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

by

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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. phosphoglucone 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 phosphoglucone 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.
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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)
a-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 discovery 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 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 derived 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 anti-serum 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):

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

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 responsible 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 distinguish 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 appropriate. 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: 

$$\begin{align*}
\text{R} \\
\text{NH}_2 & - \text{CH} - \text{COOH}
\end{align*}$$
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 ($-\text{CO.NH}-$) 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) $\text{NH}_2$ will be positively charged ($\text{NH}_2^+$), while acidic ($\text{COO}^-$) ions will be saturated and become neutral ($\text{COOH}$). 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 ($\text{pI}$). 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 appropriate 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 accommodate 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 appropriate 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 hydrolysed 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 comparison 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) **Continuous 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 continuously 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) **Discontinuous 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 continuous system (Wilkinson, 1970). Discontinuous 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 probability 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 underestimate 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 random 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:

\[ S_m = \frac{\text{number of bands of common mobility}}{\text{maximum number of bands in an individual}} \]

Figure 2, for instance, shows general protein patterns in two species. The coefficient of similarity between these two species is equal to \( \frac{4}{10} = 0.4 \). 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'.
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 straightforward. 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 subunit (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 $(a_4, b_4, a_2b_1, a_2b_2, a_1b_3)$ 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).
but differ in electrophoretic mobility are called 'isozymes' (Markert and Möller, 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, $x_n$ the expected frequency of homozygotes is simply $x_1^2 + x_2^2 + x_3^2$ \ldots + $x_n^2$. The expected frequency of heterozygotes is $H = 1 - (x_1^2 + x_2^2 + x_3^2$ \ldots + $x_n^2)$. The overall amount of variation in a population is estimated by the average frequency of heterozygotes per locus ($\bar{H}$). This is simply obtained by averaging $H$ over all loci sampled. $\bar{H}$ 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 ($\bar{P}$) 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 ($\bar{I}$) and genetic distance ($\bar{D}$) are the mean values over all loci studied, including monomorphic ones. Nei's coefficient of genetic identity (I) between two taxa is
given by:

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

where $x_i$ and $y_i$ are the frequencies of the $i$th 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. $T \text{ (years)} = 30 \times 10^6 D$ for slowly evolving loci
2. $T \text{ (years)} = 2.4 \times 10^6 D$ 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 comparison 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 geese). 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.


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 in systematics or population genetics of some invertebrates (excluding insects), arranged in chronological order.

<table>
<thead>
<tr>
<th>Author</th>
<th>Organism</th>
<th>Tissue used</th>
<th>Electrophoretic method</th>
<th>Protein/enzyme assayed</th>
</tr>
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<tbody>
<tr>
<td>Woods et al., 1958</td>
<td>crustacean and mollusc</td>
<td>serum</td>
<td>starch gel</td>
<td>haemocyanin</td>
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<td>crayfish</td>
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<td>starch gel</td>
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<td>coelomic fluid</td>
<td>starch gel</td>
<td>HAEM., EST., PROT.</td>
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<td>Manwell and Baker, 1963b</td>
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<td>serum</td>
<td>vertical starch gel</td>
<td>PROT., HAEM., CER., DO.</td>
</tr>
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<td>starch gel</td>
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<tr>
<td>Manwell et al., 1967</td>
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<td>whole animal</td>
<td>vertical starch gel</td>
<td>EST., ACP., β-GA., PEP.,MDH., ME., G6PD., 6PGD.,TPD., ALD., AMY.</td>
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<td>digestive gland</td>
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<td>EST.</td>
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<td>mollusc</td>
<td>crystalline style</td>
<td>acrylamide gel, slab</td>
<td>PROT.</td>
</tr>
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<td>Electrophoretic method</td>
<td>Protein/enzyme assayed</td>
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<td>Bowen et al., 1969</td>
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<td>haemolymph</td>
<td>agar gel and cellulose acetate</td>
<td>HAEM.</td>
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<td>Coles, 1969a</td>
<td>snail</td>
<td>liver</td>
<td>acrylamide gel</td>
<td>LDH., MDH., β-HDH., α-GPD., GDH., α-ALDH., IDH., G6PD., 6PGD.</td>
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<tr>
<td>Coles, 1969b</td>
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<td>liver</td>
<td>acrylamide gel</td>
<td>ACP., ALP., POX., LAP., β-glucosidase.</td>
</tr>
<tr>
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<td>crab</td>
<td>serum and haemolymph</td>
<td>vertical starch gel</td>
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<td>Reid and Dunnill, 1969</td>
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<td>digestive divericula</td>
<td>horizontal starch gel</td>
<td>EST.</td>
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<td>whole zooid</td>
<td>acrylamide gel in tube</td>
<td>EST., MDH., LAP.</td>
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<td>Jones, 1970</td>
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<td>acrylamide gel in tube</td>
<td>MDH., LDH., α-GPD., GDH., G6PD.</td>
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Table 1. continued

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<th>Author</th>
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<th>Electrophoretic method</th>
<th>Protein/enzyme assayed</th>
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<td>gill, gonad, heart, stomach</td>
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<td>gill, mid-gut gland, testis, hypodermis</td>
<td>acrylamide gel in tube</td>
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<td>horizontal starch gel</td>
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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 isoenzyme 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 Grasse, 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
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 comparison between the six species. Differences 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 polychaetes. 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. muchalis* 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 polychaete 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*, *Carazzi* 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 diagnosis 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 sub-distal 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.
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,
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
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<th>Ch. No.</th>
<th>ciliata</th>
<th>websteri</th>
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<th>nuchalis</th>
<th>limicola</th>
<th>aggregata</th>
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<td>7 or less</td>
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<td>8 - 12</td>
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<td>small</td>
<td>no</td>
<td>small</td>
<td>small</td>
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<tr>
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<td>present</td>
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<td>present</td>
<td>present</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>9.</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>10.</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>11.</td>
<td>0 - 4</td>
<td>0 - 4</td>
<td>4</td>
<td>2</td>
<td>0 - 4</td>
<td>4</td>
</tr>
<tr>
<td>12.</td>
<td>absent</td>
<td>absent</td>
<td>present</td>
<td>present</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>13.</td>
<td>1 or 2</td>
<td>3</td>
<td>3 or 4</td>
<td>2</td>
<td>2 or 4</td>
<td>2</td>
</tr>
<tr>
<td>14.</td>
<td>disc</td>
<td>disc</td>
<td>disc</td>
<td>disc</td>
<td>disc</td>
<td>disc</td>
</tr>
</tbody>
</table>
of 40 - 45 segments and are found in segments 7 - 10 inclusive. Gametes arise from the peritoneum, 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 penetrating 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 exemplified 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 exemplified 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 be as short as a few hours and may even be omitted (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 suitable 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).
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‰ salinity and with aeration. **Polydora** survive 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
• P. ciliata
○ P. ligni
■ P. limicola
<table>
<thead>
<tr>
<th>Species</th>
<th>Date</th>
<th>Locality</th>
<th>Grid reference</th>
<th>Habitat</th>
<th>Tide level*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. ciliata</em></td>
<td>Feb. 80</td>
<td>Plymouth, Jennycliff</td>
<td>SX 491 525</td>
<td>limpet shell</td>
<td>MTL</td>
</tr>
<tr>
<td></td>
<td>Sept. 80/Nov. 81</td>
<td>Dumpton, Pegwell Bay</td>
<td>TR 398 667</td>
<td>limestone</td>
<td>MLWN</td>
</tr>
<tr>
<td></td>
<td>Oct. 80</td>
<td>Torbay</td>
<td>SX 944 636</td>
<td>limpet shell</td>
<td>MTL</td>
</tr>
<tr>
<td></td>
<td>Oct. 80/March 81</td>
<td>Whitstable</td>
<td>TR 101 671</td>
<td>mud flat</td>
<td>MLWS</td>
</tr>
<tr>
<td></td>
<td>April 81</td>
<td>Barry, Friars point</td>
<td>ST 112 660</td>
<td>rock</td>
<td>MLWN</td>
</tr>
<tr>
<td></td>
<td>April 81</td>
<td>Sully, Swanbridge Bay</td>
<td>ST 165 672</td>
<td>rock</td>
<td>MLWN</td>
</tr>
<tr>
<td></td>
<td>June 81</td>
<td>Robin Hood's Bay</td>
<td>SE 955 055</td>
<td>limpet shell</td>
<td>MTL</td>
</tr>
<tr>
<td></td>
<td>July 81</td>
<td>Hale, Mersey estuary</td>
<td>SJ 481 813</td>
<td>mud flat</td>
<td>MLWN</td>
</tr>
<tr>
<td></td>
<td>July 81</td>
<td>New Brighton</td>
<td>SJ 313 942</td>
<td>mussel shell</td>
<td>MLWN</td>
</tr>
<tr>
<td><em>P. ligni</em></td>
<td>April 81</td>
<td>Peterstone Wentlooge</td>
<td>ST 271 792</td>
<td>mud flat</td>
<td>MLWS</td>
</tr>
<tr>
<td><em>P. limicola</em></td>
<td>June 81</td>
<td>Tees estuary</td>
<td>NZ 533 266</td>
<td>mud flat</td>
<td>MLWN</td>
</tr>
</tbody>
</table>

*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 3½ and 4½ 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 ± 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*).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Locus</th>
<th>M/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>ACP-2</td>
<td>M/P</td>
</tr>
<tr>
<td></td>
<td>ACP-3</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>ACP-4</td>
<td>M</td>
</tr>
<tr>
<td>Esterase</td>
<td>EST-2</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>EST-3</td>
<td>M/P</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>G6PD</td>
<td>M</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>MDH-1</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>MDH-2</td>
<td>M</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>ME-1</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>ME-2</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>ME-3</td>
<td>M</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>PGI</td>
<td>M/P</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>XDH</td>
<td>M</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>LDH</td>
<td>*</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>IDH</td>
<td>*</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>POX</td>
<td>*</td>
</tr>
<tr>
<td>Tetrazolium oxidase</td>
<td>TO</td>
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</tr>
</tbody>
</table>

* 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 Figure 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)
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
Plymouth  
\(n = 20\)

Dumpton  
\(n = 40\)

Torbay  
\(n = 24\)

Whitstable  
\(n = 39\)

Barry  
\(n = 36\)

Sully  
\(n = 30\)

Robin Hoods B.  
\(n = 20\)

Hale  
\(n = 35\)

N. Brighton  
\(n = 30\)

\(P. ciliata\)

Peterstone W.  
\(n = 54\)

Tees  
\(n = 46\)

\(P. ligni\)

\(P. limicola\)
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
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.
Figure 17. *P. ligni*; scanning electron micrographs of enlarged nuchal antenna. (1400 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 comparison 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 'postero-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 comparison to those *P. ciliata* populations which live in mud and to *P. ligni* and *P. limicola*. There was up to 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*.


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

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.
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 'posterior-ventral' bundle of setae on the fifth enlarged segment (1,700 x)
Figure 22. *E. ciliata* (from Barry); scanning electron micrograph of a heavy spine of the fifth enlarged segment.

HS. heavy spine; CS. companion setae

(2,500 x)
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.
Figure 24. Average number of hooded hooks per segment in different populations of *Polydora* species with standard error of the mean.

10. *P. ligni*
11. *P. limicola*
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*.

<table>
<thead>
<tr>
<th>Populations</th>
<th>n</th>
<th>anterio-dorsal</th>
<th>posterio-ventral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>present %</td>
<td>absent %</td>
</tr>
<tr>
<td><strong>P. ciliata</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plymouth</td>
<td>20</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Dumpton</td>
<td>24</td>
<td>91.7</td>
<td>8.3</td>
</tr>
<tr>
<td>Torbay</td>
<td>15</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Whitstable</td>
<td>28</td>
<td>89.3</td>
<td>10.7</td>
</tr>
<tr>
<td>Barry</td>
<td>15</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Sully</td>
<td>20</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Robin Hood’s B.</td>
<td>18</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hale</td>
<td>18</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>New Brighton</td>
<td>15</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>P. ligni</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peterstone W.</td>
<td>25</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td><strong>P. limicola</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tees</td>
<td>23</td>
<td>87.0</td>
<td>13.0</td>
</tr>
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</table>
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 25a).
   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 25b) 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 25c). 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 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 comparisons 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 individuals. 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.
Figure 28. Photograph of malate dehydrogenase zymogram of

\[ \text{P. ciliata, 1 - 3; P. ligni, 4 and P. limicola, 5.} \]

Figure 29. Photograph of xanthine dehydrogenase zymogram of

\[ \text{P. ciliata, 1 - 3; P. ligni, 4 and P. limicola, 5.} \]
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-2⁹₆ and ACP-2⁹₄, 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-2⁹₆ 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)
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.
Table 6. Summary of gene and genotype distributions and heterozygosities for the ACP-2 locus for four populations of *Polydora* which were polymorphic.

<table>
<thead>
<tr>
<th>Species &amp; Population</th>
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<th>H_o</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ACP-2⁹⁸</td>
<td>ACP-2⁹⁴</td>
<td>ACP-2⁹⁸</td>
<td>ACP-2⁹⁸/⁹⁴</td>
<td>ACP-2⁹⁴</td>
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<tr>
<td><em>P. ciliata</em> Whitstable</td>
<td>20</td>
<td>0.450</td>
<td>0.550</td>
<td>4</td>
<td>10</td>
<td>6</td>
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<tr>
<td><em>P. ciliata</em> Hale</td>
<td>17</td>
<td>0.529</td>
<td>0.471</td>
<td>5</td>
<td>8</td>
<td>4</td>
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<tr>
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<td>0.630</td>
<td>3</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td><em>P. limicola</em> Tees</td>
<td>20</td>
<td>0.400</td>
<td>0.600</td>
<td>3</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>
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)

<table>
<thead>
<tr>
<th></th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Plymouth</td>
<td>0.000</td>
<td>0.000</td>
<td>0.457</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.292</td>
<td>0.000</td>
<td>0.681</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td>2. Dumpton</td>
<td>1.000</td>
<td>0.000</td>
<td>0.457</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.292</td>
<td>0.000</td>
<td>0.681</td>
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</tr>
<tr>
<td>3. Torbay</td>
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<td>1.000</td>
<td>0.457</td>
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<tr>
<td>4. Whitstable</td>
<td>0.633</td>
<td>0.633</td>
<td>0.633</td>
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<td>0.457</td>
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<td>0.012</td>
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<tr>
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<td>1.000</td>
<td>1.000</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.292</td>
<td>0.000</td>
<td>0.681</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td>6. Sully</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.633</td>
<td>1.000</td>
<td>0.000</td>
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<tr>
<td>7. Robin Hood's B.</td>
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<td>1.000</td>
<td>1.000</td>
<td>0.633</td>
<td>1.000</td>
<td>1.000</td>
<td>0.292</td>
<td>0.000</td>
<td>0.681</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td>8. Hale</td>
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<td>0.747</td>
<td>0.747</td>
<td>0.988</td>
<td>0.747</td>
<td>0.747</td>
<td>0.747</td>
<td>0.292</td>
<td>0.041</td>
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<tr>
<td>9. N. Brighton</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.633</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.747</td>
<td>0.681</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td>10. Peterstone W.</td>
<td>0.506</td>
<td>0.555</td>
<td>0.995</td>
<td>0.555</td>
<td>0.555</td>
<td>0.555</td>
<td>0.968</td>
<td>0.555</td>
<td>0.998</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The ACP-3 (band stained brown) and ACP-4 (band stained reddish brown) loci were monomorphic for type (A) and type (B) respectively.

6. Phosphoglucone isomerase (PGI):

Figure 31 presents three types of patterns obtained. The type (A) was found in *P. ligni* and two populations 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*.

<table>
<thead>
<tr>
<th>Species &amp; population</th>
<th>n</th>
<th>gene frequency</th>
<th>genotype</th>
<th>He</th>
<th>H₀</th>
<th>X²</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PGI³</td>
<td>PGI¹³</td>
<td>PGI²⁰</td>
<td>PGI³</td>
<td>PGI⁷³</td>
</tr>
<tr>
<td><em>P. ciliata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plymouth</td>
<td>20</td>
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<td>1.000</td>
<td>0.000</td>
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<td>0</td>
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<tr>
<td>Dumpton</td>
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<td>0.000</td>
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<td>0</td>
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<tr>
<td>Torbay</td>
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<td>0.000</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Whitstable</td>
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<tr>
<td>Barry</td>
<td>16</td>
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<td>Sully</td>
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<tr>
<td>Robin Hood's B</td>
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<tr>
<td>N. Brighton</td>
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<tr>
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<td>0.273</td>
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Table 9. Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of PG1 locus among eleven populations of Polymuns (P. citella, 1 to 9; P. limni, 10; P. linfolia, 11.)

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<td>1.000 &amp; 0.000</td>
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<tr>
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<tr>
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<td>1.000 &amp; 0.000</td>
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<td>Peterstone W.</td>
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<td>0.000 &amp; 0.000</td>
<td>0.000 &amp; 0.000</td>
<td>0.000 &amp; 0.000</td>
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<td>0.936 &amp; 0.064</td>
<td>0.936 &amp; 0.064</td>
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</table>
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\textsuperscript{142} and EST-2\textsuperscript{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)
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 hydrolyzed 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 chosen 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).
Table 10. Summary of gene and genotype distributions and heterozygosities for the EST-2 locus for different populations of *P. ciliata*.

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<td>EST-2&lt;sup&gt;140&lt;/sup&gt;</td>
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<td>0.600</td>
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<td>17</td>
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<tr>
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<td>0.540</td>
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<td>11</td>
<td>0.497</td>
<td>0.500</td>
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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).

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

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Table 13: Estimates of genetic identity (below diagonal) and genetic distance (above diagonal) of EST-5 locus among eleven populations of Polystoma (P. ciliata, 1 to 9; P. ligma, 10; P. limicol, 11).
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 bores in 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 isocitrate dehydrogenase (IDH) and tetrazolium oxidase (TO) in Polydora ciliata.
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).

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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tbody>
<tr>
<td>1. Plymouth</td>
<td>0.001</td>
<td>0.001</td>
<td>0.358</td>
<td>0.001</td>
<td>0.002</td>
<td>0.337</td>
<td>0.007</td>
<td>0.349</td>
<td>0.233</td>
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<tr>
<td>2. Dumpton</td>
<td>0.999</td>
<td>0.006</td>
<td>0.361</td>
<td>0.011</td>
<td>0.003</td>
<td>0.337</td>
<td>0.007</td>
<td>0.349</td>
<td>0.233</td>
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<tr>
<td>3. Torbay</td>
<td>0.999</td>
<td>0.994</td>
<td>0.358</td>
<td>0.001</td>
<td>0.001</td>
<td>0.349</td>
<td>0.261</td>
<td>0.239</td>
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<tr>
<td>4. Whitstable</td>
<td>0.699</td>
<td>0.697</td>
<td>0.699</td>
<td>0.365</td>
<td>0.361</td>
<td>0.384</td>
<td>0.037</td>
<td>0.080</td>
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<tr>
<td>5. Barry</td>
<td>0.999</td>
<td>0.989</td>
<td>0.999</td>
<td>0.694</td>
<td>0.001</td>
<td>0.361</td>
<td>0.032</td>
<td>0.380</td>
<td>0.247</td>
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<tr>
<td>6. Sully</td>
<td>0.999</td>
<td>0.997</td>
<td>0.999</td>
<td>0.697</td>
<td>0.999</td>
<td>0.029</td>
<td>0.349</td>
<td>0.247</td>
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<tr>
<td>7. Robin Hood's B.</td>
<td>0.998</td>
<td>0.990</td>
<td>0.971</td>
<td>0.681</td>
<td>0.971</td>
<td>0.349</td>
<td>0.001</td>
<td>0.247</td>
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<tr>
<td>8. Hale</td>
<td>0.714</td>
<td>0.714</td>
<td>0.705</td>
<td>0.697</td>
<td>0.705</td>
<td>0.705</td>
<td>0.345</td>
<td>0.076</td>
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<tr>
<td>9. N. Brighton</td>
<td>0.993</td>
<td>0.993</td>
<td>0.997</td>
<td>0.686</td>
<td>0.999</td>
<td>0.708</td>
<td>0.357</td>
<td>0.241</td>
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<tr>
<td>10. Peterstone W.</td>
<td>0.705</td>
<td>0.705</td>
<td>0.694</td>
<td>0.993</td>
<td>0.697</td>
<td>0.995</td>
<td>0.700</td>
<td>0.075</td>
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<td></td>
<td></td>
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<tr>
<td>11. Tees</td>
<td>0.792</td>
<td>0.792</td>
<td>0.787</td>
<td>0.923</td>
<td>0.781</td>
<td>0.781</td>
<td>0.927</td>
<td>0.786</td>
<td>0.928</td>
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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.

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<tr>
<td>1. <em>P. ciliata</em> 'boring'</td>
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<tr>
<td></td>
<td>0.357</td>
<td>0.361</td>
<td>0.241</td>
<td></td>
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<tr>
<td>2. <em>P. ciliata</em> 'non-boring'</td>
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<tr>
<td></td>
<td>0.700</td>
<td>0.005</td>
<td>0.007</td>
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<tr>
<td>3. <em>P. ligni</em></td>
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<tr>
<td></td>
<td>0.697</td>
<td>0.995</td>
<td>0.075</td>
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<tr>
<td>4. <em>P. limicola</em></td>
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<tr>
<td></td>
<td>0.786</td>
<td>0.925</td>
<td>0.928</td>
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Table 16. Average heterozygosity ($\bar{H}$) per locus in different populations of *Polydora*.

<table>
<thead>
<tr>
<th>Species &amp; population</th>
<th>Average heterozygosity</th>
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<tr>
<td></td>
<td>expected</td>
<td>observed</td>
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<tr>
<td><strong>P. ciliata</strong></td>
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<td></td>
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<tr>
<td>Plymouth</td>
<td>0.038</td>
<td>0.031</td>
</tr>
<tr>
<td>Dumpton</td>
<td>0.038</td>
<td>0.035</td>
</tr>
<tr>
<td>Torbay</td>
<td>0.035</td>
<td>0.024</td>
</tr>
<tr>
<td>Whitstable</td>
<td>0.127</td>
<td>0.132</td>
</tr>
<tr>
<td>Barry</td>
<td>0.030</td>
<td>0.028</td>
</tr>
<tr>
<td>Sully</td>
<td>0.035</td>
<td>0.028</td>
</tr>
<tr>
<td>Robin Hood's B.</td>
<td>0.021</td>
<td>0.014</td>
</tr>
<tr>
<td>Hale</td>
<td>0.127</td>
<td>0.121</td>
</tr>
<tr>
<td>New Brighton</td>
<td>0.027</td>
<td>0.026</td>
</tr>
<tr>
<td><strong>P. ligni</strong></td>
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<td></td>
</tr>
<tr>
<td>Peterstone W.</td>
<td>0.126</td>
<td>0.108</td>
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<tr>
<td><strong>P. limicola</strong></td>
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<td></td>
</tr>
<tr>
<td>Tees</td>
<td>0.143</td>
<td>0.141</td>
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is produced (Figure 38). The phenogram shows the relationships 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

Pligni

P. ligni (non boring)

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 comparisons.

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 Ureprung, 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 posterior-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 posterior-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 Carrier (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 pennonned 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 pennoned 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 characteristically 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 than 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/few individuals with two nuchal antennae from Fort Pierce and Fort Desoto. The presence of two nuchal antennae is a characteristic 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 specific factors 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 own results and my / 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-Schrüder (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 frequencies 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), occurring 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 occurred 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 of enzymes in mud crabs from south Carolina, Turner and 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 \( (I = 0.997) \). 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 unusual 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 unsuccessful. 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 Palinscar (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).
<table>
<thead>
<tr>
<th>Taxonomic level</th>
<th>$\bar{I}$</th>
<th>$\bar{D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local populations</td>
<td>0.970 ± 0.006</td>
<td>0.031 ± 0.007</td>
</tr>
<tr>
<td>Sub species</td>
<td>0.795 ± 0.013</td>
<td>0.230 ± 0.016</td>
</tr>
<tr>
<td>Semi species</td>
<td>0.798 ± 0.026</td>
<td>0.226 ± 0.033</td>
</tr>
<tr>
<td>Sibling species</td>
<td>0.536 ± 0.023</td>
<td>0.581 ± 0.039</td>
</tr>
<tr>
<td>Non-sibling species</td>
<td>0.352 ± 0.023</td>
<td>1.056 ± 0.068</td>
</tr>
</tbody>
</table>
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 beginning 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
'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 in an 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. Phosphoglucone 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.

was obtained from Plymouth, offshore, in February, 1980. 

*N. vires* was collected from Whitstable and Blyth in May and June, 1980 respectively.

Live worms were kept in the aquarium at 10 - 15°C. *N. vires* 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. Phosphoglucone isomerase (PGI)
Figure 41. Map showing locations of *Nereis diversicolor*, *N. fucata*, and *N. virens* populations sampled.

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 phosphoglucone isomerase (PGI) and tetrazolium oxidase (TO) zymograms of different populations of $A. \text{marina}$.


Figure 43. Diagram of leucine aminopeptidase zymogram of different populations of $A. \text{marina}$.

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


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

mobility. The majority had a mean mobility of $92 \pm 0.7$ (S.D.), but a few worms showed a mean mobility $94 \pm 0.5$ (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 aminopeptidase and lactate dehydrogenase stained as a single band in all the populations of the three species of _Nereis_. Their average mobilities were $91 \pm 0.3$ (S.D.), $98 \pm 0.4$ (S.D.) and $96 \pm 0.3$ (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 $90 \pm 0.04$ (S.D.) while _N. fucata_ had a band with a mean mobility of $95 \pm 0.3$ (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
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)
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 variability 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 characteristic 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
conditions that 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 phosphoglucone 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.
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REFERENCES


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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

<table>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris*</td>
<td>36.6g</td>
</tr>
<tr>
<td>1N HCl</td>
<td>48ml</td>
</tr>
<tr>
<td>TEMED*</td>
<td>0.46ml</td>
</tr>
<tr>
<td>distilled water to 100ml</td>
<td>100ml</td>
</tr>
</tbody>
</table>

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

2) Acrylamide solution,

(A) Acrylamide

<table>
<thead>
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</thead>
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<tr>
<td>BIS*</td>
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</tr>
<tr>
<td>K\textsubscript{3}Fe(CN)\textsubscript{6}</td>
<td>0.015g</td>
</tr>
<tr>
<td>distilled water to 100ml</td>
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(B) Acrylamide

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<tr>
<td>BIS*</td>
<td>0.8g</td>
</tr>
<tr>
<td>K\textsubscript{3}Fe(CN)\textsubscript{6}</td>
<td>0.015g</td>
</tr>
<tr>
<td>distilled water to 100ml</td>
<td>100ml</td>
</tr>
</tbody>
</table>
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, 1% 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
TEMD N,N,N,N-tetramethylethylenediamine
BIS  N,N-methylenebisacrylamide
APPENDIX II

Staining recipes for making 10ml solution:

1. Acid phosphatase
   \( \alpha\)-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 BB salt 10mg
   0.5M tris-HCl, pH 7.0 1ml
   \( \alpha\)-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 MgCl₂ 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  
0.1M phosphate, pH 7  
10mg  
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 MgCl₂  
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) H₂O₂  
2ml

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

Fructose-6-phosphate (disodium salt)  14mg
0.05M MgCl₂  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*.

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<tr>
<th>Populations</th>
<th>n</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
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<tr>
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<td>2</td>
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<tr>
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<td>0</td>
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<tr>
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<td>2</td>
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<td>1</td>
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APPENDIX IV

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

<table>
<thead>
<tr>
<th>Species and Populations</th>
<th>n</th>
<th>No. of worms with caruncle covering segments</th>
<th>Mean No. of segments covered by caruncle ± S.D.</th>
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<td>Torbay</td>
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<td>18</td>
</tr>
<tr>
<td>Barry</td>
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<td>10</td>
<td>4</td>
</tr>
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</table>
APPENDIX V

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

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<th>Species</th>
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<th>( \bar{X} )</th>
<th>S.D.</th>
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</thead>
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APPENDIX VI

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

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<th>Populations</th>
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<th>observed sample range</th>
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