic technique using large-pore acrylamide gel in which is incorporated a mixture of synthetic polyamino polycarboxylic acids (carrier ampholytes) with a range of isoelectric points. When an electric current is applied to the gel, the ampholytes form a stable pH gradient from one end of the gel to the other. The ampholytes are confined to the gel by using a strong acid at the anode and a strong base at the cathode.

When a mixture of protein is introduced into this pH gradient, the various proteins will move electrophoretically until they reach the point on the gel where the pH is equal to their isoelectric point. At this point the protein is electrically neutral and will not move any further. If it diffuses from this point, it will develop charge and move back to its isoelectric point. This technique has been used in the separation of serum and other proteins, but its use in the study of isozyme is rather limited .

d) SDS and urea electrophoresis :

Urea and sodium dodecyl sulphate (SDS) are capable of solubilizing certain classes of proteins and also of breaking

polymeric molecules into constituent polypeptides. Sodium dodecyl sulphate binds to polypeptides and imparts a large negative charge which masks the individual variation in electric charge. In sodium dodecyl sulphate containing gels, polypeptide migration is dependent solely on molecular weight and with the use of suitable known markers it can be used to give an estimate of this parameter.

e) Immunoelectrophoresis :

This is a combination of electrophoresis and immuno-diffusion and is normally carried out in agar gels. Agar gels are the best medium for diffusion and antigen-antibody precipitation. The test antigens are separated by agar gel electrophoresis. When the run is completed gels are kept wet and no fixative is applied. The antiserum is now placed in trenches cut between the lines of separated antigens. After appropriate incubation precipitin arcs form between the separated antigens and antiserum trenches.

The electrophoretic step can also be carried out in starch or acrylamide followed by embedding of a strip of this gel in agar for the diffusion stage to take place.

Limitations of electrophoretic techniques :

Electrophoresis is based on three principle properties of protein; net charge, iso-electric point and size and conformation.

Sixteen of the twenty essential amino acids are electrically neutral, glutamic and aspartic acids are negatively charged while lysine and arginine are positively charged. Substitution of an amino acid by a like-charged one, will have no effect on the net charge of the protein and an insignificant change on the molecular weight. The question arises is what is the probablity that an amino acid substitution will alter the overall charge ? Examination of the genetic code shows that of the 399 possible non-redundant single base changes, 128 or 32% will result in the substitution of an amino acid of different charge (Lewontin, 1974). Another problem may arise when proteins differ by two or more substitutions involving charged amino acids, where there is a possibility of substitution of two oppositely charged amino acids which cancel each other.

It is, therefore, clear that electrophoretic studies understimate the amount of genetic variation, since not all amino acid replacements result in proteins with different electrophoretic mobilities. Moreover, most of the proteins and enzymes assayed are soluble in extraction mixtures. Genes coding for nonsoluble proteins and enzymes are generally not included in the surveys. It is very difficult to ascertain at present, whether the kinds of gene loci studied are a fair randon sample of the total genome.

Interpretation of electrophoretic data :

There are two main types of electrophoretic approach in

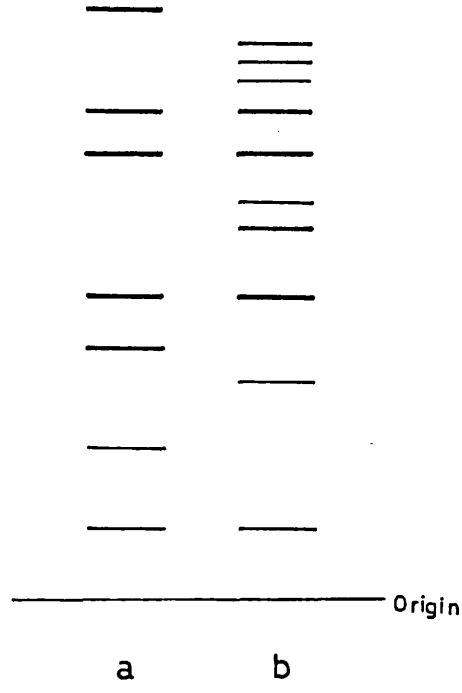
systematics, described below.

1) Band counting method : In this case the electrophorogram is treated as an overall phenotype and no attempt is made to interpret the genetic basis of variability. The differences in electrophoretic mobility of bands are used as a taxonomic character. This type of approach is useful with complex protein patterns and where separation is normally carried out by isoelectric focusing and the gels stained for general proteins. The similarity between pairs of patterns can be estimated in different ways. The most commonly used is a simple matching coefficient of similarity which is calculated as:

$$S_m = \left(\frac{\text{number of bands of common mobility}}{\text{maximum number of bands in an individual}}\right)$$

Figure 2, for instance, shows general protein patterns in two species. The coefficient of similarity between these two species is equal to 4/10 = 0.4. It is possible that the general protein bands in two species which are identical in electrophoretic mobility may represent different proteins (Ferguson, 1980). Two or more 'superimposed' polymorphic proteins can give coincidental similarity in general protein patterns. The only way to avoid this confusion is to stain for specific proteins and to examine each one individually with regard to variation.

Figure 2. Hypothetical diagram of general protein patterns of two species 'a' and 'b'.


Origin
a
b

2) Genetic analysis : According to current theories of gene action, the proteins are essentially direct gene products. If staining is carried out for specific enzymes then the zymogram is a graphic representation of the products of one or more specific genes. There are many statistics available to quantify the genetic differences between populations. In the case of monomorphic loci, a simple coefficient representing the proportion of loci with identical alleles is appropriate. Various statistics have been proposed to estimate differences based on polymorphic loci, and it seems appropriate to discuss polymorphism first before describing them.

In diploid organisms, sexually reproducing forms receive one complete set of chromosomes for each parent. The synthesis of a polypeptide is directed by gene or locus which is composed of two alleles. An 'allele' is the corresponding base sequence on each member of the homologous chromosome pair, and hence one allele comes from each parent. The two alleles code independently for the same polypeptide if they are identical. On the other hand if one of the alleles contains a different codon, then that locus will produce two polypeptides differing from each other by an amino acid substitution. When within a species, the most common allele at a locus is of a frequency of less than 0.99 or 0.95 (Dobzhansky et al., 1977) then that locus is said to be 'polymorphic'. Rare alleles at a locus lower than this frequency do not come within the definition of polymorphism. In each individual of a species there are two

possible conditions : either the alleles at a particular locus are similar (homozygous) or different (heterozygous). For example, if in a population two alleles, A and B exist for a particular locus, then there are three possible genotypes: AA, AB and BB. In case of AA and BB homozygotes one type of polypeptide will be produced but in the case of heterozygote two types will result as, at the level of structural gene expression almost all alleles are co-dominant (Ferguson, 1980), and both alleles are equally expressed. The lack of dominance at the protein phenotype level makes the genetics of protein polymorphism very straight forward. If the protein coded for by allele A and B differ by a single amino acid, and the amino acid substitution results in either a change in electric charge or in conformation of the molecule, then they will have different electrophoretic mobilities. So within the limitations of electrophoretic technique, it provides a convenient method of determining genetic variation at structural loci. To estimate such variation it is obvious that a statistically large number of individuals should be examined, and this eliminates the use of other techniques, like amino acid sequencing.

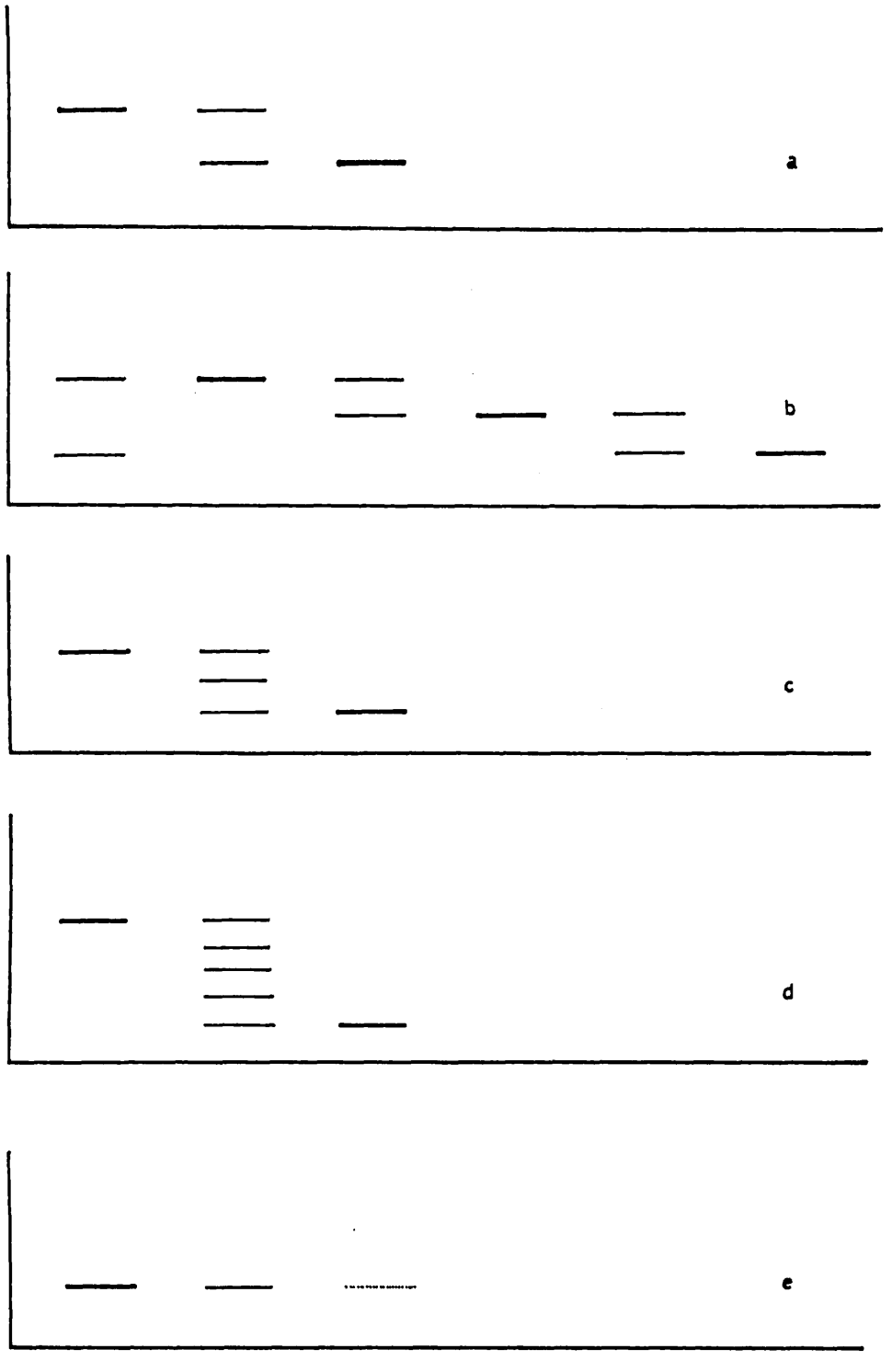
In the example above, if, from a number of individuals, the tissues where this protein is expressed are sampled, extracted and subjected to electrophoresis, then after appropriate staining of the gel, three electrophoretic patterns will be shown (Figure 3 a). In the absence of dominance and within the limitations of the electrophoretic method, phenotype

is equivalent to genotype, and the numbers of each genotype are obtained simply by counting the number of individuals with each of the three patterns.

If three alleles A, B and C exist within a species, and each results in a protein with electrophoretically distinct mobilities, then six electrophoretic patterns will be found (Figure 3 b). In the heterozygote, two bands are found on the gel if the protein consists of a single polypeptide sub-unit (monomeric). For a dimeric protein, due to the production of two different polypeptides in the heterozygote, three different dimers are found (aa, ab, bb), that is, there is a hybrid dimer produced (Figure 3 c). For tetrameric proteins five different tetramers consisting of two polypeptides are possible (a_4, b_4, a_3b_1, a_2b_2, a_1b_3) and this results in a five-banded heterozygote pattern (Figure 3 d). In some proteins there is restriction on sub-unit assembly, and hybrid molecules are not found (Ferguson, 1980). A further condition is found where a particular allele results in the production of a non-functional protein that is a null allele. Homozygotes for a null allele will not produce a band on the electrophoretic gel if the staining procedure relies on the enzyme activity. Heterozygotes will show a single band of reduced intensity (Figure 3 e). Null alleles normally are probably only found in polyploid organisms or at loci which have been duplicated that is, in those cases which have a 'spare' locus.

Different forms of enzyme which share a common substrate

Figure 3. Diagram showing patterns of protein polymorphism; a. two alleles, monomeric, b. three alleles, monomeric, c. two alleles, dimeric , d. two alleles, tetrameric, e. one normal and one null allele (from Ferguson, 1980).


a
b
c
d
e

but differ in electrophoretic mobility are called 'isozymes' (Markert and Moller, 1959) and this should not be confused with 'allozymes' (Prakash et al., 1969) which are protein products of a single genetic locus which differ in electrophoretic mobility and whose segregational behaviour in a population follows a Mendelian pattern.

A variety of measures can be used to express in a single statistic the amount of genetic variation in a population. In a random mating population, the most informative measure is the overall incidence of heterozygosity. The proportion of polymorphic loci in a population is another commonly used measure. In random mating populations the expected frequency of heterozygotes (H) at a locus can be directly calculated from the allelic frequencies. If there are 'n' alleles with frequencies $x_1, x_2, x_3, \ldots\ldots x_n$ the expected frequency of homozygotes is simply $x_1^2 + x_2^2 + x_3^2 \ldots\ldots + x_n^2$. The expected frequency of heterozygotes is $H = I-(x_1^2 + x_2^2 + x_3^2 \ldots\ldots + x_n^2)$. The overall amount of variation in a population is estimated by the average frequency of heterozygotes per locus ($\overline{H}$). This is simply obtained by averaging H over all loci sampled. $\overline{H}$ may be expressed with its standard error, which reflects the amount of heterogeneity among the loci sampled.

The proportion of polymorphic loci (P) in a population is also used to assess genetic variation. This statistic is arbitrary and imprecise (Dobzhansky et al., 1977). It is arbitrary in the sense that one has to decide first when a

locus will be considered polymorphic. As mentioned earlier, there are two criteria (a) the frequency of the most common allele in the population is less than 0.95 and (b) it is less than 0.99. Every locus which is polymorphic by the first criterion is also polymorphic by the second, but not the other way round. The proportion of polymorphic loci (P) is imprecise because it establishes only whether a locus is polymorphic or not. It does not tell you how polymorphic it is. For instance, a locus with two alleles with frequencies 0.95 and 0.05, and a second locus with 10 alleles each with a frequency 0.1 contribute equally to P, although it is clear that the second locus has more genetic variation. The average proportion of polymorphic loci ($\overline{P}_p$) can be estimated as the average of over several populations.

Nei's coefficient of 'genetic identity' (I) is another widely used statistic to quantify the genetic differences (Nei, 1972). This statistic includes both monomorphic and polymorphic loci. The 'genetic identity' ranges from zero (no alleles in common at a locus) to one (the same alleles at identical frequencies). The 'genetic distance' (D) between two populations is given by:

$$D = -\log_e I$$

The mean genetic identity ($\overline{I}$) and genetic distance ($\overline{D}$) are the mean values over all loci studied, including monomorphic ones. Nei's coefficient of genetic identity (I) between two taxa is

given by :

$$I = \frac{x_i y_i}{\sqrt{(x_i^2 \cdot y_i^2)}}$$

where x_i and y_i are the frequencies of the ith allele in populations x and y respectively. 'I' is equal to one when x and y are monomorphic for the same allele and 'I' is equal to zero when x and y are monomorphic for different alleles. The mean genetic identity ($\bar{I}$) is the mean over all loci studied (including monomorphic ones).

The time of divergence (T) of two taxa can be estimated by $T = 5 \times 10^6 D$ (Nei, 1972). If D is equal to one the populations have been isolated for approximately 5 millions years. Sarich (1977) has suggested that there are two main groups of electrophoretically studied proteins with respect to evolutionary rate. He pointed out that plasma proteins and some enzymes not involved in complex metabolic pathways appear to accumulate amino acid substitutions some ten times more rapidly than do those enzymes normally sampled in electrophoretic surveys. This bimodality in the electrophoretically observed rates of protein evolution makes it incorrect to calculate a single genetic distance (D) and relate this to a time scale. Sarich has calculated that the correct relationships are approximately:

1. T (years) = $30 \times 10^6 D$ for slowly evolving loci
2. T (years) = $2.4 \times 10^6 D$ for rapidly evolving loci.

Electrophoresis and the species problem :

The technique of electrophoresis has been applied to various taxonomic problems of vertebrate and invertebrate organisms. Generally vertebrates have received more attention than invertebrates. Results of most electrophoretic studies have confirmed the previous classification based on conventional methods. In some cases divergent classifications are produced. Sibley and his associates (1960, 1970, 1972a, 1972b, 1976) have carried out extensive comparision of the egg-white of some 1500 species of birds and their studies of over 5,000 electrophoretic profiles of avian egg-white proteins have yielded some very significant information at the level of higher taxonomic categories for example, a monophyletic as opposed to a polyphyletic origin for the ratite birds; the close relationship of the hoatzin Ophisthocomus hoazin to cuckoos of the subfamily Crotophaginae and the greater similarity of the flamingos to the Ciconiiformes (herons and storks) than to the Anseriformes (ducks and gees). Their study was based on the assumption that " 1. protein structure is genetically determined, 2. the number of amino acids in a protein chain is probably approximately equal to the number of Mendelian genes which determine its structure, 3. protein tends to be 'conservative' in the evolutionary sense, 4. similarity in protein structure reflects similarity in genetic structure, that is DNA sequence and hence phylogenetic relationship, and 5. electrophoretic 'profiles' are indices to some aspects of protein structure" (Sibley, 1960).

Dessauer and Fox (1956) made an extensive taxonomic study of plasma proteins, using electrophoretic techniques, of more than 100 species and sub-species of amphibians and reptiles. On the basis of electrophoretic patterns they constructed a 'tentative key' to the orders of these two classes. A more refined biochemical key was presented by Avise (1975) to nine species of genus Lepomis (sunfish) using Lepomis macrochirus macrochirus as a standard, and taking electrophoretic mobilities of enzymes in other species relative to this species.

Other studies on vertebrate animals include rodents (Baur and Pattie, 1968; Johnson and Selander, 1971; Avise et al., 1974; Kilpatrick and Zimmerman, 1975; Chan et al., 1979), fishes (Sharp, 1969; Jones and Mackie, 1970; Dando, 1970, 1974; Ferguson, 1974; Menzel, 1977; Garlick and Terwilliger, 1978; Mackie and Jones, 1978), squirrels (Nadler and Hughes, 1966; Nadler, 1968; Seaman and Nash, 1977), lizards (Gorman and Dessauer, 1966; Guttman, 1970) and snakes (Dessauer and Pough, 1975; Foote and McMahon, 1977; McDermid et al., 1977).

Among invertebrates extensive work has been carried out on the fruit fly Drosophila (see Dobzhansky et al., 1977). Table 1 presents a summary of electrophoretic studies of invertebrates excluding insects. The malaria mosquito Anopheles and the fruit fly Drosophila are the classical cases of sibling species and a number of other characters besides conventional

Table 1. A summary of electrophoretic methods used is systematics or population genetics of some invertebrates (excluding insects), arranged in chronological order.

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Woods *et al.*, 1958	crustacean and mollusc	serum	starch gel	haemocyanin
Whittaker, 1959	crayfish	serum	starch gel	haemocyanin
Manwell and Baker, 1963a	sea-cucumber	coelomic fluid	starch gel	HAEM., EST., PROT.
Manwell and Baker, 1963b	crabs	serum	vertical starch gel	PROT., HAEM., CER., DO.
Manwell, 1966	sea-cucumber	body fluid	starch gel	HAEM.
Manwell *et al.*, 1967	copepods	whole animal	vertical starch gel	EST., ACP., β-GA., PEP., MDH., ME., G6PD., 6PGD., TPD., ALD., AMY.
Wright and File, 1968	gastropod	digestive gland	starch gel	EST.
Bedford and Reid, 1969	mollusc	crystalline style	acrylamide gel, slab	PROT.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Bowen *et al.*, 1969	brine shrimp	haemolymph	agar gel and cellulose acetate	HAEM.
Coles, 1969a	snail	liver	acrylamide gel	LDH., MDH., β-HDH.,α-GPD., GDH., α-ALDH., IDH., G6PD., 6PGD.
Coles, 1969b	snail	liver	acryalmide gel	ACP., ALP., POX., LAP., β-glucosidase.
Horn and Kerr, 1969	crabs	serum and haemolymph	vertical starch gel	PROT., HAEM.
Reid and Dunnill, 1969	bivalve	digestive diverticula	horizontal starch gel	EST.
Gooch and Schopf, 1970	ectoprocta	whole zooid	acrylamide gel in tube	EST., MDH., LAP.
Jones, 1970	polychaete	whole worm	acrylamide gel in tube	MDH., LDH., α-GPD., GDH., G6PD.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Wium-Anderson, 1970	gastropod	buccal mass	acrylamide gel	PROT., HAEM.
Gooch and Schopf, 1971	ectoprocta	whole zooids	acrylamide gel	EST., MDH., LAP., ALP., TO.
Jelnes et al., 1971	bivalve	digestive gland	starch gel	EST.
Michelson and Dubois, 1971	mollusc	crystalline style	acrylamide gel in tube	PROT., AMY., ALP., EST., CAT., POX.
Berger, 1973	gastropod	whole snail	acrylamide gel	EST.
Milbrink and Nyman, 1973	oligochaetes	whole worm	starch gel	EST.
Schopf and Murphy, 1973	sea star	hepatopancreas	acrylamide gel, slab	SDH., PGI., HK., LAP., PROT., MDH., FK., EST., TO., GOT., NK., NDH.
Synder and Gooch, 1973	gastropod	whole snail	starch gel	PGI., PEP.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Grassle and Grassle, 1974	polychaete	whole worm	vertical starch gel	MDH.
Ayala et al., 1975	krill	whole animal	starch gel	ACP., AO., ALD., ALP., EST., FUM., G6PD., G3PD., α-GPD., HK., IDH., LAP., ME., MDH., ODH., PGM., TO., PGI., TPI., XDH., GOXT.
Battaglia and Bisol, 1975	copepod and amphipod	whole animal	acrylamide gel in tube, vertical slab	PROT., EST., LAP., PGM., GPI., G6PD., MDH., LDH., ADH., GOT., ACP., ALP., TO.
Levinton and Fundiller, 1975	bivalve	muscle	starch gel	LAP.
Grassle and Grassle, 1976	polychaete	whole worm	vertical starch gel	PHI., XDH., PGM., IDH., MDH., α-GPD.
Fujino and Nagaya, 1977a	bivalve	adductor muscle and digestive diverticula	horizontal starch gel	MYOG., EST.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Fujino and Nagaya, 1977b	bivalve	digestive diverticula	horizontal starch gel	TO., LAP.
Jelnes, 1977	gastropod	whole snail	starch gel	PGI.
Marcus, 1977	sea urchin	gut	acrylamide gel, slab	ALP., ACP., ME., MDH., HK., PGM., PGI., PEP., EST., GOT., AMY.
Nyman and Skoog, 1977	gastropod	whole snail	horizontal starch gel	EST., POX.
Steiner et al., 1977	isopod	whole animal	horizontal starch gel	PGI., ALP., PROT., EST., GOT., PGM., 6PGD., MDH., α-GPD.
Bowen and Sterling, 1978	brine shrimp	whole animal	horizontal starch gel	EST., MDH.
Chambers, 1978	gastropod	whole snail	horizontal starch gel	ACP., ALD., ALP., EST., TO., GOT., G3PD., HEXD., LAP., ME. 6PGD., PGI., PGM., SDH.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Massaro and Cohen, 1978	crab	gill, gonad, heart, hepatopacreas, lung, intestine, pericardial gland, muscle, sperm duct	starch gel	LDH., MDH., G6PD., IDH., SDA., PGM., GK., GPK., AK., CA., EST.
Murphy, 1978	limpet	digestive gland	starch gel	LAP., PGI.
Nagaya et al., 1978	bivalve	adductor muscle, digestive diverticula	horizontal starch gel	MYOG., EST., TO., LAP.
Sywula and Bartkowiak, 1978	ostracod	whole animal	acrylamide gel in tube	EST., ACP., POX., MDH., LDH.
Dando et al., 1979	barnacle	muscle	vertical starch gel	MDH., GAPD., SOD., PK.,EN., PGK., ARK., PGM., FBA., MPI., GPI.
Gooch and Hetrick, 1979	amphipod	whole animal	starch gel	MDH., PGI., PEP.
Jelnes, 1979	gastropod	whole snail	starch gel	EST., PGI., GOXT., 6PGD.,NP., IDH., α-GPD., XDH., FK.

Table 1. continued

Author	Organism	Tissue used	Electrophoretic method	Protein/enzyme assayed
Lassen, 1979	gastropod	whole snail	horizontal starch gel	ACP., EST., GOT., LAP., MDH., PGM., TO.
Lyerla *et al.* 1979	isopod	whole animal	horizontal starch gel	MDH.
Dando and Southward, 1980	barnacle	muscle	vertical starch gel	MDH., PGD., GAPD., SOD., EN., HK., PK., PGK., ARK., PGM., ALD., MPI., GPI.
Dendinger, 1980	crab	gill, mid-gut gland, testis, hypodermis	acrylamide gel in tube	LDH., 6PGD., G6PD.
Rice and Simon, 1980	polychaete	whole worm	horizontal starch gel	PGI., GOT., XDH., MDH., IDH., EST.
Turner *et al.*, 1980	crab	whole animal	horizontal starch gel	AMY., CAT., GLU., GOT., TO., GPT., G6PD., 6PGD., ALP., ALD., IDH., MDH., PGI., PGM. ME., EST., PROT.

morphology and interbreeding success were found to be useful in discriminating between the sibling species of these genera (Mayr, 1963; Dobzhansky et al., 1977). Among the most important characters were breeding behaviour, karyotype, habitat and electrophoretically determined genetic similarity of genotypes. The later character is especially important as differences in all other traits such as morphology, physiology and reproduction should be reflected in genetic differences if an adequate sample of the genome is obtained. A survey of literature shows that the assumption that electrophoretically detectable isozyme variation is a true reflection of genetic differentiation between populations and species, has been made by numerous investigators, who have studied local or regional genetic variation.

There are many instances where gene frequency data obtained by electrophoretic analysis provides the main source of evidence for variation with little or no contribution at all from morphological, physiological or other parameters (Manwell and Baker, 1963a; Manwell et al., 1967; Milkman and Beaty, 1970; Gooch and Schopf, 1971; Jelnes et al., 1971; Gooch et al., 1972; Synder and Gooch, 1973; Berger, 1973; Battaglia and Bisol, 1975; Tracy et al., 1975; Ayala et al., 1975; Grassle and Grassle, 1976; Marcus, 1977; Rutherford. 1977; Theisen, 1978; Cole and Morgan, 1978; Chambers, 1978).

The first example of the discovery of sibling species by electrophoresis was that of the holothurian Thyonella gemmata

(Manwell and Baker, 1963a). This species of sea-cucumber is common in the muddy sand flats at Aligator harbour, Florida. While surveying the haemoglobin of T. gemmata, they found two very different electrophoretic patterns of haemoglobin. Close examination of T. gemmata revealed two types. One variety is quite scarce, has slightly less tapering oral and aboral ends and possess thicker tube feet while the other variety which is common, is slightly thinner. These were identified as 'stout' and 'thin' forms respectively. The spicules, which are usually used for species identification in different species of sea-cucumber, were found to be identical in these two forms. When the two different patterns of electrophoretically detectable haemoglobin were correlated with 'stout' and 'thin' forms, it was discovered that both have distinct and consistent patterns. The electrophoretic data suggested that these two forms do not interbreed in nature although they live sympatrically.

One particularly well known case of sibling species is that of marine polychaete Capitella capitata (Fabricius), reported by Grassle and Grassle (1976). C. capitata is a small benthic polychaete and is regarded as a pollution indicator species. It is a cosmopolitan species, and has been reported throughout most of the world from coastal waters, often in areas of high organic content (Wass, 1967). In an extensive study of C. capitata from New England, Grassle and Grassle (1974, 1976, 1977) found that they were really dealing with not one, but at least six different species which were

identical morphologically but quite different in isozyme pattern as shown by starch gel electrophoresis. They found no more than two alleles, out of eight loci examined, were shared in any pairwise comparision between the six species. Difference in reproductive biology between the sibling species were discovered and differences were found in the number and size of the eggs produced and in the variation in the length of planktonic larval stages in laboratory cultures. Some of these forms appeared to be more opportunistic than others, which questions the use of this 'species' collectively referred to as C. capitata for laboratory pollution bioassay experiments and as a field indicator of pollution (Bellan et al., 1972; Reish, 1973).

The organism chosen for the present study was Polydora ciliata (Johnston), a member of the family Spionidae and sub-family Spioninae. The genus Polydora, Boccardia, Tripolydora and Pseudopolydora of sub-family Spioninae comprise the polydorid complex (Fauchald, 1977), in which the fifth segment is modified and includes specialized setae. The genus Polydora is the largest of the complex and the family, containing over 70 species which are generally distinguishable from each other on the basis of setal structure, location of branchiae and habitat.

The specific identification of P. ciliata has been under considerable confusion ever since it was described by Johnston (1838) from crevices of slaty rocks in Berwick Bay, England.

Rasmussen (1973) has discussed the separation of P. ciliata from closely related polydorids. He showed that the characters used for separation of the species, like the number of modified setae on the fifth segment, is not stable, even in P. ciliata from the same region. He, therefore, suggested that some other species of Polydora, such as P. ligni, P. websteri, P. cirrosa and P. nuchalis may prove to be only varieties of P. ciliata. More recently Kendall (1980) reported morphological variations in P. ciliata and showed that the modified setae of the fifth segment, a diagnostic character of the species, are not consistent either in number or in shape, even in a single individual. He identified his polydorid as P. ciliata by using Fauvel's key (1927) but when he sought confirmation in the key of Hartman-Schröder (1971) a second identification, that of P. limicola (Annenkova) was obtained, although he used the same specimen. When he tried to resolve this discrepancy by sending these animals to specialist taxonomists for identification, they were equally divided between those favouring P. ciliata and those favouring P. limicola. Kendall (1980), however suggested that P. ciliata may be more closely related to P. limicola than P. ligni.

Polydora species such as 'P. ligni' (= P. ciliata Rasmussen, 1973) have an opportunistic life history' (Grassle and Grassle, 1974). They are able to exploit an open habitat, because of their high rate of production and high dispersal ability. In addition to its opportunistic nature and its proposed role as a pollution indicator, Polydora species are

important to the commercial oyster industry. Unlike P. websteri and P. ciliata which seek out oysters and burrow into their shells, P. ligni may settle in masses on top of an oyster bed in such numbers that as the worms grow and build larger tubes the entire oyster bed may become buried and the oysters smothered (Nelson and Stauber, 1941; Mortensen and Galtsoff, 1944).

In spite of its commercial importance and the confused state of its taxonomy, little has been done to resolve the problem by employing modern techniques. On the basis of morphology and population genetics (using electrophoretic technique) of P. ciliata it is intended to assess the degree of intra and inter-specific variation that is found or that has occurred in this species complex. Relevant morphological characters have been considered in detail using a scanning electron microscope since, in practice, it is these characters that must be used to separate species if separation is necessary. Gene frequencies inferred from isozyme systems using acrylamide slab gel electrophoresis have been compiled for each ~~each~~ population and used to calculate genetic distance and genetic identity between populations.

Evidence from the above observations combined with other information available in published reports is incorporated to produce an overview of intra and inter-specific variation.

In addition to Polydora species, other polychaetes, like

Nereis diversicolor, *N. fucata*, *N. virens* and *Arenicola marina* were also investigated electrophoretically. It is surprising in the light of the abundance in the marine environment and their variety of adaptations, that more polychaetes have not been used in population genetics studies. From 27 non-insect invertebrate species listed by Nevo (1978) which have been analyzed by population geneticists, none were polychaetes.

SECTION I

Polydora ciliata complex

(A literature review)

Systematics :

The type species of the genus Polydora was described by Bosc in 1802 as P. cornuta from Charleston, South Carolina. Bosc's description was superficial and it is almost impossible today to determine the species, hence the type species of Polydora is indeterminable (Blake, 1971). The genus Polydora Bosc has been under considerable confusion and other genera such as Boccardia, Carazzia and Pseudopolydora have on more than one occasion been brought out of synonymy and used as genera or subgenera. The major systematic treatments of the polydorids include those of Carazzi (1895), Mensil (1896), Söderström (1920). Fauvel (1927), Okuda (1937), Hartman (1941), Berkeley and Berkeley (1952), Kirkegaard (1959), Day (1967), Blake (1969a, 1971), Foster (1971a), Hartman-Schröder (1971) and Rainer (1973).

Specific diagonosis in some cases is more confusing. New species have been described on the basis of a few specimens, sometimes in poor condition. For instance, Light (1969) described P. narica as a new species on the basis of a single specimen. Similarly the single specimen on which Webster's (1879) description was based about P. caeca is not known to exist and the description itself is faulty and misleading (Loosanoff and Engle, 1943). Verrill (1880) erected four species of

Polydora but failed to provide adequate descriptions and figures except for P. concharum. P. gracilis Verrill and P. tubifex Verrill were described but never figured. Later on, in 1944, Hartman published a number of Verrill's plates containing figures of P. concharum and P. littorea, a nomen nudum. P. gracilis Verrill was however described as a synonym of P. ciliata, later by Carazzi (1895).

Polydora hamata and P. caeca were described by Webster (1879) for the first time. P. hamata was referred to Boccardia by Blake (1966) and P. caeca was renamed by Hartman (1943) as P. websteri. Recently, Rasmussen (1973) suggested that this P. websteri (Webster) may be a synonym of P. ciliata (Johnston). Rasmussen (1973) also suspected that P. ciliata (Johnston) and P. ligni Webster are not two distinct species. More recently Kendall (1980), while surveying the fauna of Seal Sands, a mud-flat in the estuary of the river Tees, north east England, found P. limicola Annenkova, but due to considerable variation he lumped it with P. ciliata (Johnston). He investigated various populations of P. ciliata including limicola and ligni forms, and on the basis of morphology he speculated that limicola and ciliata are forms of the same highly variable species. P. limicola was originally described as a sub-species of P. ciliata by Annenkova (1934) but Hartman (1961) elevated it to species rank. This species is also closely related to P. aggregata Blake. Blake now believes that P. aggregata is probably the same as P. limicola or at the most only a sub-species (personal communication).

Morphology :

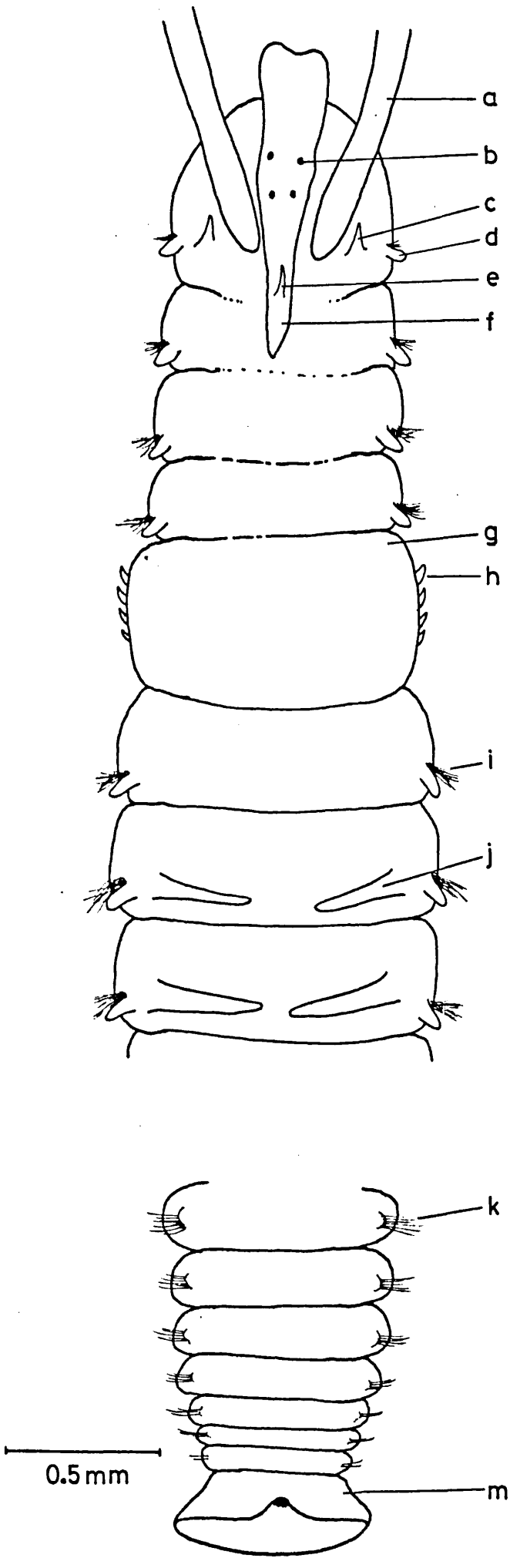
Figure 4 is a generalized diagram of a Polydora. The morphological characters which have been and are still being used for taxonomic purposes can be divided into 'setal' and 'non-setal' characters.

Among the setal characters, the modified setae of the fifth, enlarged, segment is considered as the most important morphological character. In P. ciliata and closely related species this character has been found most controversial. Typically, a series of heavy or modified spines is present on the lateral side of the fifth segment and is accompanied by 'companion setae'. The heavy spines usually have a subdistal tooth or projection of varying size and shape. In addition to these there are other bundles of setae on the fifth segment. These additional setae are located anterior and dorsal (notosetae) or posterior and ventral (neurosetae) or both relative to heavy spines (Figure 19).

The other important setal character is the presence or absence of notosetae in the first segment. The first segment has well formed parapodial lobes, but only neurosetae are present in P. ciliata and closely related species, although they have a well developed notopodial lobe. Other species, like P. socialis, P. flava and P. concharum have both neuro- and notosetae on their first segments. The occurrence of bidentate hooded hooks is also considered an important character

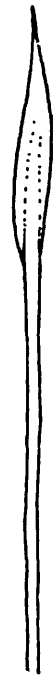
Figure 4. Diagram showing the morphological features of a generalized Polydora. 1. anterior and posterior end in dorsal view, 2. heavy spines of fifth segment, 3. neuropodial hooded hook, 4. companion setae of fifth segment and 5. posterior notopodial setae.

(a) palp, (b) eye, (c) 1st notopodial lobe, (d) neurosetae, (e) nuchal antenna, (f) caruncle, (g) fifth segment, (h) heavy spine, (i) notosetae, (j) branchia, (k) notosetae, (m) pygidium.

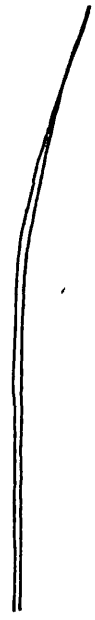


2

3



4



5

for the discrimination of species. These usually start from the 7th segment in P. ciliata complex while in P. commensalis these start from the 10 - 17 segments (Blake, 1971). The number of hooded hooks varies from segment to segment within an individual. The shaft of the hooded hook has a constriction in P. ciliata complex but it is lacking in other species like socialis, tetrabranchia and commensalis . The notosetae on the posterior most segments are sometimes modified and therefore, constitute an important taxonomic character. In P. ciliata complex these setae are simple capillaries and not modified, while in quadrilobata and hoplura these are modified as awl-shaped and falcate spines respectively (Blake, 1971; Read, 1975).

The non-setal characters which are generally held to be of taxonomic importance in Polydora are: 1) the presence or absence of a nuchal antenna on the caruncle, 2) the posterior extent or length of the caruncle, 3) the number of eyes on the prostomium, 4) the presence of pigments, especially in the anterior region, 5) the first segment to bear branchiae and 6) the shape of the pygidium. These non-setal characters along with setal characters are summarized in table 2 for P.ciliata and the other closely related species of the complex.

Life history :

Detailed descriptions of the development of the gametes in polydorid worms are given by Söderström (1920) and Dorsett (1961). The gametes in P. ciliata first become visible in worms

Table 2. Some taxonomic characters of P. ciliata (Johnston) and closely related species. The characters are:

1. first segment notosetae
2. number of heavy spines (fifth segment)
3. sub-distal projection on the heavy spine
4. companion setae (fifth segment)
5. notosetae on the fifth segment
6. neurosetae on the fifth segment
7. number of segment bearing 1st hooded hook
8. constriction on shaft (hooded hook)
9. modified posterior notosetae
10. pigments on the anterior region
11. number of eyes
12. nuchal antenna
13. number of segments covered by caruncle
14. shape of pygidium

Ch. No.	species of Polydora					
	ciliata	websteri	ligni	nuchalis	limicola	aggregata
1.	absent	absent	absent	absent	absent	absent
2.	7 or less	6	8 - 12	?	8 - 15	8 - 15
3.	distinct tooth	flange	small tooth	no tooth	small tooth	small elevation
4.	spatu-late	spatu-late	penici-llate	spatu-late	spatu-late	spatu-late
5.	present	present	absent	present	present	present
6.	present	present	absent	present	present	present
7.	seven	seven	seven	seven	seven	seven
8.	present	present	present	present	present	present
9.	absent	absent	absent	absent	absent	absent
10.	absent	absent	absent	absent	present	present
11.	0 - 4	0 - 4	4	2	0 - 4	4
12.	absent	absent	present	present	absent	absent
13.	1 or 2	3	3 or 4	2	2 or 4	2
14.	disc	disc	disc	disc	disc	disc

of 40 - 45 segments and are found in segments 7 - 10 inclusive. Gametes arise from the peritoneium, just inside the ventral epithelium and migrate to the gonads where they remain until they reach a size of 20 - 30 µm in diameter. They are then released into the coelom by the rupture of the epithelium covering the gonads and are transported posteriorly to the region of maturation. After maturation, the gametes are released through modified nephridia which serve as sperm and oviduct in male and female respectively.

According to Dorsett (1961) and Blake (1969b) increase in temperature initiates spawning of Polydora species. However, the spawning periods are variable, and depend on the species of Polydora and geographical region. For instance, P. ciliata from Kiel (Germany) spawn from October to April with a maximum spawning in July, while P. ciliata from Plymouth (England) spawn from January to October with maximum spawning in March (Thorson, 1946). Generally those species that have a long planktonic phase like P. ciliata (Daro and Polk, 1973; Dorsett, 1961; Wilson, 1928), P. ligni (Watling, 1975) and P. websteri (Blake, 1969b) have a spawning period in spring or early summer, though they may have a secondary spawning in autumn. On the other hand those species that have a short planktonic phase like P. hoplura (Wilson, 1928), P. nuchalis (Woodwick, 1960) and P. quadrilobata (Blake, 1969b) often spawn in late autumn or early winter.

In P. ciliata it is thought that females only lay eggs

in the presence of a ripe male. The spermatozoa are released into the water where they find their way to the burrow of the female and are carried in with the respiratory current. Since the eggs are surrounded by an egg capsule, fertilization takes place by the sperm peneterating the capsule (Dorsett, 1961). Protandry and neoteny have been reported for P. hermaphroditica (Hannerz, 1956) and P. nuchalis (Woodwick, 1960). A reciprocal transfer of sperms occurs between two protandric males P. nuchalis and sperms are stored. These sperms fertilize the eggs which develop in the later female stage (Woodwick, 1960).

The fertilized eggs develop inside the capsule. There are two main types of egg development. The first is examplified by P. ciliata in which almost all of its eggs in the capsule develop into larvae. These larvae hatch at about the three setiger stage and have a long planktonic phase (Wilson, 1928; Dorsett, 1961). The second type of egg development is examplified by P. hoplura in which only a small proportion of its eggs, usually less than one in ten, develops into a larva. The other eggs, nurse eggs, are used as food by the developing larvae. This type of feeding is known as 'adelophagia' (Wilson, 1928) and it allows larvae of P. hoplura to remain within the protective brood longer than P. ciliata larvae. P. hoplura, therefore, pass through a short planktonic phase, if any, before settling and metamorphosing. P. quadrilobata has both types of development, depending on its geographical region, a feature known as

'poecilogony' (Blake, 1969b).

P. ciliata and P. commensalis may have a pelagic phase as long as six weeks (Dorsett, 1961 and Hatfield, 1965, respectively), and their larvae can be carried over great distances by water currents. Hannerz (1956) estimated that larvae, such as P. ciliata and P. commensalis, that spend a month or more in the plankton could be carried away more than 670 kilometers from their brood tubes by currents with a speed as low as 1 knot. In P. hoplura the pelagic phase may ~~may~~ be as short as a few hours and may even be ommitted (Wilson, 1928) and it is obvious that the larvae are not carried far from their brood tubes.

The development of Polydora larvae have been dealt with in detail by Wilson (1928), Hannerz (1956, 1961) and Blake (1969b). Larvae of many Polydora species exhibit habitat selection and they search for a suitable substratum on which to settle (Wilson, 1928; Dorsett, 1961, Blake, 1969b). Some species like P. flava which settles only in the presence of Lithothamnium crusts, are specific in their search for a permanent home. Others, such as P. ciliata which settles on a wide variety of substrata, are less specific in their requirements (Hannerz, 1956). Many factors direct Polydora larvae to a suitable habitat, or induce metamorphosis once a siutable habitat is reached. The clearest indication to the larvae of the suitability of a substratum for settlement is the presence of adults of the same species. It is therefore,

not surprising that some polydorid larvae such as P. ciliata prefer settling near P. ciliata adults (Blake, 1969b; Kiselva, 1967). Many Polydora species are able to delay metamorphosis until they find a suitable substratum, and these include P. ciliata (Dorsett, 1961; Wilson, 1928), P. commensalis (Hatfield, 1965), P. flava, P. caeca, P. ligni and P. hermaphroditica (Hannerz, 1956). After metamorphosis, the Polydora grow mainly by increasing their segment number. P. ciliata, which settles after about three weeks in the plankton when it has reached 16 segments, may grow at the rate of one segment per day. It may lay eggs about three weeks after settlement, when it has reached forty segments. Thus P. ciliata may complete its life cycle within six weeks. P. ciliata may lay a second batch of eggs two weeks after the first batch, but then it usually dies (Daro and Polk, 1973).

MATERIALS AND METHODS

Collection and maintenance of worms :

Samples of three different species of Polydora were collected from the intertidal zone of various localities of England and Wales (Figure 5). Nine populations of P. ciliata and one population each of P. ligni and P. limicola were sampled. P. ciliata was collected from a variety of habitats such as rock, shell, limestone and mud flat. The identifications were confirmed by use of the key devised by Hartman-Schröder (1971). Data on locality and type of substratum are summarized in Table 3.

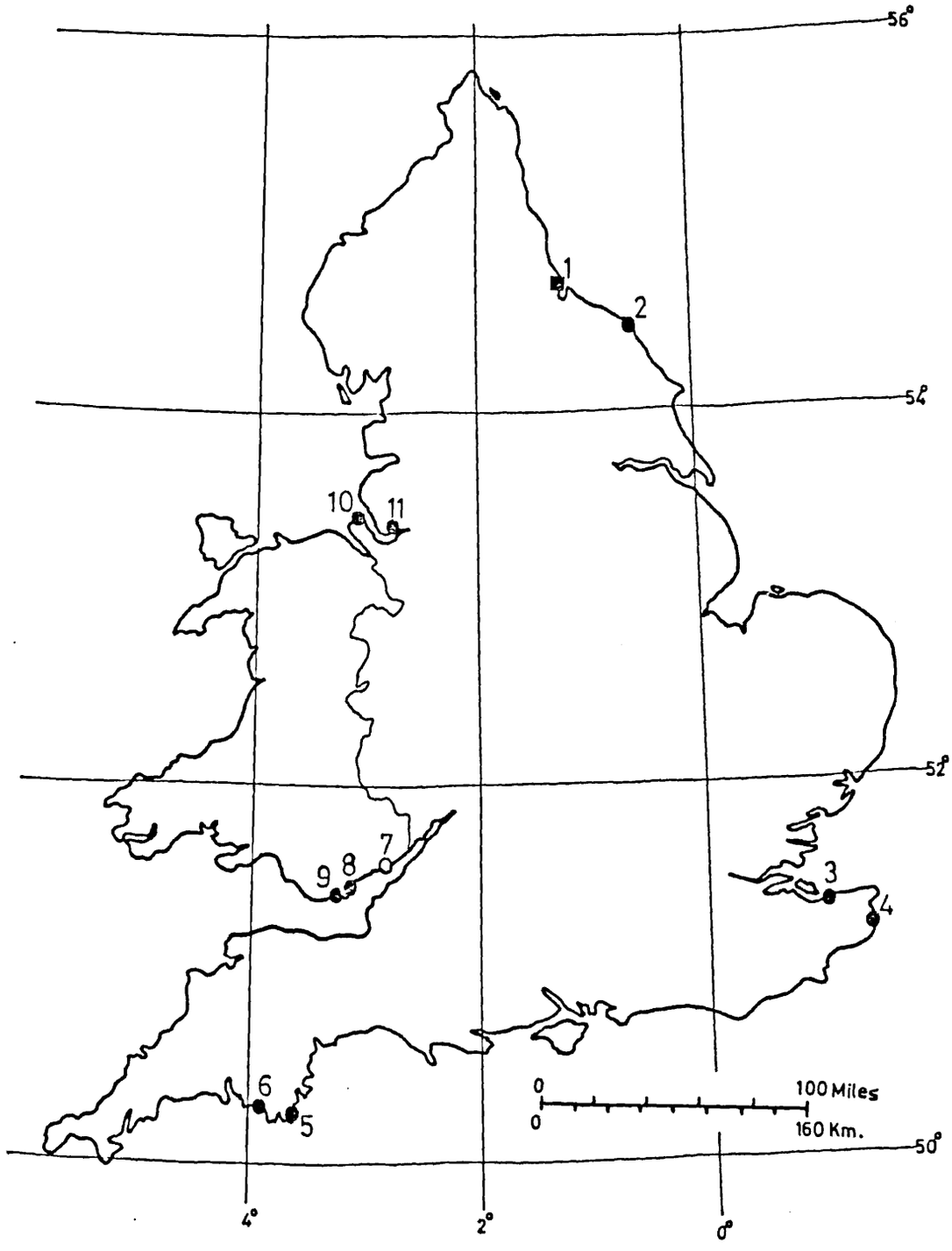
Live worms were transported to the laboratory where they were kept in tanks in the aquarium at 10 - 15°C. These tanks were provided with circulated artificial sea water of 32°/oo salinity and with aeration. Polydora servive several weeks in the tanks if left in their burrows. Worms were hand sorted from the mud as needed. Shell and limestone were cracked while small pieces of rock were put in 0.05% (w/v) solution of phenol in sea water to remove the worms from their burrows.

Polyacrylamide gel electrophoresis :

Sample preparation : Each worm, with the gut cleared, was homogenised inside a microcentrifuge tube by inserting a glass rod attached to an electric motor and moving the glass rod up and down in the tube. A homogenisation mixture was made

Figure 5. Map showing locations of Polydora populations sampled.

1. Tees estuary, 2. Robin Hood's Bay, 3. Whitstable 4. Dumpton, 5. Torbay, 6. Plymouth, 7. Peterstone Wentlooge, 8. Sully (Swanbridge Bay), 9. Barry (Friars point), 10. New Brighton, 11. Hale, Mersey river.



- ● P. ciliata
- o P. ligni
- ■ P. limicola

Table 3. Populations of *Polydora* investigated

Species	Date	Locality	Grid reference	Habitat	Tide level*
P. ciliata	Feb. 80	Plymouth, Jennycliff	SX 491 525	limpet shell	MTL
	Sept.80/Nov.81	Dumpton, Pegwell Bay	TR 398 667	limestone	MLWN
	Oct. 80	Torbay	SX 944 636	limpet shell	MTL
	Oct.80/March 81	Whitstable	TR 101 671	mud flat	MLWS
	April 81	Barry, Friars point	ST 112 660	rock	MLWN
	April 81	Sully, Swanbridge Bay	ST 165 672	rock	MLWN
	June 81	Robin Hood's Bay	SE 955 055	limpet shell	MTL
	July 81	Hale, Mersey estuary	SJ 481 813	mud flat	MLWN
	July 81	New Brighton	SJ 313 942	mussel shell	MLWN
P. ligni	April 81	Peterstone Wentlooge	ST 271 792	mud flat	MLWS
P. limicola	June 81	Tees estuary	NZ 533 266	mud flat	MLWN

*MTL. mid tide level, MLWN. mean low water neap, MLWS. mean low water spring.

up of 40% sucrose in electrode buffer, tris-glycine, pH 8.3 (Appendix I) and 20 - 100 µl solution were used depending on the size of the worm. Homogenisation was carried out in an ice bath and stopped after every two or three minutes to avoid local heating due to friction. Homogenates were stored in a deep freeze at -20°C until required for enzyme assay.

Pooled samples of different populations were also prepared. Twenty worms from the same populations were pooled and weighed. An equal amount of homogenisation mixture was added and homogenisation was then carried out in a 15 ml centrifuge tube, as described above. Aliquots were kept at -20°C until needed for enzyme assay.

Slab-gel preparation : Stock solutions which were used in preparing 7.5% and 15% acrylamide gels are described in Appendix I. A gel-slab casting apparatus and a gel cassette kit supplied by Pharmacia Fine Chemicals were used for setting the gels.

The required number of gel cassettes (82 x 82 mm) were prepared and placed in the appropriate compartment of the gel casting apparatus. Exact amounts of stock solutions were mixed in a 250 ml vacuum flask and 10 to 20 anti-bumping granules or glass pieces were added. The solution was deaerated with the help of a rotary vacuum pump for 5 to 10 minutes or until bubbles cease to come out. The deaerated mixture was gently poured down the gap between the cassettes and the side

wall of the casting apparatus, taking care to avoid bubble formation. If, however, bubbles formed, they were dislodged by gently tapping the apparatus.

All gels were poured the day before their intended use and were precooled in a refrigerator prior to the electrophoresis.

Electrophoretic run : The Pharmacia gel electrophoresis apparatus GE-4 was used in the present study. Electrophoresis was performed at low temperature (8 - 10°C) for $3\frac{1}{2}$ and $4\frac{1}{2}$ hours for 7.5% and 15% acrylamide gels respectively. Electrode buffer and gel were precooled in a refrigerator. During electrophoresis cooled water was circulated through the coolant circulation plate of the apparatus by means of a Grant flow cooler. A Shandon power pack was employed to supply a constant current of 8mA per slab for the first fifteen minutes and then 16mA per slab for the rest of the run.

The electrode buffer used was tris-glycine, pH 8.3 (Appendix I) and this was diluted ten times before use and was used once only. As many as 4 slabs were run at a time. Each slab had six wells and was loaded with standard protein and five samples. Usually 20 - 50 µl samples were applied by means of a Terumo micro syringe. After the run was completed gels were sliced into three slices by using a gel slicer and a slicing frame. The portion of the gel containing standard protein was cut and developed separately for protein, while

the slices were used for three different enzyme assays. The worm homogenate was sometimes applied on two different gel slabs. Thus as many as six different enzyme assays could be obtained from an individual worm.

Two internal standards, horse-heart myoglobin (Type III) and horse-spleen ferritin (Type I) supplied by Sigma Chemical Co, were used (Johnson, 1971). The ferritin and myoglobin ratio on 7.5% gel was 5.25 $\pm$ 0.18 (S.D.). No measurements were made on gels for which this ratio was outside of the range 5.1 - 5.4. Enzyme mobilities were measured with reference to myoglobin which was given a nominal mobility of 100. In 15% acrylamide gel, which was used for separating acid phosphatase bands, only myoglobin was run as the standard protein.

Gels were developed for the enzymes listed in Table 4. Staining procedures were adapted from Shaw and Koen (1968) and Shaw and Prasad (1970). These methods are outlined in Appendix II. After the appearance of the enzyme bands, the reaction was stopped by washing the gel with distilled water and adding a fixing solution (7% acetic acid). Most gels keep their original appearance for many months following this fixation. However, with stains using NBT or MTT (p 272) the bands start to fade after a few weeks.

A Pharmacia slab gel drier was used to dry some of the gels in their original shapes for a permanent record.

Light microscopy :

Polydora were narcotized in 7.5% magnesium chloride solution and fixed in 4% formalin in sea water, before transferring them to 70% alcohol for storage. They were then mounted in lactophenol in which they were allowed to clear for 2 - 10 days. Worms were examined under a Zeiss photomicroscope, for the variability of their morphological characters.

Scanning electron microscopy :

Worms from each population were prepared for examination with a scanning electron microscope. Worms were relaxed and fixed as above. Specimens were dehydrated to absolute alcohol and then acetone. All worms were then critical-point dried in a Polaron drying apparatus. Individuals were mounted on aluminium stubs covered with double sided sticky tape and coated with gold in a Polaron cool sputter coater. Samples were examined on a Cambridge S4-10 scanning electron microscope and photographed.

Table 4. List of enzymes assayed for 11 populations of *Polydora* (*P. ciliata*, *P. ligni*, *P. limicola*).

Enzymes	Locus	M/P
Acid phosphatase	ACP-2	M/P
	ACP-3	M
	ACP-4	M
Esterase	EST-2	P
	EST-3	M/P
Glucose-6-phosphate dehydrogenase	G6PD	M
Malate dehydrogenase	MDH-1	M
	MDH-2	M
Malic enzyme	ME-1	M
	ME-2	M
	ME-3	M
Phosphoglucose isomerase	PGI	M/P
Xanthine dehydrogenase	XDH	M
Lactate dehydrogenase	LDH*	*
Isocitrate dehydrogenase	IDH*	*
Peroxidase	POX*	*
Tetrazolium oxidase	TO*	*

*pooled sample had to be used because of their low activity.

RESULTS

Morphology :

The morphological characters which are generally considered to be of taxonomic importance in P. ciliata were compared between the nine populations. The morphological characters of P. ligni and P. limicola were also studied and these two species were compared with P. ciliata. Hartman-Schröder's (1971) key was used for the identification of species. The characters studied include: (1) the presence or absence of setae in the notopodium of the first segment, (2) the nature of the setae of the fifth enlarged segment, (3) the number and shape of neuropodial hooded hooks, (4) the shape of the setae in posterior notopodia, (5) the number of eyes on the prostomium, (6) the presence or absence of a nuchal antenna on the caruncle, (7) the presence or absence of pigments in the first four segments, (8) the length or posterior extent of the caruncle, (9) the first segment to bear branchia, (10) the first segment having neuropodial hooded hooks and (11) the shape of the pygidium.

Each of the above characters is defined below and compared between all populations. Some of these characters were found to be consistent both within and between populations and are described below as 'monomorphic characters' while other characters which were found to be variable are described

under the heading of 'polymorphic characters'. Although it was possible to score each of the above characters by the use of a compound microscope, the scanning electron microscopy was useful in defining several of the above mentioned structures, particularly the setae of the fifth enlarged segment.

Monomorphic characters :

Several of the features analyzed showed no variation within or between populations. These include the absence of notosetae in the first segment. The first segment had parapodia with well developed notopodial lobes in all the eleven populations studied but no one individual was observed with notosetae. Only neurosetae were present (Figure 6). No variation was observed in the position of neuropodial hooded hooks, which began in the seventh segment (Figure 7). The shape and structure of the hooded hook was similar in all the populations investigated and no intra or interspecific variation was observed. Figure 8 shows a typical neuropodial hooded hook, as seen by scanning electron microscope, while Figur 9 shows a neuropodial hooded hook, as seen by a compound microscope after clearing with lactophenol. Each neuropodial hooded hook had a bifid tip with the main fang slightly acute to the shaft. A constriction and curve were prominent on the shaft. The number of the neuropodial hooded hooks per segment varied from species to species and from population to population and this will be described later on.

The presence of only capillary setae in the notopodium of the parapodia of the posterior segments and the position of the branchiae, which first appear on the seventh segment, were also found consistent within and between all the populations studied. The shape of the pygidium was always disc-like in all populations although slight differences were observed in the shape of the disc (Figure 10). With the exception of the P. limicola population, all other populations lacked pigmentation in the anterior region, especially in the first four segments. Not a single individual of the P. limicola population (n = 50) was found without black pigments (Figure 11).

Polymorphic characters :

The number of eyes present on the prostomium are presented for each population in Figure 12. Each of the nine populations of P. ciliata displayed considerable variation in number of eye spots. The Plymouth, Barry and Sully populations had the highest incidence of individuals with no eye spots at all. The majority of individuals in each population had two eye spots, except the Whitstable and Hale populations in which case the majority had four eye spots. P. ligni and P. limicola also showed variation but the individuals with four eyes were found to be more common. Very few individuals with one eye spot were observed. The three populations of P. ciliata showed less than ten percent of worms with one eye spot. No individual was found with one

Figure 6. P. ciliata; first parapodium as seen with the scanning electron microscope.(400 x)

NS. neurosetae, NP. notopodial lobe, P. palp

Figure 7. P. ligni; scanning electron micrograph showing 7th segment bearing neuropodial hooded hooks (HH). (100 x)


P
NP
NS


HH

Figure 8. P. ciliata (from Dumpton); neuropodial hooded hook as seen with the scanning electron microscope. (3,500 x)

Figure 9. P. ciliata (from Dumpton); neuropodial hooded hook as seen with the compound microscope after clearing in lactophenol. (800 x)

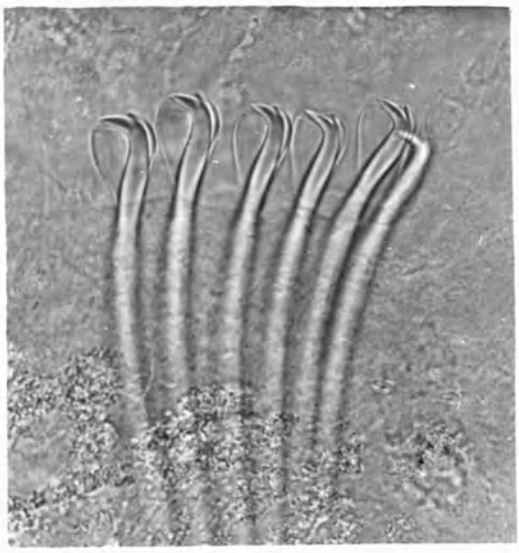


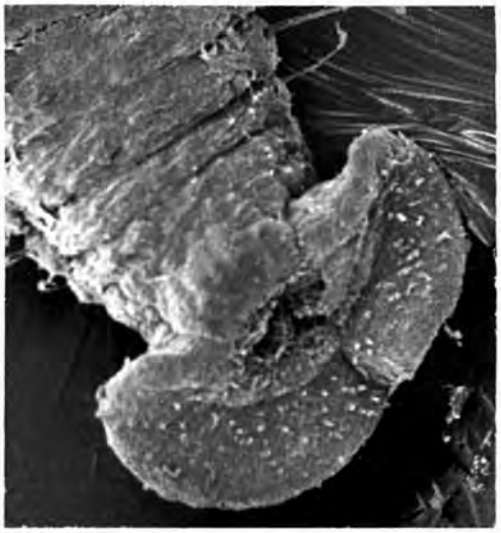
Figure 10. Variation in the shape of disc-like pygidium in Polydora.

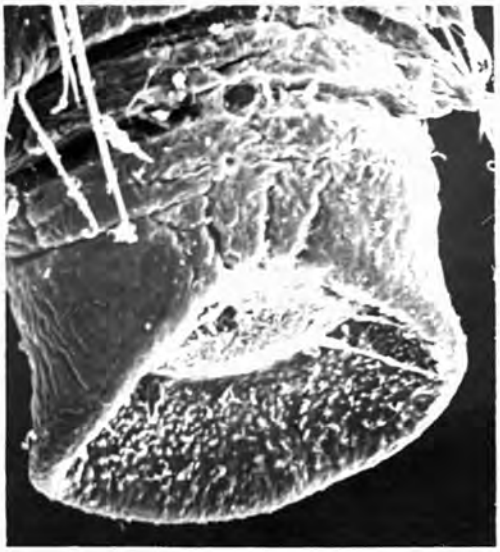
A. P. limicola

B. P. ciliata (from Dumpton)

C. P. ciliata (from Dumpton)

(200 x)





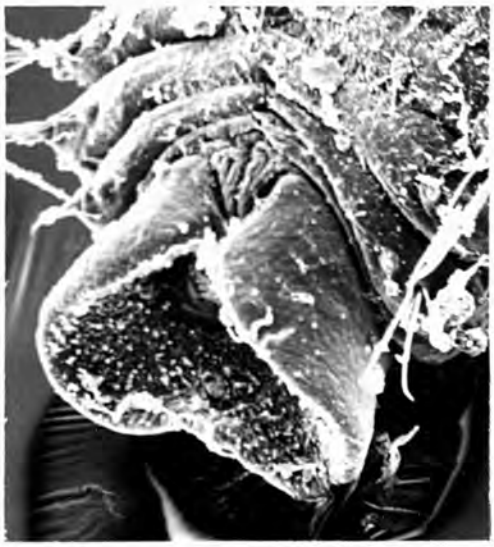
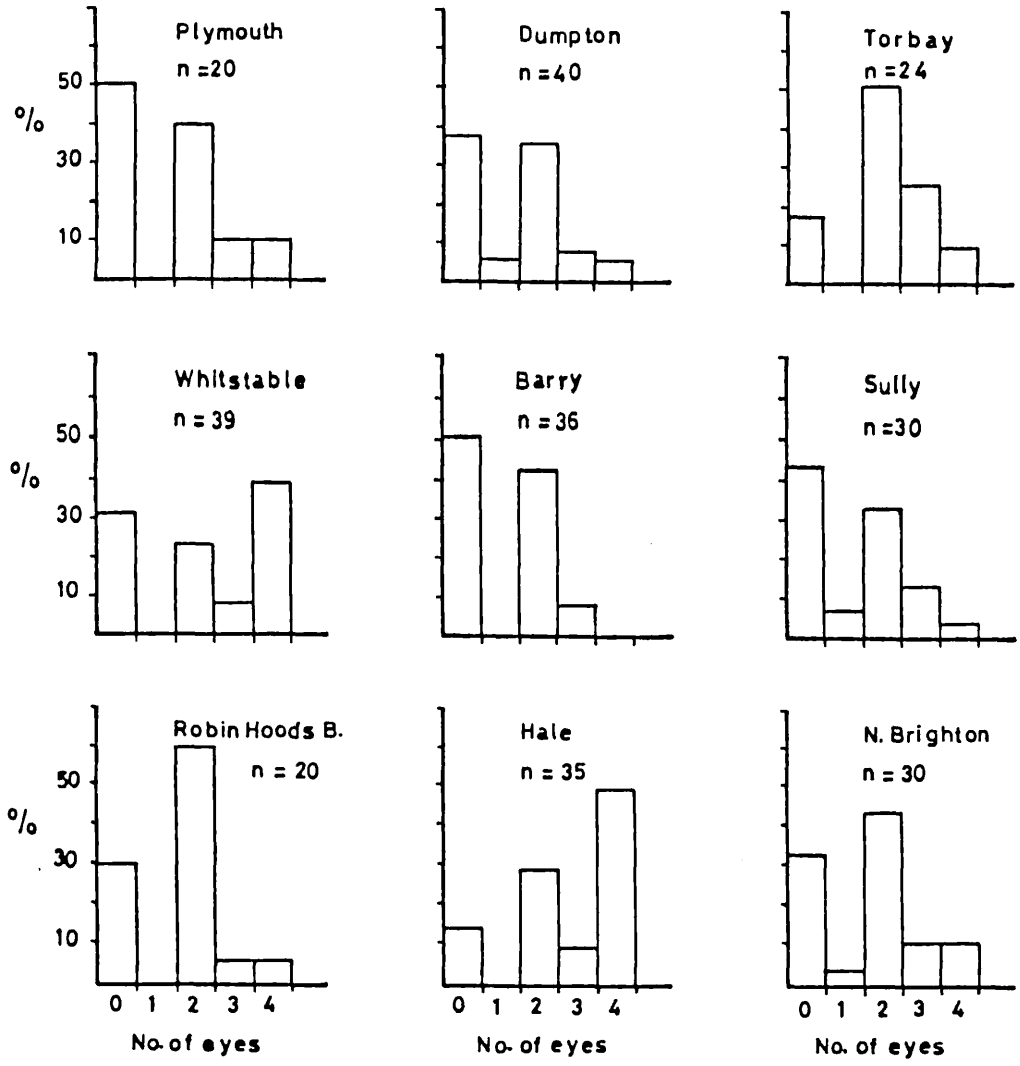
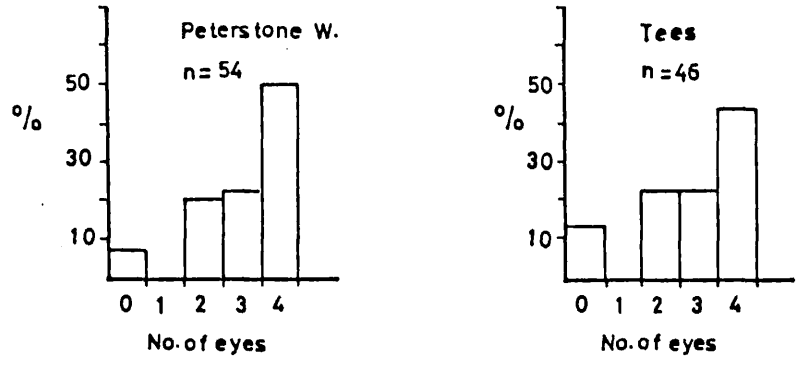


Figure 11. The anterior region of P. limicola (A) and P. ciliata (B) from Barry, showing presence and absence of black pigments. Photomicrographs were taken after clearing in lactophenol. (110 x)

Figure 12. Histograms showing the percent occurrence of eye spots in different populations of Polydora



P. ciliata



P. ligni

P. limicola

eye spot in either P. ligni (n = 54) or P. limicola (n = 46).

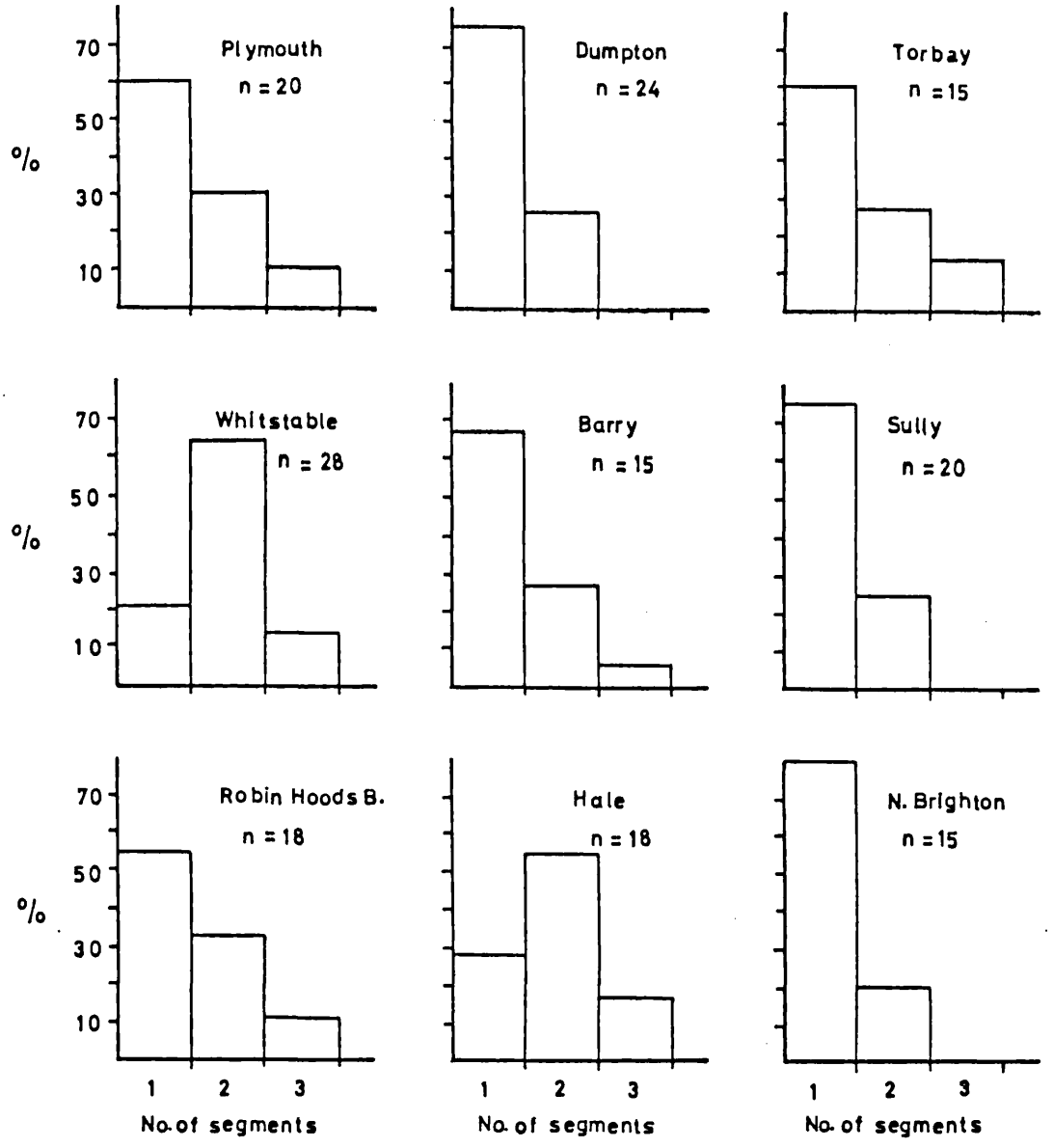
The caruncle is a mid-dorsal extension of the prostomium which passes between the palps in Polydora (Figures 14,15 and 16) and continues posteriorly for a number of segments. The length of the caruncle is generally species specific. However, considerable variation was observed within and between populations of P. ciliata. In P. ligni and P. limicola, the length of the caruncle was also variable. The length of the caruncle was determined by its posterior extent relative to the segments. The distribution of caruncle lengths for all the populations studied is presented in Figure 13. All measurements were made with the scanning electron microscope. Caruncle length varied in all populations examined. Among nine populations of P. ciliata the highest proportion of worms with long caruncles was observed in worms from the Whitstable and Hale populations. Among the three species, P. ligni had the highest proportion of worms with long caruncles. The New Brighton worms had the highest proportion of short caruncle. In most cases the caruncle was partially surrounded by cilia (Figure 14 A) which were often observed to have modified tips. The extent of the ciliation seemed to vary with the length of caruncle and did not show any uniform pattern between populations. Very few specimens were observed without these cilia (Figure 14 B).

The nuchal antenna is a conical projection located on the

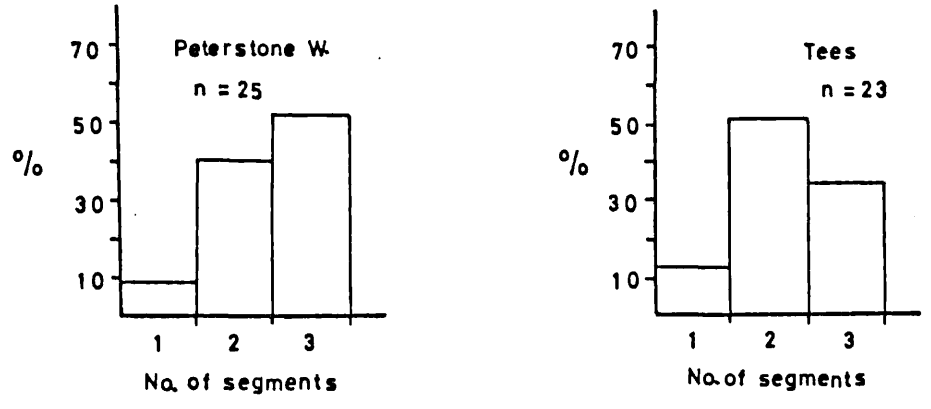
the caruncle just posterior to the palp or at the point where the first and second segment meet. The individuals examined did not possess a nuchal antenna in P. ciliata, except in the Whitstable population, where three worms out of thirty-nine (7.7%) had this structure. In P. ligni fortysix worms out of fifty-four (85%) possessed a nuchal antenna. No individual was observed with a nuchal antenna in P. limicola. The size and shape of the nuchal antenna varied between individuals, from a small bump to a long thin projection upto 30µm in length (Figure 15, 16,17).

The setae of the fifth enlarged segment are one of the most important morphological characters in the genus Polydora. Typically a series of heavy spines is present accompanied by companion setae. Sometimes additional bundles of setae are present and are called 'anterio-dorsal' (notosetae) and 'posterio-ventral' (neurosetae) according to their position with respect to heavy spines. Considerable variation was observed in the number of heavy spines in P. ciliata populations. Figure 18 shows the average number, with standard deviation and observed sample range, of heavy spines in the fifth enlarged segment. The number of heavy spines was counted, after clearing the worm with lacto-phenol, by the use of the compound microscope. Seven populations of P. ciliata, out of nine, had 4 - 7 heavy spines while other two populations, namely Whitstable and Hale had a range of 6 - 10 and 5 - 10 respectively. P. ligni had the maximum number of heavy spines with a range of 9 - 13 spines.

Figure 13. Histograms showing percent distribution of caruncle length with respect to segment number in different populations of Polydora.



P. ciliata



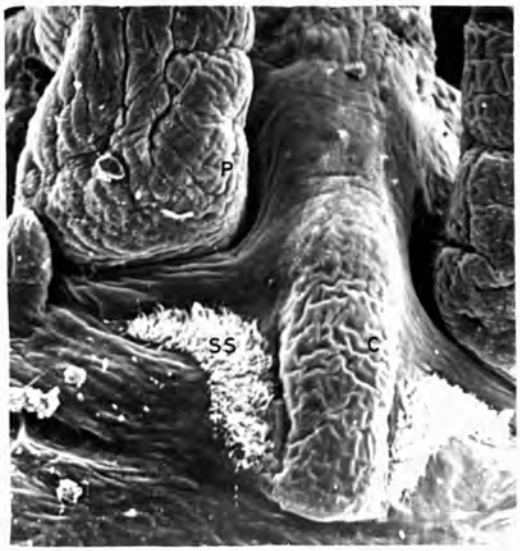
P. ligni

P. limicola

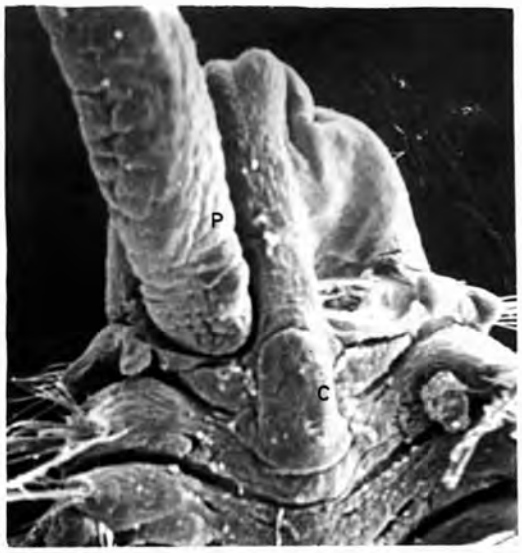
Figure 14. Scanning electron micrographs of the anterior region (dorsal view) of P. ciliata from Dumpton, showing caruncle. (180 x)

A. with sensory cilia and B. without sensory cilia surrounding the caruncle.

C. caruncle, SS. sensory cilia P. palp



A



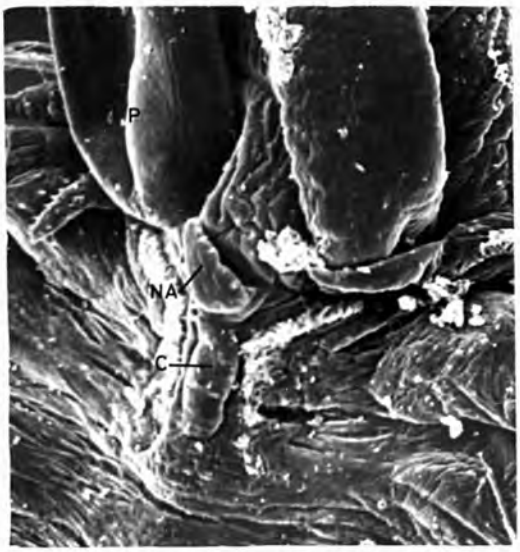
B

Figure 15. P. ligni; scanning electron micrograph of a caruncle bearing nuchal antenna.(150 x)

C. caruncle, NA. nuchal antenna, P. palp.

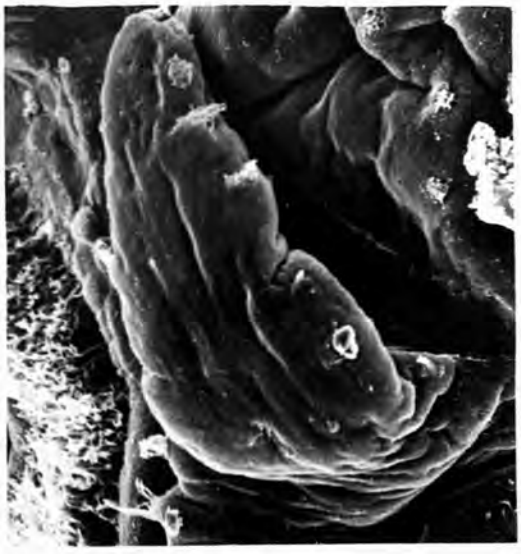
Figure 16. P. ciliata (from Whitstable); scanning electron micrograph of a caruncle bearing nuchal antenna.(150 x)

C. caruncle, NA. nuchal antenna, P. palp.


P
NA
C


NA
C

Figure 17. P. ligni; scanning electron micrographs of enlarged nuchal antenna. (1,400 x)



In P. limicola 7 - 10 heavy spines were observed.

The heavy spines are accompanied with 'companion setae'. The examination of these setae by scanning electron microscope showed no variation in structure among P. ciliata populations. All individuals had pennoned companion setae in all the nine populations of P. ciliata investigated (Figures 19, 22). In P. ligni fringed or brush-like companion setae were observed (Figure 23) and no pennoned setae was found. In P. limicola the companion setae were identical with P. ciliata and no intrapopulation variation was observed (Figure 19).

The tip or distal end of the heavy spines carry some forms of accessory structure. This could be a weakly developed shelf or flange to a prominent tooth. These structures were examined by scanning electron microscope. Variation in the shape is shown in Figures 19, 22 and 23. Generally these accessory structures were slightly ill-developed in P. ligni and P. limicola in comparision to P. ciliata. Some heavy spines could not be seen by scanning electron microscope as they were totally internal and they were examined with a compound microscope after clearing the worms in lactophenol. The additional bundles of setae namely 'anterio-dorsal' and 'posterio-ventral' were examined by scanning electron microscope (Figures 19 and 21). These bundles consisted of one to five setae and were found in most cases in P. ciliata except the Hale population, where

majority did not have these setae. In the case of P. ligni, no individual worm was found with this additional bundle of setae while in P. limicola the situation was more or less identical with that of P. ciliata. Table 5 shows the percent occurrence of these additional setae in different populations of Polydora.

The number of neuropodial hooded hooks may vary from segment to segment in a single individual, but considerable variation was observed between some populations of P. ciliata. Generally, those populations of P. ciliata which live in hard substrata, had fewer hooded hooks in comparision to those P. ciliata populations which live in mud and to P. ligni and P. limicola. There was upto a maximum of 6, 10, 18 and 11 neuropodial hooded hooks per segment in P. ciliata (hard substrate), P. ciliata (mud), P. ligni and P. limicola respectively. The average number of neuropodial hooded hooks per segment in all the populations studied is presented in Figure 24. It was difficult and time consuming to count hooded hooks in all the segments and it was therefore, decided to take a few sample segments, say 15 segments from each individual distributed equally (5 segments each from anterior, middle and posterior region) and to estimate the average number of hooks per segment. Usually in P. ligni hooded hooks were arranged in two rows (Figure 7) while in P. ciliata only a single row was observed.

Figure 18. Population range diagram showing variation in the number of heavy spines in the fifth enlarged segment of *Polydora*.

1. *P. ciliata*, Plymouth; 2. Dumpton; 3. Torbay; 4. Whitstable; 5. Barry; 6. Sully; 7. Robin Hood's Bay; 8. Hale; 9. New Brighton.

10. *P. ligni*

11. *P. limicola*

In each sample the vertical line indicates the total variation of the sample, the broad portion of the line indicates the standard deviation and the crossbar, the mean.

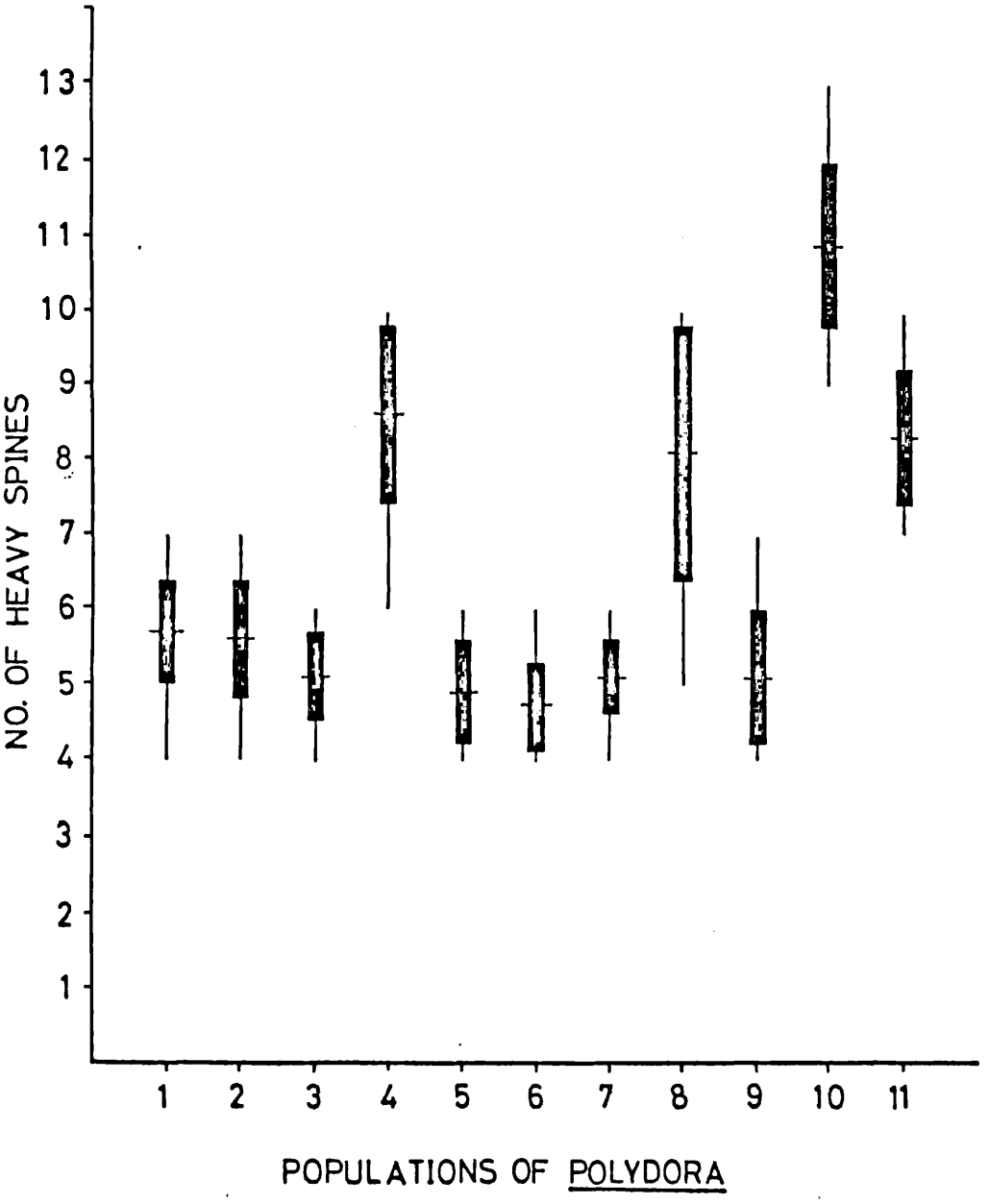

NO. OF HEAVY SPINES
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
POPULATIONS OF POLYDORA

Figure 19. Scanning electron micrographs of Polydora species showing arrangement of various types of setae on the fifth enlarged segment.

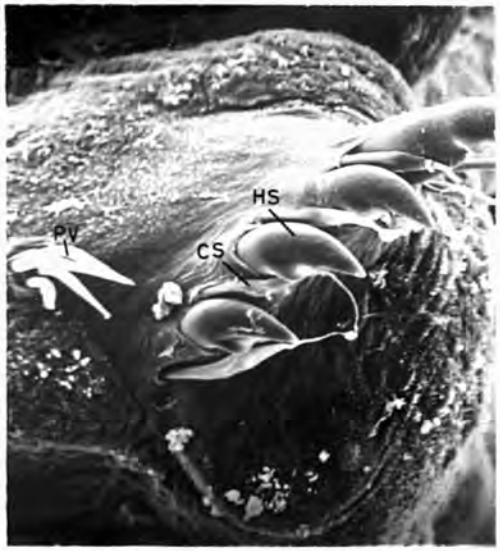
a. P. ciliata from Barry (1,000 x)

b. P. ciliata from Dumpton (700 x)

c. P. limicola (700 x)

AD. 'anterio-dorsal' setae; CS. companion setae; HS. heavy spine; PV. 'posterio-ventral' setae.


AD
HS
CS


HS
PV
CS

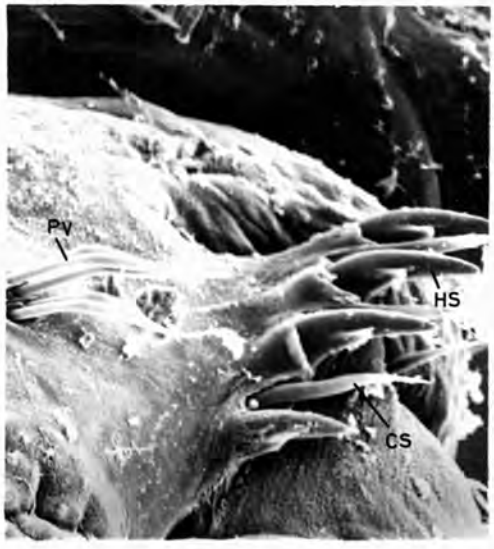

PV
HS
CS

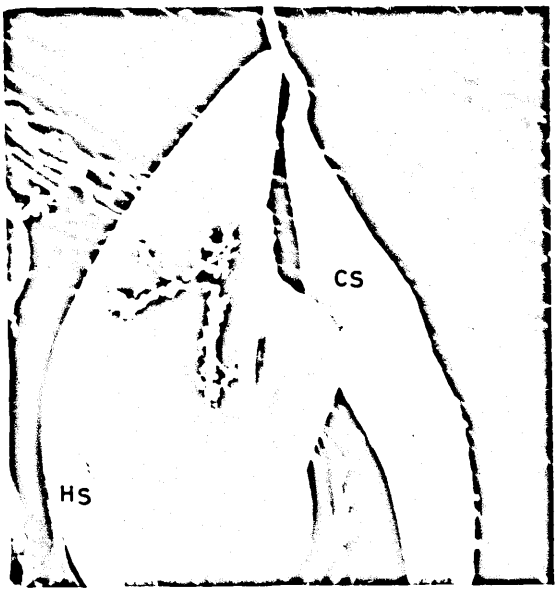
Figure 20. P. ligni; Scanning electron micrograph of the fifth enlarged segment; arrow indicates an extra ordinary long heavy spine. (180 x)

Figure 21. P. limicola; Scanning electron micrograph of the 'posterio-ventral' bundle of setae on the fifth enlarged segment (1,700 x)

Figure 22. P. ciliata (from Barry); scanning electron micrograph of a heavy spine of the fifth enlarged segment.

HS. heavy spine; CS. companion setae

(2,500 x)


CS
HS

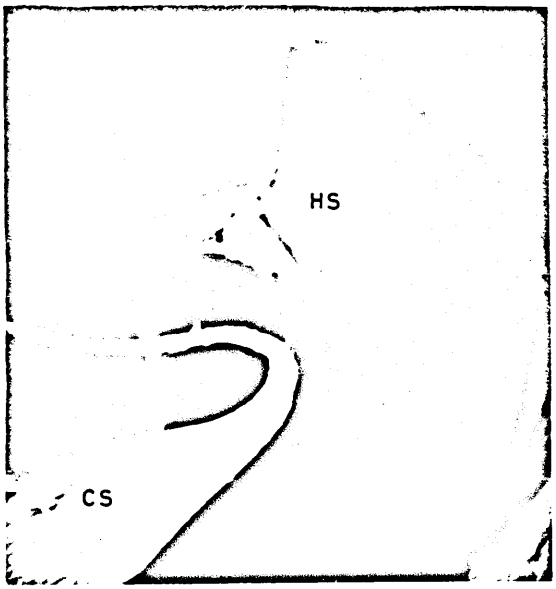
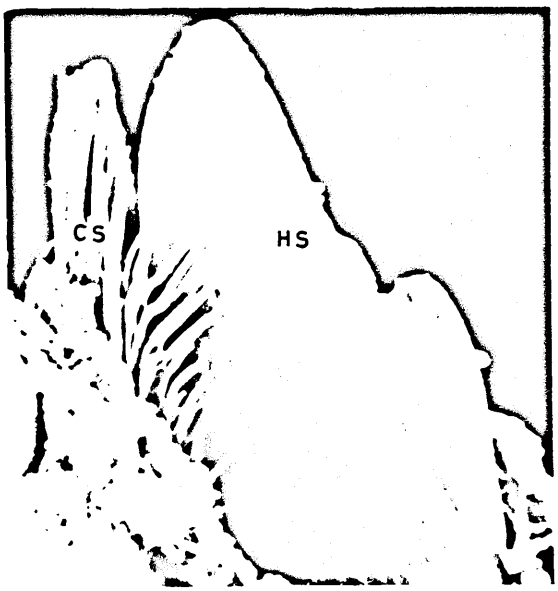

HS
CS

Figure 23. P. ligni; scanning electron micrograph of a heavy spine of the fifth enlarged segment.(3,000 x)

HS. heavy spine, CS. companion setae.


CS
HS

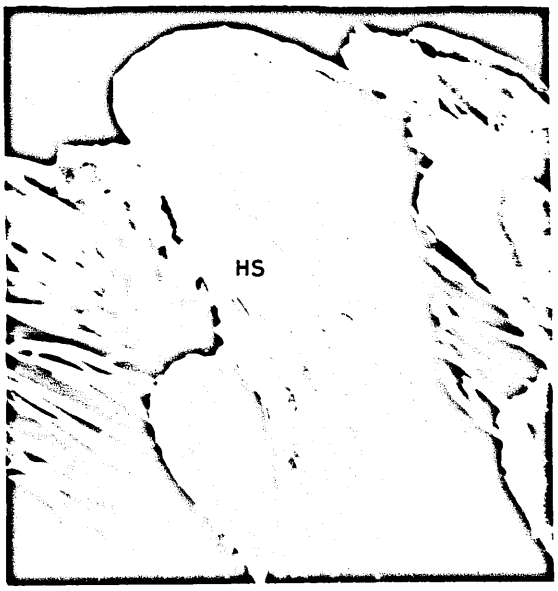

HS

Figure 24. Average number of hooded hooks per segment in different populations of *Polydora* species with standard error of the mean.

1. *P. ciliata*, Plymouth; 2. Dumpton, 3. Torbay 4. Whitstable, 5. Barry, 6. Sully, 7. Robin Hood's Bay, 8. Hale, 9. New Brighton,

10. *P. ligni*

11. *P. limicola*

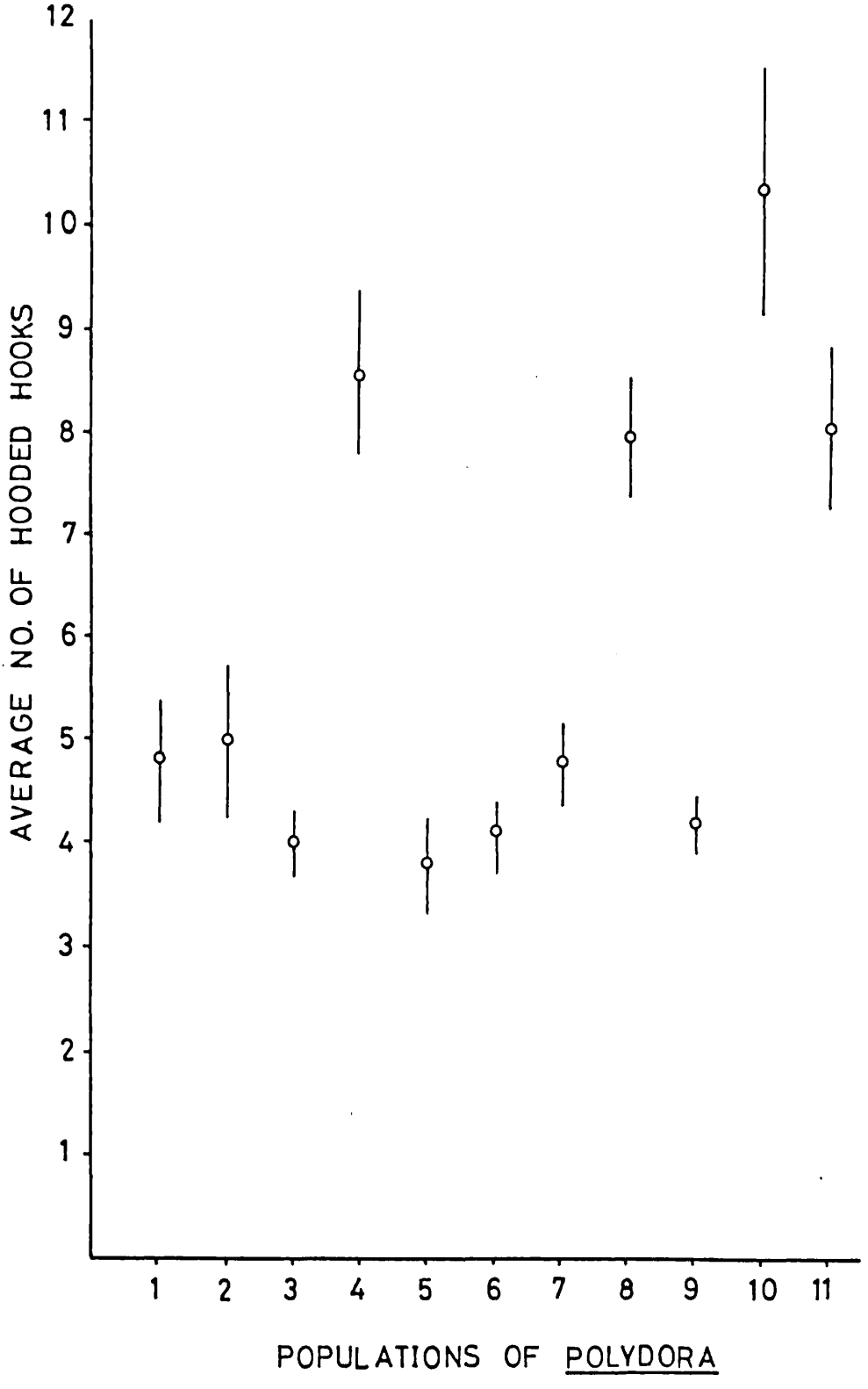

12
11
10
9
8
7
6
5
4
3
2
1
AVERAGE NO. OF HOODED HOOKS
1
2
3
4
5
6
7
8
9
10
11
POPULATIONS OF POLYDORA

Table 5. Percent occurrence of 'anterio-dorsal' and 'posterio-ventral' bundles of setae with respect to heavy spines on the fifth enlarged segment in Polydora.

Populations	n	anterio-dorsal		posterio-ventral	
		present %	absent %	present %	absent %
P. ciliata					
Plymouth	20	90.0	10.0	100.0	0.0
Dumpton	24	91.7	8.3	100.0	0.0
Torbay	15	100.0	0.0	100.0	0.0
Whitstable	28	89.3	10.7	92.9	7.1
Barry	15	100.0	0.0	100.0	0.0
Sully	20	100.0	0.0	100.0	0.0
Robin Hood's B.	18	100.0	0.0	100.0	0.0
Hale	18	0.0	100.0	11.0	88.9
New Brighton	15	100.0	0.0	100.0	0.0
P. ligni					
Peterstone W.	25	0.0	100.0	0.0	100.0
P. limicola					
Tees	23	87.0	13.0	78.0	22.0

Electrophoresis :

The criteria which were utilized in this study in estimating the numbers of genetic loci are as follows:

1. A codominant diallelic or triallelic locus in which each allele forms or produces a single enzyme band of a particular mobility is easily recognised (Figure 25 a). In heterozygotes the band is usually lighter than the corresponding band in a homozygote, since it is produced by a single polypeptide. This type of band pattern has been noted repeatedly in Drosophila and other organisms (Wright, 1963; Scandalios, 1969). Such a pattern in this study was regarded as a reliable criterion for the existence of a polymorphic genetic locus. In multimeric enzymes, polypeptide sub-units may form one or more intermediate bands approximately equally spaced between the bands of homozygote mobilities. Commonly a single intermediate band is found in heterozygotes (Figure 25 b) which presents no problem in interpretation. However, the genetic relationships of multimeric enzymes produced by several alleles of different electrophoretic mobility can be complicated. Complex loci of this multimeric type was not found in the present study.

2. Bands are usually localized into discrete zones and are not randomly scattered over the gels. A zone is considered representative of a single locus if variation within it appears independent of other zones of the gel (Figure 25 c). Therefore,

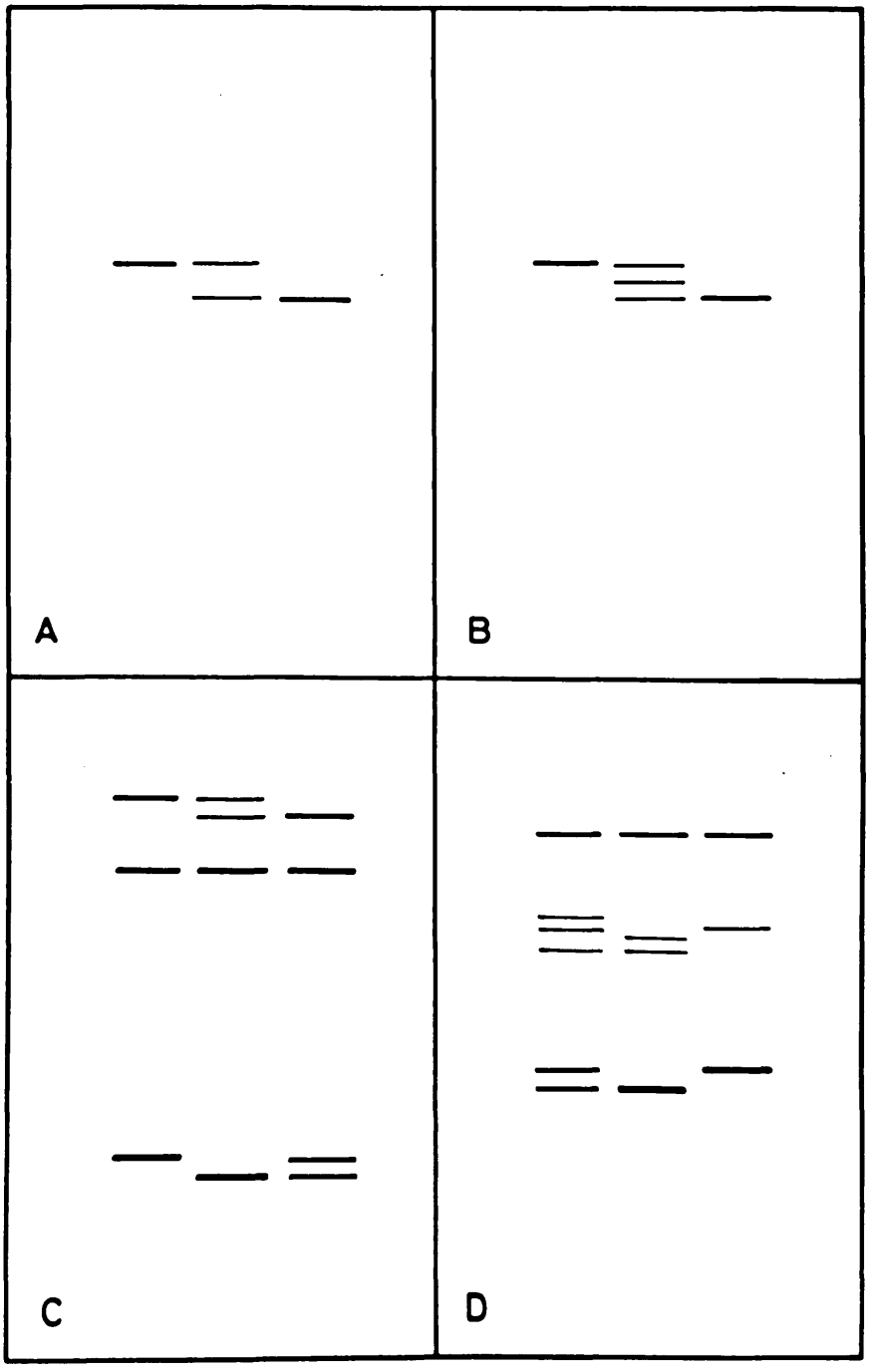
Figure 25. Diagram showing various criteria used to distinguish different locus on zymogram.

a. single locus, monomeric

b. single locus, dimeric

c. three loci, two polymorphic and one monomorphic

d. three loci, two polymorphic and one monomorphic


A
B
C
D

a zone of activity appearing as a band of uniform mobility is regarded as monomorphic if it is adjacent to zones definitely identified as polymorphic (Hubby and Lewontin, 1966). Similarly zones containing bands of variable mobility but difficult to interpret genetically are considered polymorphic if they are adjacent to zones identified as either monomorphic or polymorphic by other criteria (Figure 25 d).

3. The use of alternate substrates and specific inhibitors also permits the recognition of loci. The rationale is that a band or a band complex differentially affected by these agents in comparisions to adjacent zones probably includes polypeptides that originate from a single locus. This conclusion does not extend to groups of bands with widely different mobilities that happen to respond to the same inhibitors or alternate substrates.

Gels stained for non-specific esterases with α-naphtyl acetate (as is the case in the present study) usually show numerous zones of activity that may represent several enzymes. Alternate substrates and various inhibitors with partial specificity have been used by Allen (1961) Ogita and Kasai (1965) and Manwell et al. (1967) to distinguish classes of esterases.

It is common practice to assign a number to each specific band of isozyme for easy reference. Usually the fastest moving anodal band is numbered 1 and the numbering continues in cathodal

direction. It is generally accepted that in a figure the anode of the gel should be either at the top or at the right hand side of the figure (Brewer, 1970). In the present study gels are figured with anode at the top. The numbering of bands starts from anode towards cathode. For example, in P. ciliata there are four zones of acid phosphatase activity under the control of separate genetic loci (Figure 30 and 32). These enzymes are designated acid phosphatase - 1 to acid phosphatase - 4 in order of decreasing mobility and distance from the origin. The corresponding loci are termed ACP-1 to ACP-4. Thus the second zone of acid phosphatase activity from the anodal end is the isozyme acid phosphatase - 2 and it is controlled by the locus ACP-2. The ACP-2 locus has two mobility variants that migrate distances of 98 and 94 relative to that of myoglobin standard, which was given an arbitrary mobility of 100. The mobility variants belong to two alleles of ACP-2 locus and are designated ACP-2^{98} and ACP-2^{94}. The heterozygote is designated as ACP-$2^{98/94}$.

Out of seven enzyme systems assayed (Table 4) four enzyme systems were monomorphic in all the eleven populations of Polydora species. The other three enzyme systems comprising six loci, were either monomorphic or polymorphic. These enzyme systems are described below separately. No bands were observed when incubated without substrates.

Monomorphic enzyme system :

1. Glucose-6-phosphate dehydrogenase (G6PD):

The glucose-6-phosphate dehydrogenase stains as a single band system (Figure 26) in all the populations of Polydora studied. In some cases a second band was found which was very light and took a very long time to develop. This light band is not regarded as glucose-6-phosphate dehydrogenase because of its doubtful nature. The glucose-6-phosphate dehydrogenase reaction produces 6-phosphogluconate which may serve as a substrate for a 6-phosphogluconate dehydrogenase upon prolonged staining (Brewer, 1970). When the gel was incubated in staining solution having no substrate no band was found which suggests that the second light band which appeared after prolonged staining is not 'nothing dehydrogenase' band.

2. Malate dehydrogenase (MDH):

The NAD-dependent malate dehydrogenase stains as a two band system (Figures 26 and 28). The fast moving band (MDH-1 locus) had an average mobility 99.9 $\pm$ 0.3 (S.D.). This locus does not show polymorphism and was found monomorphic in all populations of Polydora studied. The second slow moving band (MDH-2 locus) had an average mobility 90.4 $\pm$ 0.76 (S.D.) and was monomorphic as well. The MDH-1 locus stained darker than MDH-2 locus and both loci were

revealed on all gels stained for MDH.

3. Malic enzyme (ME):

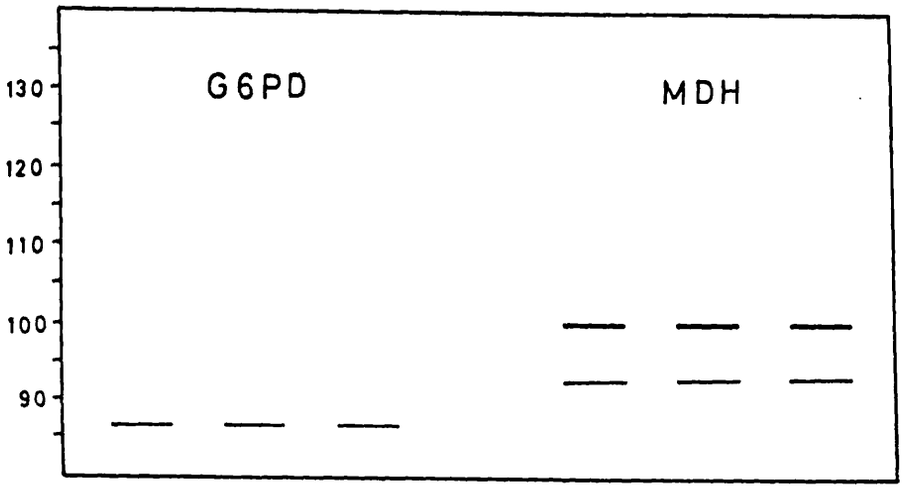
The malic enzyme (NADP-dependent malate dehydrogenase) shows three zones of activity, all represented by a single band system (Figure 27). The two fast moving bands (ME-1 and ME-2 loci) were stained darker than the slow moving band (ME-3 locus) which was stained as a very light and thin band. The two fast moving bands were screened in all individuals assayed while the slow moving band was either too faint or absent in some indivduals. All populations were identical with respect to these three loci. The average mobilities of these bands were 101.1 $\pm$ 0.2 (S.D.), 90.5 $\pm$ 0.3 (S.D.) and 81.7 $\pm$ 0.2 (S.D.) for ME-1, ME-2 and ME-3 locus respectively.

4. Xanthine dehydrogenase (XDH):

Xanthine dehydrogenase stains as a single band system in all the eleven populations of Polydora studied (Figures 27 and 29). No inter- or intra-population variation was found and all populations were genetically identical with respect to XDH locus.

Figure 26. Diagram showing position of bands of glucose-6-phosphate dehydrogenase (G6PD) and malate dehydrogenase (MDH) found in eleven populations of Polydora.

Figure 27. Diagram showing position of bands of malic enzyme (ME) and xanthine dehydrogenase (XDH) found in eleven populations of Polydora.


G6PD
MDH
130
120
110
100
90

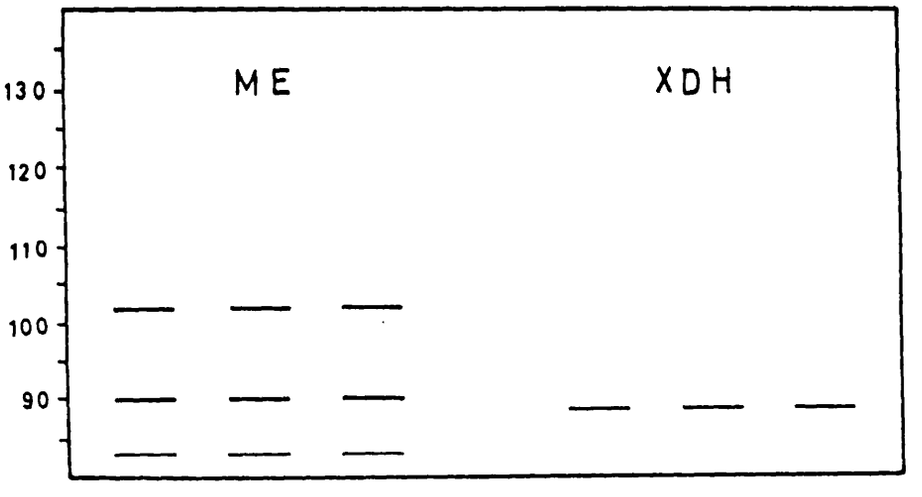
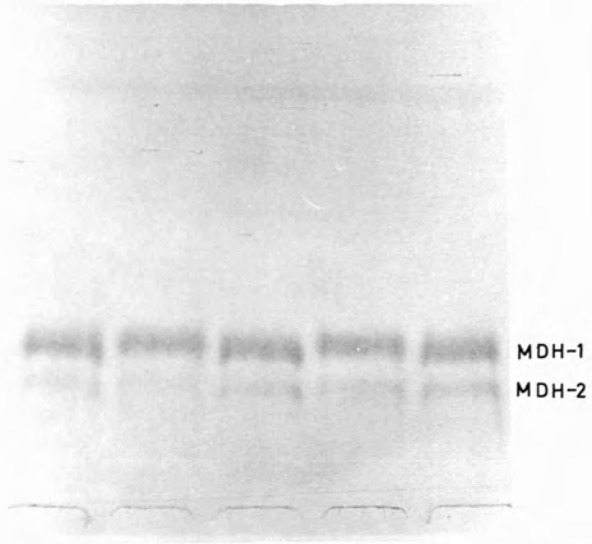
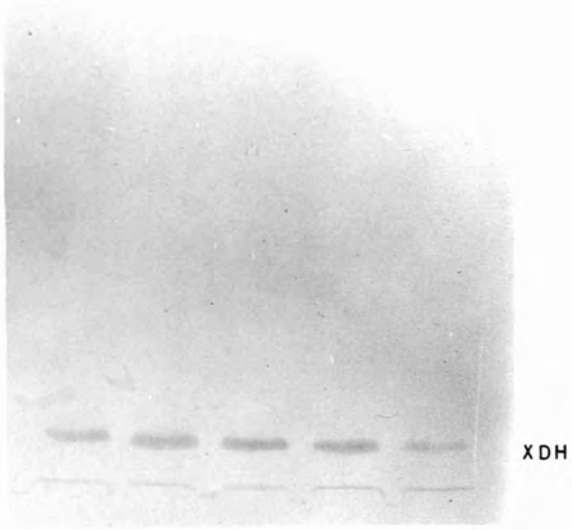

ME
XDH
130
120
110
100
90

Figure 28. Photograph of malate dehydrogenase zymogram of *P. ciliata*, 1 - 3; *P. ligni*, 4 and *P. limicola*, 5.

Figure 29. Photograph of xanthine dehydrogenase zymogram of *P. ciliata*, 1 - 3; *P. ligni*, 4 and *P. limicola*, 5.


MDH-1
MDH-2


XDH

Polymorphic enzyme system :

5. Acid phosphatase (ACP) :

Figure 30 diagrams the positions of different zones of acid phosphatase activity. The ACP-1 locus was difficult to interpret genetically. It was stained lightly and it was not revealed consistently and therefore not included in the estimation of genetic identity or distance. The ACP zymogram can be classified into two types (A) and (B) according to the pattern of bands. The type (A) was observed in P. ligni, P. limicola and two populations of P. ciliata namely Hale and Whitstable. The type (B) was observed in seven populations of P. ciliata namely Plymouth, Dumpton, Torbay, Barry, Sully, Robin Hood's Bay and New Brighton.

The ACP-2 locus was found polymorphic in type (A). It involved two alleles ACP-2^{98} and ACP-2^{94}, which were monomeric and therefore the heterozygote had two bands. The gene frequency and genotype are summarized in Table 6. The expected and observed heterozygosities were found in accordance to Hardy-Weinberg equilibrium and X^2 test shows no significant difference ($P<0.05$). In type (B) ACP-2 locus was monomorphic. The mobility of the single band was identical with ACP-2^{98} of type (A).

Table 7 presents estimates of genetic identity and genetic distance of ACP-2 locus between pairs of eleven populations.

Figure 30. Diagram of acid phosphatase zymogram showing two types of patterns found in Polydora species.

A. P. ciliata (non-boring form), P. ligni and P. limicola.

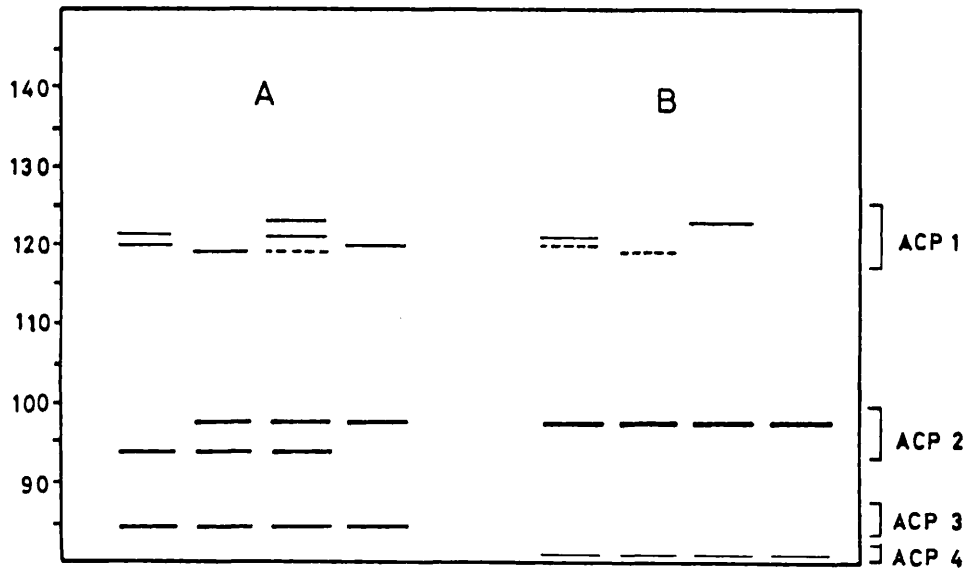
B. P. ciliata (boring form)

Figure 31. Diagram of phosphoglucose isomerase zymogram showing three different types of patterns found in Polydora species.

A. P. ciliata (non-boring form) and P. ligni.

B. P. limicola

C. P. ciliata (boring form)


140
130
120
110
100
90
A
B
ACP 1
ACP 2
ACP 3
ACP 4

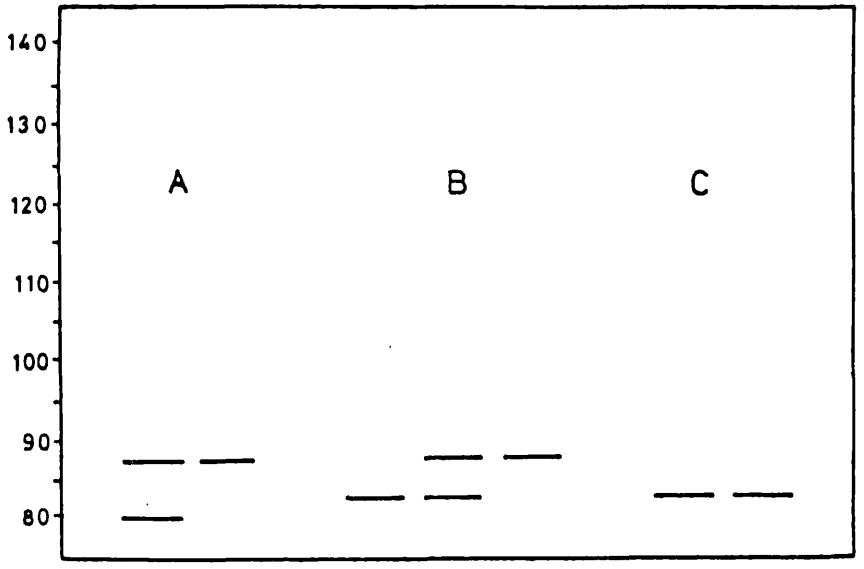
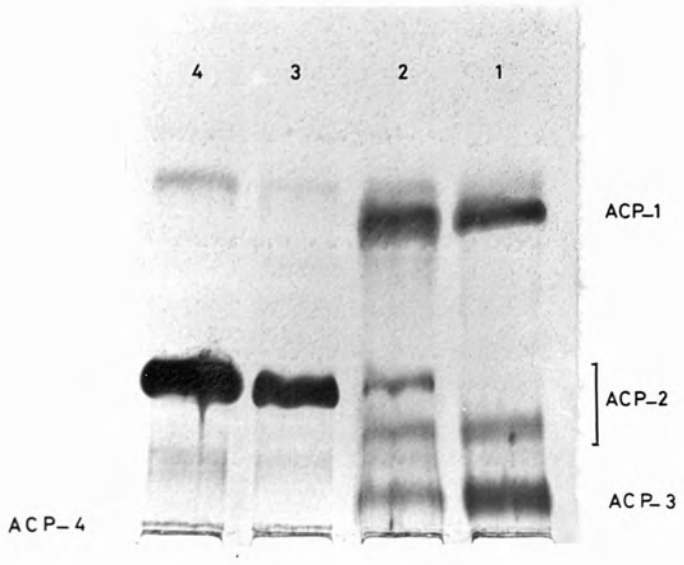

140
130
120
110
100
90
80
A
B
C

Figure 32. Photograph of acid phosphatase zymogram showing two types of patterns found in Polydora species.

1 and 2. P. ligni

3 and 4. P. ciliata (boring form)

Figure 33. Photograph of phosphoglucose isomerase zymogram of P. ligni, 1; P. limicola, 2; P. ciliata (boring form), 3 to 5.


4
3
2
1
ACP-1
ACP-2
ACP-3
ACP-4

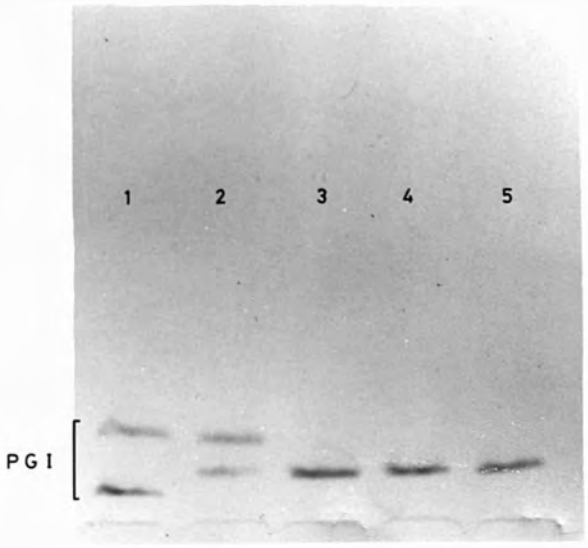

1
2
3
4
5
PGI

Table 6. Summary of gene and genotype distributions and heterozygosities for the ACP-2 locus for four populations of Polydora which were polymorphic.

Species & Population	n	gene frequency		genotype			H_e	H_o	X^2
		ACP-2^{98}	ACP-2^{94}	ACP-2^{98}	ACP-$2^{98/94}$	ACP-2^{94}			
P. ciliata Whitstable	20	0.450	0.550	4	10	6	0.495	0.500	0.000
P. ciliata Hale	17	0.529	0.471	5	8	4	0.498	0.470	0.002
P. ligni Peterstone W.	23	0.370	0.630	3	11	9	0.466	0.478	0.001
P. limicola Tees	20	0.400	0.600	3	10	7	0.480	0.500	0.001

Table 7. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of ACP-2 locus among eleven populations of Polydora (P. ciliata, 1 to 9; P. ligni, 10; P. limicola, 11)

	1	2	3	4	5	6	7	8	9	10	11
1. Plymouth		0.000	0.000	0.457	0.000	0.000	0.000	0.292	0.000	0.681	0.589
2. Dumpton	1.000		0.000	0.457	0.000	0.000	0.000	0.292	0.000	0.681	0.589
3. Torbay	1.000	1.000		0.457	0.000	0.000	0.000	0.292	0.000	0.681	0.589
4. Whitstable	0.633	0.633	0.633		0.457	0.457	0.457	0.012	0.457	0.012	0.005
5. Barry	1.000	1.000	1.000	0.633		0.000	0.000	0.292	0.000	0.681	0.589
6. Sully	1.000	1.000	1.000	0.633	1.000		0.000	0.292	0.000	0.681	0.581
7. Robin Hood's B.	1.000	1.000	1.000	0.633	1.000	1.000		0.292	0.000	0.681	0.589
8. Hale	0.747	0.747	0.747	0.988	0.747	0.747	0.747		0.292	0.041	0.032
9. N. Brighton	1.000	1.000	1.000	0.633	1.000	1.000	1.000	0.747		0.681	0.589
10. Peterstone W.	0.506	0.555	0.555	0.995	0.555	0.555	0.555	0.968	0.555	0.998	

The ACP-3 (band stained brown) and ACP-4 (band stained reddish brown) loci were monomorphic for type (A) and type (B) respectively.

6. Phosphoglucose isomerase (PGI) :

Figure 31 presents three types of patterns obtained. The type (A) was found in P. ligni and two population of P. ciliata (Whitstable and Hale). In type (A) the locus had two alleles PGI^{87} and PGI^{80}. The homozygote PGI^{87} and heterozygote $PGI^{80/87}$ were observed while homozygote for slow moving allele PGI^{80} was not found. The type (B) pattern was found in P. limicola where both homozygotes and heterozygote were observed. The two alleles PGI^{83} and PGI^{87} were identified. This type shared PGI^{87} allele with type (A). The type (C) pattern was observed in seven populations of P. ciliata, namely Plymouth, Dumpton, Torbay, Barry, Sully, Robin Hood's Bay and New Brighton. This type was monomorphic and had single band which was similar in mobility with allele PGI^{83} of type (B). The result of gene frequency and genotype are summarised in Table 8, and the estimates of genetic identity and genetic distance are presented in Table 9 for PGI locus.

7. Esterase (EST) :

Figure 34 diagrams the various zones of esterase activity on the gel. There were many slow moving bands which usually

Table 8. Summary of gene and genotype distributions and heterozygosities for the PGI locus for different populations of Polydora.

Species & population	n	gene frequency			genotype					H_e	H_o	X^2
		PGI^{87}	PGI^{83}	PGI^{80}	PGI^{87}	$PGI^{87/83}$	PGI^{83}	$PGI^{87/80}$	PGI^{80}			
P. ciliata												
Plymouth	20	0.000	1.000	0.000	0	0	20	0	0	0.000	0.000	
Dumpton	34	0.000	1.000	0.000	0	0	34	0	0	0.000	0.000	
Torbay	8	0.000	1.000	0.000	0	0	8	0	0	0.000	0.000	
Whitstable	25	0.122	0.000	0.880	0	0	0	6	19	0.211	0.240	0.003
Barry	16	0.000	1.000	0.000	0	0	16	0	0	0.000	0.000	
Sully	20	0.000	1.000	0.000	0	0	20	0	0	0.000	0.000	
Robin Hood's B	11	0.000	1.000	0.000	0	0	11	0	0	0.000	0.000	
Hale	19	0.105	0.000	0.895	0	0	0	4	15	0.198	0.210	0.001
N. Brighton	12	0.000	1.000	0.000	0	0	12	0	0	0.000	0.000	
P. ligni												
Peterstone W.	28	0.107	0.000	0.893	0	0	0	6	22	0.191	0.214	0.003
P. limicola												
Tees	22	0.727	0.273	0.000	12	8	2	0	0	0.397	0.364	0.003

Table 9. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of PGI locus among eleven populations of *Polydora* (*P. ciliata*, 1 to 9; *P. ligni*,10; *P. limicola*, 11)

	1	2	3	4	5	6	7	8	9	10	11
1. Plymouth		0.000	0.000	1.000	0.000	0.000	0.000	1.000	0.000	1.000	0.066
2. Dumpton	1.000		0.000	1.000	0.000	0.000	0.000	1.000	0.000	1.000	0.066
3. Torbay	1.000	1.000		1.000	0.000	0.000	0.000	1.000	0.000	1.000	0.066
4. Whitstable	0.000	0.000	0.000		1.000	1.000	1.000	0.001	1.000	0.001	2.045
5. Barry	1.000	1.000	1.000	0.000		0.000	0.000	1.000	0.000	1.000	0.066
6. Sully	1.000	1.000	1.000	0.000	1.000		0.000	1.000	0.000	1.000	0.066
7. Robin Hood's B.	1.000	1.000	1.000	0.000	1.000	1.000		1.000	0.000	1.000	0.066
8. Hale	0.000	0.000	0.000	0.999	0.000	0.000	0.000		1.000	0.001	1.754
9. N. Brighton	1.000	1.000	1.000	0.000	1.000	1.000	1.000	0.000		1.000	0.066
10. Peterstone W.	0.000	0.000	0.000	0.999	0.000	0.000	0.000	0.999	0.000		2.198
11. Tees	0.936	0.936	0.936	0.129	0.936	0.936	0.936	0.173	0.936	0.111	

occupy half of the gel on cathode side. These bands were not found in all individuals and sometimes they were too faint to measure precisely. Sometimes these zones did not appear as bands due to streaking and diffusion. On the other hand the resolution of fast moving or anodal bands was good and they were resolved consistently. Two different types of patterns were found in esterase zymograms. The type (A) was represented by P. ligni, P. limocola and two populations of P. ciliata, namely Whitstable and Hale. Type (B) was represented by other seven populations of P. ciliata. The EST-1 locus which was stained as a single band in type (A) is not included in the calculations of genetic identity or genetic distance because of its inconsistency. This locus was never observed in the type (B) zymogram.

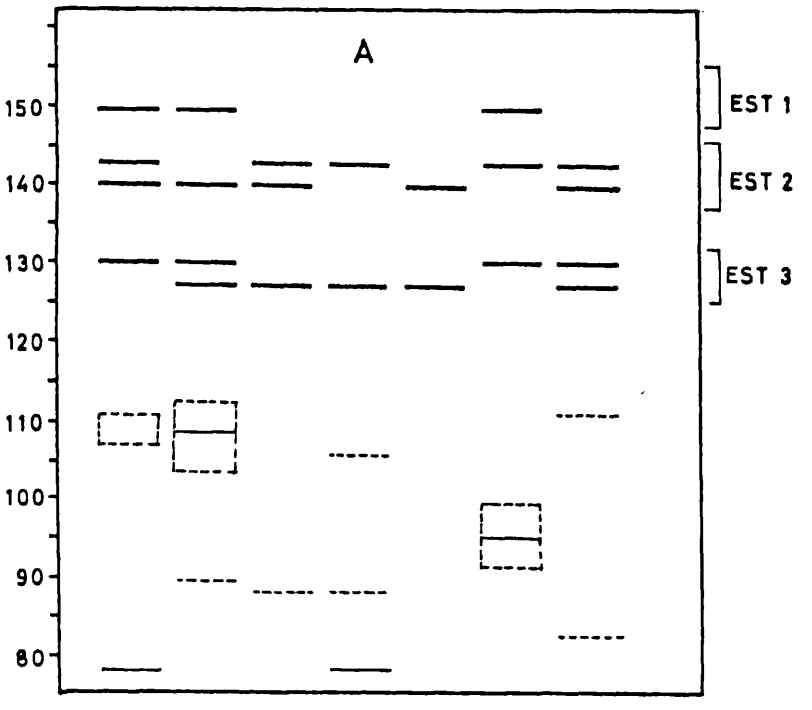
The EST-2 locus was polymorphic in both types and heterozygotes had two bands corresponding to two homozygotes, EST-2^{142} and EST-2^{140}. Table 10 shows the gene frequency and genotypes for this locus for eleven populations of Polydora. P. ciliata from Robin Hood's Bay population had the lowest number of heterozygote individuals. No significant difference was found between expected and observed heterozygosities in all populations ($P<0.05$). The genetic identity and genetic difference are presented in Table 11 for EST-2 locus.

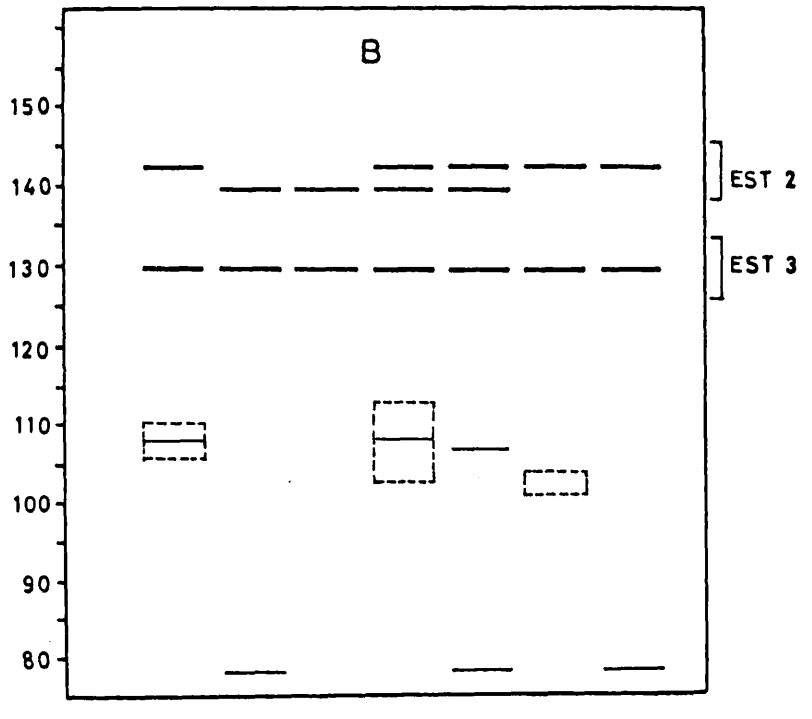
The EST-3 locus was found polymorphic in type (A) but was monomorphic in type (B). The mobility of monomorphic EST-3 locus in type (B) was the same as that of the fast moving allele,

Figure 34. Diagram of esterase zymogram showing two types of patterns found in Polydora species.

A. P. ligni, P. limicola and P. ciliata (non-boring form).

B. P. ciliata (boring form)


A
150
140
130
120
110
100
90
80
EST 1
EST 2
EST 3

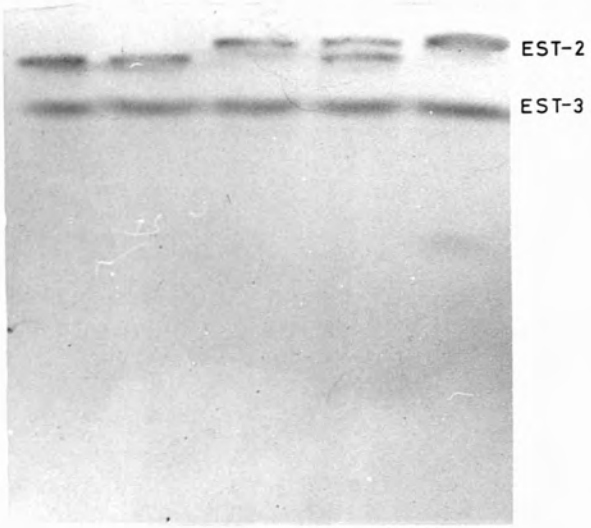

B
150
140
130
120
110
100
90
80
EST 2
EST 3

EST-3^{130}, of polymorphic locus in type (A). The frequency of gene and genotypes are presented in Table 12 for four populations which were polymorphic. In Table 13 genetic identity and genetic distance are shown for different pairs of eleven populations, for EST-3 locus.

An attempt was made to stain gel for non-specific esterases by using inhibitors and more specific substrate like acetylthiocholine in place of α-naphthyl acetate, which is commonly used for non-specific esterase. The acetylthiocholine is hydrolized by acetylcholine and pseudocholine esterases. By using specific inhibitors such as tetraisopropylpyrophosphoramide and 1:5 bis-(4-trimethyl ammonium phenyl)pentan-3-one diiodide, two different esterases can be differentiated. When the gel was incubated after electrophoresis for the two specific esterases, the whole gel became dark brown and no band was seen.

In addition to enzyme systems described above, other enzymes such as lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH), peroxidase (POX) and tetrazolium oxidase (TO) were also separated electrophoretically. These enzymes were too weak in their activity to separate from individual worms. Pooled samples were therefore used to find out if they indicate any significant difference between populations of P. ciliata. Three populations were choosen for this purpose. The Whitstable population, a non-boring form which lives at low tide level where it builds mud tubes.

Figure 35. Photographs of esterase zymogram of P. ciliata from Dumpton (boring form).


EST-2
EST-3

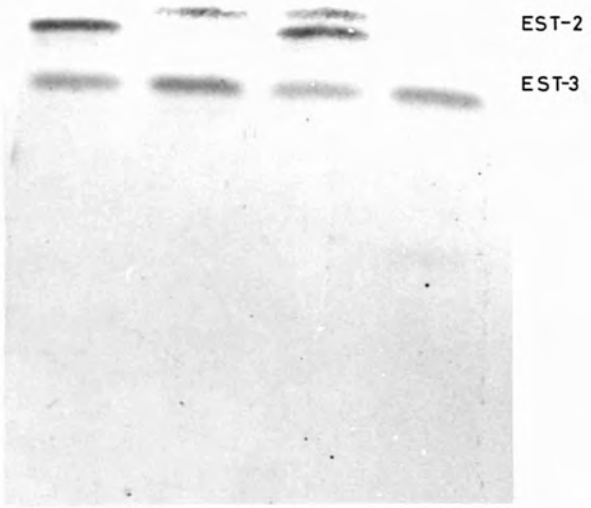

EST-2
EST-3

Table 10. Summary of gene and genotype distributions and heterozygosities for the EST-2 locus for different populations of Polydora.

Species & population	n	gene frequency		genotype			H_e	H_o	X^2
		EST-2^{142}	EST-2^{140}	EST-2^{142}	EST-$2^{142/140}$	EST-2^{140}			
P. ciliata									
Plymouth	25	0.440	0.560	5	12	8	0.493	0.480	0.000
Dumpton	48	0.458	0.542	11	22	15	0.496	0.458	0.002
Torbay	16	0.656	0.344	7	7	2	0.451	0.437	0.000
Whitstable	40	0.550	0.450	12	20	8	0.495	0.500	0.000
Barry	25	0.740	0.260	14	9	2	0.385	0.360	0.001
Sully	30	0.650	0.350	13	13	4	0.455	0.433	0.001
Robin Hood's B.	28	0.161	0.839	1	7	20	0.270	0.250	0.001
Hale	20	0.425	0.575	4	9	7	0.489	0.450	0.003
N. Brighton	15	0.233	0.767	1	5	9	0.357	0.333	0.002
P. ligni									
Peterstone W.	45	0.400	0.600	8	20	17	0.480	0.444	0.003
P. limicola									
Tees	38	0.460	0.540	8	19	11	0.497	0.500	0.000

Table 11. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of EST-2 locus among eleven populations of Polydora (P. ciliata, 1 to 9; P. ligni, 10; P. limicola, 11).

	1	2	3	4	5	6	7	8	9	10	11
1. Plymouth		0.001	0.002	0.001	0.002	0.002	0.021	0.001	0.009	0.001	0.001
2. Dumpton	0.999		0.076	0.017	0.148	0.036	0.134	0.002	0.085	0.007	0.001
3. Torbay	0.998	0.927		0.021	0.011	0.001	0.473	0.105	0.354	0.130	0.075
4. Whitstable	0.999	0.983	0.979		0.060	0.018	0.264	0.031	0.185	0.045	0.016
5. Barry	0.992	0.862	0.989	0.940		0.012	0.687	0.190	0.526	0.224	0.146
6. Sully	0.998	0.965	0.999	0.982	0.988		0.460	0.104	0.344	0.124	0.070
7. Robin Hood's B.	0.979	0.872	0.623	0.768	0.503	0.631		0.103	0.006	0.081	0.139
8. Hale	0.999	0.998	0.900	0.969	0.827	0.901	0.902		0.059	0.002	0.003
9. N. Brighton	0.991	0.918	0.702	0.831	0.591	0.709	0.994	0.942		0.044	0.087
10. Peterstone W.	0.999	0.993	0.878	0.956	0.799	0.883	0.922	0.998	0.957		0.007
11. Tees	0.999	0.999	0.928	0.984	0.864	0.932	0.870	0.997	0.917	0.993	

Table 12. Summary of gene and genotype distributions and heterozygosities for the EST-3 locus for four populations of Polydora which were polymorphic.

Species & population	n	gene frquency		genotype			H_e	H_o	X^2
		EST-3^{130}	EST-3^{127}	EST-3^{130}	EST-$3^{130/127}$	EST-3^{127}			
P. ciliata Whitstable	40	0.337	0.663	4	19	17	0.447	0.475	0.002
P. ciliata Hale	20	0.375	0.625	3	9	8	0.468	0.450	0.001
P. ligni Peterstone W.	45	0.455	0.545	10	21	14	0.496	0.467	0.002
P. limicola Tees	38	0.579	0.421	13	18	7	0.488	0.470	0.001

Table 13. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of EST-3 locus among eleven populations of Polydora (P. ciliata, 1 to 9; P. ligni, 10; P. limicola, 11).

	1	2	3	4	5	6	7	8	9	10	11
1. Plymouth		0.000	0.000	0.792	0.000	0.000	0.000	0.665	0.000	0.445	0.212
2. Dumpton	1.000		0.000	0.792	0.000	0.000	0.000	0.665	0.000	0.445	0.212
3. Torbay	1.000	1.000		0.792	0.000	0.000	0.000	0.665	0.000	0.445	0.212
4. Whitstable	0.453	0.453	0.453		0.792	0.792	0.792	0.003	0.792	0.025	0.115
5. Barry	1.000	1.000	1.000	0.453		0.001	0.000	0.665	0.000	0.445	0.212
6. Sully	1.000	1.000	1.000	0.453	1.000		0.000	0.665	0.000	0.445	0.212
7. Robin Hood's B.	1.000	1.000	1.000	0.453	1.000	1.000		0.665	0.000	0.445	0.212
8. Hale	0.514	0.514	0.514	0.997	0.514	0.514	0.514		0.665	0.012	0.083
9. N. Brighton	1.000	1.000	1.000	0.453	1.000	1.000	1.000	0.514		0.445	0.212
10. Peterstone W.	0.641	0.641	0.641	0.975	0.641	0.641	0.641	0.988	0.641		0.030
11. Tees	0.809	0.809	0.809	0.891	0.809	0.809	0.809	0.920	0.809	0.970	

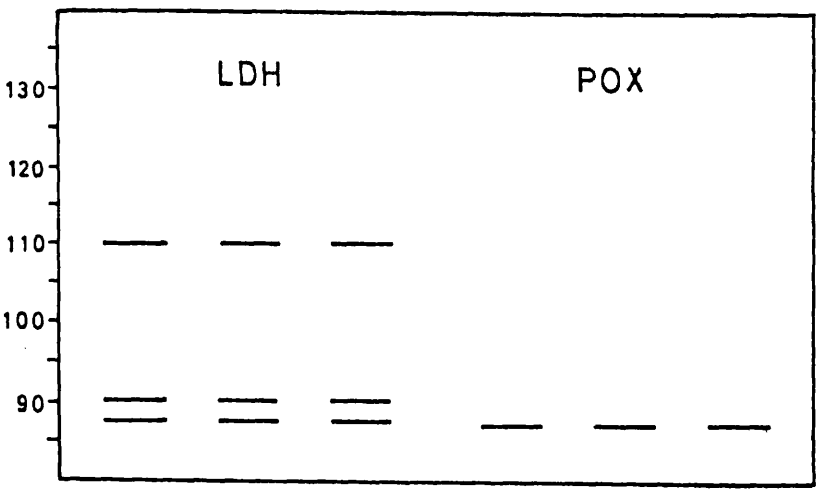
The second population was from Dumpton, a boring form which bores in limestone (chalk) and lives at mid tide level. The third population was from Torbay, another boring form which boresin Patella shell and can be collected from high to mid tide level of the intertidal zone.

Figures 36 and 37 diagrams the four enzyme systems investigated from pooled samples. The lactate dehydrogenase zymogram showed three bands in all the three populations of P. ciliata. One dark fast moving band and two light slow moving bands. Their mobilities were 110, 90 and 87 respectively. With peroxidase a single cathodal band was observed in all samples. The same was the case with isocitrate dehydrogenase where a single light band was found. In tetrazolium oxidase two bands were revealed. In this system the whole gel stained blue leaving the tetrazolium oxidase zone white. The fast and slow moving bands had a mobility of 105 and 95 respectively.

The mean genetic identity and genetic distance for 13 loci between pairs of eleven populations are presented in Table 14. It is obvious from the table that the Plymouth, Dumpton, Torbay, Barry, Sully, Robin Hood's Bay and New Brighton populations of P. ciliata are genetically similar to one another. It is also clear from the table that Whitstable and Hale populations of P. ciliata are slightly different from the other seven populations of the same species. The first seven populations bore in hard substrate like shell, limestone and rock and are therefore called

Figure 36. Diagram showing position of bands of lactate dehydrogenase (LDH) and peroxidase (POX) in Polydora ciliata

Figure 37. Diagram showing position of bands of isocitarate dehydrogenase (IDH) and tetrazolium oxidase (TO) in Polydora ciliata.


LDH
POX
130
120
110
100
90

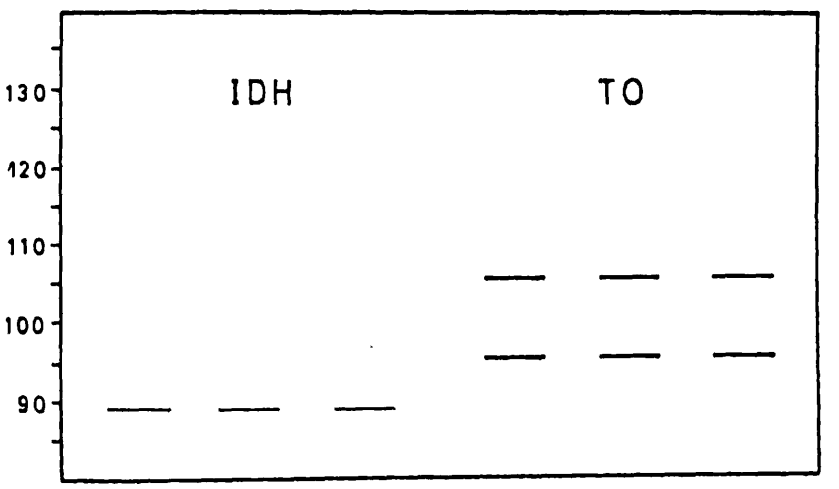

IDH
TO
130
120
110
100
90

Table 14. Estimates of mean genetic identity (below diagonal) and genetic distance (above diagonal) of 13 loci among eleven populations of Polydora (P. ciliata, 1 to 9; P. ligni, 10; P. limicola, 11).

	1	2	3	4	5	6	7	8	9	10	11
1. Plymouth		0.001	0.001	0.358	0.001	0.001	0.002	0.337	0.007	0.349	0.233
2. Dumpton	0.999		0.006	0.361	0.011	0.003	0.010	0.337	0.007	0.349	0.233
3. Torbay	0.999	0.994		0.358	0.001	0.001	0.029	0.349	0.261	0.365	0.239
4. Whitstable	0.699	0.697	0.699		0.365	0.361	0.384	0.003	0.377	0.007	0.080
5. Barry	0.999	0.989	0.999	0.694		0.001	0.040	0.361	0.032	0.380	0.247
6. Sully	0.999	0.997	0.999	0.697	0.999		0.029	0.349	0.021	0.365	0.247
7. Robin Hood's B.	0.998	0.990	0.971	0.681	0.961	0.971		0.349	0.001	0.361	0.247
8. Hale	0.714	0.714	0.705	0.997	0.697	0.705	0.705		0.345	0.005	0.076
9. N. Brighton	0.993	0.993	0.997	0.686	0.968	0.979	0.999	0.708		0.357	0.241
10. Peterstone W.	0.705	0.705	0.694	0.993	0.684	0.694	0.697	0.995	0.700		0.075
11. Tees	0.792	0.792	0.787	0.923	0.781	0.787	0.781	0.927	0.786	0.928	

hereafter as "boring form". The later two populations of *P. ciliata* namely, Whitstable and Hale, live in mud and are called as "non-boring form".

The data of genetic identity and genetic distance is pooled for the two forms of *P. ciliata* and is presented in Table 15 with other two species. The mean of mean genetic identity between 21 pairs of *P. ciliata* 'boring form' is 0.989 ± 0.012 (S.E.) and evidently they are very similar genetically. The mean genetic identity between 14 pairs of 'boring' and 'non-boring' forms is 0.700 ± 0.009 (S.E.). This figure is very similar to 0.697 ± 0.007 (S.E.) which is the estimated mean genetic identity between 7 pairs of *P. ligni* and *P. ciliata* 'boring' form. On the other hand the mean genetic identity between 3 pairs of *P. ligni* and *P. ciliata* 'non-boring' form is 0.995 ± 0.002 (S.E.). The mean genetic identity between seven pairs of *P. ciliata* 'boring' form and *P. limicola* is 0.786 ± 0.004 (S.E.) while the mean genetic identity between two pairs of *P. ciliata* 'non-boring' form and *P. limicola* is 0.925 ± 0.001 (S.E.).

The average heterozygosity per locus ($\bar{H}$) is presented in Table 16. The greater heterozygosity is shown by 'non-boring' form of *P. ciliata*, *P. ligni* and *P. limicola*.

From data presented in Table 15 for four groups of *Polydora*, a phenogram by unweighted pair-group arithmetical average (UPGMA) clustering method (Sneath and Sokal, 1973)

Table 15. Estimates of mean genetic identity (below diagonal) and genetic distance (above diagonal) of pooled data of 'boring' and 'non-boring' forms of *P. ciliata*, *P. ligni* and *P. limicola* for 13 loci.

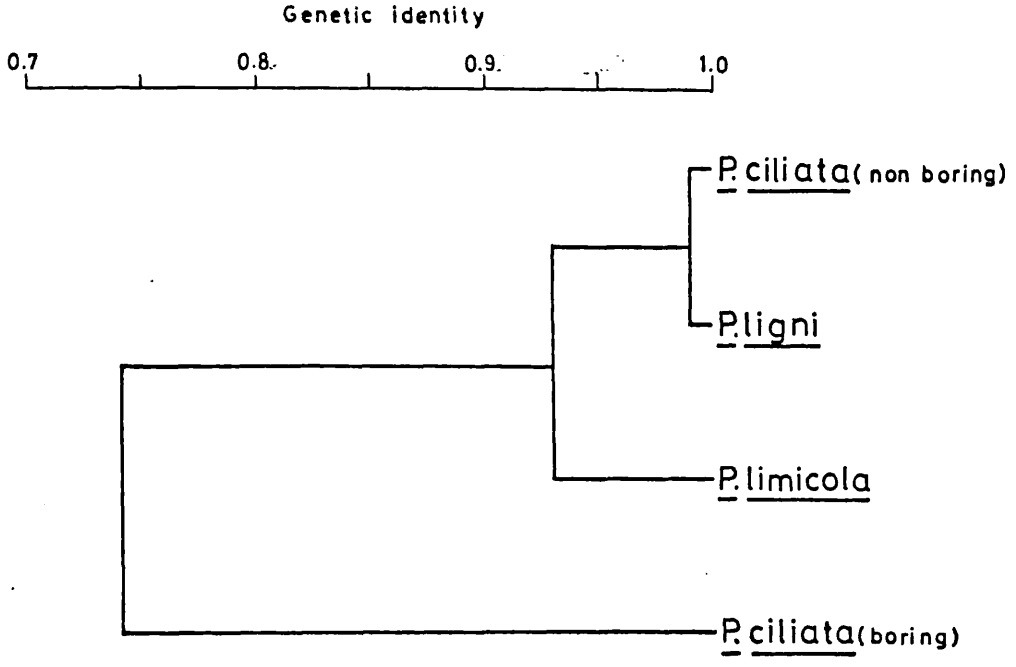
	1	2	3	4
1. *P. ciliata* 'boring'		0.357	0.361	0.241
2. *P. ciliata* 'non-boring'	0.700		0.005	0.007
3. *P. ligni*	0.697	0.995		0.075
4. *P. limicola*	0.786	0.925	0.928	

Table 16. Average heterozygosity ($\overline{H}$) per locus in different populations of Polydora.

Species & population	Average heterozygosity	
	expected	observed
P. ciliata		
Plymouth	0.038	0.031
Dumpton	0.038	0.035
Torbay	0.035	0.024
Whitstable	0.127	0.132
Barry	0.030	0.028
Sully	0.035	0.028
Robin Hood's B.	0.021	0.014
Hale	0.127	0.121
New Brighton	0.027	0.026
P. ligni		
Peterstone W.	0.126	0.108
P. limicola		
Tees	0.143	0.141

is produced (Figure 38). The phenogram shows the realtion-ships between four groups of Polydora based on genetic similarity. P. ciliata 'non-boring' form and P. ligni are more closely related to each other than any other group. P. ciliata 'boring' form and P. ciliata 'non-boring' form are more distantly related to any other group.

Figure 38. Phenogram showing relationships of four groups of *Polydora* generated according to UPGMA method of cluster analysis and based on the data in Table 15.


Genetic identity
0.7
0.8
0.9
1.0
P. ciliata (non boring)
P. ligni
P. limicola
P. ciliata (boring)

DISCUSSION

The present study was initiated because the high degree of variability displayed by Polydora ciliata (Johnston) is well known. The aim was to assess the degree of variation in order to find out whether it exhibits sufficient variation to warrant separation into "sibling" species. The variation in a species may occur at different levels such as morphology, ecology, physiology, reproduction, development or genetics. The last category is presumably the basis of most of the variation in preceding divisions. With the advent of new techniques, genetic variation can be more directly and independently assessed. In Polydora ciliata no study has yet been made on its genetics. It was thought that the other closely related species of P. ciliata such as P. ligni and P. limicola which are found in British waters may also be included so that their separation from P. ciliata can also be re-assessed. The separation of these species is questioned by Rasmussen (1973) and Kendall (1980) who believe that P. ligni and P. limicola may be simply varieties of P. ciliata.

In the following discussion morphology and population genetics of these species with main emphasis on P. ciliata, is analysed.

Morphology :

In polychaetous annelids the number of traits which are

used in taxonomy varies from family to family. Families such as Psammodrilidae, Lumbrineridae and Capitellidae lack definite head appendages and have poorly developed parapodia. This results in few discrete characters upon which to base taxonomic distinction. Other families such as Nereidae, Arenicolidae, Syllidae and Spionidae have numerous structures commonly used in taxonomic description. It is reasonable for the purpose of examining intraspecific variation to choose a species which shows a relatively large number of definite morphological characters. In addition to number, the reliability of a trait or groups of traits must be considered. Some characters among polychaetous annelids vary with the age or reproductive state of the individual, as in the Nereidae, where adults may undergo dramatic morphological changes at sexual maturity. Such morphological characters would not be suited for any taxonomic work, unless enough care was taken to ensure that each individual examined was in the same reproductive state. Most polychaetous annelids which produce planktonic larvae exhibit change in setae types accompanying the transition from a planktonic to a benthic existence. Juveniles and newly settled adults often possess both larvae and adult setae which may result in taxonomic confusion. Thus, morphological characters in order to be of maximum taxonomic value must be reliable and variation due to the age and reproductive state must be excluded from intraspecific comparisions.

In the present study a relatively large number of

populations from a variety of environments and substrata were analysed. A comparatively large number of morphological characters were utilized. Individual worms were also examined alive by slowing down their movement in a dilute solution of alcohol or isotonic magnesium chloride in sea water.

The scanning electron microscope has made it possible to study structure at a magnification and resolution not possible before. The magnification range of the scanning electron microscope is extraordinary wide. It ranges from 10 times to 100,000 times or more. It overlaps the stereoscopic and compound microscope at one end and the medium range TEM at the other. Although scanning electron microscopes only became available commercially towards the end of 1965, taxonomists were quick to appreciate their value in studying surface details in different organisms. By 1969 an extensive series of papers describing the application of scanning electron microscopy to systematic and related research had been published. The great depth of focus of the scanning electron microscope is very important. This combined with the method of image formation, which makes recessed areas appear dark and projecting areas cast shadows and allows the human eye to interpret and comprehend the images obtained, gives its most valuable characters. The only disadvantage is that it is unable to give an indication of internal structures that lie below the surface.

Studies of the fine structure of setae by the scanning electron microscope have revealed some interesting taxonomic characters in the Spioniidae (Foster, 1971b), Trochochaetae, Pectinariidae (Orrhage, 1971) and Capitellidae (Warren, 1976). In the present study morphological characters were examined by scanning electron microscope and also by the compound microscope to reveal internal parts of setae which lie below the surface.

The setae of polychaetous annelids are believed to have evolved as adaptations for anchorage within tubes and during burrowing (Fauchald, 1974). They probably originated as roughened thickenings of the cuticle. Later becoming associated with musculature and eventually acquiring the various characters we see today. Setae are important taxonomic characters on the basis of their durability. The particular shapes of polychaete setae have been shown to depend on the dynamic pattern of microvilli associated with the secretion of a glycoprotein matrix within the setal sac (Gustus and Cloney, 1973; O'Clair and Cloney, 1974). The authors concluded that the microvilli pattern, which changes temporally to produce the specific characters of a seta, must be genetically controlled, thereby accounting for species specific setation patterns. It is thought that the presence or absence of setae at a particular location on the body of a polychaete is probably also genetically determined, since the fate of an ectodermal cell or derivative is usually determined by that cell's genome following a specific

inductive stimulus (Balinsky, 1975). Whether or not an ectodermally derived cell becomes a primary setae secreting cell or 'chaetoblast' would depend upon two factors. First, upon its capability or competency to do so by virtue of having the genetic information capable of producing the proper secretion. Secondly, upon its receiving the proper inductive stimulus to differentiate. Most embryologists agree that the inductive processes which result in differentiation of tissues and organs are themselves a result of genetic programmes which are species specific (Markert and Ursprung, 1971). Thus the presence or absence of setae as well as their specific morphology can be interpreted as having their bases in the genetic complement of the species.

Generally three basic types of setae are found in the genus Polydora. They are capillary setae, heavy spines and hooded hooks. Capillary setae are found throughout the body in parapodia. Heavy spines are found in the fifth segment and in some species in the posterior notopodia. Hooded hooks are found in the neuropodium and they are located posterior to the fifth segment. Hooded hooks are common in tube-dwelling polychaetes. They are presumed to function as anchors to hold the worm inside its tube. The position and structure of hooded hooks are used as taxonomic characters. Among polydorids the position or the segment at which the hooded hooks first appeared is taken as a generic character (Read, 1975). Although some workers, like Foster (1971b), believe that this character is less reliable than some other features.

In the present study no intraspecific or interspecific variation was observed in the position or in the fine structure of the hooded hooks, however the number of hooded hooks per segment showed considerable differences (Figure 24). Those populations of P. ciliata which were collected from calcareous substrata had fewer hooded hooks per segment than those populations which were collected from mud. The other two mud living species, P. ligni and P. limicola also had higher numbers of hooded hooks per segment. Worms which bore in calcareous substrata have a more protected and less disturbed tube and hence need a smaller number of hooded hooks than those worms which form a tube by accumulating mud.

The arrangement and shape of the setae on the fifth enlarged segment of polydorids is generally considered as species specific. A single row of heavy spines is accompanied by a series of fine companion setae in close proximity to the spines, and one or more bundles of capillary setae above or below the row of heavy spines (Hartman, 1945, 1951; Blake, 1971). In P. ciliata two bundles of capillary setae were present which were located anterio-dorsal and posterio-ventral with respect to the heavy spines (Figure 19). The functional significance of these setae is unknown. Rasmussen (1973) reported that the majority of P. ciliata had these bundles of capillary setae. His table showed that only one out of fifteen worms lacked the anterio-dorsal and another one lacked the posterio-ventral. Kendall (1980) reported

that the majority of P. ciliata from Robin Hood's Bay had both bundles and very few individuals had one bundle. He also reported that the majority of worms from the Mersey, England did not have these bundles at all. Out of fifty, only eleven worms had a posterio-ventral bundle and thirty nine were without any bundle of capillary setae. The result of the present study agrees with Kendall's result. Table 5 shows that the majority of P. ciliata worms from Robin Hood's Bay had these setae and the reverse is true for those from Hale (the Mersey estuary).

In P. ligni, these bundles of capillary setae were not reported until 1973, when Rasmussen showed that few individuals of P. ligni had these structures. More recently Rice and Simon (1980) reported that the majority of individuals did not have these setae with the exception of one population where the majority had them. In the present study none of the P. ligni worm had these bundles of capillary setae (Table 5). This character was also found to be variable in P. limicola populations, where the majority had these setae Kendall (1980) reported that 44 out of 50 worms of P. limicola had both bundles of capillary setae and the rest had only one bundle. It is clear from the foregoing discussion that the presence or absence of anterio-dorsal or posterio-ventral bundles of setae is not constant and hence they do not constitute reliable taxonomic characters.

The functional significance of the heavy spines and

companion setae in polydorids has not been adequately explained. Blake and Evans (1973) reviewed the literature and summarized much of the evidence concerning the utility of these heavy spines in boring. They also supported Söderström's (1923) view that the heavy spines of the fifth enlarged segment may be used to secure the worm in place during periods of feeding and respiration. However, Zottoli and Carriker (1974), after prolonged observation of living specimens in artificial burrows during all phases of their activity, revealed that the heavy spines are not used in the manner suggested by Blake and Evans (1973). Zottoli and Carriker (1974) believe that burrow formation in Polydora is achieved chemically and not mechanically. They presented firm evidence from ultrastructural studies of P. websteri burrows, that the spines are not used to excavate burrows. Haigler (1969) reported that individuals of Polydora websteri are able to penetrate calcareous substrata after removal of heavy spines, thus strongly supporting the idea that heavy spines are not used in shell penetration.

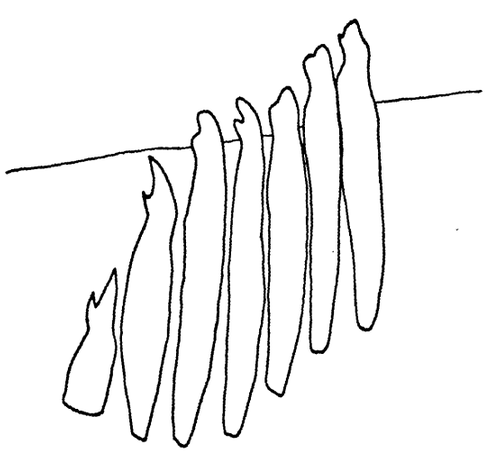
The eleven populations of Polydora species studied here comprise seven of the 'boring' form and four of the 'non-boring'. The result shows that the 'boring' form had few spines in the fifth enlarged segment while the 'non-boring' form had a large number of heavy spines (Figure 18). This could also be taken as evidence that these heavy spines are not used in boring activity otherwise the 'boring' form would have been expected to have a greater number. Zottoli

and Carriker (1974), however, suggested that the heavy spines are used to keep the diameter of the inner tube constant throughout its length in P. websteri, and they also appear to perform the same function in 'non-boring' forms. Blake and Evans (1973) hypothesized that heavy spines with bristle-like tips in Boccardia columbiana, B. berkeleyorum and Pseudopolydora reishi may remove attached organisms from the inside of the tube, but presented no evidence to support this view.

The heavy spines have a sub-distal tooth or projection. The shape of heavy spines and their projections is considered as species specific. Much emphasis have been given to this sub-distal projection in the taxonomy of Polydora and many new species have been erected on the basis of this structure such as P. aggregata (Blake, 1971) and P. websteri (Hartman, 1943). Rasmussen (1973) and Kendall (1980) suspected its reliability as taxonomic characters. They showed that the variation of sub-distal tooth was considerable. Even a single individual displayed variation in shape of heavy spines and sub-distal tooth (Figure 39). Rice and Simon (1980) reported some individuals of P. ligni having two instead of one sub-distal tooth on the heavy spines of the fifth enlarged segment.

In the present study each population had almost constant shapes of heavy spines. However, interpopulational differences in P. ciliata were observed. The 'boring' form of P. ciliata had a projection which was not an acute tooth but a flange

Figure 39. Camera lucida sketch of the heavy spines of the fifth enlarged segment in a specimen of P. ciliata from Robin Hood's Bay which demonstrate the diversity of shape of the heavy spines (from Kendall, 1980).



(Figure 22). The sub-distal projection in P. ligni (Figure 23) was a tooth and this was very similar to the'non-boring' form of P. ciliata. In P. limicola a small elevation was present on the fifth segment heavy spines.

The companion setae of the fifth enlarged segment are believed to be species specific. In P. ciliata the setae are pennoned or spatulate and in P. ligni fringed or penicillate (brush-like). Rasmussen (1973) showed that this criterion is not reliable for the separation of the two species. He observed individuals having both types of setae and could not distinguish P. ciliata from P. ligni on this basis. Kendall (1980) supported Rasmussen's view and believed that this character is highly variable. However, this view is not supported by Michaelis (1978), who in a study of the morphology and ecology of P. ciliata and P. ligni showed that the two species had different types of companion setae. In the present study all populations of P. ciliata including 'boring' and 'non-boring' forms had both pinnoned and spatulate companion setae (Figure 19 and 22) while P. ligni had fringed or penicillate setae (Figure 23). This result agrees with Michaelis (1978) and Rice and Simon (1980) who found only brush like companion setae in P. ligni.

The distribution of capillary setae on the first four segments of P. ciliata, P. ligni and P. limicola was found to be identical in all populations. Only the first notopodium lacked these setae (Figure 6). Blake (1969b) reported that

seven out of twelve species of Polydora from eastern North America characterstically had capillary setae in the first notopodium. If environmental conditions can induce setae to form in a position where they do not occur normally, then the first notopodium would seem to be a prime candidate for such induction.

Apart from setal characters, many of the important taxonomic features of spionid and other polychaetes are soft parts which may change shape, fade colour, shrink or otherwise deteriorate during preservation. However, if care is taken in the collection, relaxation and preservation of samples, most of the above problems can be eliminated. Soft parts and pigmentation pattern are important features in the taxonomy of larval and adult spionid polychaetes (Hannerz, 1956; Blake, 1969b).

Branchiae are common in spionid polychaetes and range from complex branched structures in Paraprionospio to simple strap-like notopodial derivatives in Polydora. The biological function of polychaete branchiae is well established (Sander, 1976). Foster (1971b) considered the position of the first branchia as a more reliable generic character than the position of the first hooded hooks. Out of twelve species of Polydora from east coast of North America, Blake (1971) described four as being variable in the position of the first branchia.

The functional significance of having branchial appendages is not difficult to see considering that Polydora is a tube-dwelling animal often found in low oxygen environments. The particular advantage of having the first branchia on segment six (as in P. commensalis) or on segment eight (as in P. socialis and P. normalis) rather than on segment seven is not clear. These various interspecific branchial patterns are probably the result of genetic differences which are selectively neutral.

The caruncle of polychaetes is a sensory organ, projecting posteriorly from the prostomium (Day, 1967; Fauchald, 1977). Ameyaw-Akumfi (1976) suggested that the caruncle of an amphinomid polychaete functioned mainly as a tactile receptor. Examination of the caruncle with the scanning electron microscope revealed the presence of sensory tufts or setae except in a few individuals (Figure 14). The variation observed in the length of the caruncle (Figure 13) is difficult to evaluate in terms of functional significance unless those individuals with a longer caruncle are simply better equipped to sense their environment then those with shorter caruncles. Sensory structures similar to those found on the caruncle were also observed on the palps and pygidium (Figure 10). The caruncle, therefore does not act alone as a sensory receptor.

In a classical study of polychaete nuchal organs, Rullier (1951) was able to trace nerve bundles from the ventral nerve

trunk and cerebral ganglia to the area of the caruncle. He examined three species of spionids and found considerable innervation of the nuchal organ (ciliated region alongside the caruncle) and the caruncle itself, even though he could not find any sensory receptors on the caruncle. His conclusion was that the nuchal organ served a chemosensory function. Whittle and Zahid (1974) presented ultrastructural evidence indicative of a chemoreceptive function for the nuchal organs in four families of polychaetes. It may be possible that the sensory cilia observed on the caruncle, palps and pygidium are probably also related to chemoreception.

The length of the caruncle is used as a taxonomic character (Hartman 1943, 1961; Webster, 1886; Blake, 1971). Rice and Simon (1980) showed that the length of caruncle is variable in P. ligni. Same was the case in P. ciliata, P. ligni and P. limicola studied here (Figure 13). This character should be used with caution for discriminating Polydora species.

The caruncle often bears an antenna which is also used as a species specific character in Polydora (Hartman, 1951; Woodwick, 1953; Blake, 1971). The nuchal antenna is not found in P. ciliata and P. limicola, but P. ligni shows a distinct nuchal antenna. Rice and Simon (1980) revealed that 22% of P. ligni from Ft. Desoto, Florida, United States, lacked it. In the present study 15% of P. ligni worms lacked this antenna. Polydora ciliata and P. limicola did not have any nuchal antenna except the Whitstable population where

7.7% of the worms had this antenna. Rice and Simon (1980) also reported a few individuals with two nuchal antennae from Fort Pierce and Fort Desoto. The presence of two nuchal antennae is a characterstic feature of P. maculata Day (1967). In the light of these results this character should also be used with caution.

The number of eye spots usually reported for P. ciliata is 0 to 4 (Johnston 1838; Söderström, 1920; Fauvel, 1927; Rasmussen, 1973). In the present study the number of eye spots observed for P. ciliata also varies from 0 to 4. Very few individuals had one eye spot and six out of eleven populations did not have any individual with one eye spot (Figure 12). The specificfactors responsible for the loss or maintenance of the eye spot in the adults are unknown. Dean (1969) found that several species of Polydora failed to form eyes after decapitation and subsequent anterior regeneration.

The number of eye spots for P. limicola is the same as for P. ciliata (Hartman, 1961) but for P. ligni it is reported as 4 (Webster, 1886; Hartman, 1969; Blake, 1971) but variation in the number of eye spots in P. ligni was reported by Rasmussen (1973) who showed that the number varies from 2 to 4. More recently Rice and Simon (1980) reported that the number of eye spots varies from 0 to 6 in P. ligni. In the present study P. ligni had either no eye spot or 2 to 4 eye spots (Figure 12). The present result agrees with Rasmussen (1973) and Rice and Simon (1980) and

in the light of these results it is obvious that the number of eye spots does not constitute a reliable taxonomic character.

The presence of pigment in the anterior region in adult P. ciliata and P. ligni has not been reported. Kendall (1980) however showed that out of 25 individuals of P. ciliata from Robin Hood's Bay, Yorkshire, England, 21 had dark pigment. He also found pigmented individuals of P. ciliata from the Mersey estuary, England. The planktonic larvae of P. ciliata have pigments on most segments (Hannerz, 1956). This pigmentation is lost soon after the larvae settle from the plankton. In this study P. ciliata and P. ligni did not show any pigmentation (Figure 11) either in the anterior, or in the posterior region. Even the population of P. ciliata collected from Robin Hood's Bay had no pigment. The only possibility which could explain this disagreement between Kendall's results and my own / is that the P. ciliata for the present study were collected from Patella shells while Kendall collected P. ciliata from rock crevices where they make tubes of sand and mud and do not bore in the rock. If this is the case, a study of that population from Robin Hood's Bay which do not bore, using scanning electron microscopy and enzyme electrophoresis, would be valuable.

An attempt was made to obtain some individuals of P. ciliata which live in crevices. The worms were not abundant and only eight worms were collected after considerable effort and time. Out of eight worms five turned out to be

P. flava and only three were P. ciliata. However these were not pigmented.

Hartman-Schröder (1971) considered the possession of dark pigmentation as species specific character in separating P. ciliata (Johnston) and P. limicola (Annenkova). In the present study all P. limicola individuals had dark pigments (Figure 11), the pigments generally being present on the peristomium and in the first four segments. Kendall (1980) reported that 90% individuals of P. limicola from the Tees estuary had pigments in the anterior segments. However, results in the present study show that P. limicola from the Tees estuary had 100% worms with dark pigments in the anterior region. These pigments do not fade when preserved, either in alcohol or in formalin (at least, after six months, as observed during the present study).

The size and shape of the pygidium (or terminal 'segment') is also used as a taxonomic feature in Polydora. P. ciliata , P. ligni and P. limicola have a disc shaped pygidium (Figure 10). They showed slight variation in the shape of the disc. It also varies within species depending upon age and the stage of regeneration in incomplete worms. For these reasons, size and shape of the pygidium was not found to be a useful feature for interpopulation comparision.

Population genetics :

The electrophoretic technique has made it possible to study variation at a very basic level, that of gene expression. The genetics of allozyme variation was first described for the esterase-6 locus in Drosophila melanogaster (Wright, 1963). The esterase zymogram showed many bands that occupy different positions. However, these bands were grouped into clusters. The cluster designated esterase-6 was occupied by a fast moving band, and a slow moving band or by both. Wright designated these as F/F, S/S and F/S in genotype. He verified the correctness of this genotype designation by making a number of single pair matings in which the two parents and progeny were tested by gel electrophoresis. No electrophoretic patterns of esterase-6 locus were found contrary to expectation based on Wright's classification. Each mating type produced only those types of individuals expected under Mendelian inheritance, and these appeared in the correct proportion.

Subsequent to Wright's pioneer analysis, his findings have been confirmed by numerous other population geneticists. It is assumed today that the enzyme variation observed by electrophoretic methods is genetic in origin and that it is represented at individual gene loci. The organisms which have been most extensively studied for electrophoretic enzyme variants are the house mouse (Mus musculus),the fruit fly (Drosophila spp.), the deer mouse (Peromyscus) and man (Shaw, 1965).

In the case of marine invertebrates, especially in polychaetous annelids, very few works have been published. In many cases the electrophoretic study of isozymes led to the discovery of sibling species. Manwell and Baker (1963a) reported for the first time a sibling species of sea-cucumber (see page 66 and 67). Selander et al.(1971) determined gene frequencies encoded by eight genetic loci for populations of fiddler crabs from Cape Cod to Florida. They found an undescribed sympatric species previously considered a colour morph of Uca pugilator Bosc. They found that allele composition at PGI-1, EST-1 and EST-2 loci were different in two sibling species. In addition these authors were able to confirm the existence of two species of U. pugnax (Smith) from the Gulf coast which had been previously suspected of being separate species on behavioural grounds. The Gulf coast populations of U. pugnax were also found to differ biochemically from the same morphological species from the Atlantic coast.

Murphy (1978) described a new species of limpet, Collisella austrodigitalis, from California, based on gene frequency differences between northern and southern populations. The two groups had different allelic frquencies at a leucine aminopeptidase locus and that individuals can be correctly assigned to their geographic entity with a 98.8% probability on the basis of their genotype. The groups also had slight differences in allelic frequencies at a phosphoglucose isomerase locus. An intermediate geographical

location was found having both species, C. austrodigitalis (southern) and C. digitalis (northern), occuring sympatrically. The genotype frequency analysis revealed that the two species were not interbreeding. After determining the species electrophoretically, examination of shell morphology from sites where these species occured together revealed some subtle interspecific differences.

In Goniobasis floridensis, a gastropod from Florida, considerable genetic differences were observed by Chambers (1978). The genetic identity calculated between these two different groups was 0.468 for eighteen loci. This value is in the range of values found when comparing different non-sibling species of Drosophila (Ayala et al., 1974), although specific identification of these gastropod species was not always possible based solely on morphological characters. However, they showed habitat preferences. The G. floridensis was found on vegetation while the reference species was found on rock.

Dando and Southward (1980) described a new species of barnacle Chthamalus proteus based on electrophoretic results of enzymes. This new species differs from the related species C. fragilis in three out of sixteen enzymes. It has only been possible to devise morphological criteria to distinguish these two species after determining them electrophoretically. Although the two species slightly overlap in morphological characters, electrophoretic results showed no evidence of hybridization.

In an electrophoretic survey ofenzymes in mud crabs from south Carolina, Turnerand Lyerla (1980) found that the two morphological varieties or 'forms' of Panopeus herbstii, namely 'simpsoni' and 'obesa' were different at two loci. Using Nei's index to estimate genetic identity, however, the two forms turned out to be genetically identical ($\bar{I}$ = 0.997). This was due to large numbers of monomorphic loci at which the two forms shared electromorphs. Twenty out of twenty three loci were monomorphic (86.9%). On an individual gene basis, the two forms had different esterase zymogram patterns and at the amylase locus two alleles were found in 'obesa' but not in 'simpsoni'. They concluded that the esterase and amylase variation indicate a greater genetic difference between these two populations or morphological 'forms' than that implied by their designation as 'form' variants.

Within polychaetes, at least three cases of sibling species are known which are based upon or supported by electrophoretic evidence. George (1967) observed an unusal population of Cirriformia tentaculata (Montagu) at Plymouth, Devon, England, which utilized a different reproductive strategy from other nearby populations. Electrophorograms of esterase obtained using starch gel electrophoresis revealed considerable polymorphisms between individuals of Plymouth populations. This variation in esterase pattern was taken as evidence for populational polymorphism in genetic structure which would support a possible genetic basis for reproductive

differences. This species is found throughout Europe and the United States and would be a good subject for studies of geographical divergence.

Capitella capitata is another example of sibling species in polychaetes identified by electrophoresis (Grassle and Grassle, 1974, 1976, 1977). Following an oil spill and the resultant defaunation the composition of the recolonizing community was traced. Capitella was the most opportunistic species to arrive at the scene. Study of Capitella populations from several sites over the next few years revealed the presence of at least six sibling species. These six sibling species could be identified on the basis of their life histories, genotypes and to a lesser extent, morphology. From electrophoretic results it was observed that out of eight loci, no more than two alleles were common between any pair of species. They made extensive attempts to crossbreed pairs of males and females between species but were unsuccesful. In some cases as many as five sibling species were found inhabiting the same locality. However, the different species showed wide fluctuation in temporal abundance. Even though all of the sibling species of Capitella capitata could be considered opportunistic, some were more opportunistic than others. It was concluded that these siblings represent temporal adaptations based on the dispersal capability of the larval stages.

Rice and Simon (1980) analysed five populations of

Polydora ligni Webster from the United States. The electrophoretic study of ten loci revealed some differences between populations. Four out of five populations did not show considerable genetic differences but the fifth population was significantly different from the rest. It is interesting to note that the fifth population came from a different habitat. This population lived on bivalve shells but it is not clear whether it really bores into the shells or not. The genetic distance and genetic identity for the fifth population varied from all others at a level corresponding to sibling species.

In the present study a total of thirteen loci were studied. In P. ciliata 'boring' form, twelve out of thirteen loci were monomorphic (92.3%) and only one was polymorphic (7.7%). In P. ciliata 'non-boring' form, P. ligni and P. limicola nine out of thirteen loci were monomorphic (69.2%) and four were polymorphic (30.8%).

On the basis of individual loci, ACP-3, ACP-4 and PGI were found completely different in the two forms of P. ciliata and they had no alleles in common at these three loci which indicates a greater genetic difference. The ACP-3 locus was monomorphic for the 'non-boring' form. This ACP-3 locus was never observed in the 'boring' form. The reverse was true for the ACP-4 locus. This ACP-4 locus was consistently resolved in the 'boring' form but never found in the 'non-boring' form of P. ciliata. The acid phosphatase is a lysosomal

enzyme and Doering and Palincsar (1978) found that there was a relationship between changes in acid phosphatase activity and the life cycle stages of the nematode , Panagrellus silusiac. Apart from differences between larvae and adult nematodes, they showed that a twenty days-old adult nematode had six acid phosphatase bands when separated electrophoretically on polyacrylamide gel while a twenty-five-days old adult nematode had seven bands of acid phosphatase. The bands varied in colour, they were either red, faint red or yellow. Further tests showed that each coloured band was truly acid phosphatase. In the light of their findings it would be premature to describe acid phosphatase bands (ACP-3 and ACP-4) as 'diagnostic' for the particular forms of P. ciliata. A study of seasonal variation of acid phosphatase in these populations by electrophoresis would be valuable. Furthermore, this enzyme system has not been found 'diagnostic' in any case of sibling species in marine invertebrates.

In the PGI locus two alleles were observed for 'non-boring' form and a single band was found in 'boring' form of P. ciliata which was different in mobility from the two alleles. This lack of common alleles between the two forms may mean that these could be considered as two genetically distinct populations. The phosphoglucose isomerase (PGI) locus has been studied in many organisms including marine and fresh water invertebrates. This enzyme system was regarded as 'diagnostic' between the two species of gastropod,

Bulinus tropicus and B. permembranaceus (Jelnes, 1977). It was revealed consistently in more than.200 individuals. This enzyme system was also studied by Murphy (1978) who described sibling species of Goniobasis and by Chambers (1978) who described sibling species of Collisella. These authors were able to resolve the PGI locus consistently like many others. In the present study, this PGI locus may be considered as 'diagnostic' and the two forms of P. ciliata can be assigned without any hesitation on the basis of their genotype.

On an overall basis, the mean genetic identity data which is presented in Table 15, shows that the two forms of P. ciliata has a $\bar{I} = 0.700 \pm 0.009$ (S.E.). This figure does not come in the range of sibling species as described for Drosophila by Ayala et al. (1974). The genetic identity or distance estimates are not available for six sibling species of Capitella capitata but Rice and Simon (1980) gave this estimate for P. ligni. The sibling species of P. ligni had a mean genetic identity ($\bar{I}$) 0.597. This estimate comes under the range of sibling species as described by Ayala et al. (1974) for Drosophila (see Table 17).

The mean genetic identity in the present study shows that the P. ciliata 'non-boring' form, P. ligni and P. limicola come under the range of local populations while the P. ciliata 'boring' form comes under the range of sub or semi-species.

Table 17. Average genetic similarity, $\bar{I}$ and genetic distance, $\bar{D}$ between taxa of various levels of evolutionary divergence in the Drosophila willistoni group (from Ayala et al., 1974).

Taxonomic level	$\bar{I}$	$\bar{D}$
Local populations	0.970 ± 0.006	0.031 ± 0.007
Sub species	0.795 ± 0,013	0.230 ± 0.016
Semi species	0.798 ± 0.026	0.226 ± 0.033
Sibling species	0.536 ± 0.023	0.581 ± 0.039
Non-sibling species	0.352 ± 0.023	1.056 ± 0.068

The existence of genetic differences between the two forms of P. ciliata reported here are not the result of sampling error. The two independent samples of P. ciliata were collected from Whitstable ('non -boring' form) six months apart (Table 3) and both resulted in similar gene frequencies. Another population from Dumpton ('boring' form) was sampled twice, thirteen months apart (Table 3) and both samples gave similar results. Possible explanations for the existence of such genetic differences could be due to environmental differences, habitat or reproductive isolation.

The two forms live in different environmental conditions. The 'boring' form lives under pure 'marine' conditions while 'non-boring' form lives under less 'marine' or under estuarine conditions. It may well be possible that low oxygen and salinity may have resulted in considerable selection pressure for certain genotypes in estuarine populations. It would be interesting to raise larvae from a stock laboratory culture under different salinity, temperature and oxygen conditions and to determine the magnitude and direction of the selective forces.

The habitat may have been related to the observed genotypic differences. The two forms live on different substrata and also at different tidal levels in the intertidal zone. It is well known that Polydora exhibit habitat selection and their larvae search for a suitable substratum on which to settle.

They prefer to settle where the adults of the same species are present (Wilson, 1928; Dorsett, 1961; Blake, 1969b). Graham and Gay (1945) also reported some spionods for their preference to settle on hard substrata. Makay and Doyle (1978) found interpopulational differences in the affinity of spirorbid polychaetes for various substrate depending upon the environmental conditions. P. ligni also shows interpopulational differences in gene frequencies with respect to their habitat (Rice and Simon, 1980).

The lack of a common allele between closely related species is taken as an evidence of reproductive isolation (Grassle and Grassle, 1976; Dando and Southward, 1980) and the genetic difference observed in P. ciliata could be explained as due to reproductive isolation.

The model of speciation recognizes two stages in the formation of species. In the first stage, allopatric populations become genetically sufficiently different for natural selection to favour the development of reproductive isolation between the populations if they were to come in contact. The second stage occurs after sympatry when reproductive isolation is being completed. The two forms of P. ciliata may be in the first stage of speciation.

The question raised at the begining was whether P. ciliata, which lives on a variety of substrata, is a single homogeneous species or not. The answer depends upon the relative weight

placed upon each portion of the foregoing evidence. A museum taxonomist, working with a few preserved individuals might conclude that P. ciliata is a single species showing minor variation in some of its taxonomic characters. Some taxonomists working with large samples taken from different populations might go further and synonomize P. ciliata with P. ligni and P. limicola, as did Rasmussen (1973) and Kendall (1980). An ecologist studying ecology and morphology of two populations which occur at different habitats might conclude that P. ciliata and P. ligni are two distinct species as Michaelis (1978) did. A reproductive or population biologist, considering the variation in breeding period and fecundity might split a single population of P. ciliata into two groups as Dorsett (1961) did.

The generally accepted definition of a biological species is that actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr et al., 1953). This view of a species sometimes poses practical difficulties especially in those cases where speciation is in progress and this is what appears to be the case in polychaetes (Clark, 1977).

Looking at the population genetics and the foregoing morphological evidence the conclusion would probably be reached that the P. ciliata 'non-boring' form and 'boring' form are genetically different populations and that the 'non-boring' form should be referred to as P. ligni. The

P. limicola, is genetically very similar to *P. ligni* and not to *P. ciliata* 'boring' form.

It is, however, desirable that more loci should be studied so that it could be ascertained whether the large number of monomorphic loci found during the present study has not given a false impression.

SECTION II

INTRODUCTION

'Polychaete taxonomy is still largely at the descriptive "alpha" stage' (Clark, 1977), although they are abundant in the marine environment. Polychaetes are generally sluggish in nature and not very mobile. Many species of polychaetes are cosmopolitan and have a wide geographical range. It is, therefore, not surprising if minor differences in morphology and other traits exist between members of the same species living in different geographical areas. If these minor differences do not exist in those characters which are considered taxonomically important (like setae, paragnaths) then they may easily be overlooked by taxonomists and this also applies to sympatric species.

The use of characters, not previously considered taxonomically important, has led to the separation of many species. Healy and Wells (1959) were able to separate Abarenicola pacifica and A. vagabunda from A. claparedii on the basis of breeding biology. Gibson (1978) studied eleven species of the genus Dodecaceria. He found that the reproductive biology, especially the capacity for asexual reproduction, as well as morphological characters had to be considered in order to separate these species. Three species in the genus Platynereis were found to be reproductively isolated although they were morphologically similar (Just, 1914, 1915, 1929). It is evident that it has only been possible to separate species, which are morphologically similar, by studying them

in detail and where such studies have not been carried out, there is taxonomic confusion. Since the concept of a species turns upon reproductive isolation, variation in reproductive biology or in genetic materials are considered more important than any other factors. As mentioned elsewhere the high resolving power and ease of application of electrophoretic methods to species identification offers a potentially valuable tool. However, this is largely an unexploited tool in polychaete taxonomy.

It was thought some preliminary observations of isozymes by electrophoresis in some other polychaetes would be helpful and might provide an incentive for future work. Arenicola marina (Linnaeus) shows striking differences in the time of spawning in British waters (Duncan, 1960). Duncan concluded that environmental factors are not necessarily responsible for such differences but these could be due to genetic differentiation and this is not surprising for the comparatively sedentary habits of Arenicola species living in a discontinuously distributed habitat. Small samples of Arenicola marina were obtained and analysed electrophoretically in the present study and since no work has been published on Arenicola isozymes to date, results are presented in this section.

Nereis diversicolor Müller is another example of complex species in Polychaeta. N. limnicola and N. japonica are morphologically identical with N. diversicolor except for the arrangement of the paragnaths, a character which has

proved to be somewhat unreliable (Smith, 1958) and variation in paragnath numbers in relation to sediment type has been reported by Barnes (1978). However, the reproductive isolation of the three species is total on behavioural grounds (Clark, 1977). No attempt has been made so far to study the nature of variation in genetic materials by using modern techniques of these worms. During the present study it was felt that some preliminary observations ought to be made and electrophorograms of some enzymes of Nereis diversicolor and other species are presented.

MATERIALS AND METHODS

Arenicola marina :

Small samples of A. marina were obtained from six different localities in England, Wales and Scotland. (Figure 40). All samples were collected in summer, 1980 except from the Plymouth locality, which was collected in winter, 1980. Live worms were kept in a tank with sand and sea water inan aquarium at 10 - 15°C. These tanks were provided with circulated artificial sea water and with aeration. Worms were used for preparing samples within two weeks of collection. Live worms obtained from Millport were frozen in liquid nitrogen and stored in a deep freeze (-20°C).

Each worm, with the gut cleared,was relaxed by adding magnesium chloride crystals (to 7%) and then dissected in ice-cold sea water. The body wall was cut open and the coelomic cavity was washed thoroughly with ice-cold sea water to remove any developing gametes. Worms were blotted dry and weighed. An equal amount of homogenisation mixture was added and worm was then homogenised in a tissue grinder, immersed in ice-cold water. The homogenisation mixture was the same as described for Polydora species earlier. The resulting homogenate was spun in a refrigerated centrifuge at 0 - 4°C for 90 minutes at 28,000 g. The clear supernatant was carefully removed and aliquots were stored at -20°C until required.

The Millport worms were defrozen and dissected in ice-cold sea water to remove coelomic fluid and gametes if any. They were then homogenised and spun as described above.

The electrophoretic procedure was the same as for Polydora species. Samples of 5 - 10 µl were applied to the gel and after electrophoresis gels were stained for the following enzymes using the staining methods given in Appendix II.

1. Acid phosphatase (ACP)
2. Leucine aminopeptidase (LAP)
3. Phosphoglucose isomerase (PGI)
4. Tetrazolium oxidase (TO)
5. Esterase (EST)
6. Malate dehydrogenase (MDH)

Nereis species :

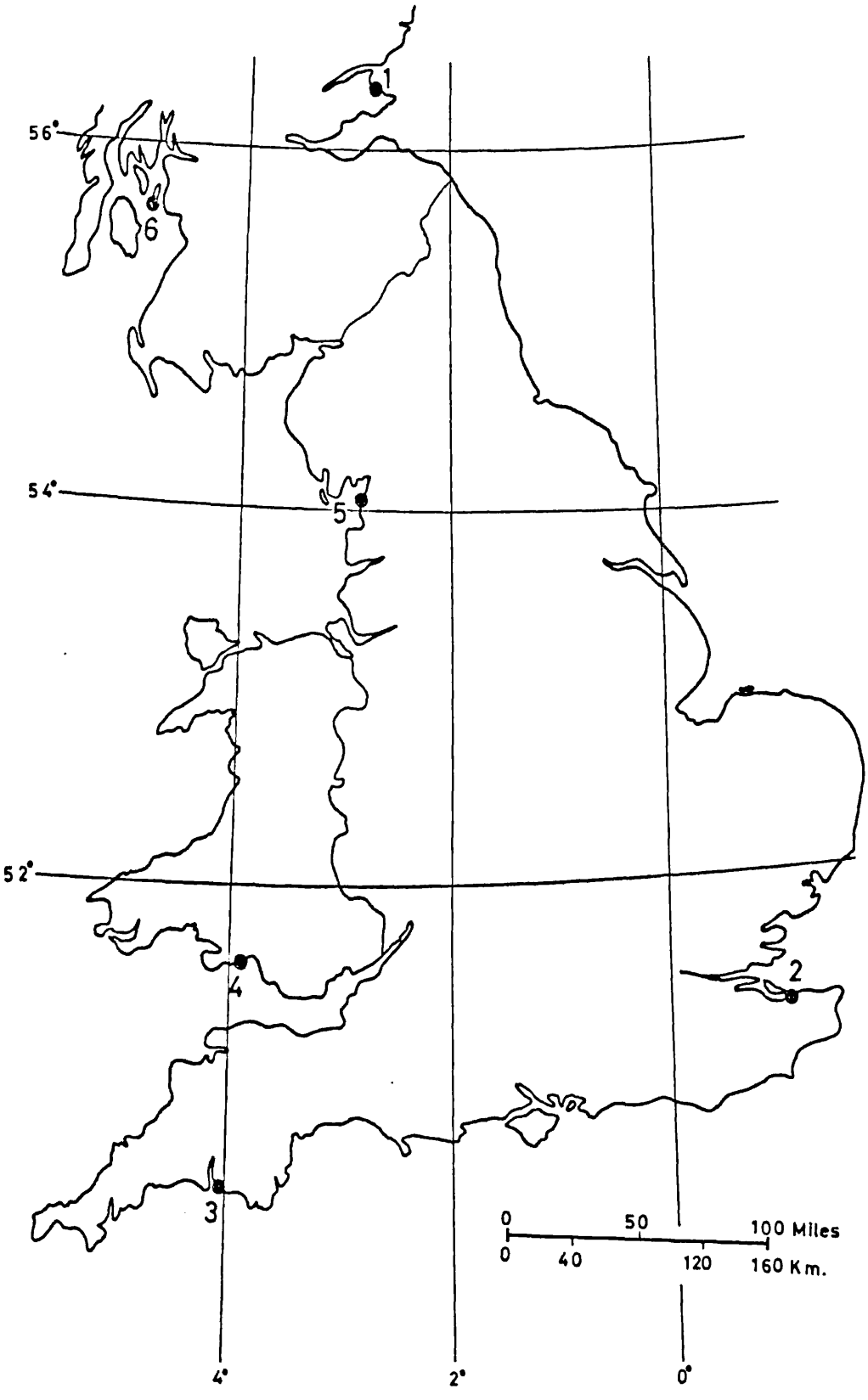
Three different species of Nereis were obtained. N. diversicolor was obtained from Swansea, Plymouth, Whitstable and Blyth. The Blyth sample was divided into two age groups, those which were approximately younger than a year and those older than a year. All samples were collected in summer, 1980 except that from Plymouth, where collection was made in winter.

N. fucata from shells housing hermit crabs (Eupagurus sp.), were obtained from Millport in summer, 1980 and another sample

Figure 40. Map showing locations of Arenicola marina populations sampled.

1. St. Andrews, 2. Whitstable, 3. Plymouth,

4. Swansea, 5. Lancaster and 6. Millport.


56°
54°
52°
4°
2°
0°
1
2
3
4
5
6
0
50
100 Miles
0
40
120
160 Km.

was obtained from Plymouth, offshore, in Febraury, 1980. N. virens was collected from Whitstable and Blyth in May and June, 1980 respectively.

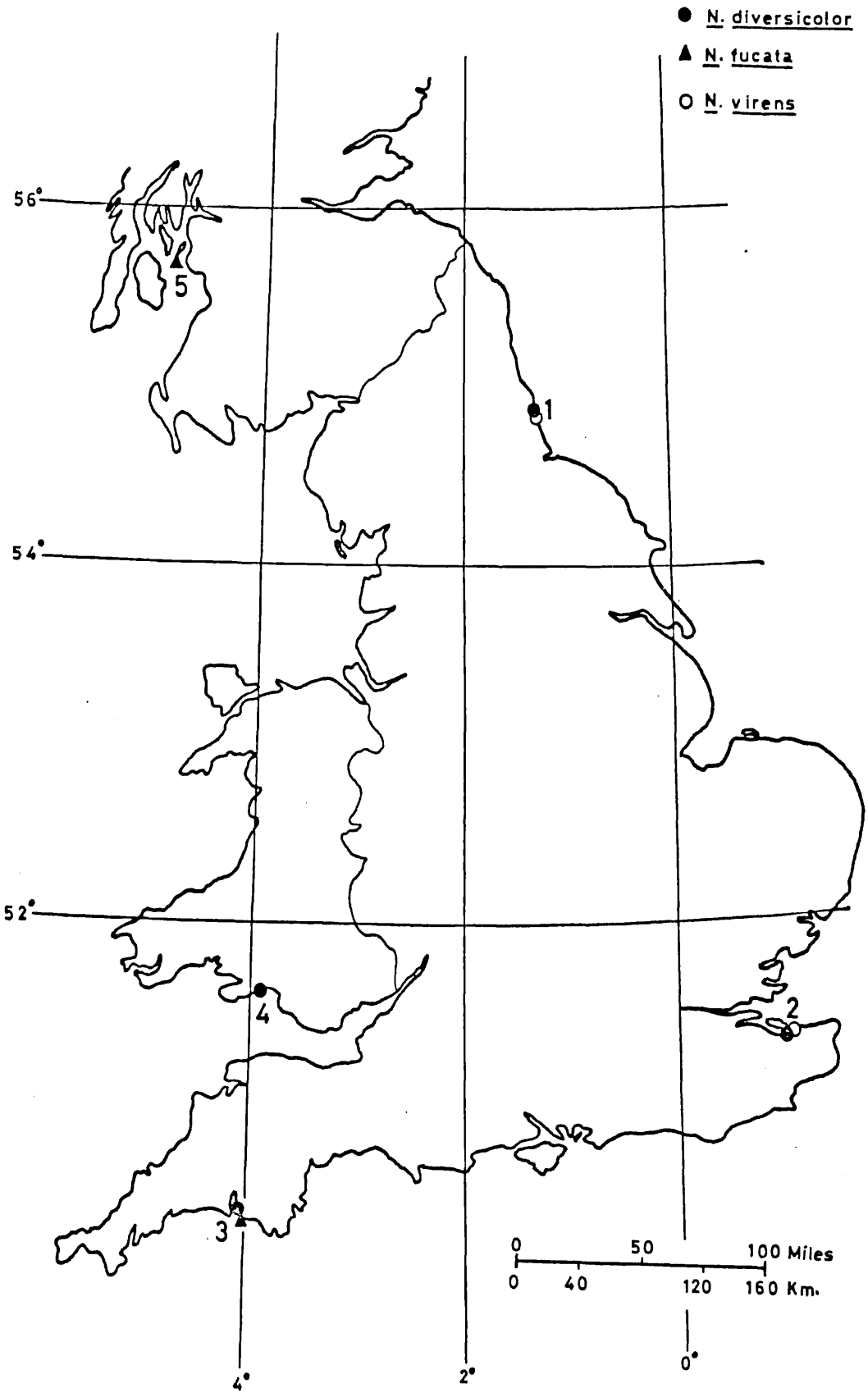
Live worms were kept in the aquarium at 10 - 15°C. N. virens and N. diversicolor do not survive well in circulated sea water and they were either kept in little sea water or over wet filter paper. Shells housing hermit crabs were kept in a tank and provided with circulated sea water and aeration. Shells were cracked open to get the worms out.

Samples for electrophoresis were made in the same way as described for Arenicola marina and were stored at -20°C until needed. The electrophoretic procedure was the same as for Polydora species. Samples of 10 - 15 µl were applied to the gel, and after electrophoresis they were stained for:

1. Esterase (EST)
2. Glucose-6-phosphate dehydrogenase (G6PD)
3. Leucine aminopeptidase (LAP)
4. Lactate dehydrogenase (LDH)
5. Malate dehydrogenase (MDH)
6. Phosphoglucose isomerase (PGI)

Figure 41. Map showing locations of *Nereis diversicolor*, *N. fucata*, and *N. virens* populations sampled.

1. Blyth, 2. Whitstable, 3. Plymouth, 4. Swansea and 5. Millport.


● N. diversicolor
▲ N. fucata
○ N. virens
56°
54°
52°
5
1
4
2
3
0 50 100 Miles
0 40 120 160 Km.
4°
2°
0°

RESULTS

The electrophoretic results presented hereunder, are based on small samples and therefore, no attempt has been made to calculate allele frequencies or genetic identity or distance. The electrophoretic results of Arenicola marina and Nereis species are presented separately.

Arenicola marina :

The six enzyme systems which were studied are presented in Figures 42 to 47. The phosphoglucose isomerase stained as a single band in all populations (Figure 42) and no difference was observed. The average mobility of the PGI band was 90 ± 0.4 (S.D.) and the sample size was 30 from six populations. The malate dehydrogenase was also found to have a single band on the zymogram which usually stained very dark. The average mobility of this band for 30 worms was 100 ± 0.8 (S.D.). Figure 46 is a photograph of a malate dehydrogenase zymogram. The acid phosphatase zymogram showed 3 bands (Figure 44). The two fast moving bands stained lightly and had mean mobilities 112 ± 0.6 (S.D.) and 108 ± 0.4 (S.D.) respectively. The slow moving band which stained darkly had a mean mobility 94 ± 0.3 (S.D.). A total of 35 worms were screened for this enzyme system from six different populations.

The leucine aminopeptidase zymogram showed great variability (Figures 43 and 45). Three worms from St. Andrews

had three bands, LAP^{120}, LAP^{112} and LAP^{105} while the other two worms had only two bands and lacked LAP^{120}. In the Whitstable, Plymouth and Millport populations only LAP^{120} was found (n = 18). The worms from Swansea showed slight variation in the mobility of the single band. This band was not very sharp and due to diffusion it was not possible to measure the mobility precisely. The average mobility for 5 worms was 108 $\pm$ 0.7 (S.D.). The Lancaster population showed two bands LAP^{120} and LAP^{117}, (n = 4).

The tetrazolium oxidase zymograms showed three different types of patterns (Figure 42). Type 1 was found in the Whitstable population where three bands were observed. The two slow moving bands TO^{92} and TO^{103} were resolved in all six worms from Whitstable. The third fast moving band had either a mobility 119 or 116. Out of six worms three had TO^{119} and the other three had TO^{116}. Type 2 was found in Millport, Swansea, Plymouth and St. Andrews in which case only a single band TO^{103} was observed. A third type was found in the Lancaster population. In this, 4 out of 5 had a double banded pattern, TO^{92} and TO^{103}. The fifth worm had a single band TO^{103}.

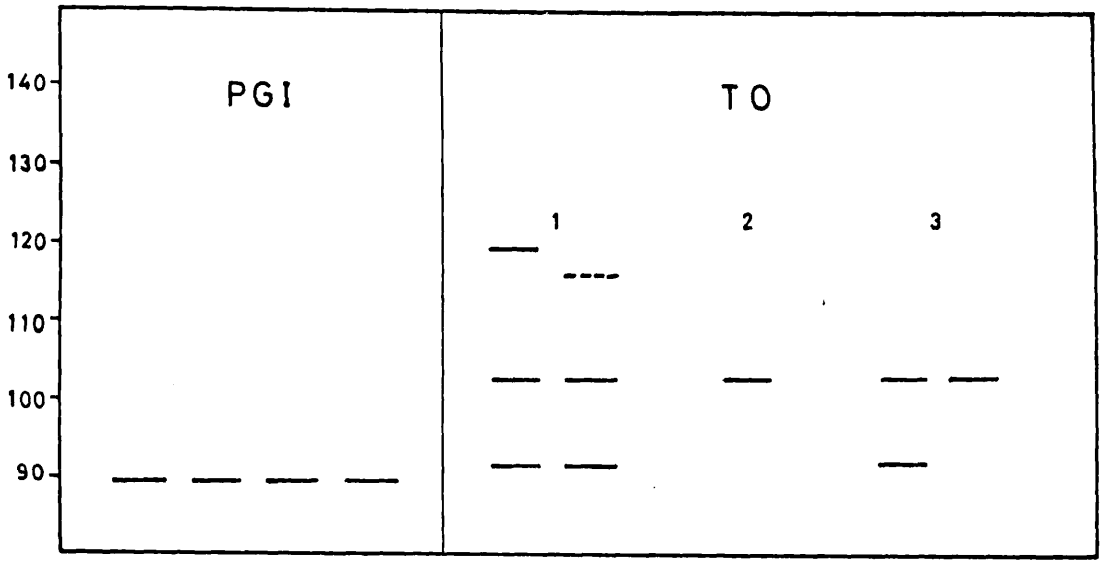
The esterase zymograms showed two zones of activity (Figure 47). The fast moving band had a mean mobility of 130 $\pm$ 0.9 (S.D.). In some individuals this band stained very dark and was thick, while in others it was more lightly stained. The slow moving band showed slight variation in

Figure 42. Diagram of phosphoglucose isomerase (PGI) and tetrazolium oxidase (TO) zymograms of different populations of A. marina.

1. Whitstable, 2. Plymouth, Millport and St. Andrews, 3. Lancaster.

Figure 43. Diagram of leucine aminopeptidase zymogram of different populations of A. marina .

1. St. Andrews, 2. Plymouth, 3. Whitstable, 4. Swansea, 5. Millport and 6. Lancaster.


PGI
TO
140
130
120
110
100
90
1
2
3

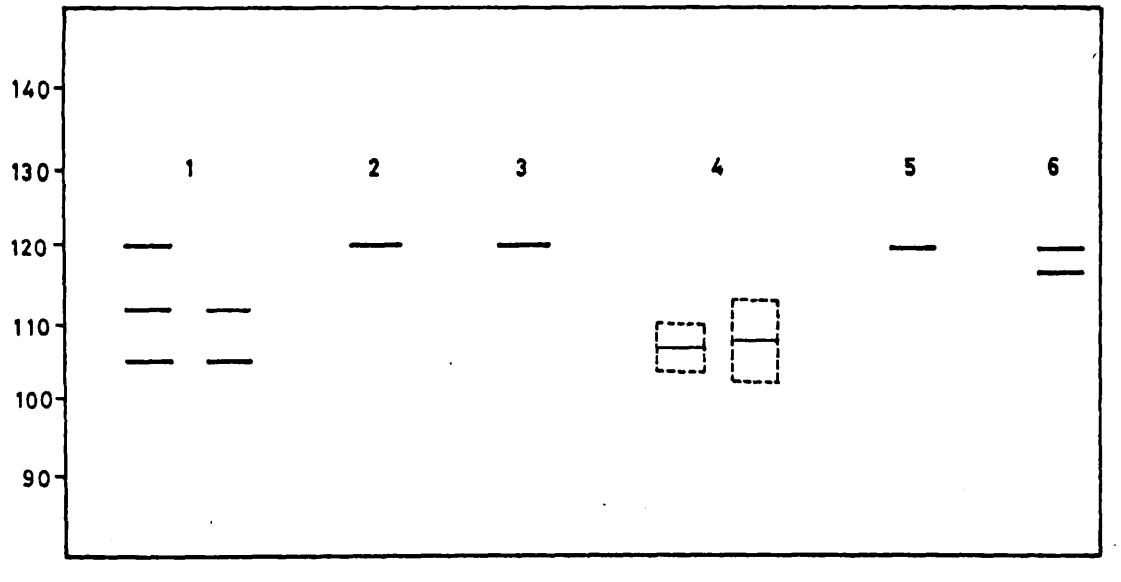
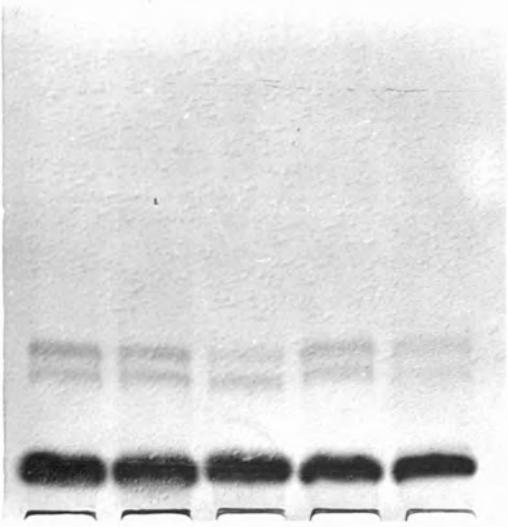

140
130
120
110
100
90
1
2
3
4
5
6

Figure 44. Photograph of acid phosphatase zymogram of different populations of A. marina.

Figure 45. Photograph of leucine aminopeptidase zymogram of different populations of A. marina.

1. St. Andrews, 2. Whitstable, 3. Swansea and 4. Millport.



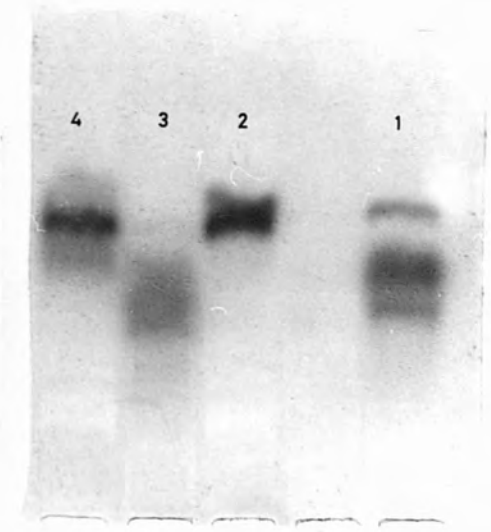
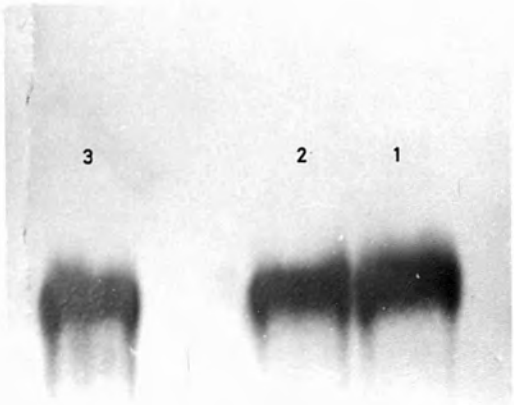

4
3
2
1

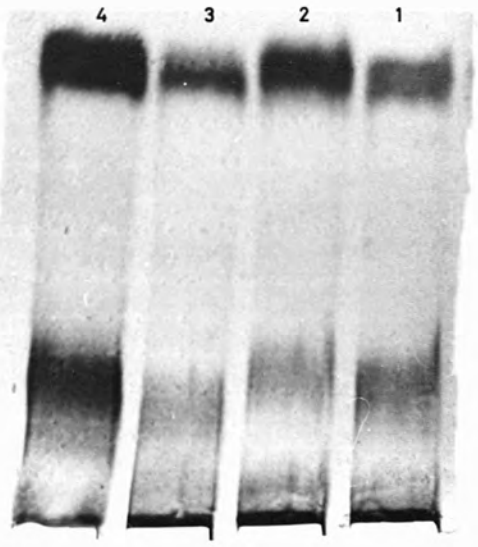
Figure 46. Photograph of malate dehydrogenase zymogram of different populations of A. marina.

1. Plymouth, 2. Whitstable and 3. Lancaster.

Figure 47. Photograph of esterase zymogram of different populations of A. marina.

1. Whitstable, 2. Plymouth, 3. Millport and 4. Swansea.


3
2
1


4
3
2
1

mobility. The majority had a mean mobility of 92 ± 0.7 (S.D.), but a few worms showed a mean mobility 94 ± 0.5 (S.D.).

Nereis species :

Figure 48 diagrams the esterase zymogram of different species of Nereis. In N. diversicolor the fast moving or anodal zone had three different bands located close to each other but sometimes with two or even only one band. This zone is referred as EST-1 and the alleles are EST-1^{138}, EST-1^{135} and EST-1^{132}. In four worms from Plymouth and Swansea these three alleles were found which were located equidistantly. The homozygote for EST-1^{138} was also observed from the same populations. The homozygote EST-1^{132} was not quite clear because it was always resolved with EST-1^{135}, which creates confusion about its designation as a single dimeric locus. But the homozygote for EST-1^{132} was observed in the Blyth population. It seems likely that this locus is polymorphic and dimeric, which produces the three banded heterozygote (Figure 25 b). The other slow moving bands in N. diversicolor were difficult to interpret, but it is obvious that they were not monomorphic and a large sample could reveal the pattern which would fit a genetic system. The two age groups from the Blyth population do not show any striking differences with respect to this enzyme system. In fact, they do not show any difference in any other enzyme systems discussed hereafter.

The esterase zymogram of N. fucata showed only one zone of activity which is anodal and appears polymorphic. In N. virens two distinct zones were observed and both appear polymorphic. The three different species had some bands in common but the overall pattern of the zymograms was different for the three species.

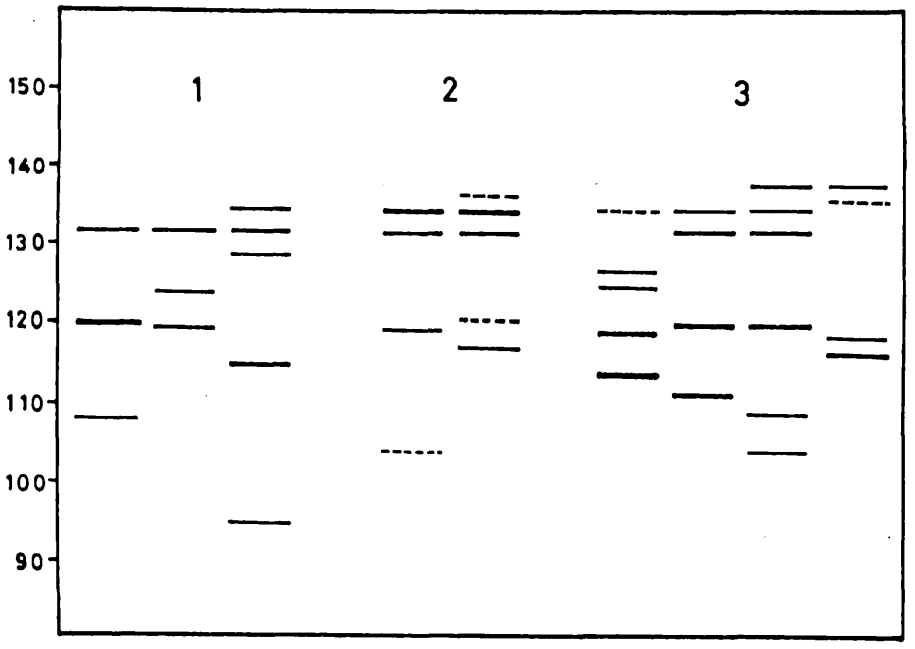
The glucose-6-phosphate dehydrogenase, leucine amino-peptidase and lactate dehydrogenase stained as a single band in all the populations of the three species of Nereis. Their average mobilities were 91 ± 0.3 (S.D.), 98 ± 0.4 (S.D.) and 96 ± 0.3 (S.D.) respectively.

Figure 50 shows interspecific variation found in malate dehydrogenase. The N. fucata had different mobilities from N. diversicolor and N. virens. Two types of zymogram were observed in N. fucata; one had a double-banded pattern $MDH^{91/101}$ and the other type had only one band, MDH^{91}. Only seven N. fucata were screened and in the absence of a homozygote for the fast moving allele it could not be described with certainty as polymorphic.

The phosphoglucose isomerase also exhibited interspecific variation. N. diversicolor and N. virens had identical bands with a mean mobility of 90 ± 0.04 (S.D.) while N. fucata had a band with a mean mobility of 95 ± 0.3 (S.D.). The phosphoglucose isomerase is probably monomorphic in the three species. No interpopulation difference was found.

Figure 48. Diagram of esterase zymogram of *Nereis* species .

1. *N. diversicolor* from Blyth (2nd year age-group)
2. *N. diversicolor* from Blyth (1st year age-group)
3. *N. diversicolor* from Plymouth
4. *N. fucata* from Millport
5. *N. virens* from Whitstable


150
140
130
120
110
100
90
1
2
3

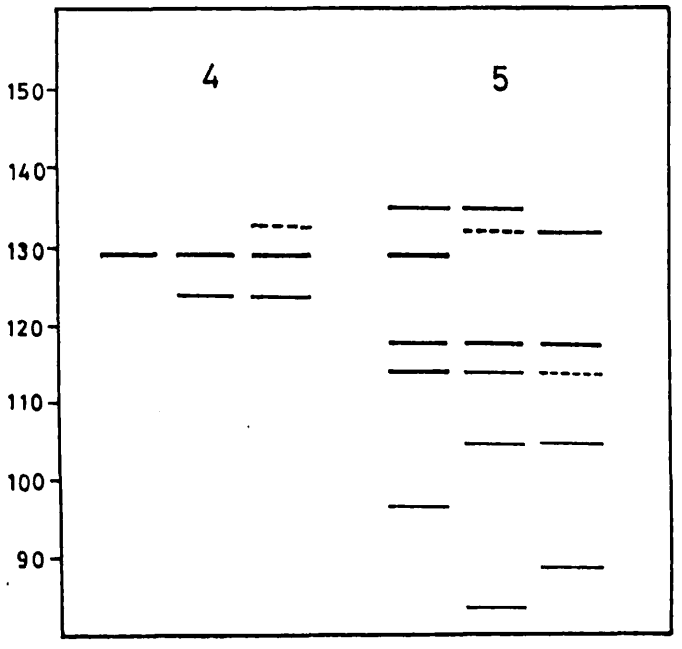
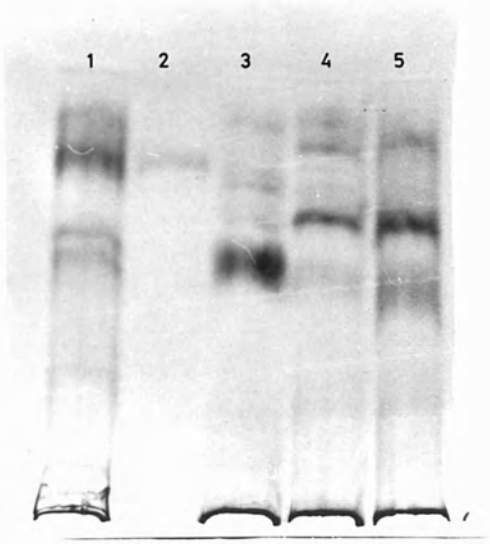

150
140
130
120
110
100
90
4
5

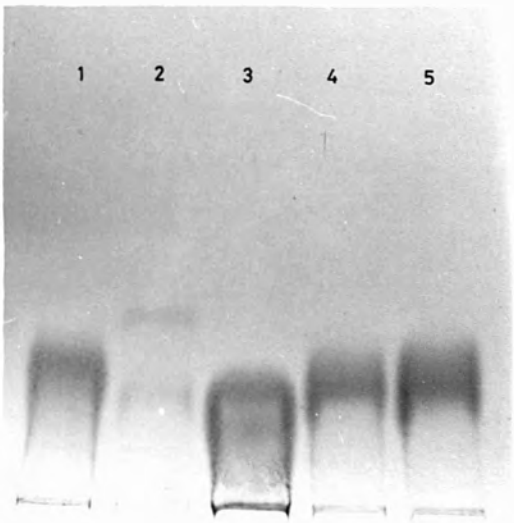
Figure 49. Photograph of esterase zymogram of *Nereis* species.

1. *N. virens* from Blyth
2. *N. fucata* from Millport
3. *N. diversicolor* from Plymouth
4. *N. diversicolor* from Blyth (2nd year age-group)
5. *N. diversicolor* from Blyth (1st year age-group)

Figure 50. Photograph of malate dehydrogenase zymogram of *Nereis* species .

1. *N. virens* from Blyth
2. and 3. *N. fucata* from Millport
4. *N. diversicolor* from Blyth (2nd year age-group)
5. *N. diversicolor* from Blyth (1st year age-group)


1
2
3
4
5


1
2
3
4
5

DISCUSSION

The main objective of this work was to provide some preliminary information about enzyme electrophoresis in polychaetes. The results show that in Arenicola marina as well as in Nereis species a good deal of genetic variablity may be detected by electrophoresis.

Arenicola marina inhabits the tidal zone and burrows in sandy beaches which may be separated by stretches of unsuitable coast and possibly causing local isolation. The worm is neither an active swimmer nor an energetic traveller at any stage of its life-history. The adult worm swims slowly and clumsily and they do it very seldom. If conditions are favourable, an individual will remain in the same burrow for several months (Wells, 1957). The eggs and sperm are discharged on the surface of the sand, at low tide. The fertilized eggs and larvae are carried up shore by the tide and deposited in the Fucus zone where they live and develop into small worms. There is no pelagic larval phase (Newell, 1948). In the light of these facts, genetic differentiation between populations is not unexpected. Duncan (1960) compared the spawning periods of A. marina from different beaches of Britain and concluded that each population had its own characterstic spawning period, though always in October, November or December. It is however, not known whether these differences in spawning period are due to genetic or environmental causes or to a combination of both factors.

The result of the present study shows variation in leucine aminopeptidase, esterase and tetrazolium oxidase. Few worms were analysed from each population and, therefore, the possibility of other enzyme systems being variable cannot be ruled out. A. marina can be collected in large numbers from suitable beaches and further light could be thrown on the problem by extensive study of electrophoretically separated isozymes.

Many examples of reproductive isolation without morphological differentiation in the Nereidae have been reviewed by Clark (1977). Three species of Platynereis, P. dumerilii (Audouin and Milne Edwards), P. megalops Verrill, and P. massiliensis (Moquin-Tandon) exhibit complete reproductive isolation in spite of their morphological similarity. They inhabit different geographical areas or different depth of tidal zones. P. dumerilii is found in European waters where it inhabits the subtidal zone while P. megalops is found along the New England coast. These two species are also similar in the sense that they both undergo epitokal metamorphosis and swarm at the sea surface. However the mode of fertilization is different in the two species. In P. dumerilii, fertilization is external while in P. megalops it is internal. The third species P. massiliensis is found in European waters and inhabits the intertidal zone. This species does not undergo metamorphosis but breeds within its tube. A similar example is found in the genus Nereis, in which three species are reproductively isolated in spite of their

morphological similarity. N. diversicolor breeds on the mud-flats in which it lives. N. japonica swarms at the sea surface to breed and N. limnicola is a viviparous, self fertilizing hermaphrodite which breeds within its tube. These examples and many others reported by Clark (1952, 1977) show that speciation is an ongoing process within the polychaetes and numerous examples of different stages of speciation probably remain to be discovered.

N. diversicolor typically lives in estuarine mudflats and local races have been described which suggest genetic differences (Smith, 1977; Barnes, 1978). The study of population genetics of Nereidae would be interesting since the family is strictly monotelic, that is, they spawn once in their life time and die soon after spawning. Olive and Garwood (1981) studied a population of N. diversicolor from Blyth, Northumberland, England and suggested a life span of 3 years. They also suggested that successive year groups would be genetically isolated. A sample of the same population was collected in summer, 1980 and analysed electrophoretically. The result shows that the two age groups (which were kindly separated by Dr Garwood himself) are not genetically different. The result is not surprising. The same authors (Olive and Garwood, 1981) observed under laboratory conditionsthat worms can reach maturity when they are 2 years old. They also discuss the possibility that males can breed and die when 2 years old, while females do not reach maturity until 3 years old and this would obviously break down genetic

isolation between successive age groups.

With the exception of esterase, no other enzyme system was found to be variable in 3 different populations of N. diversicolor. Interspecific differences were, however, observed in two other enzyme systems namely, malate dehydrogenase and phosphoglucose isomerase. It is however, interesting to note that the two species, namely N. diversicolor and N. virens which live on mud-flats, were found to be almost identical in all enzyme systems studied while N. fucata which lives offshore and in deep water was different in three enzyme systems out of six studied here.

REFERENCES

REFERENCES

Allen, S.L. (1961), Genetic control of the esterases in the protozoan Tetrahymena puriformis. Ann. N.Y. Acad. Sci., 94: 753-773.

Ameyaw-Akumfi, C. (1976), The function of the caruncle of an amphinomid Eurythoe sp., a histological investigation. Zool. Anz., 196: 318-322.

Annenkova, N. (1934), Kurze Ubersicht der Polychaetes der Literalzone der Bering Insel (Kommander Insel) nebst Beschreibung neuer Arten. Zool Anz., 106: 322-331.

Avise, J.C. (1975), Systematic value of electrophoretic data. Syst. Zool., 23: 465-481.

Avise, J.C., Smith, M.H., Selander, R.K., Lawlor, T.E. and Ramsey, P.R. (1974), Biochemical polymorphism and systematics in the genus Peromyscus. V. Insular and mainland species of the subgenus Haplomylomys. Syst. Zool., 23: 226-238.

Ayala, F.J., Tracey, M.L., Hedgecock, D. and Richmond, R.C. (1974), Genetic differentiation during the speciation process in Drosophila. Evolution, 28: 576-592.

Ayala, F.J., Valentine, J.W. and Zumwalt, G.S. (1975), An electrophoretic study of the antarctic zooplankton Euphausia superba. Limnol. Oceanogr., 20: 635-640.

Balinsky, B.I. (1975), An introduction to embryology. W.B. Sanders Co., Philadelphia, 648pp.

Barnes, R.S.K. (1978), Variation in paragnath number of Nereis diversicolor in relation to sediment type and salinity regime. Est. coast. mar. Sci., 6: 275-284.

Battaglia, B. and Bisol, P.M. (1975), Biochemical polymorphism in marine crustacea in relation to their ecology. Proc. 9th Europ. mar. biol. Symp., 573-585.

Baur, E.W. and Pattie, D.L. (1968), Lactate dehydrogenase genus in rodents. Nature, 218: 341-343.

Bedford, J.J. and Reid, M.S. (1969), Gel electrophoresis of proteins in the crystalline style of certain mollusca. Comp. Biochem. Pysiol., 29: 659-664.

Bellan, C., Reish, D.J. and Foret, J.P. (1972), The sublethal effects of a detergent on the reproduction, development and settlement in the polychaetous annelid Capitella capitata. Mar. Biol., 14: 183-188.

Berger, E.M. (1973), Gene enzyme variation in three sympatric species of Littorina. Biol. Bull., 145: 83-90.

Berkeley, E. and Berkeley, C. (1952), Annelida, polychaeta sedentaria. Can. Pac. Fauna, No.9: 1-139.

Blake, J.A. (1966), On Boccardia hamata (Webster), new combination (Polychaeta, Spionidae). Bull. south.

Calif. Acad. Sci., 65: 176-184.

Blake, J.A. (1969a), Systematics and ecology of shell boring polychaetes from New England. Am.Zool., 9: 813-820.

Blake, J.A. (1969b), Reproduction and larval development of Polydora from northern New England (Polychaeta, Spionidae). Ophelia, 7: 1-63.

Blake, J.A. (1971), Revision of the genus Polydora from the east coast of North America (Polychaeta, Spionidae). Smithson. contrib. Zool., 75: 1-32.

Blake, J.A. and Evans, J.W. (1973), Polydora and related genera as borers in mollusc shells and other calcareous substrates (Polychaeta, Spionidae). Veliger, 15: 235-249.

Bosc, L.A.G. (1802), Histoire naturelle des vers contenant leur description et leurs moeurs, avee figures dessinees d'après nature. Volumes 1-3: 324pp. Paris.

Bowen, S.T., Lebherz, H.G., Poon, M., Chow, V.H.S. and Grigliatti, T.A. (1969), The haemoglobins of Artemia salina - I. Determination of phenotype by genotype and environment. Comp. Biochem.Physiol., 31: 733-747.

Bowen, S.T. and Sterling, G. (1978), Esterase and malate dehydrogenase in 15 Artemia populations. Comp. Biochem. Physiol., 61B: 593-595.

Boyden, A.A. (1942), Systematic serology: A critical appreciation. Physiol. Zool., 15: 109-144.

Boyden, A.A. (1953), A half century of systematic serology. Bull. serol. Museum No. 6: 1-3.

Boyden, A.A. (1958), Comparative serology: Aims, methods and results. In W.H.Cole (ed.), Serological and Biochemical comparision of proteins. Rutgers Univ. Press, New Brunswick, N.J. 3-24pp.

Brewer, G.J. (1970), An introduction to isozyme techniques. Academic Press, N.Y., 186pp.

Brock, V. (1978), Morphological and biochemical criteria for the separation of Cardium glavcum from Cardium edule. Ophelia, 17: 207-214.

Buzzati-Traverso, A.A. (1953), Identification of recessive gene heterozygotes by means of paper-partition chromatography. Nature, 171: 575-576.

Carazzi, D. (1895), Revisione del genero Polydora Bosc. ecenni su due specie che vivono sulle ostriche. Mittheilungen aus der Zoologischen St. Neapel, 11: 4-45.

Chambers, S.M. (1978), An electrophoretically detected sibling species of Goniobasis floridensis (Mesogastropoda, Pleuropoceridae). Malacologia, 17: 157-162.

Chank, K.L., Dhaliwal, S.S. and Youg, H.S. (1979), Protein variation and systematics of three subgenera of

Malayan rats (Rodentia: Muridae, genus Rattus Fischer). Comp. Biochem. Physiol., 64B: 329-337.

Clark, R.B. (1952), Speciation of polychaetes in the Clyde sea area. Nature, 169: 975.

Clark, R.B. (1977), Reproduction, speciation and polychaete taxonomy. In D.J.Reish and K.Fauchald (eds.), Essays on polychaetous annelids in memory of Dr Olga Hartman, Allan Hancock Foundation, Univ. south. Calif., 477-501.

Clarke, J.T. (1964), Simplified "Disc" (polyacrylamide gel) electrophoresis. Ann. N.Y. Acad. Sci., 121: 428-436.

Cole, M.A. and Morgan, R.P. (1978), Genetic variation in two populations of blue crab Callinectes sapidus. Estuaries, 1: 202-205.

Coles, G.C. (1969a), Isoenzyme of snails liver-I. Hydrolysing enzymes and peroxidase. Comp. Biochem. Physiol., 29: 403-411.

Coles, G.C. (1969b), Isoenzymes of snail livers-II. Dehydrogenases. Comp. Biochem. Physiol., 31: 1-14.

Consden, R., Gordon, A.H. and Martin, A.J.P. (1944), Quantitative analysis of proteins: a partition chromatographic method using paper. Biochem. J., 38: 224-232.

Cronquist, A. (1964), The old systematics. In C.A.Leone (ed.), Taxonomic biochemistry and serology. Ronald Press,N.Y.

Dando, P.R. (1970), Mergim (Lepidorhombus whiff-iagonis) populations in the English channel and approaches - Lactate dehydrogenase and glycerol-3-phosphate dehydrogenase polymorphism. J. mar. biol. Ass. U.K., 50: 801-818.

Dando, P.R. (1974), Distribution of multiple glucosephosphate isomerase in teleostean fishes. Comp. Biochem. Physiol., 47B: 663-679.

Dando, P.R. and Southward, A.J. (1980), A new species of Chthamalus (Crustacea: Cirripedia) characterized by enzyme electrophoresis and shell morphology: with a revision of other species of Chthamalus from the western shores of the Atlantic ocean. J. mar. biol. Ass. U.K., 60: 787-831.

Dando, P.R., Southward, A.J. and Crisp, D.J. (1979), Enzyme variation in Chthamalus stellatus and Chthamalus montagui (Crustacea: Cirripedia): evidence for the presence of C. montagui in the Adriatic. J. mar. biol. Ass. U.K., 59: 307-320.

Daro, M.H. and Polk, P. (1973), The autecology of Polydora ciliata along the Belgian coast. Neth. J. Sea. Res., 6: 130-140.

Darwin, C. (1872), On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London 502pp.

Day, J.H. (1967), A monograph of the Polychaeta of southern Africa. Part 2. Sedentaria. Brit. Mus. (nat. Hist.) London, pub. 656: 459-878.

Dean, D. (1969), Relationship between eyes and anterior regeneration in spionid polychaetes. Am. Zool., 9: 1146.

Dendinger, J.E. (1980), Electrophoretic separation of three dehydrogenases from various tissues of the Atlantic blue crab Callinectes sapidus. Comp. Biochem. Physiol., 66B: 431-433.

Dessauer, H.C. and Fox, W. (1956), Characterstic electrophoretic patterns of plasma proteins of orders of amphibia and reptilia. Science, 124: 225-226.

Dessauer, H.C. and Pough, F.H. (1975), Geographic variation of blood protein and the systematics of King snakes (Lampropeltis getulus). Comp. Biochem. Physiol., 50B: 9-12.

Dobzhansky, T. Ayala, F.J., Stebbins, G.L. and Valentine, J.W. (1977), Evolution. W.H.Freeman and Co., San Francisco, 572pp.

Doering,G.N. and Palincsar, E.E. (1978), Acid phosphatase during the life cycle of the Nematode, Panagrellus silusiae. Biol. Bull., 154: 374-382.

Dorsett, D.A. (1961), The reproduction and maintenance of

Polydora ciliata (Johnston) at Whitstable. J. mar. biol. Ass. U.K., 41: 383-396.

Duncan, A. (1960), The spawning of Arenicola marina (L) in the British Isles. Proc. zool. Soc. London, 134: 1347-156.

Fauchald, K. (1974), Polychaete phylogeny: a problem in protostome evolution. Syst. Zool., 23: 493-506.

Fauchald, K. (1977), The polychaete worms. Nat. Hist. Mus. Los Angeles, Sci. ser. , 28: 1-190.

Fauvel, P. (1927), Polychétes sédentaires. Faune Fr., No. 16: 494pp.

Ferguson, A. (1974), The genetic relationships of the coregonid fishes of Britain and Ireland indicated by electrophoretic analysis of tissue proteins. J. Fish. biol., 6: 311-315.

Ferguson, A. (1980), Biochemical systematics and evolution. Blackie & Son Ltd. Glasgow, 194pp.

Fisher, R.A. (1930), The genetical theory of natural selection. Clarendon Press, London, 272pp.

Flynn, F.V. (1968), Application of starch gel electrophoresis to the study of serum and urine proteins. In I. Smith, (ed.), Chromatographic and electrophoretic techniques Vol. II Zone electrophoresis. William Heinemann-Medical Books Ltd. 246-291.

Foote, R. and McMahon, J.A. (1977), Electrophoretic studies of rattlesnake (Crotalus and Sistrurus) venom: Taxonomic implications. Comp. Biochem. Physiol., 57B: 235-241.

Foster, N.M. (1971a), Spionidae (Polychaeta) of the Gulf of Mexico and the Caribbean sea. Uitg. natuurw. Studkring Suriname, 63: 1-183.

Foster, N.M. (1971b), Notes on spionid (Polychaeta) setae. Trans. Am. microsc. Soc., 90: 34-42.

Fujino, K. and Nagaya, N. (1977a), Biochemical polymorphism in the Pacific oyster - I. Variants in myogen and esterases. Bull. Jap. Soc. scient. Fish., 43: 983-988.

Fujino, K. and Nagaya, N. (1977b), Biochemical polymorphisms in the Pacific oyster - II. Variants in tetrazolium oxidase and leucine aminopeptidase. Bull. Jap. Soc. scient. Fish., 43: 1455-1459.

Garlick, W.S. and Terwilliger, R.C. (1978), The lactate dehydrogenase isozymes of six species of cottid fishes. Comp. Biochem. Physiol., 60B: 261-266.

George, J.D. (1967), Cryptic polymorphism in the cirratulid polychaetes Cirriformia tentaculata. J. mar. biol. Ass. U.K., 47: 75-79.

Gibbson, P.H. (1978), Systematics of Dodecaceria (Annelida: Polychaeta) and its relation to the reproduction of its species. J. Linn. Soc. (zool.), 63: 275-287.

Gooch, J.L. and Hetric, S.W. (1979), The relation of genetic structure to environmental structure: Gammarus minus in a karst area. Evolution, 33: 192-206.

Gooch, J.L. and Schopf, T.J.M. (1970), Population genetics of marine species of the phylum Ectoprocta. Biol. Bull., 138: 138-156.

Gooch, J.L. and Schopf, T.J.M. (1971), Genetic variation in the marine ectoproct Schizoporella errata . Biol. Bull., 141: 235-246.

Gooch, J.L., Smith, B.S. and Knupp, D. (1972), Regional survey of gene frequencies in the mud snail Nassarius obsoletus. Biol. Bull., 142: 36-48.

Gordon, A.H., Keil, B. and Sebesta, K. (1949), Electrophoresis of proteins in agar jelly. Nature, 164: 498-499.

Gorman, G.C. and Dessauer, H.C. (1966), The relationships of Anolis of the Roquet species group (Sauria: Iguanidae)-I. Electrophoretic comparision of blood protein. Comp. Biochem. Physiol., 19: 845-853.

Graham, H.W. and Gay, H. (1945), Season of attachment and growth of sedentary marine organisms at Oakland, California. Ecology, 26: 375-386.

Grassle, P.F. and Grassle, J.P. (1974), Opportunistic life histories and genetic system in marine benthic polychaetes. J. mar. Res., 32: 253-284.

Grassle, J.P. and Grassle, J.F. (1976), Sibling species in the marine pollution indicator Capitella (Polychaeta). Science, 192: 567-569.

Grassle, J.F. and Grassle, J.P. (1977), Temporal adaptation in sibling species of Capitella. In B.C. Coull (ed.), Ecology of marine benthos. Publ. Univ. south Carolina Press. 177-189.

Gustus, R.M. and Cloney, R.A. (1973), Ultrastructure of the larval compound setae of the polychaete Nereis vexillosa Grube. J. Morph., 10: 355-366.

Guttman, S.I. (1970), Haemoglobin electrophoresis and relationships within the lizard genus Sceloporus (Sauria: Iguanidae). Comp. Biochem. Physiol., 34: 563-568.

Haigler, S.A. (1969), Boring mechanism of Polydora websteri inhabiting Crassostrea virginica. Am. Zool., 9: 821-828.

Hannerz, L. (1956), Larval development of the polychaete families Spionidae Sars, Disomidae Mesnil, and Poecilochaetidae n.fam. in the Gullmar Fjord, Sweden. Zool. Bidr. Upps., 31: 1-204.

Hannerz, L. (1961), Polychaeta: larvae. Families: Spionidae, Disomidae, Poecilochaetidae. Fich. Ident. Zooplanct., 19: 1-12.

Hartman, O. (1941), Polychaetous annelids. Part III Spionidae from California, with keys to species and genera and description of two new form. Allan. Hancock Pac. Exped., 7: 289-323.

Hartman, O. (1943), Description of Polydora websteri, n.sp. In V.L. Loosanoff and J.B. Engle, Polydora in oysters suspended in the water., Biol. Bull., 85: 70-72.

Hartman, O. (1944), New England Annelida, Part 2. including the unpublished plates by Verrill with reconstructed captions., Bull. Am. Mus. mat. Hist., 82: 331-343.

Hartman, O. (1945), The marine annelids of North Carolina. Bull. Duke Univ. mar. Sta., 21: 1-53.

Hartman, O. (1951), The littoral marine annelids of the Gulf of Mexico. Publs Inst. mar. sci. Univ. Tex., 2: 7-124.

Hartman, O. (1961). Polychaetous annelids from California. Allan Hancock Pac. Exped., 25: 1-226pp.

Hartman. O. (1969), Atlas of the sedentariate polychaetous annelids from California. Allan Hancock found., Univ. S. California, Los Angeles, 812pp

Hartman-Schröder, G. (1971), Annelida, borstenwurmer Polychaeta. Tierwelt Deutschlands, 58: 594pp

Hatfield, P.A. (1965), Polydora commensalis Andrews larval development and observations on adults. Biol. Bull. 128: 356-368.

Healy, E.H. and Wells, G.P. (1959), Three new lugworms (Arenicolidae, Polychaeta) from the north Pacific area. Proc. zool. Soc. London, 133: 315-335.

Horn, E.C. and Kerr, M.S. (1969), The haemolymph proteins of the blue crabs, Callinectes sapidus - I. Hamocyanins and certain other major protein constituents. Comp. Biochem. Physiol., 29: 493-508.

Hubby, J.L. and Lewontin, R.C. (1966), A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in Drosophila pseudoobscura. Genetics, 54: 577-594.

Hunter, R.L. and Markert, C.L. (1957), Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science, 125: 1294-1295.

Huxley, J. (1940), The new systematics. Syst. Ass., London, 582pp.

Jelnes, J.E. (1977), An electrophoretic character useful in the distinction between Bulinus tropicus and B. permembranaceus (Gastropoda, Planorbidae). Steenstrupia, 4: 139-141.

Jelnes, J.E. (1979), Experimental taxonomy of Bulinus (Gastropoda: Planorbidae). II. Recipes for horizontal starch gel electrophoresis of ten enzymes in Bulinus and description of the internal

standard systems and of two new species of the Bulinus forskalii complex. J. Chromat., 170: 405-411.

Jelnes, J.E., Peterson, G.H. and Russell, P.J.C. (1971), Isoenzyme taxonomy applied on four species of Cardium from Danish and British waters with a short description of the distribution of the species. Ophelia, 9: 15-19.

Johnson, G.B. (1971), Analysis of enzyme variation in natural populations of the butterfly Colias eurytheme. Proc. natn. Acad. Sci. U.S.A., 68:997-1001.

Johnson, W.E. and Selander, R.K. (1971), Protein variation and systematics in kangaroo rats (genus Dipodomys). Syst. Zool. 20: 377-405.

Johnston, G. (1838), Miscellanea zoologica. Aricidae. Magazine of zoology and botany, Edinburgh, 2: 63-73.

Jones, M.L. (1970), The presence of certain dehydrogenase among polychaetous annelids as shown by disc electrophoresis. Comp. Biochem. Pysiol., 36: 605-611.

Jones, B.W. and Mackie, I.M. (1970), An application of electrophoretic analysis of muscle myogens to taxonomic studies in the genus Merluccius. Comp. Biochem. Physiol., 32: 267-273.

Just, E.E. (1914), Breeding habits of the heteronereis form of Platynereis megalops at Woods Hole. Biol. Bull., 27: 201-212.

Just, E.E. (1915), An experimental analysis of fertilization in Platynereis megalops. Biol Bull., 28: 93-114.

Just, E.E. (1929), Breeding habits of Nereis dumerilii at Naples. Biol. Bull., 57: 307-310.

Kendall, M.A. (1980), Variations in some morphological characterstics of Polydora ciliata (Johnston). J. nat. Hist., 14: 405-411.

Kilpatric, C.W. and Zimmerman, E.G. (1975), Genetic variation and systematics of four species of mice of the Peromyscus boylii species group. Syst. Zool., 24: 143-162.

Kirkegaard, J.B. (1959), The Polychaeta of West Africa. Atlantide Rep., 5: 7-117.

Kiselva, G.A. (1967), Settlement of Polydora ciliata (Johnston) larvae on different substrates. In Benthic biocoenosis and the biology of benthonic organisms in the Black sea. Ukranian Akad. Sci. Kiev., 85-90.

Kohn, J. (1957), A cellulose acetate supporting medium for zone electrophoresis. Clin. Chim. Acta., 2: 297.

Lassen, H.H. (1979), Electrophoretic enzyme patterns and breeding experiments in Danish mudsnails (Hydrobiidae). Ophelia, 18: 83-87.

Levinton, J.S. and Fundiller, D.L. (1975), An ecological approach to the study of biochemical polymorphisms. Proc. 9th Europ. mar. biol. Sym., 165-178.

Lewontin, R.C. (1974), The genetic basis of evolutionary change. Columbia Univ. Press, 346pp.

Light, W.J. (1969), Polydora narica, new species and Pseudopolydora kempi new subspecies, two new spionids (Annelida;Polychaeta) from central California. Proc. Calif. Acad. Sci., 36: 531-550.

Linnaeus, C. (1758), Systema naturae per regna tria naturae, secundum classes, ordines, genera, species cum characteribur, differetiis, synonymis, locis, Editio decima, reformata, Tomus I. Laurentii Salvii, Holmiae, 824pp.

Loosanoff, V.L. and Engle, J.B. (1943), Polydora in oyster suspended in the water. Biol. Bull., 85: 69-78.

Lyerla, J.H., Lyerla, T.A. and Johnson, S.E. (1979), Malate dehydrogenase isozyme patterns in species of terrestrial isopods. Comp. Biochem. Physiol., 63B: 19-24.

Mackay, T.F.C. and Doyle, R.W. (1978), An ecological genetic analysis of the settling behaviour of a marine polychaete I. Probablity of settlement and gregarious behaviour. Heredity, 40: 1-12.

Mackie, I.M. and Jones, B.W. (1978), The use of electrophoresis of the water soluble (sarcoplasmic) proteins of fish muscle to differentiate the closely related species of hake (Merluccius sp.). Comp. Biochem. Physiol., 59B: 95-98.

Mainardi, D. (1959), Immunological distances among some gallinaceous birds. Nature, 184: 913-914.

Manwell, C. (1966), Sea cucumber sibling species: polypeptide chain types and oxygen equilibrium of haemoglobin. Science, 152: 1393-1395.

Manwell, C. and Baker, C.M.A, (1963a), A sibling species of sea cucumber discovered by starch gel electrophoresis. Comp. Biochem. Physiol., 10: 39-53.

Manwell, C. and Baker, C.M.A. (1963b), Starch gel electrophoresis of sera from some marine arthropods: Studies on the heterogeneity of hemocyanin and on a 'ceruloplasmin-like protein'. Comp. Biochem. Physiol., 8: 193-208.

Manwell, C., Baker, C.M.A., Ashton, P.A. and Corner, E.D.C. (1967), Biochemical differences between Calanus finmarchicus and C. helgolandicus. J. mar. biol. Ass. U.K., 47: 145-169.

Marcus,N.H. (1977), Genetic variation within and between geographically separated populations of the sea urchin

Arbacia punctulata. Biol. Bull., 153: 560-576.

Markert, C.L. and Moller, F. (1959), Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. Proc. natn. Acad. Sci. U.S.A., 45: 753-763.

Markert, C.L. and Ursprung, H. (1971), Developmental genetics. Prentice-Hall, Inc., New Jersey, 214pp.

Massaro, E.J. and Cohen, E. (1978), An electrophoretic analysis of selected enzymes of the coconut crab *Birgus latro*. Comp. Biochem. Physiol., 60B: 151-152.

Mayr, E. (1963), Animal species and evolution. Harvard Univ. Press, Cambridge, Mass., 797pp.

Mayr, E., Linsley, E.G. and Usinger, R.L. (1953), Methods and principles of systematic zoology. McGraw-Hill, N.Y. 328pp.

McDermid, E.M., Board, P.G. and Agar, N.S. (1977), Studies on the blood of Australian elapid snakes - III. electrophoretic analysis of serum proteins and red cell enzymes. Comp. Biochem. Physiol., 56B: 361-365.

Menzil, B.W. (1977), Morphological and electrophoretic identification of a hybrid cyprinid fish *Notropis cerasinus* x *Notropis C. cornutus*, with implications on the evolution of *Notropis albeolus*. Comp.

Biochem. Physiol., 57B: 215-218.

Mesnil, F. (1896), Etudes de morphologie externe chez les Annelides. Bull. scient. Fr. Belgique, 29: 110-287.

Michaelis, H. (1978), Zur morphologie und ökologie von Polydora ciliata und P. ligni (Polychaeta, Spionidae). Helgol. Wiss. Meeresunters, 31: 102-116.

Michelson, E.H. and Dubois, L. (1971), An electrophoretic analysis of the crystalline style of Oncomelania (Mollusca:Hydrobidiae). Comp. Biochem. Physiol., 38B; 263-268.

Milbrink, G. and Nyman, L. (1973), On the protein taxonomy of aquatic oligochaetes. Zoon., 1: 29-35.

Milkman, R. and Beaty, L.D. (1970), Large-scale electrophoretic studies of allelic variation in Mytilus edulis. Biol. Bull., 139:430.

Mortensen, E. and Galtsoff, P.S. (1944), Behaviour and tube building habits of Polydora ligni. Biol. Bull., 87: 164-165.

Murphy, P.G. (1978), Collisella austrodigitalis sp. nov.: A sibling species of limpet (Acmaedae) discovered by electrophoresis. Biol. Bull., 155: 193-206.

Nadler, C.F. (1968), The serum proteins and transferrins of the ground squirrel subgenus Spermophilus. Comp.

Biochem. Physiol. 27: 487-503.

Nadler, C.F. and Hughes, I.H. (1966), Serum protein electrophoresis in the taxonomy of some species of the ground squirrel subgenus Spermophilus. Comp. Biochem. Physiol., 18: 639-651.

Nagaya, N., Sasaki, K. and Fujino, K. (1978), Biochemical polymorphism in the Pacific oyster - III. Local populations in Hokkaido and the north east districts of Japan. Bull. Jap. Soc. scient. Fish., 44: 1041-1046.

Nei, M. (1972), Genetic distance between populations. Am. Nat., 106: 283-292.

Nelson, T.C. and Stauber, L.A. (1941), Further observations on the mud worm, Polydora ligni Webster, on oyster beds in Delaware Bay. Natn. Shellfish Ass., 4pp.

Nevo, E. (1978), Genetic variation in natural populations: Patterns and theory. Theoret. popul. biol., 13: 121-177.

Newell, G.E. (1948), A contribution to our knowledge of the life history of Arenicola marina (L). J. mar. biol. Ass. U.K., 27: 554-580.

Nuttall, G.H.F. (1901), The new biological test for blood in relation to zoological classification. Proc. R. Soc. Lond., 69: 150-153.

Nuttall, G.H.F. (1904), Blood immunity and blood relationship. Cambridge Univ. Press, London, 444pp.

Nyman, L. and Skoog, G. (1977), Species specific enzymes in some snail species (Mollusca, Gastropoda) from fresh and brackish water in Sweden. Zoologica Scripta, 6: 327-330.

O'Clair, R.M. and Cloney, R.A. (1974), Patterns of morphogenesis mediated by dynamic microvilli: Chaetogenesis in Nereis vexillosa. Cell. Tiss. Res., 151: 141-157.

Ogita, Z and Kasai, T. (1965), Genetico-biochemical analysis of specific esterases in Musca domestica. Jap. J. genet., 40: 173-184.

Okuda, S. (1937), Spioniform polychaetes from Japan. J. Fac. Sci. Hokkaido Imper. Univ., 5: 217-254.

Olive, P.J.W. and Garwood, P.R. (1981), Gametogenic cycle and population structure of Nereis (Hediste) diversicolor and Nereis (Nereis) pelagica from north-east England. J. mar. biol. Ass. U.K., 61: 193-213.

Ornstein, L. and Davis, B.J. (1962), Disc electrophoresis. Distillation products industries, Div. Eastman Kodak Co., Rochester, N.Y.

Orrhage, L. (1971). Light and electron microscope studies of some annelid setae. Acta Zool., Stockh. 52: 157-169.

Prakash, S., Lewontin, R.C. and Hubby, J.L. (1969), A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genic variation in central, marginal and isolated populations of Drosophila pseudoobscura. Genetics, 61: 841-858.

Rainer, S. (1973), Polydora and related genera (Polychaeta, Spionidae) from Otago waters. J. R. Soc. N.Z., 3: 545-564.

Rasmussen, E. (1973), Systematics and ecology of the Isefjord marine fauna (Denmark). Ophelia, 11: 1-495.

Raymond, S. (1964), Acrylamide gel electrophoresis. Ann. N.Y. Acad. Sci., 121: 350-365.

Raymond, S. and Weintraub, L. (1959), Acrylamide gel as a supporting medium for zone electrophoresis. Science, 130: 711.

Read, G.B. (1975), Systematics and biology of polydorid species (Polychaeta:Spionidae) from Wellington harbour. J. R. Soc. N.Z., 5: 395-419.

Reid, R.G.B. and Dunnill, R.M. (1969), Specific and individual differences in the esterases of members of the genus Macoma (Mollusca:Bivalvia). Comp. Biochem. Physiol., 29: 601-610.

Reish, D.J. (1973), The use of benthic animals in monitoring the marine environment. J. environ. planning Pollut. cont., 1: 32-38.

Rice, S.A. and Simon, J.L. (1980), Intraspecific variation in the pollution indicator polychaete Polydora ligni (Spionidae). Ophelia, 19: 79-115.

Rullier, F. (1951), Etude morphologique, histologique et physiologique de l'organe nucal chez les annelids polychaetes sedentaires. Inst. oceanogr. Monaco Ann., 25: 207-341.

Rutherford, J.C. (1977), Geographical variation in morphological and electrophoretic characters in the holothurian Cucumaria curata. Mar. Biol. 43: 165-174.

Salthe, S.N. (1969), Geographic variation of the lactate dehydrogenase of Rana pipens and Rana palustris. Biochem. genet., 2: 271-304.

Sander, F. (1976), Therespiratory significance of the Sabellastarte magnifica branchial crown. Comp. Biochem. Physiol. 53A: 263-264.

Sanger, F. and Thompson, E.O.P. (1953), The amino acid sequence in the glycyl chain of insulin. Biochem. J., 53: 353-374.

Sarich, V.M. (1977), Rates, sample sizes and the neutrality

hypothesis for electrophoresis in evolutionary studies. Nature, 265: 24-28.

Scandalios, J.G. (1969), Genetic control of multiple molecular forms of enzymes in plants: A review. Biochem. genet., 3: 37-79.

Schopf, T.J.M. and Murphy, L.S. (1973), Protein polymorphism of the hybridizing sea stars Asterias forbesi and A. vulgaris and implications for their evolution. Biol. Bull., 145: 589-597.

Seaman, R,N. and Nash, D.J. (1977), An electrophoretic description of five species of squirrel. Comp. Biochem. Physiol., 58B: 309-311.

Selander, R.K., Johnson, W.E. and Avise, J.C. (1971), Biochemical population gentics of fiddler crabs (Uca). Biol. Bull., 141: 402.

Sharp, G.D. (1969), Electrophoretic study of tuna hemoglobins. Comp. Biochem. Physiol., 31: 749-755.

Shaw, C.R. (1965), Electrophoretic variation in enzymes. Science, 149: 936-942.

Shaw, C.R. and Koen, A.L. (1968), Starch gel zone electrophoresis of enzymes. In I. Smith (ed.), Chromatographic and electrophoretic technique, Vil.II . Zone electrophoresis, William Heinemann-Medical Books Ltd. 325-364.

Shaw, C.R. and Prasad, R. (1970), Starch gel electrophoresis of enzymes - A compilation of recipes. Biochem. genet. 4: 297-320.

Sibley, C.G. (1960), The electrophoretic patterns of Avian egg-white proteins as taxonomic characters. Ibis, 102: 215-284.

Sibley, C.G. (1970), A comparative study of the egg-white proteins of passerine birds. Bull. Peabody Mus. nat. Hist., 32: 1-131.

Sibley, C.G. (1976), Protein evidence of the relationships of some Australian passerine birds. Proc. Inst. orinthological Congr., 16: 557-570.

Sibley, C.G. and Ahlquist, J.E. (1972). , A comparative study of the egg-white proteins of non-passerine birds. Bull. Peabody Mus. nat. Hist., 39: 1-276.

Sibley, C.G. and Frelin, C. (1972), The egg-white protein evidence for ratite affinities. Ibis, 114: 377-387.

Smith, I. (1968), Paper electrophoresis at low voltage: Techniques of paper electrophoresis. In I. Smith (ed.), Chromatographic and electrophoretic techniques, William Heinemann-Medical Books Ltd., 17-43.

Smith, R.I. (1958), On reproductive pattern as a specific characterstics among nereid polychaetes. Syst. Zool., 7: 60-73.

Smith, R.I. (1977), Physiological and reproductive adaptations of Nereis diversicolor to life in the Baltic sea and adjacent waters. In D.J.Reish and K.Fauchald, (eds.), Allan Hancock Foundation, Univ. S. California Press.

Smithies, O. (1955), Zone electrophoresis in starch gels: Group variations in serum proteins of normal human adults. Biochem. J. 61: 629-641.

Smithies, O. (1959), An improved procedure for starch gel electrophoresis: Further variations in the serum proteins of normal individuals. Biochem. J., 71: 585-587.

Sneath, P.H.A. and Sokal, R.R. (1973), Numerical taxonomy. W.H. Freeman and Co., San Francisco.

Söderstrom, A. (1920), Studien über die Polychaeten Familie Spionidae. Inaug. Diss. Uppsala.

Söderstrom, A. (1923), Über das Bohren der Polydora ciliata. Zool. Bidr. Upps., 8: 319-340.

Steiner, W.W.M., Lisowski, E.A. and Osterbur, D. (1977), Biochemical differences in sympatric colour morphs of an aquatic isopod (Asellus brevicauda). Comp. Biochem. Physiol., 56B: 371-374.

Stevens, P.J. (1969), Principles of chromatography. In D.R. Browning (ed.), Chromatography, McGraw Hill, London.

Synder, T.P. and Gooch, J.L. (1973), Genetic differentiation in Littorina saxatilis (Gastropoda). Mar. Biol., 22: 177-182.

Sywula, T. and Bartkowiak, S. (1978), Preliminary study of the isoenzymes of ostracoda. Crustaceana, 35: 265-272.

Theisen, B.F. (1978), Allozyme clines and evidence of strong selection in three loci in Mytilus edulis L. (Bivalvia) from Danish waters. Ophelia, 17: 135-142.

Thorson, G. (1946), Reproductive and larval development of Danish marine bottom invertebrates with special reference to the planktonic larvae in the sound (Oresund). Medd. Komm. Danm. Fiskeri-og. Havunders kbl. ser. Plankt., 4: 1-523.

Tracey, M.L., Nelson, K., Hedgecock, D., Shleser, R.A. and Pressick, M.L. '1975), Biochemical genetics of lobsters: Genetic variation and the structure of American lobster (Homarus americanus) populations. J. Fish. Res. Bd. Canada, 32: 2091-2101.

Turner, K and Lyerla, T.A, (1980), Electrophoretic variation in sympatric mud crabs from north inlet, South Carolina. Biol. Bull., 159: 418-427.

Verrill, A.E. (1880), Notice on recent addition to the marine invertebrata of the northeastern coast of America, with description of new genera and species

and critical remarks on others - Part II. Annelida, Gephyrea, Nemertina, Nematoda, Polyzoa, Tunicata, Echinodermata, Porifera. Proc. U.S. natn. Mus., 2: 165-205.

Warren, L.M. (1976), A review of the genus Capitella (Polycheta: Capitellidae). J. Zool., 180: 195-209.

Wass, M.L. (1967), Biological and physiological basis of indicator organisms and communities. In T.A. Olsen and F.J. Burges (eds.), Pollution and marine ecology. John Willey and Sons, N.Y. 212-283.

Watling, L. (1975), Analysis of structural variations in a shallow estuarine deposit feeding community. J. exp. mar. Biol. Ecol., 19: 275-313.

Webster, H.E. (1879), On the Annelida chaetopoda of the Virginian coast. Trans Albany Inst., 9: 202-269.

Webster, H.E. (1886), The Annelida chaetopoda of New Jersey. A. report N.Y. State Mus. nat. Hist., 39: 128-159.

Wells, G.P. (1957), The life of the lug worm. New Biology, 22: 3-19.

Whittaker, J.R. (1959), Localization of haemocyanin on starch gel electrophoresis patterns. Nature, 184: 193-194.

Whittle, A.C. and Zahid, Z.R. (1974), Fine structure of nuchal organs in some errant polychaetous annelids. J. Morph., 114: 167-184.

Wieme, R.J. (1959), Studies on agar-gel electrophoresis. Arscia, Brussels.

Wieme, R.J. (1965), Agar-gel electrophoresis. Amsterdam: Elsevier.

Wilkinson, J.H. (1970), Isoenzymes. Chapman and Hall, London, 369pp.

Wilson, D.P. (1928), The larvae of Polydora ciliata Johnston and Polydora hoplura Claparede. J. mar. biol. Ass. U.K., 15: 567-603.

Wium-Anderson, G. (1970), Haemoglobin and protein variation in three species of Littorina. Ophelia, 8: 267-273.

Woods, K.R., Paulsen, E.C., Engle, R.L. and Pert, J.H. (1958), Starch gel electrophoresis of some invertebrate sera. Science, 127: 519-520.

Woodwick, K.H. (1953), Polydora nuchalis, a new species of polychaetous annelid from California. J. Wash. Acad. Sci., 43: 381-383.

Woodwick, K.H. (1960), Early larval development of Polydora nuchalis Woodwick, a spionid polychaete. Pac. Sci., 14: 122-128.

Woodwick, K.H. (1963), Taxonomic revision of two polydorid species (Annelida, Polychaeta, Spionidae). Proc. biol. Soc. Wash., 76: 209-216.

Wright, C.A. (1959), The application of paper chromatography to a taxonomic study in the molluscan genus Lymnaea. J. Linn. Soc.(Zool.), 44: 222-237.

Wright, C.A. (1974), Techniques. In C.A. Wright (ed.), Biochemical and immunological taxonomy of animals. Academic Press, London, 417-434.

Wright, C.A. and File, S.K. (1968), Digestive gland esterases in the genus Bulinus (Mollusca, Planorbidae). Comp. Biochem. Physiol., 27: 871-874.

Wright, T.R.F. (1963), The genetics of an esterase in Drosophila melanogaster. Genetics, 48: 787-801.

Zottoli, R.A. and Carriker, M.R. (1974), Burrow morphology, tube formation and microarchitecture of shell dissolution by the spionid polychaete, Polydora websteri. Mar. Biol., 27: 307-316.

APPENDICES

APPENDIX I

Stock solutions used for gel preparation:

Deaerated distilled water was used throughout and the solutions were stored in amber glass bottles in a refrigerator with the exception of ammonium persulphate solution which was made fresh each time.

1) Tris-buffer, pH 8.9

Tris*	36.6g
1N HCl	48ml
TEMED*	0.46ml
distilled water to	100ml

Accurate pH was ensured by titration in the HCl to tris and TEMED dissolved in 40ml distilled water.

2) Acrylamide solution,

(A)	Acrylamide	30g
	BIS*	0.8g
	$K_3Fe(CN)_6$	0.015g
	distilled water to	100ml
(B)	Acrylamide	60g
	BIS*	0.8g
	$K_3Fe(CN)_6$	0.015g
	distilled water to	100ml

3) Initiator

Ammonium persulphate	0.14g
distilled water to	100ml

For making gel, 7.5% acrylamide concentration:

1 part tris-buffer

2 parts acrylamide solution (A)

4 parts initiator

1 part distilled water

For making gel, 15% acrylamide concentration:

Acrylamide solution (B) was taken in place of (A).

Electrode buffer:

Tris-glycine, pH 8.3

Glycine	28.8g
Tris*	6.0g
distilled water to	100ml

*Abbreviations:

Tris	Tris(hydroxymethyl)aminomethane
TEMED	N,N,N',N'-tetramethylethylenediamine
BIS	N,N'-methylenebisacrylamide

APPENDIX II

Staining recipes for making 10ml solution:

1. Acid phosphatase

α-naphthyl acid phosphate(Na-salt)	10mg
Fast garnet GBC salt	10mg
0.05M acetate, pH 5.0	10ml

Filter the solution and incubate gel in this for one hour at room temperature.

2. Esterase

Fast blue RR salt	10mg
0.5M tris-HCl, pH 7.0	1ml
α-naphthyl acetate(1% w/v in acetone)	0.5ml
distilled water to	10ml

Filter the solution and incubate gel at room temperature for 30 to 60 minutes. Change the staining solution if it becomes cloudy.

3. Glucose-6-phosphate dehydrogenase

NADP*	7mg
NBT*	5mg
PMS*	0.2mg
Glucose-6-phosphate(disodium salt)	0.03g
0.5M tris-HCl, pH 7.0	1ml
distilled water to	10ml

Incubate gel in dark at 37°C for 30 minutes.

4. Isocitrate dehydrogenase

NADP*	2mg
NBT*	0.8mg
PMS*	1.3mg
Isocitrate(trisodium salt)	30mg
0.05M $MgCl_2$	0.4ml
1M tris-HCl, pH 8	1ml
distilled water to	10ml

Incubate gel in dark at 37°C for 30 to 60 minutes.

5. Lactate dehydrogenase

substrate:

DL-lactic acid (sodium salt)	1ml
0.06M phosphate, 7.5 pH	1ml

Stain:

NAD*	6.6mg
NBT*	3.5mg
PMS*	0.2mg
substrate	2ml
0.5M tris-HCl, pH 7	1ml
distilled water to	10ml

Incubate gel in dark at 37°C for 30 minutes.

6. Leucine aminopeptidase

(A)	L-Leucyl-β-naphthylamide HCl	2mg
	Dimethyl formamide	0.5ml
	0.1M phosphate, pH 7 to	10ml

(B)	Black K salt	10mg
	0.1M phosphate, pH 7	10ml

Incubate gel in solution (A) for 4 hours at room temperature. Pour off solution (A) and replace with solution (B). Incubate in this solution for 15 to 30 minutes at room temperature.

7. Malate dehydrogenase

NAD*	4mg
NBT*	2mg
PMS*	0.6mg
L-malic acid	16mg
0.05M tris-HCl, ph 9.2 to	10ml

Incubate gel in dark at 37°C for 30 minutes.

8. Malic enzyme

NADP*	2mg
NBT*	2mg
PMS*	0.6mg
L-malic acid	16mg
0.05M $MgCl_2$	0.5ml
0.05M tris-HCl, pH 9.2 to	10ml

Incubate gel in dark at 37°C for 1 hour.

9. Peroxidase

o-dianisidine	2mg
0.1M tris-citrate, pH 5	6ml
0.1% (v/v) H_2O_2	2ml

Incubate gel in cold room (10°C) for 30 to 60 minutes.

10. Phosphoglucose isomerase

Fructose-6-phosphate(disodium salt)	14mg
0.05M $MgCl_2$	1ml
Glucose-6-phosphate dehydrogenase (Type XI, Sigma)	10units
NADP*	1.5mg
PMS*	0.4mg
MTT*	1mg
0.04M tris-HCl, pH 8 to	10ml

Incubate gel in dark at 37°C for 15 to 30 minutes.

11. Tetrazolium oxidase

NAD*	2.5mg
NBT*	2mg
PMS*	0.5mg
0.05M tris-HCl, pH 8.5 to	10ml

Incubate gel at room temperature and expose to light until white bands appear on blue background.

12. Xanthine dehydrogenase

NAD*	6.6mg
NBT*	3.5mg
PMS*	0.2mg
Hypoxanthine	20mg
1M tris-HCl, pH 8	2ml
distilled water to	10ml

Incubate gel in dark at 37°C for 1 hour.

*Abbreviations

MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
PMS	Phenazine methosulphate
Tris	Tris(hydroxymethyl)aminomethane

APPENDIX III

Number of Eye spots in different populations of *Polydora*.

Populations	n	No. of eye spots				
		0	1	2	3	4
P. ciliata						
Plymouth	20	10	0	8	2	0
Dumpton	40	15	2	18	3	2
Torbay	24	4	0	12	6	2
Whitstable	39	12	0	9	3	15
Barry	36	18	0	15	3	0
Sully	30	13	2	10	4	1
Robin Hood's B.	20	6	0	12	1	1
Hale	35	5	0	10	3	17
New Brighton	30	5	0	10	3	17
P. ligni						
Peterstone W.	54	4	0	11	12	27
P. limicola						
Tees	46	6	0	10	10	20

APPENDIX IV

Length of caruncle with respect to segment number in different populations of Polydora.

Species and Populations	n	No. of worms with caruncle covering segments			Mean No. of segments covered by caruncle ± S.D.
		1	2	3	
P. ciliata					
Plymouth	20	12	6	2	1.50 ± 0.67
Dumprton	24	18	6	0	1.25 ± 0.43
Torbay	15	9	4	2	1.50 ± 0.74
Whitstable	28	6	18	4	1.93 ± 0.60
Barry	15	10	4	1	1.40 ± 0.61
Sully	20	15	5	0	1.25 ± 0.43
R. Hood's B.	18	10	6	2	1.55 ± 0.68
Hale	18	5	10	3	1.89 ± 0.65
N. Brighton	15	12	3	0	1.20 ± 0.40
P. ligni					
Peterstone W.	25	2	10	13	2.44 ± 0.63
P. limicola					
Tees	23	3	12	8	2.22 ± 0.65

APPENDIX V

Frequency table of the number of heavy spines in different populations of *Polydora*.

Species	Populations	number of heavy spines (X)										n	$\bar{X}$	S.D.
		4	5	6	7	8	9	10	11	12	13			
P. ciliata	Plymouth	2	3	8	2	0	0	0	0	0	0	15	5.7	0.7
	Dumpton	2	7	9	2	0	0	0	0	0	0	20	5.6	0.8
	Torbay	1	6	2	0	0	0	0	0	0	0	9	5.1	0.6
	Whitstable	0	0	2	1	6	7	7	0	0	0	23	8.6	1.2
	Barry	4	7	3	0	0	0	0	0	0	0	14	4.9	0.7
	Sully	4	5	1	0	0	0	0	0	0	0	10	4.7	0.6
	Robin Hood's B.	1	5	2	0	0	0	0	0	0	0	8	5.1	0.5
	Hale	0	2	0	1	3	4	2	0	0	0	12	8.1	1.7
	New Brighton	3	5	2	1	0	0	0	0	0	0	11	5.1	0.9
P. ligni	Peterstone W.	0	0	0	0	0	2	5	8	2	3	20	10.9	1.1
P. limicola	Tees	0	0	0	3	9	5	2	0	0	0	19	8.3	0.9

APPENDIX VI

Mean number ± standard deviation and observed sample range of neuropodial hooded hooks per segment in different populations of *Polydora*.

Populations	n	mean No. of hooded hook	S.D.	observed sample range
P. ciliata				
Plymouth	7	4.8	0.06	4 - 6
Dumpton	18	5.0	0.72	4 - 6
Torbay	12	4.0	0.34	3 - 4
Whitstable	15	8.6	0.78	6 - 10
Barry	9	3.8	0.54	3 - 4
Sully	12	4.1	0.36	3 - 5
R. Hood's B.	8	4.8	0.39	4 - 6
Hale	10	8.0	0.62	6 - 9
New Brighton	14	4.2	0.31	4 - 6
P. ligni				
Peterstone W.	20	10.4	1.20	8 - 18
P. limicola				
Tees	14	8.1	0.82	7 - 11