Aspects of the Genetics and Taxonomy of Marine Nemerteans.

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor of Philosophy.

by

Alex David Rogers B.Sc. Honours

(University of Liverpool)

Port Erin Marine Laboratory,

Department of Environmental and Evolutionary Biology,

School of Life Sciences,

University of Liverpool,

Port Erin,

Isle of Man,

British Isles.

December, 1992.

"It's only when you miss the connection you know how vital it is,

or when something slips through your fingers you know how precious it is"

The Chameleons

Contents

Acknowledgements

It's not difficult to know where to start with this but it is difficult to know where to finish. I owe such a great deal to my two supervisors it is difficult to put the right words on paper. I don't think I could have had two friendlier bosses than Ray and John. Ray - who so kindly welcomed me to the 'TEAM' back in 1989 and with whom I've shared several adventures to darkest North Wales and finally to New Zealand in '92. What a trip! Hunting tree frogs and ghost crabs at one in the morning on a tropical island - Speight's, Bintang and Johnny Walkers in profusion (Whoops! Sorry Land Rover - but you did say we were all insured to drive). Ray was my initial source of my fascination in nemerteans (at least one person read your book) and I now count him well and truly amongst friends. I look forward to many future years joint work on our not so insignificant slimey beasts that "look like something that has just come out of your nose" (Cheers Trevor).

John likewise deserves a medal for putting up with my cries of "Wow! Another species - Oh - perhaps not" and still providing constant encouragement. In particular I must thank him for putting up with the chocolate devouring deviant that so messily occupied an enclave in the corner of his room in the latter part of this wormy work. Again I hope our paths remain closely connected in the future - especially since John's humour is nearly as warped as mine.

Without the N.E.R.C.'s grant GR3/7168 I would have been severely skint over the past three years - I thank them for their support over the duration of our three years work on nemerteans of which the partial result is this tome.

I must also thank Trevor Norton for the use of his hard won facilities at Port Erin. May the place remain as it is for many years despite the current climate in which everything is measured in terms of cash and people seem little interested in the pursuit of truth (gosh where did that come from!). Oh and by the way thanks for your endearing comments on nemerteans - I'll take them as near a compliment to my fine beasts as an algal ecologist can manage!

While I'm about the lab I must thank all the so-called non-academic staff of

PEML. You've made this place such a pleasant place to work you wouldn't believe (it!). I am thoroughly indebted to Pat and Barbara (I'm still not sure how that's spelt). Pat has maintained sanity despite all my efforts over the last year to pester her round the bend and back. Thanks especially for 'keeping me' over the viva (and Brian, Dave and Chris) Your all welcome down South anytime. Barbara likewise has never failed to provide help when I've needed it (usually in a panic). Thanks Derek A. for all your help in such a difficult job - especially in the area of the electrophoresis labs. Joyce, Gloria and Joyce - what can I say but who could fail to be cheerful when faced with your smiles (and may be singing) in the morning. The same goes for Eddy, Woody, Beau, Derek and Chris. I hope to see you all in the future.

Special thanks must go to Mike Bates - who dived himself to destruction on our Azores adventure. Ben and I sincerely apologise for the Piri Piri in Lisbon - NOT! No - I'm joking. I have great admiration for Mike's endless enthusiasm for a job which over the winter months must on occasions be less than inspiring - I'd dive with you anytime (but would you dive with me!).

Concerned with the aforementioned adventure I must bring in the A team - Steve Hawkins who provided me with my first opportunity of overseas work - I'm sure it won't be the last - and thanks for allowing the deviant to set up temporary den in Thie Ny Grumpy. It's a shame I never got that photo during a certain car chase in the early hours across Faial. To Ben with whom I have shared several often bizarre trips involving large dark women, sausage eaters from hell, haggis's from hell, worms and a rather dubious green Land Rover. I have also shared a RIB with him and Dickey - which though a pain at times was truly great at others. You're both good mates and better keep in touch (for the next expedition). Also thanks to Elaine - one of the truly nice people of this world and another Azorean veteran and of course Richard - whose coolness as he tried to shake Ben off of his expensive minirover was amazing. Richard also deserves a meddle for reading this thesis and for his retension comments. At this point I must mention Ricardo, Edwardo and Noberto (even if at 5ft nothing he had the cheek to call me Lettle Alex). Of course I must also mention Olavo - who was so tragically killed after the expedition. I think he touched us all with his kindness and

generosity and I'll remember him always.

To friends past and present I must say thanks for bearing with me. No person is an island and I thrive off the good company you've all provided. To the postgraddys - and associates Marcus, Stuart, Stuart, Andy, Pol, Helena, Bruce, Bill, Ulli, Joel, Jill, Ben, Dickey (again), Dave, Rowan, Kirsty, Debbie, Bev, Jan, Chris, Sara L., Sarah D., Sarah P.,Sarah B. Wanni (good luck my friend), Richard (or is it Ri..... no better not) Tim E., Tim H., Terry, Salma, Marcia, Kate, EZ, Graeme, Sean, John B. To the lecturers not so far mentioned - Andy B., Richard H., Audrey G, John S., Joe J. and Don W.

Special mentions not so far made must go to the terrible trio and occassional quad Jim, Graham, Sara and Phil. What are you like - come and see me or else. Also to Ruth, Dave and again Sara who helped me when I needed it badly - you're very dear to me.

Also to the current honours year with whom I've had such a great time in the short time I've known you - I won't go into all the names but you know who you are - I count you all as friends and look forward to seeing you again. What a good bunch of people you really are.

Last but absolutely not last I must thank my Mum and Dad (and Tracey) who provided me with such an invaluable fascination into all things natural at such a young age. Who also provided me with unceasing encouragement throughout my academic career. I think this work must be dedicated to you in the end.

Aspects of the genetics and taxonomy of marine nemerteans.

A.D. Rogers

Abstract

Starch gel electrophoresis was used to investigate the taxonomy and population genetics of several species of common littoral or shallow sublittoral nemerteans found around the North Atlantic coasts of the U.S.A., Canada, Sweden and Europe.

Levels of heterozygosity were estimated for eight heteronemertean and a single hoplonemertean species over a maximum of 13 enzyme loci. Mean observed heterozygosity was found to range between 0.044 for *Lineus ruber* (a low value for marine invertebrates) to 0.278 for *Riseriellus occultus* (a high level of genetic variability). For most species investigated, mean observed fell in a range between 0.08 - 0.12, values which are within the normal range for invertebrate phyla.

Genotype frequency data was used to investigate the genetic structure of populations of four common species of nemertean found around the North Atlantic. These were the heteronemerteans *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and the hoplonemertean *Amphiporus lactifloreus*. These species all undergo a form of development which does not include a pelagic larval phase and *Lineus sanguineus* is reputed to only reproduce asexually by fragmentation. Contrary to expectations from other workers, genetic data suggested that in at least two species, *Lineus ruber* and *Lineus viridis* moderate levels of gene-flow were maintained between conspecific populations over a distance of 6,000 km or more. Passive dispersal is suggested as a possible explanation for this.

Lineus sanguineus did not show genetic evidence of asexual reproduction (e.g. a large deviation from genotype frequencies expected under Hardy-Weinberg equilibrium conditions i.e. large F_{IS} values).

A cryptic species and new genus of heteronemertean, *Riseriellus occultus*, discovered by use of electrophoresis, is described. This species resembles two known species of heteronemertean, *Lineus longissimus* and *Lineus viridis*. Genetic evidence for another cryptic species found occurring sympatrically with populations of *Lineus*

ruber and *Lineus viridis* is presented. A new species of palaeaonemertean, *Callinera monensis*, discovered in a harbour on the Isle of Man, is described.

A genetic investigation in to how related members of the genera *Lineus* and *Micrura* were revealed very few common alleles between congeneric species. It is proposed that this is due to fundamental flaws in the taxonomy of the Heteronemertea and that the order is in need of urgent revision.

Chapter One

General Introduction

1.1 The Phylum Nemertea; Biology, Ecology and Commercial Importance.

Nemerteans comprise a phylum of elongate vermiform animals with soft bodies capable of extreme contraction and elongation (Gibson, 1982b). They are bilaterally symmetrical, generally unsegmented, and vary in length from a few millimetres (Gibson, 1982a) to over 30 meters (McIntosh, 1873-74) in the case of *Lineus longissimus* Gunnerus 1770. Nemerteans vary in colour from dull uniform hues to bright and often intricate patterns of yellow, orange, red and green (e.g. *Quasilineus pulcherrimus* Gibson 1981).

The anatomical features which distinguish nemerteans from other phyla may be summarised as follows:

They possess an eversible muscular proboscis housed, when retracted, in a unique dorsal fluid-filled chamber, the rhynchocoel (see Gibson, 1972, 1982b). This proboscis may be armed, as in the members of the order Enopla, with a single large central stylet (see Stricker & Cloney, 1982; Stricker, 1983, 1984) or a pad of smaller more conical stylets. Alternatively it may be unarmed, as in members of the order Anopla, but furnished with large numbers of epithelial barbs (Jennings & Gibson, 1969). The proboscis is used almost exclusively for the capture of prey (Gibson, 1972), though it may be used as a defensive weapon (Gibson, 1972) and in a few species for specialised locomotion (Moore & Gibson, 1985).

A ciliated alimentary tract with separate mouth and anus. This may simply comprise an unspecialised straight-through gut, but in most species it is morphologically and functionally divisible into foregut and intestine (Kirsteuer, 1967; Jennings & Gibson, 1969). Most nemertean species have intestinal diverticula and some species have additional caeca (Gibson, 1972).

A closed blood-vascular system which runs through the parenchymatous connective tissue of the body. The system may comprise a pair of lateral longitudinal vessels connected anteriorly and posteriorly by cephalic and anal loops respectively, as in the cephalothricid archinemerteans. It may be more complicated, with the addition of further

longitudinal vessels, transverse connectives and vascular networks (see Gibson, 1985). The blood-vascular system of nemerteans, with its increased capabilities of internal transport and circulation, has allowed the animals to become large, robust, very active and capable of complex feeding and digestive activities (Brusca & Brusca, 1990).

A well developed nervous system consisting of a pair of bilobed cerebral ganglia, connected by dorsal and ventral transverse commissures. They also have a pair of ganglionated longitudinal nerve cords and a complex of minor nerves (Gibson, 1982b).

A ciliated epidermis and a body wall composed of a number of longitudinal and circular muscle layers. The arrangement of these muscle layers and the position of the nervous system with respect to them are considered to be important in the higher classification of nemerteans (Gibson, 1982a).

Until recently the phylum has been regarded as an acoelomate group derived from the turbellarian flatworms (Hyman, 1951). Evidence for this theory came from morphological similarities between the two phyla, observed by light microscopy and also from serological tests (Schepotieff, 1913). Recent ultrastructural studies of the integument indicate that nemerteans have little in common with turbellarians (Norenberg, 1985). Furthermore, ultrastructural studies of the muscle cells, development of the rhynchocoel, development of gonads and the organisation of the blood vessels (Turbeville & Ruppert, 1985) have suggested that nemerteans have evolved from coelomate rather than acoelomate ancestors. Results from ribosomal RNA sequencing support this hypothesis (Turbeville *et al.*, 1992). In such a case the rhynchocoel, digestive and blood circulatory systems of nemerteans are regarded as remnants of an ancestral coelom.

Over one thousand species of nemerteans have now been described from different parts of the world (Prof. R. Gibson *pers. comm.*, Liverpool John Moores University, Department of Natural Sciences, Byrom Street, Liverpool, L3 3AF). It is likely that this represents only a fraction of the true number of species that exist, since the nemertean faunas of many regions have not been studied. Most nemerteans are free living organisms which commonly occur littorally and sublittorally beneath boulders, in crevices, on algal holdfasts, amongst algal fronds, on colonial sessile invertebrates and on or in gravel beds

(Gibson, 1982b). Many species also burrow in mud or sand and some are interstitial (Kirsteuer, 1971; Norenburg, 1988). They have been shown to form a substantial component in the fauna of many marine ecosystems (e.g. Gibson, 1983; Koukouras & Russo, 1991; Grassle & Maciolek, 1992). Nemerteans have successfully invaded freshwater and terrestrial environments (Pantin, 1969; Moore & Gibson, 1985; Gibson & Moore, 1989) and one group is entirely pelagic (Gibson, 1982a)

With the exception of a few commensal species nemerteans are believed to be almost exclusively carnivores and scavengers. The few ecological studies on nemerteans have shown that they are important predators in many littoral and subtidal benthic ecosystems.

Roe (1970, 1976, 1979) studied the feeding behaviour and ecology of the intertidal hoplonemertean, *Paranemertes peregrina* Coe 1901 which was found to emerge on to mudflats when the tide recedes and to feed preferentially on nereid polychaetes. Predation by *Paranemertes* was found to predate significant proportions of populations of nereid species in some areas to the extent that their recruitment was lowered. Roe suggested such an effect on recruitment could help maintain the densities of other polychaete species in the community. Nordhausen (1988) observed similar effects on experimental populations of nereid polychaetes on tidal flats in the Wadden Sea following predation by the heteronemertean *Lineus viridis* Müller 1774. In Britain nereid polychaetes have been shown to be an important food source to certain species of wading birds (Goss-Custard *et al.*, 1977).

Bartsch (1973) studied feeding of the hoplonemertean *Tetrastemma melanocephalum* Johnston 1837 on the amphipod *Corophium volutator* Pallas on mudflats. She estimated that *Tetrastemma* consumed over 10,000 amphipods m^{-2} $month^{-1}$. The high density of *Corophium* (118,000 m^{-2}) could certainly support such levels of predation. McDermott (1984) studied the feeding biology of another hoplonemertean, *Nipponnemertes pulcher* Johnston 1837 on the amphipods *Haploops tubicola* Liljeborg and *Haploops tenuis* Kanneworff in the Danish Øresund. The study suggested that *Nipponnemertes* also had a significant impact on its amphipod prey species.

McDermott (1976) also described predation of the razor clam, *Ensis directus* Conrad by the heteronemertean, *Cerebratulus lacteus* Leidy 1851 in New Jersey and North Carolina, U.S.A.. Rowell & Woo (1990) carried out further studies on predation by *Cerebratulus lacteus* but on a commercially important species of bivalve, *Mya arenaria* Linnaeus in Nova Scotia. They found that when *Mya* populations were reduced by fishing and recruitment was lowered by environmental factors, large populations could be devastated by *Cerebratulus lacteus*. Foraging efficiency in *Cerebratulus lacteus* was a contributory factor in its ability to destroy clam populations since the nemertean was able to locate prey even when present at very low densities.

A number of studies on benthic ecology in Antarctica have reported on the feeding activities of *Parborlasia corrugatus* McIntosh 1876. *Parborlasia corrugatus* is an extremely large nemertean, reaching lengths of up to 2 meters (Andriashev, 1966; Knox, 1970) and ranging in weight from 15.1 to 103.9g (Dearborn, 1965; Pearse & Giese, 1966). This species is both a necrophagous scavenger and a voracious predator (Gibson, 1983; Heine *et al.*, 1991) and like *Cerebratulus lacteus* it is very efficient at locating potential prey at a distance. Dayton *et al.* (1974) described how the asteroid *Acodontaster conspicuus* Koehler if injured was attacked by the asteroid *Odontaster validus* Koehler and enormous numbers of *Parborlasia corrugatus*. The *Acodontaster* were buried under piles of nemerteans and *Odontaster* as high as 20cm within a few hours of the initial attack. After two weeks nothing remained of the *Acodontaster conspicuus* but a star shaped pile of ossicles. Dayton *et al.* (1974) estimated from similar piles of ossicles found on the substratum that at least 3.5% of the *Acodontaster conspicuus* population was killed in this way in one year. Gut contents of *Parborlasia corrugatus* have also been found to include faecal pellets, diatoms, sponge spicules, anemones, polychaetes, gastropods, pelecypods, isopods, amphipods and fish (Dearborn, 1965). *Parborlasia corrugatus* is considered a very abundant and ecologically important species in Antarctic and Sub-Antarctic waters (Gibson, 1983; Heine *et al.*, 1991).

Three genera of nemerteans, *Carcinonemertes* Coe 1902, *Pseudocarcinonemertes* Fleming & Gibson 1981 and *Alaxinus* Gibson *et al.*, 1990 have been found living

epizootically on decapod crustaceans. These species prey on eggs which are brooded by their host decapods. The life history of nemertean egg predators is closely synchronised to the life cycle of the host.

Worms are deposited as egg strands on the host broods where they develop and hatch into planktonic larvae (Wickham & Kuris, 1985). The larval nemerteans can remain in the plankton for long periods of time (at least nine months in the case of *Carcinonemertes errans* Wickham 1978 [Wickham & Kuris, 1985]). The larvae then find their host by an unknown mechanism (*Carcinonemertes errans* may cue on chemicals released in the host crabs urine [Wickham & Kuris, 1985]).

Juvenile worms may encyst in the gills of the host, as in *Carcinonemertes carcinophila* Kölliker 1845 on the blue crab *Callinectes sapidus* Rathbun (Humes, 1942). Alternatively they may be found in the limb joints, axillae and under the abdomen of the host crab, as in *Carcinonemertes epialti* Coe 1902 on the shore crab *Hemigrapsus oregonensis* Dana (Kuris, 1978; Wickham *et al.*, 1984). During ecdysis in at least some crab species nearly all the nemerteans on a single host are able to transfer from the exuvium to the new exoskeleton before moulting is complete (Wickham *et al.*, 1984). Juvenile worms have not been observed feeding on the host exoskeleton (Wickham & Kuris, 1985). Evidence suggests that the juvenile worms take up sufficient dissolved organic compounds (which have leaked from the host crab) across the body wall to meet with their respiratory needs (Roe *et al.*, 1981; Crowe *et al.*, 1982).

When the host crabs become ovigerous, juvenile nemerteans invade the broods and feed on the eggs. The nemerteans penetrate the egg membrane using the stylet of the proboscis (Wickham, 1978; Roe, 1984) and then feed suctorially on the egg contents (Roe, 1984). Feeding rates of nemerteans on crustacean eggs have been given at approximately one egg per nemertean per day (Wickham, 1979). This can give rise to considerable rates of mortality when a single crab may be infected with up to 85,000 nemerteans (*Carcinonemertes regicides* Shields *et al.* 1989 on the red king crab, *Paralithodes camtschatica* Tilesius [Kuris *et al.*, 1991]).

Increased rates of egg mortality are experienced by many host species as the

brooding period proceeds due to the increased growth and feeding rates of the adult nemerteans (Kuris, 1978; Kuris & Wickham, 1987). In *Paralithodes camtschatica* increased rates of egg mortality are associated with increased numbers of *Carcinonemertes regicides*. This species of crustacean egg predator matures at a very small size and has a fast generation time and in this case autoinfection is thought to rapidly increase the intensity of infection through the brooding period (Kuris *et al.*, 1991).

Mortality in broods of infected crab species can be as high as 100% (Kuris & Wickham, 1987). The results of such high levels of egg mortality can be devastating to populations of the host decapod species, especially when combined with the effects of commercial fishing (Wickham, 1986). Epizootic infestations by nemerteans on crustaceans have been implicated in the collapse and subsequent non-recovery of several populations of commercially important crustacean species. The Dungeness crab (*Cancer magister* Dana) fishery of central California collapsed in 1960 and has as yet shown no indication of recovery. Elevated levels of egg loss due to *Carcinonemertes errans* are the only documented source of extraordinary mortality found in this population of crabs (Wickham, 1986). The red king crab (*Paralithodes camtschatica*) fishery of Alaska was one of the most valuable crab fisheries in the world (Otto, 1981), yielding over 80,000 tonnes in 1981. Three years later (1983-1984) the fishery collapsed. The nemertean *Carcinonemertes regicides* is considered to be a major contributory factor in this collapse.

Nemerteans are not only important to man in their adverse effects on commercially important food species but may also provide a useful source of biologically active compounds. Kem (for review see 1973) isolated a number of toxins from several species of nemerteans. Nemerteans use toxin to either paralyse their prey (Roe, 1970) or it is incorporated into their tissues as a defensive mechanism (Kem, 1973; Heine *et al.*, 1991). Many of the striking colour patterns in nemerteans are probably aposematic (e.g. *Tubulanus annulatus* Montagu 1804 (Sundberg, 1979a)). Nemertean toxins are active in minute concentrations, as low as 2×10^{-10} M (Kem, 1973). One toxin, anabaseine, isolated from *Amphiporus angulatus* Müller 1774, *Amphiporus lactifloreus* Johnston 1827-1828, *Paranemertes peregrina* and *Tetrastemma workii* Corrêa 1961 has been shown

to have nicotine like properties (Kem, 1973). This toxin is now being studied for use in the treatment of Parkinson's disease (W.R. Kem *pers. comm.*, Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, FL32610, U.S.A.).

1.2 Problems In Nemertean Taxonomy

Clearly nemerteans are ecologically and commercially important animals, yet the phylum remains a poorly known and obscure group (Gibson, 1985), even among professional biologists. The main reason for this is the difficulty involved in classifying them (Brunberg, 1964; Gibson, 1985). Nemertean taxonomy involves the detailed description of the internal morphology of specimens from serial sections. This requires a great deal of time and a certain level of expertise. Furthermore most biological specimens are fixed in formalin solutions without prior narcotisation. Nemerteans are highly contractile animals and such procedures render samples virtually useless for detailed taxonomic study. But there are also fundamental problems in nemertean taxonomy itself.

It was the Reverend W. Borlase (1758) who first described a nemertean from Mount's Bay in Cornwall as a "long worm" (*Lineus longissimus*); an illustration accompanies the work. Over the next hundred years a large number of nemertean species were described on the basis of their external characteristics. No reference was made to internal structure since the functional morphology of nemerteans was poorly understood at this time (Gibson & Crandall, 1989). External morphology of nemerteans has been noted as being very variable (Joubin, 1894; Punnett, 1901; Stephenson, 1911; Coe, 1940; Friedrich, 1960; Berg, 1972; Cantell, 1975; Gibson, 1985) and many early descriptions were probably based on only a few specimens. On the basis of inadequate description, these species were classified into a number of major genera. These included *Cerebratulus* Renier 1804, *Lineus* Sowerby 1806, and *Amphiporus*, *Micrura* and *Tetrastemma* Ehrenberg 1831.

Schultze (1851, 1853) described the morphology of the proboscis and correctly determined the use of the stylet apparatus as an aggressive organ for delivering poison into

prey. He also suggested that the function of the accessory stylet pouches was to supply the armed proboscis with replacement stylets, a view contested by his contempory workers such as McIntosh (1873-74). In the same works Schultze described the circulatory and digestive system of nemerteans, accurately locating the position of the mouth and anus. Furthermore, he proposed a classification of nemerteans based on proboscis morphology and the structure of the nervous system, which still remains in use today, though in a highly refined form:

Table 1.1 Classification of Schultze (1853) as abstracted from McIntosh (1873-1874).

Classis Turbellaria. Sub-classis *Proctucha*.

Ordo *Nemertinea*.

Central nervous system consisting of two ganglia on each side, an anterior and posterior, which have two commissures, a superior and inferior, between which the proboscis passes.

Anopla

Proboscis without stylets

The ganglia united at their anterior border by a long and slender dorsal commissure. The lateral nerve-trunk springing from the anterior portion of the anterior ganglion, so that the posterior end is rounded. The ventral commissure common to both ganglia.

On each side of the head is a large and often very shallow furrow, having a small ciliated pit at the posterior end.

Enopla

Proboscis with stylets.

Anterior border of the ganglia rounded. The dorsal commissure in the form of a small band between the dorsal surfaces of the ganglia. The lateral nerve-trunks forming a continuation of the posterior ganglia. The ventral commissure common to both ganglia.

The long cephalic furrow absent; but there are ciliated pits.

The next major advance in nemertean systematics came with the work of W.C. McIntosh (1873-74), in his *Ray Society Monograph, British Marine Annelids*. As well as describing in detail the anatomy of nemerteans McIntosh put forward a classification scheme elaborating on that of Schultze (1851, 1853) by the inclusion of new internal morphological details in his descriptions of nemertean families.

Table 1.2 The classification scheme of McIntosh (1873-74).

Class Turbellaria.

Order Nemertinea.

Sub-Order Enopla.

Proboscis with stylets.

Family I. Amphiporidae. Ganglia rather rounded. Lateral nerves within the muscle layers of the body wall. Mouth opening in front of the ganglionic commissures.

Sub-Family Amphiporinae. Proboscis proportionally large.

Genus I *Amphiporus*, Ehrenberg 1831.

Genus II *Tetrastemma*, Ehrenberg 1831.

Genus III *Prosorhochmus*, Keferstein 1862.

Sub-Family Nemertinae. Proboscis proportionally small.

Genus IV Nemertes.

Sub-Order Anopla.

Proboscis without stylets.

Family II. Lineidae. Ganglia elongated. Muscular layers of the body wall three in number, viz. external longitudinal, circular and internal longitudinal. Proboscis furnished with five coats, viz. external elastic, longitudinal and accessory bands, circular, basement and glandular layers. Snout with deep lateral fissure on each side.

Genus V. *Lineus* Sowerby 1806.

Genus VI. *Borlasia* Oken 1815.

Genus VII. *Cerebratulus* Renier 1804.

Genus VIII. *Micrura* Ehrenberg 1831.

Genus IX. *Meckelia* Leuckart 1828.

Family III. Carinellidae. Lateral nerves placed between the basement-layer of the cutis and the external (circular) muscle coat of the body wall, or in the substance of the longitudinal layer close to the circular. There are only two muscle coats. The proboscis has four muscle layers, viz. external elastic, circular, longitudinal and glandular.

Genus X. *Carinella* Johnston 1833.

Genus XI. *Valencinia* Quatrefages 1846.

Family IV. Cephalothricidae. Commissures of the ganglia separated by a distinct antero-posterior interval. Lateral nerves placed between the longitudinal muscular coat and an isolated inner band of fibres. Proboscis has an external circular (or elastic), an internal longitudinal, and a glandular layer supplied with acicular papillae.

Genus XII. *Cephalothrix* Örsted 1843.

The definition of nemertean groups by the arrangement of the body wall and proboscis muscle layers was further elaborated by Bürger (1895). In his beautifully illustrated text *Die Nemertinen des Golfes von Neapel und der Angrenzenden Meeres-Abschnitte*, Bürger divided the nemerteans into four orders on the basis of the position of the lateral nerve cords within the layers of the body wall and on the organisation of the body wall musculature.

Table 1.3 The classification scheme of Bürger (1895).

Order I. **Protonemertini**. Lateral stems outside the body wall musculature. Body wall musculature two layered. Mouth opening behind the brain. Proboscis without stylet. Blind gut caeca absent.

Order II. **Mesonemertini**. Lateral stems within body wall musculature; this is two layered. Mouth opening behind the brain. Proboscis without stylet. Blind gut caeca absent.

Order III. **Metanemertini**. Lateral stems inside the body wall musculature in the body parenchyma. Body wall musculature two layered. Mouth in front of the brain. Proboscis with stylet. Blind gut caeca present.

Order IV. **Heteronemertini**. Lateral stems in the body wall musculature, which has meanwhile become three layered, by the three layered tube forming another longitudinal muscle layer. The lateral stems lie between the newly added and the old layers. Mouth behind the brain. Proboscis without stylet. Blind gut caeca absent.

Bürger (1895) also gave, for the first time, complete descriptions of the internal morphology of many species. However, he made no attempt to reorganise many of the long standing nemertean genera on the basis of the internal structure of their members. Diagnoses for genera and even families were based on external characters or on very superficial internal morphological characters. For example:

Holorhynchocoelomia. The rhynchocoelom extends up to the anus.

Family 6. Prosorhochmidae. With four eyes; Body long and thin.

Family 7. Amphiporidae. With many eyes.

Family 8. Tetrastemmatidae. With four eyes; Body short and fat.

The number of ocelli, a highly variable external character, is clearly unsound, as the basis for the diagnoses of families. Only six years later Punnett (1901) expressed his reservations about the family Lineidae, stating that " ....the three genera, *Lineus*, *Cerebratulus* and *Micrura* are exceedingly ill defined. Many anatomical differences are found in the family, amongst which may more particularly be mentioned the following:-

(a) A diagonal muscle layer, neurochord cells, eyes and frontal organ may be either present or absent.

(b) A well marked cephalic vascular head loop may be present, or the cephalic vessels may form an anastomosing network.

(c) The excretory system shows great variations in its backwards extent; the position of the tubules may be dorsal or ventral, or both; they may reach forward to the cerebral organ, or may commence some way behind it. Also some species possess a number of ducts whilst only one pair is present in others.

For the majority of the Lineidae, and indeed in many British forms, we are as yet in ignorance with regard to many of these points, and until they have been determined it is useless to attempt to place the classification of the family on a more satisfactory basis."

Coe (1943) proposed a system of classification that remains in use to the present day. This was largely based on Bürger's (1895) system of classification and finally established the nemerteans as an independent phylum.

Table 1.4 The classification scheme of Coe (1943).

Phylum Rhynchocoela (Nemertea)

Class I: **Anopla.**

Mouth below or posterior to the cerebral ganglia; central nervous system situated within the body wall (epidermis, dermis, or body wall musculature); proboscis not differentiated into three regions and either not armed or provided with large numbers of rhabdite like epithelial barbs.

Order 1: Palaeonemertea (Palaeonemertini). Body wall musculature either of two layers (outer circular and inner longitudinal) or three layers (outer circular, middle longitudinal, inner circular); dermis gelatinous or absent; central nervous system either in inner longitudinal musculature or external to body wall muscles.

Order 2: Heteronemertea (Heteronemertini). Body wall musculature of three layers (outer longitudinal, middle circular, inner longitudinal), sometimes with additional thin inner circular and outer oblique muscle layers; dermis well developed and fibrous; central nervous system in middle circular muscle layer.

Class II. **Enopla.**

Mouth anterior to cerebral ganglia; central nervous system internal to the body wall musculature, which is two layered (outer circular, inner longitudinal); proboscis regionally differentiated and armed with one or more needle like central stylets (except in order Bdellonemertea).

Order 3: Hoplonemertea (Hoplonemertini). Proboscis armed with one or more central stylets; intestine straight with paired lateral diverticula; no posterior ventral sucker.

Suborder 1: Monostilifera. Central stylet armature a single structure carried in a large cylindrical basis.

Suborder 2: Polystilifera. Proboscis armature consists of a pad or shield bearing numerous small stylets.

Tribe 1: Reptantia. Body adapted for crawling or burrowing; rhynchocoel with caecal outgrowths; cerebral organs and nephridial system present.

Tribe 2: Pelagica. Bathypelagic, with body adapted for free swimming or floating in deep water; rhynchocoel without caeca; cerebral organs and nephridial system absent.

Order 4: Bdellonemertea (Bdellonemertini or Bdellomorpha). Proboscis not armed with stylets; intestine sinuous and without lateral diverticula; foregut doliiform, with many papillae; with a posterior ventral sucker.

Only a few changes have been proposed to this system of classification:

Iwata (1960a) proposed that the order Palaeonemertea be split into the orders Archinemertea and Palaeonemertea on morphological grounds. He also proposed in the same paper that the order Bdellonemertea be reduced to a sub-order of the Hoplonemertea. The splitting of the Palaeonemertea has gained general acceptance among nemertean taxonomists (Gibson *pers. comm.*, Liverpool John Moores University).

Gibson (1988) proposed a reorganisation of the class Enopla, replacing the orders Hoplonemertea and Bdellonemertea with the orders Distromatorhynchocoela and Urichorhynchocoela on the basis of the organisation of the rhynchocoel musculature. This proposal is currently the subject of vigorous debate (Gibson, 1990; Sundberg, 1990).

The legacy of this taxonomic history is that nemertean systematics is plagued by a multitude of inadequately described taxa at the levels of species, genera and family. Gibson (1985) estimated that out of 900 named species of nemerteans he had reviewed, over 40% remained in the four old genera of *Amphiporus*, *Cerebratulus*, *Lineus* and *Tetrastemma.* Gibson & Crandall (1989) reviewed the literature that related to the 145 species that have been included in the genus *Amphiporus*. A translation of Ehrenberg's original diagnosis of the type species *Amphiporus albicans* Ehrenberg 1831 was given:

" A rather slender filiform body, soft, variable, without distinct annulations; a simple papillate gut, with terminal mouth and anus; no separate genital aperture; four frontal patches of very many eyes converging anteriorly."

Gibson & Crandall (1989) state " There is nothing in this definition which has any taxonomic significance except for the reference to the papillate proboscis (illustrated)." They subsequently designated the type species for the genus, *Amphiporus albicans*, a nomen dubium and proposed a new type species, *Amphiporus lactifloreus* (now approved as the type species [International Commission of Zoological Nomenclature, 1992]). Most other *Amphiporus* species were identified as nomina nuda, nomina dubia, species inquirendae or synonomous with other taxa.

Friedrich (1960) said of the genus *Lineus* that "...a clear diagnosis of the genus is at present not possible, as data are incomplete and totally contradictory." Gibson (1981)

came to similar conclusions about the genus *Cerebratulus* "Although some 100 or more species of *Cerebratulus* have been described, there remain insufficient morphological data for a definitive diagnosis of the genus."

One solution to these problems, proposed by Gibson (1985), was an investigation of nemertean taxa using a standard method for description of the morphology of each species based upon a series of transverse sections. A standard comprehensive list of characters to be recorded in such a study could be prepared so that interspecific comparisons and comparisons of the results of different workers could be made.

Such an approach has inherent difficulties. Friedrich (1960) expressed the following doubts concerning his attempt at defining the genus *Micrura*. "Do these anatomical differences lie within species specific variability or do they mark out different species. If we inferred the first case then subsequently it would be impossible to ascertain different species by differentiation of anatomical characteristics....It remains scarcely feasible, from the present material to crystallize out a uniform agreement, to derive at least one anatomical basis to give a genus diagnosis while the question of species specific differences still remains, until from extensive material the importance of anatomical differences is established."

Berg (1972) redescribed the two similar hoplonemertean species, *Amphiporus lactifloreus* and *Amphiporus dissimulans* Riches 1893. He found that as well as variation in external characteristics, the internal morphology varied. In *Amphiporus lactifloreus* the number of proboscidial nerves varied from 11-14. There were also variations in the morphology of the cerebral organs, layout of the blood system and the number of nephridial efferent ducts. Sundberg (1979b, 1980) found substantial variation in taxonomic characters within the species *Tetrastemma laminariae* Uschakow 1928.

Is the description of nemertean species based on external and internal morphology valid given this large intraspecific variation? A large number of specimens may be examined to try and account for intraspecific variation and this may be incorporated into a species description. A large number of characters may be recorded in an attempt to distinguish between similar species. It may be the case that some nemertean species are so

similar, when intraspecific variation is taken into account, that it is impossible to differentiate between them on morphological grounds (suspected in some *Carcinonemertes* species [Gibson pers. comm., Liverpool John Moores University.]) Artefacts occurring during the fixation and sectioning of material may further complicate descriptions of internal morphology.

It is not even known which morphological characters are significant at the species or generic level and therefore the same data on the internal morphology of nemertean taxa may be interpereted in different ways. e.g. Sundberg, (1989) reunited the genera *Pantinonemertes* Moore & Gibson 1981, and *Geonemertes* Semper 1863, separated on the basis of a detailed study of internal morphology, concluding that several of the characters used to distinguish the genera were plesiomorphic.

Defining species and genera solely on the basis of the description of internal morphology as put forward by Gibson (1985) is dubious. More objective techniques are required to address the problems in nemertean systematics at all taxonomic levels. Molecular genetic techniques provide a more objective guide to systematic relationships between taxa since they measure genotypic rather than phenotypic divergence. This removes the need to select or emphasise characters when inferring relationships between species (Thorpe, 1982), eliminates environmental influences on phenotypic characters (e.g. Crothers, 1973, 1985; Beaumont & Wei, 1991) and removes the possibility of artifacts resulting from methods of study in morphological investigations. One such technique is starch gel electrophoresis.

Electrophoresis is based on the differential migration of water soluble protein molecules, extracted from tissues through a gel (e.g. hydrolysed starch, polyacrylamide) across which is an electrical potential (Thorpe, 1982). The rate at which these proteins migrate is determined by their net charge, size and shape. Rate of migration also depends on the viscosity or pore size of the electrophoretic medium and the strength of the electric field across the gel (Alberts *et al.*, 1983; Murphy *et al.* 1990). Following electrophoresis proteins can be marked by specific histochemical stains applied to the electrophoretic medium (Shaw & Prasad, 1970; Harris & Hopkinson, 1976; Murphy *et al.*, 1990). Details

of methodology employed in electrophoresis are provided by a number of authors (Shaw, 1965; Bier, 1967; Scopes, 1968; Brewer, 1970; Gordon, 1975; Sargent & George, 1975; Siciliano & Shaw, 1976; Smith, 1976; Gottlieb, 1977; Harris & Hopkinson, 1976; Ferguson, 1980; Gaal *et al.*, 1980; Richardson *et al.*, 1986; Leary & Brook, 1990; Murphy *et al.*, 1990).

When an amino acid substitution occurs in a protein molecule, this may alter net charge, or cause conformational changes, altering the rate of protein migration during electrophoresis (only about one in three of such changes are considered to be detectable over the usual pH ranges used in electrophoresis; e.g. Selander, 1976). Since the molecular structure of proteins is determined genetically and is likely to be free from environmental influences, such a substitution must be the result of genetic change, i.e. mutation. Therefore the level of difference between homologous proteins in related populations or species provides an indication of the levels of genetic differentiation between them (Thorpe, 1982).

The level of genetic differentiation between two species or populations over a range of enzyme loci may be reduced to a single figure using measures of genetic distance or genetic identity (Smith, 1977). There are many such measures, including Nei's (1972, 1978) genetic distance and genetic identity, Roger's (1972) genetic distance, the Cavalli-Sforza & Edwards (1967) arc distance and the Manhattan distance (Wright, 1978). These measurements use different models for rates of evolutionary change in gene frequencies (see Swofford & Olsen, 1990 for discussion on genetic distance measures). The most commonly used measures of genetic distance and identity are those of Nei (1972).

Nei's (1972) measure of genetic identity between two populations is given by :

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

where x_i and y_i are the frequencies of the i'th allele in populations x and y respectively.

The mean genetic identity over all loci studied is calculated as:

$$I = \frac{Ixy}{\sqrt{(I_x I_y)}}$$

where Ixy, Ix and Iy are the arithmetic means, over all loci of $\sum$xi yi, xi2 and yi2 respectively (Nei, 1972).

The genetic distance (D) is given by the equation D = -ln I.

Nei's (1972) genetic identity (I) is on a scale of zero (allele frequencies of populations show no similarity) to one (allele frequencies of populations identical). Nei's (1972) genetic distance (D) is on a scale of zero (populations identical) to infinity (populations show no similarity).

Nei's (1972) measure of genetic distance, D, is said to estimate the number of substitutions per locus (Thorpe, 1982; Swofford & Olsen, 1990). Since the rate of amino acid substitution is approximately constant (Zuckerkandl & Pauling, 1965) then the accumulated number of substitutions in a protein must be approximately proportional to time (see Wilson *et al.*, 1977; Kimura, 1983). In this case divergence between homologous proteins may be related to evolutionary time (Thorpe, 1982). This is the basis of the molecular clock theory (Zuckerkandl & Pauling, 1965; Fitch, 1976; Kimura, 1983; Hartl & Clark, 1989). Since enzyme variation is related to evolutionary time, there should be a relationship between taxonomic separation and Nei's (1972) genetic distance.

Thorpe (1982) suggested that conspecific populations, congeneric species and species of different genera should on average have been isolated for different lengths of time. They should therefore have different probabilities of having certain values of Nei's (1972) genetic identity (I). Thorpe (1982) using published data plotted the probability of populations within species, between species and between genera, falling into certain values of Nei's (1972) genetic identity (I). He concluded that there was a clear relationship between taxonomic divergence and genetic distance. The critical I value for distinguishing between genera was given as 0.35, e.g. a genetic identity below 0.35 would indicate that two species are likely to be in separate genera. The critical value for congeneric species was given as between 0.35 and 0.85 and that of conspecific populations as above 0.85 (about

80% of conspecific I values were above 0.95).

This has obvious relevance to systematic studies. By the use of enzyme electrophoresis a measure of the genetic distance between populations can be obtained. This can be used as an objective guide to systematic relationships. If two sympatric populations have a low identity, e.g. I=0.5, then it is likely that they constitute separate species. This technique has already successfully been used to study the systematics of several groups of marine invertebrates (e.g., sponges - Solé-Cava & Thorpe, 1986, 1987a; Solé-Cava *et al.*, 1991a,b, 1992; sea anemones - Carter & Thorpe, 1981; Bucklin & Hedgecock, 1982; Haylor *et al.*, 1984; Smith & Potts, 1987; Solé-Cava & Thorpe, 1987b; Solé-Cava *et al.*, 1985; Bryozoa - Thorpe *et al.*, 1978a,b; Thorpe & Mundy, 1980; molluscs - Ward & Warwick, 1980; Skibinski *et al.* 1980, 1983; Johannesson & Johannesson, 1990; Munksgaard, 1990; Gardner, 1992; Brierly, 1992; Crustacea - McKinnon *et al.*, 1992).

Starch gel electrophoresis has also been successfully used to study nemertean systematics. Cantell & Gidholm, (1977) found species specific differences in isoenzyme mobilities for malate dehydrogenase and non-specific esterases between the two species *Micrura fasciolata* Ehrenberg 1831 and *Micrura baltica* Cantell 1975.

Williams *et al.* (1983) compared isoenzyme patterns in six enzyme loci for *Lineus ruber* Müller 1774, *Lineus viridis* and *Lineus sanguineus* Rathke 1799. Genetic identity values between these three species were all found to be extremely low. Nei's (1972) genetic identity was well below 0.2 between all three species, a very low value for intrageneric comparisons. Furthermore, no allelic diversity was observed in any of the three *Lineus* species. This is very unusual especially for marine invertebrates (Powell, 1975; Manchenko & Balakirev, 1984; Bucklin, 1985; Solé-Cava *et al.*, 1985; Solé-Cava & Thorpe, 1987b, 1990).

Sundberg & Janson (1988) studied the hoplonemertean species *Oerstedia dorsalis* Abildgaard 1806. Three forms of this species could be distinguished on the basis of their external colouration. Isozyme differences at six enzyme loci indicated that one of these colour forms was a separate species, subsequently described as *Oerstedia striata* Sundberg, 1988. Mean heterozygosity in the three colour morphs of *Oerstedia* ranged from 0.132 to

0.316. These values are very high for eukaryotic organisms in which heterozygosity usually lies in the range of 0.05 to 0.15 (Selander, 1976; Nevo, 1978; Nevo *et al.* 1984).

In the present study starch gel electrophoresis was used to investigate the systematics of one of the oldest nemertean genera, *Lineus* Sowerby 1806. The genus lacks a sound diagnosis and many of its species (there are over 90; Gibson, 1982a) are poorly described. As such the genus typifies many of the problems commonly encountered in nemertean systematics. Seven species from the genus *Lineus* are found on British shores (Gibson, 1982b), of these four are common. These four species, *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and the type species for the genus, *Lineus longissimus* were the main subject of this investigation.

Chapters four and five refer to two cryptic species found by starch-gel electrophoresis amongst *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and *Lineus longissimus* in populations along the coasts of Britain, France and Spain. Chapter six describes an electrophoretic investigation of the phylogenetic relationships between the four species from the genus *Lineus* mentioned above and two species from the genus *Micrura* namely *Micrura fasciolata* and *Micrura purpurea* Dalyell 1853. The implications of these findings to nemertean systematics are discussed in Chapters 4, 5, 6 and 8.

A hoplonemertean, *Amphiporus lactifloreus* was also investigated to contrast with *Lineus* species (heteronemerteans). *Amphiporus lactifloreus*, has been recently accepted as the new type species for the genus *Amphiporus* (Gibson & Crandall, 1989; International Commission of Zoological Nomenclature, 1992). Electrophoretic investigations described in Chapter 3 indicate that this species is a single monophyletic species.

Isoenzyme data from Chapters 3,4,5,6 and 8 provide estimates of genetic variability in nemertean species. In Chapter 3 the population genetics of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* are investigated. Observed fits of genotype frequencies to Hardy-Weinberg expectations within populations and estimations of gene-flow between conspecific populations of nemertean species over a geographic range of 6000 km are detailed in this chapter. These data are compared and contrasted with data on gene-flow between conspecific populations of other taxa of marine organisms from

previous studies.

Chapter seven contains the description of a new species of palaeonemertean, *Callinera monensis* (Rogers *et al.*, 1992) discovered on the Isle of Man during the course of the present investigations.

Chapter 2
General Materials and methods

2.1 Nemertean Collection.

Most of the nemertean species under investigation were collected intertidally. The only exceptions to this were *Lineus longissimus*, *Micrura fasciolata* and *Micrura purpurea* which were collected subtidally by SCUBA divers.

Lineus ruber, *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* commonly occurred underneath stones and rocks lying in silt, muddy sand, sand and fine shelly gravel (*pers. obs.*). Sediments beneath stones tended to be clean though *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* were collected from beneath stones lying in sediments which were black and smelt of hydrogen sulphide (*per. obs.*). Sediments also tended to be well drained (*pers. obs.*). Suitable shores for these nemertean species were of a semi-rocky to boulder shore type (*pers. obs.*). Beaches consisting of a lot of small stones (8cm +) were also occasionally suitable (*pers. obs.*). Organic enrichment from sewage discharge or rotting algal material seemed to increase numbers of *Amphiporus lactifloreus* and *Lineus sanguineus* and to a lesser extent *Lineus viridis* (*pers. obs.*).

Specimens were collected by carefully scraping them off the substrate using a small knife. Occasionally a small pair of forceps would be employed to remove worms from holes or polychaete tubes. The use of forceps often broke the specimen.

Lineus longissimus was collected intertidally only at extreme low water from beneath large boulders or mooring blocks, not too firmly embedded in mud or muddy sand. It was also occasionally found crawling across the substratum on gravel beds, in a depth of 10 - 30 m, by divers around the west coast of the Isle of Man (*pers. obs.*).

Specimens were placed in plastic buckets with lids (height = 9cm, diameter = 13cm) half filled with fresh sea water, subsequent to collection. During collecting trips sea water in each bucket was changed every day to prevent a harmful build up of waste products. Buckets were placed in a cool box with ice packs and the specimens were transported to the Isle of Man alive. Specimens of *Lineus ruber* and *Lineus viridis* from the U.S.A. and France were sent in waterproof packages containing filter paper soaked in seawater; survival of specimens was high (>80%). Specimens were also sent in closed

containers of seawater. Survival of these specimens was poor (<30%). *Lineus ruber* showed a much higher level of mortality than *Lineus viridis* during transport from overseas.

At Port Erin specimens were kept alive in plastic tanks (length = 32cm, width = 21cm, height = 22cm) prior to study. These were filled with sea-water, aerated and kept in a temperature controlled room at ambient sea-water temperature. Specimens from separate sites were kept in separate tanks. Tanks were only two thirds full of sea-water to prevent nemerteans from escaping.

2.2 Nemertean Sample Sites.

Sampling sites for the present study were located on the west coast of the U.S.A, the coasts of Sweden, the United Kingdom, France and Spain. Figure 2.1 shows the distribution of sites in the North Atlantic area.

United Kingdom.

Sites were chosen along the west coast of the United Kingdom running from north to south (Fig. 2.2). Populations of given nemertean species could then be compared at varying distances along the same coast line. A study of population structure and of the distances over which these species could maintain gene flow was then possible.

Oban, West Scotland (Fig. 2.3). 56°24'N, 05°32'W.

Sampling date 1-7/11/90

(i) Dunstaffnage beach. 56° 26'N, 05° 26'W.

Large sheltered beach composed of grey, soft mud penetrated by many small holes. Approximately 20 *Lineus viridis* found foraging across mud surface in daylight. Several dead *Lineus viridis* were observed on the mud surface.

(ii) Oban Harbour. 56° 24'N, 05° 32'W.

Southern half of harbour was composed of many stones (10-40 cm across) lying in a fine dark gravel. This also contained silt, leaf litter and evidence of sewage pollution.

Many *Amphiporus lactifloreus* found. Also *Lineus viridis*, *Lineus ruber* and *Cephalothrix* spp. No nemerteans were found in the north end of the harbour. There was evidence of oil pollution here.

Cumbria, North West England.

Sampling date 8-14/9/91.

(i) Whitehaven (Fig. 2.4, Fig. 2.5). 54°34'N, 03°35'W.

Collected in area 100 - 200m south of Whitehaven harbour. Area consisted of boulders lying in damp silt, gravel and sand with a lot of dead organic material (but not anoxic). Area grossly polluted by sewage discharges and an outfall from a chemical plant further south. Evidence of raw sewage on shore. Large amounts of *Enteromorpha* spp. and large numbers of *Capitella capitata* Fabricius, turbellarians and some *Carcinus maenas* Linnaeus. Very large numbers of *Amphiporus lactifloreus* found. Also *Lineus viridis*, *Lineus ruber*, *Lineus sanguineus* and *Procephalothrix filiformis* Johnston 1828-29.

(ii) Gutterby (Fig. 2.6). 54°19'N, 03°28'W.

Long unbroken shore composed of large areas of *Mytilus edulis* Linnaeus beds and reefs of *Sabellaria alveolata* Linnaeus. Some areas of stones lying in sediment. Large numbers of turbellarians. Few nemerteans, but specimens included *Amphiporus lactifloreus*, *Lineus ruber*, *Lineus viridis* and *Emplectonema gracile* Johnston 1837. [Caution - this shore is on the edge of an M.O.D. bombing range.]

(iii) Walney Island, Barrow-in-Furness (Fig. 2.7). 54°06'N, 03°16'W.

Sampled areas between Mill Scar and Tummer Hill Scar and at Bent Haw Scar. Nemerteans were found under stones (up to 30cm diameter), some of which were covered in *Enteromorpha* spp, lying in sand and fine gravel from mid tide level to high water neaps. Species found included *Amphiporus lactifloreus*, *Lineus ruber*, *Lineus viridis* and *Emplectonema gracile*. A lot of turbellarians were also present.

Sampled South East point area. This was composed of rocks and stones lying in sand. Only two specimens of *Amphiporus lactifloreus* found.

Isle of Man (Fig. 2.8).

Sampling Date - various throughout study.

(i) Port Erin Bay. 54°05'N, 04°46'W.

Within Port Erin Bay four areas can be defined for the collection of nemerteans.

(a) Traie Vane, below the ruins of the old marine biology laboratory. *Amphiporus lactifloreus*, *Lineus ruber* and *Lineus sanguineus* were collected from beneath stones and rocks lying in fine gravel from low water to upper mid tide levels (October - December, 1989). Specimens for much of the initial work on electrophoretic techniques were collected here. After a year's collecting these nemertean species became locally very rare.

(b) Port Erin beach. The sandy shore at Port Erin shows a gradient in particle size from coarse at the north end of the strand to fine at the southern end (Fincham, 1968). The hoplonemertean *Psammamphiporus elongatus* Stephenson 1911, was found in sand at the southern end of Port Erin beach (October, 1989).

(c) South shore between the jetty and life boat station. This shore comprise areas of gravel and areas of boulders lying in fine gravel, sand or silt. At low water *Lineus longissimus*, *Emplectonema neesii* Örsted 1843 and occasionally *Emplectonema gracile* were found under rocks. *Tetrastemma herouardi* Oxner 1908 was obtained from the holdfasts of *Laminaria digitata* Hudson. Between low water and mid tide level *Amphiporus lactifloreus*, *Lineus ruber* and *Lineus viridis* were found beneath stones (throughout 1989 - 1992).

(d) Subtidally around Port Erin breakwater. Two species were found crawling amongst the concrete blocks that made up Port Erin breakwater, these were *Lineus longissimus* and *Tubulanus superbus* Kölliker 1845 (March, 1991).

(ii) Port Erin Bay area - Calf of Man. 54°03'N, 04°49'W.

Specimens of *Lineus longissimus*, *Emplectonema neesii* and *Tubulanus superbus* were collected from 5-30 metres depth on pearl nets used for scallop cultivation in Bay Fine (April, 1991).

Two specimens of *Nipponnemertes pulcher* and one of *Cerebratulus fuscus* McIntosh 1873-74 were dredged from beds of shelly gravel west of Port Erin Bay (April,

1991).

A single specimen of *Amphiporus allucens* Bürger 1895 (species inadequately described [Gibson, 1982b]) was obtained from beneath a stone by SCUBA divers from around the Calf of Man (June, 1991). A single specimen of *Cerebratulus roseus* Delle Chiaje 1841 was obtained from beneath a stone lying in course gravel mixed with mud at 18m behind Thousla Rock in the Calf Sound (June, 1991). This habitat was associated with large numbers of the ophiuroid *Amphiura securigera* Düben & Koren.

Lineus longissimus was recorded frequently by SCUBA divers around the Calf of Man, particularly in the vicinity of the Buroo and the Puddle (throughout 1989-1992).

Large numbers of *Oerstedia dorsalis* were collected from clumps of the hydroid *Tubularia indivisa* Linnaeus removed from Chicken Rock, Calf of Man by SCUBA. divers (August, 1992).

(iii) Chapel Beach, Port St Mary. 54°04'N, 04°41'W.

A single specimen of *Amphiporus hastatus* McIntosh 1873-74 (species inadequately described [Gibson, 1982b]) was obtained by digging in sand near low water (September, 1990).

(iv) Bay Ny Carrickey. 54°05'N, 04°41'W.

Some specimens of *Lineus viridis* were obtained from between Black rocks and Poyll Brein under stones and rocks lying in sand or fine gravel (May, 1992).

(v) Castletown Bay. 54°04'N, 04°39'W.

Specimens were found south west of Castletown on the shore 50 - 100m east of the old pier at Scarlet under stones and rocks lying in muddy sand and gravel. Specimens included *Lineus ruber*, *Lineus viridis* and *Amphiporus lactifloreus* (May, 1992). A single specimen of *Amphiporus hastatus* was obtained by digging in coarse sand at low water in Castletown estuary (September, 1990).

(vi) Derbyhaven. 54°04'N, 04°37'W.

Specimens were obtained from two areas in Derbyhaven.

(a) Derbyhaven Harbour. Area comprised of a large tidal mudflat with some gravel and larger stones. Type locality of new palaeonemertean species *Callinera monensis* Rogers

et al. 1992, obtained from the mud surface and from 8-10cm down in mud (October, 1989 and March 1991). A single specimen of *Tubulanus annulatus* was obtained from near low water beneath a stone lying in mud (October, 1989).

(b) Beach site, Derbyhaven Bay. Specimens of *Lineus ruber*, *Lineus viridis* and *Amphiporus lactifloreus* obtained from beneath stones and rocks lying in sand next to rocky outcrops opposite old lime kilns, south of Derbyhaven (November, 1989). *Lineus acutifrons* Southern 1913 (species inadequately described [Gibson, 1982b]) was obtained by digging in sand at low water in Derbyhaven Bay (October, 1989).

Llandudno, North Wales (Fig. 2.9). 53°19'N, 03°49'W.

Sampling date 6-9/9/90.

(i) East shore, from Pen-trwyn to area south east of landing stage below cable car station. Shore comprised stones and rocks lying in silty gravel. Also beds of *Mytilus edulis* to the north west of the landing stage. Nemerteans were found under rocks mainly below the cable car station and also just above the upper level of the *Mytilus edulis* beds. Species found included *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*, *Amphiporus lactifloreus*, a new genus and species *Riseriellus occultus* Rogers *et al.* 1993, *Emplectonema gracile*, *Prosorhochmus claparedii* Keferstein 1862 and *Cephalothrix* spp.

(ii) West shore, from Llandudno to the ruins of a bishop's palace. Nemerteans were found under stones lying in shelly gravel containing silt at approximately mid tide level. Species found included *Lineus ruber*, *Lineus viridis*, *Amphiporus lactifloreus* and *Emplectonema gracile*. *Lineus bilineatus* Renier 1804 was also found in empty tubes of *Lanice conchilega* Pallas lying under stones.

Anglesey, North Wales (Fig. 2.10). 53°18'N, 4°02'W.

Sampling date 25-29/3/91

(i) Black Point. Collecting area was below coast guard look out to the south of two outfall pipes. Specimens were collected from beneath stones and rocks lying in clean silty sand. Species obtained were *Lineus ruber*, *Lineus viridis*, *Amphiporus lactifloreus* and *Riseriellus occultus*, all at approximately mid tide level. Higher on the shore *Prosorhochmus claparedii* could be found beneath stones.

(ii) Penmôn. Collecting area was around Trwyn y penrhyn. Specimens were found in muddy sand beneath stones and boulders. Species included *Lineus ruber*, *Lineus viridis* and a few *Lineus bilineatus*.

Trevaunance Cove, St Agnes, Cornwall (Figs. 2.11, 2.12). 50°20'N, 05°11'W.

Date sampled 20/1/92.

Large areas of the northern side of the cove were searched for nemerteans. This consisted of large lumps of rock interspersed with large rockpools and clean shelly gravel. No nemerteans were found in this area. In the area where rocks on the north end of the bay mixed with sand that formed the greater part of Trevaunance Cove many nemerteans were found. These were collected from beneath rocks lying in shelly gravel with silt and sand. *Lineus viridis* was most common with small numbers of *Amphiporus lactifloreus* and a single specimen of *Lineus bilineatus*. Specimens were particularly common where substrate was loosely packed.

Kingsand and Cawsand Bay. 50°20'N, 04°11'W.

Date sampled 23/1/92.

Very poor area for nemerteans. A few specimens of *Lineus viridis* and *Amphiporus lactifloreus* found under rocks in Cawsand Bay.

Plymouth Sound (Fig. 2.13). 50°20'N, 04°09 'W.

Date sampled 19 and 21/1/92.

(i) Plymouth Hoe. Area below Citadel hill and west of Citadel Hill was searched for nemerteans. A few specimens of *Lineus viridis* and *Amphiporus lactifloreus* were found under stones lying in muddy sand.

(ii) Jennycliff Bay (Fig. 2.14). Very polluted shore. A few nemerteans found to the north of a sewage pipe. These were *Lineus viridis*, *Amphiporus lactifloreus* and what appeared to be *Lineus ruber* (see Chapter 5). Specimens were obtained from beneath stones lying in silty gravel.

(iii) Renney Rocks. Very poor site for nemerteans; only a single specimen of *Amphiporus lactifloreus* found.

Wembury Bay (Fig. 2.15). 50°19'N, 04°05'W.

Date sampled 22/1/92.

Sampled area between Wembury village (church) and Blackstone rocks. Nemerteans were mainly found to the east of a small stream running out from Wembury, under boulders lying in silt and gravel. What appeared to be *Lineus ruber* (see Chapter 5), *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* were found but in fairly low numbers, occurring in patches.

Yealm Estuary (Fig. 2.16). 50°19'N, 04°02'W.

Date sampled 22/1/92.

East bank of estuary, west of the village of Noss Mayo was sampled. Nemerteans were found under stones lying on the muddy banks of the estuary at approximately mid tide level. Specimens included what appeared to be *Lineus ruber* (see Chapter 5) and *Amphiporus lactifloreus*.

Salcombe Estuary (Fig. 2.17, 2.18). 50°13'N, 03°45'W.

Date sampled 24/1/92.

Sampled from Island Quay on Batson Creek, along Salcombe Harbour on the west side of the estuary. Along Batson Creek a few specimens of what appeared to be *Lineus ruber* (see Chapter 5) and *Amphiporus lactifloreus* were found under stones lying in mud. Past the ferry landing large stones became more common and the sediment became more sandy. Larger numbers of what appeared to be *Lineus ruber* (see Chapter 5), *Lineus viridis* and *Amphiporus lactifloreus* were found along this stretch of shore. Moving south along the estuary beds of *Sargassum muticum* Yendo and *Zostera marina* Linnaeus were found. Associated fauna included *Crepidula fornicata* Linnaeus, large numbers of *Trivia monacha* da Costa, *Carcinus maenas* and various sponges, tunicates and Bryozoa. Underneath large mooring blocks with chain at low water were found 7 very large specimens of *Lineus longissimus*.

Sweden.

Vattenholmen, Tjärnö (Fig. 2.20). 58°03'N, 11°07'E.

Sampling date 1-7/6/92.

(i) Nemerteans were collected by dredging over shelly gravel at approximately 20m depth, around Vattenholmen Island near Tjärnö. Dredge material which included shells, tunicates, hydroids, *Alcyonium digitatum* Linnaeus, *Asterias rubens* Linnaeus and *Branchiostoma* spp (amphioxus) was placed in fish boxes to deoxygenate; nemerteans were removed from the water's surface after a few hours and for a further 48 hours. Specimens obtained included *Micrura fasciolata*, *Micrura purpurea*, *Lineus bilineatus* and *Nipponnemertes pulcher*.

(ii) *Pomatoceros triqueter* Linnaeus tubes were collected from mud at a depth of 4-8m in a narrow channel to the south of Tjärnö. These were placed in a bucket of seawater and left to deoxygenate. Specimens of *Micrura purpurea* and *Micrura fasciolata* were collected from the waters surface a few hours later and for 48 hours thereafter.

France.

Pointe de Barfleur, Normandy (Fig 2.21). 49°18'N, 00°25'W.

Sampling date 6/1992.

Specimens of *Lineus ruber*, *Lineus viridis* and what appeared to be *Lineus ruber* were collected on the upper shore beneath cobbles and boulders, lying in sand.

Spain.

Ría de Foz, northern Spain (Fig. 2.22). 43°34'N, 7°14'W.

Sampling date 9/90

Specimens of *Riseriellus occultus* were collected from the upper shore among the roots of *Spartina* spp. and from muddy sand in beds of *Zostera noltii* Hornemann.

Densities of *Riseriellus* were recorded at 6-131 worms m^{-2}; associated fauna included polychaetes (*Capitella capitata* Fabricius, *Streblospio benedicti* Webster, *Pygospio elegans* Claparède, *Heteromastus filiformis* Claparède, *Alkmaria romijni* Horst, *Nereis diversicolor* Müller and *Malacoceros fuliginosus* Claparède) molluscs (*Hydrobia ulvae* Pennant and *Scrobicularia plana* da Costa) crustaceans (*Melita palmata* Montagu, *Idotea chelipes* Pallas, *Chaetogammarus marinus* Leach, *Hyale nilssoni* Rathke and *Carcinus maenas*) and unidentified Oligochaeta and Chironomidae. Mean grain sizes of sediments were in the range of 0.047-0.17 mm, the silt clay fraction varied from 16.8-73.15% and the organic content had a range of 3.72- 9.02% (Rogers *et al.*, in press). *Lineus longissimus* was also found near low water.

United States of America

Manset, Southwest Harbour, Mount Desert Island, Maine (Fig. 2.19). 44°22'N, 68°19'W.

Sampling date 11-12/1991.

Specimens of both *Lineus ruber* and *Lineus viridis* were collected intertidally at this locality.

Canada

St Croix River, Brandy Cove, St Andrews, New Brunswick (Fig. 2.19). 45°05'N, 67°04'W.

Sampling date 8/1991

Specimens were collected from Brandy Cove near the mouth of the St. Croix river at St Andrews. *Lineus ruber* and *Lineus viridis* were both collected from this area. Unusually both species appear very dark or even black in this locality, making them difficult to separate on external morphology.

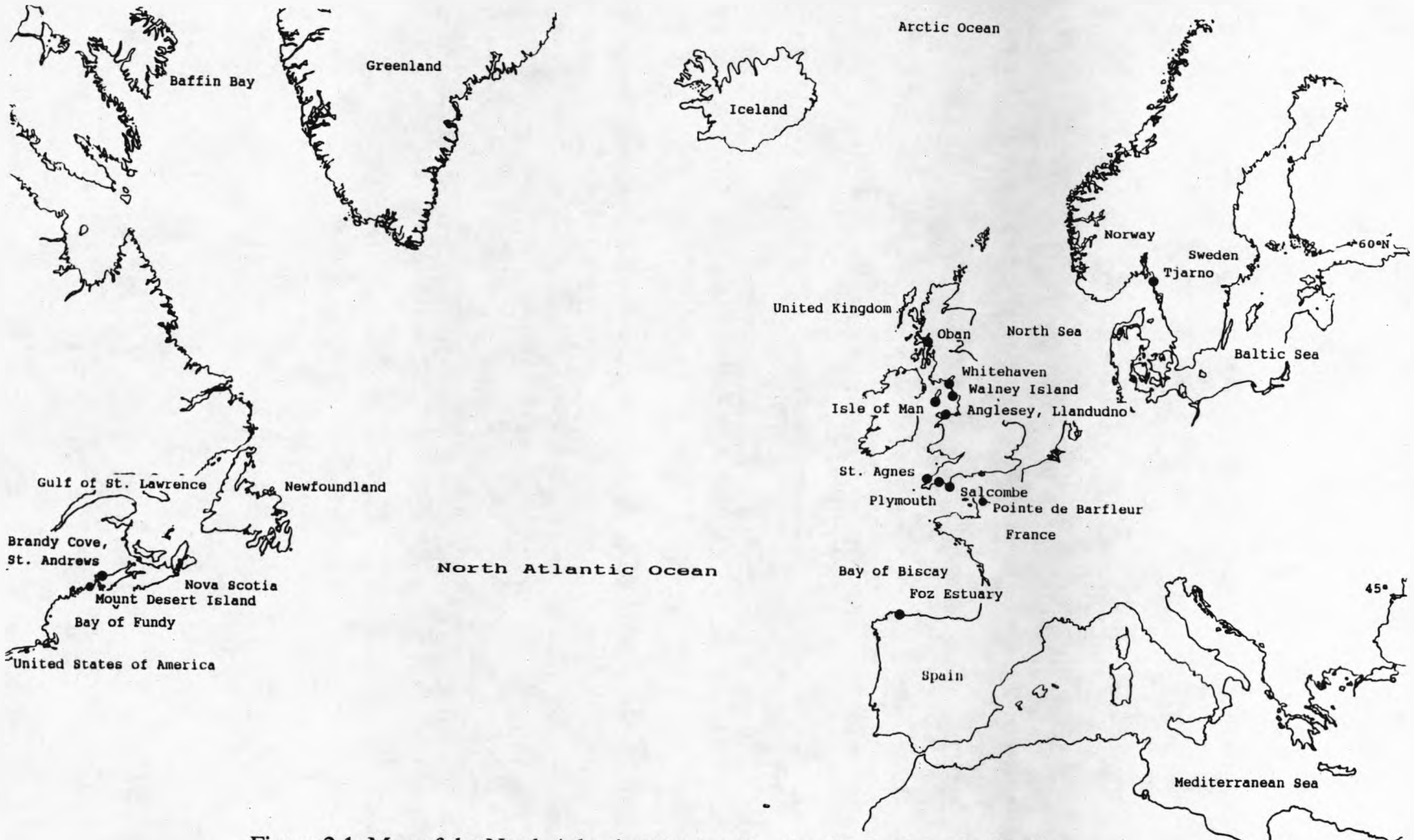


Figure 2.1. Map of the North Atlantic area showing the distribution of collecting sites.

Figure 2.2. Map of the United Kingdom showing collecting sites.

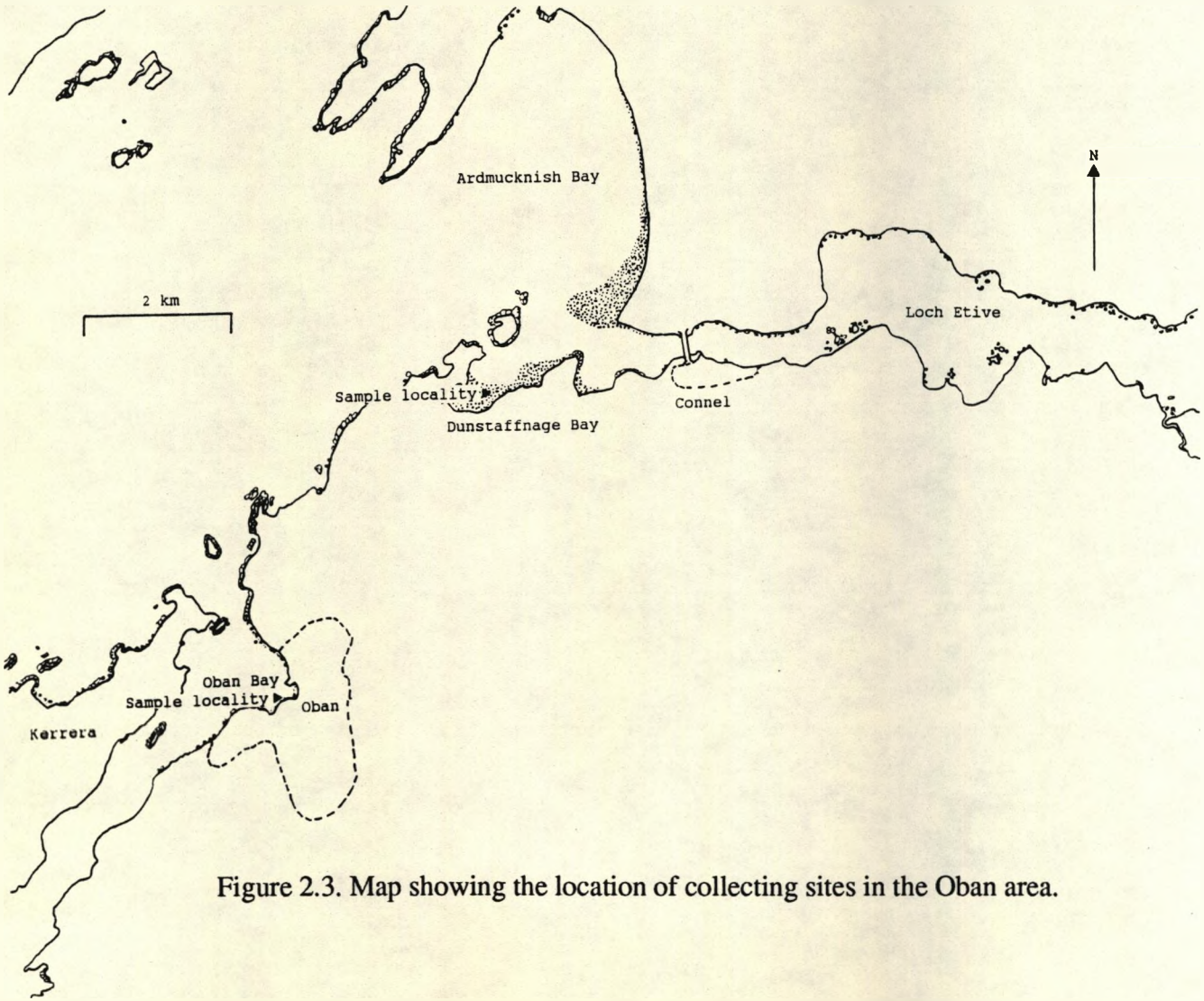


Figure 2.3. Map showing the location of collecting sites in the Oban area.

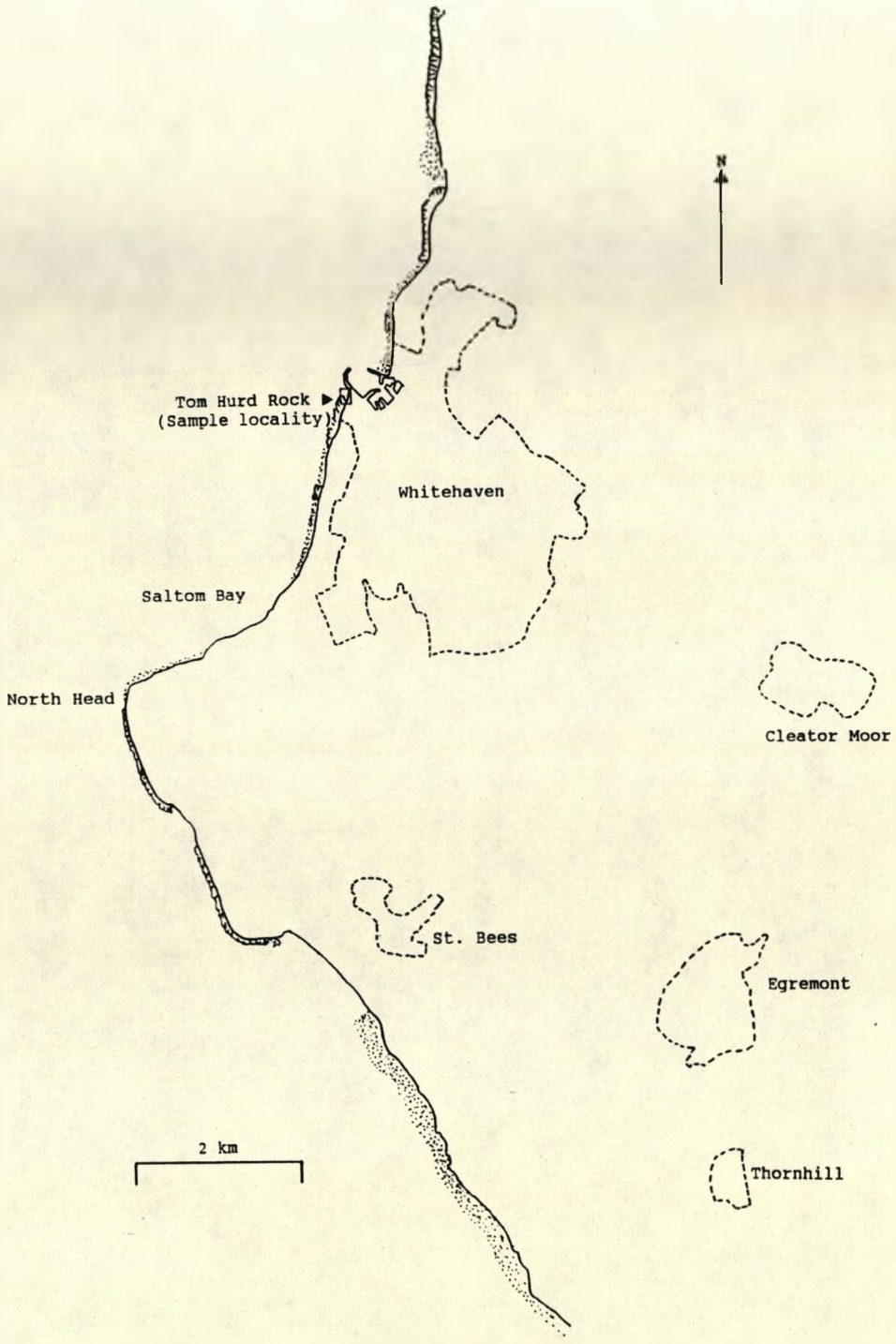


Figure 2.4. Map showing the location of the Whitehaven collecting site.

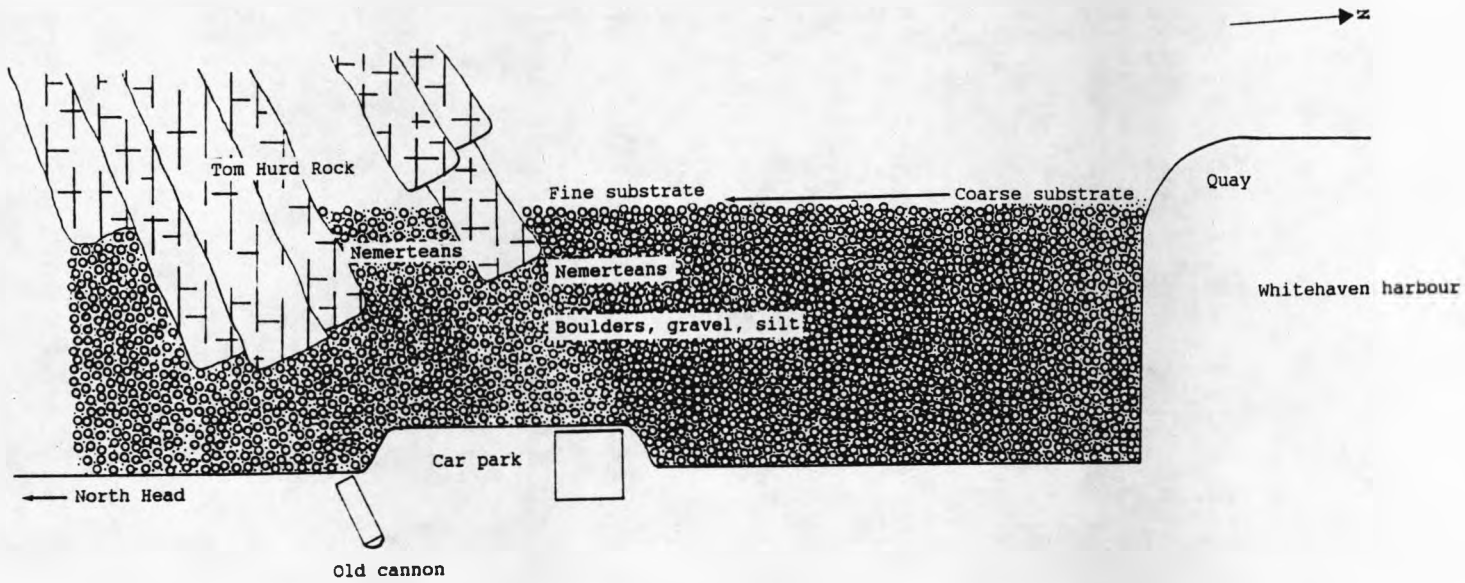


Figure 2.5. Sketch map showing the Whitehaven collecting site. Not to scale.

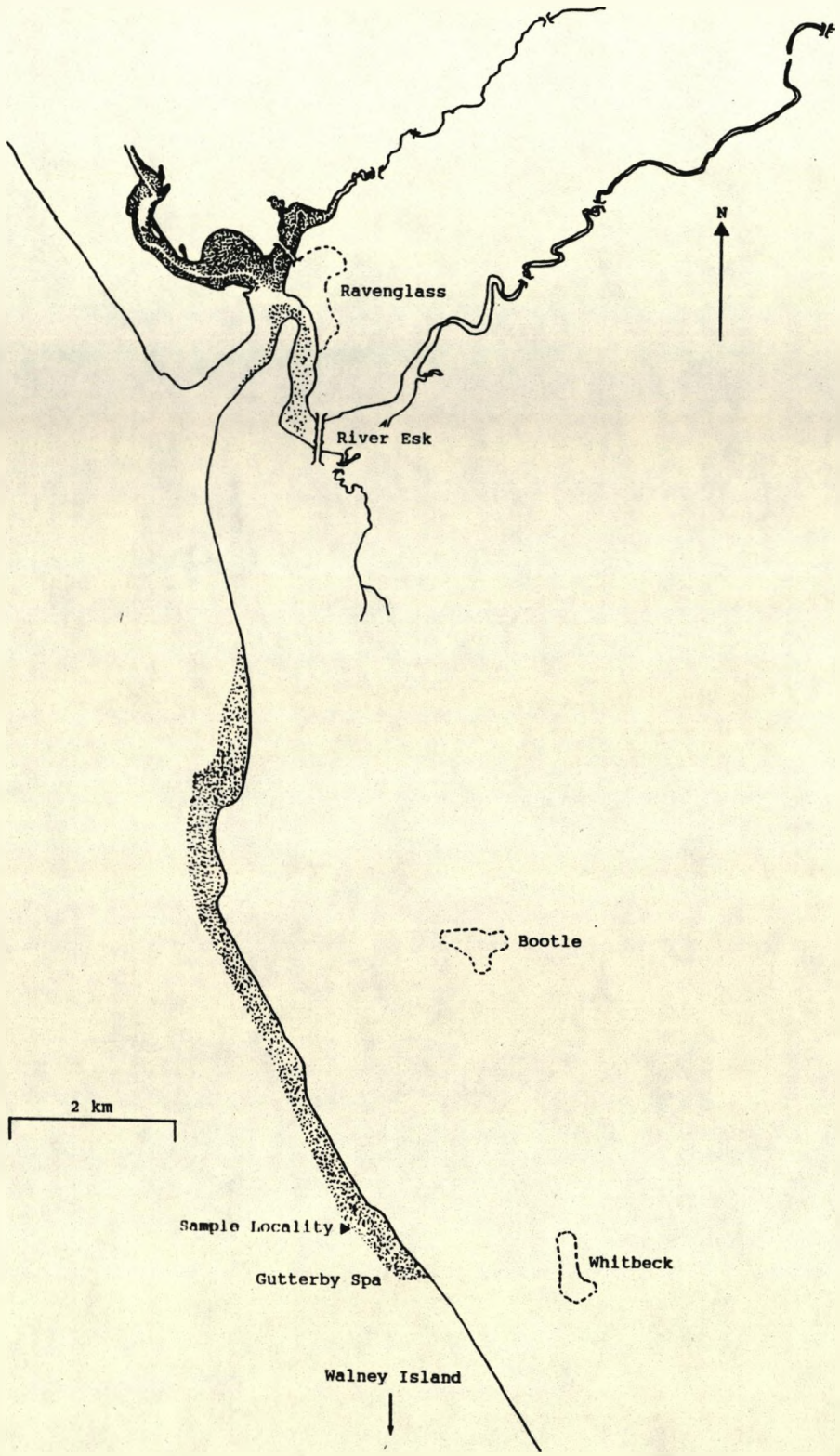


Figure 2.6. Map showing the location of the Gutterby collecting site.

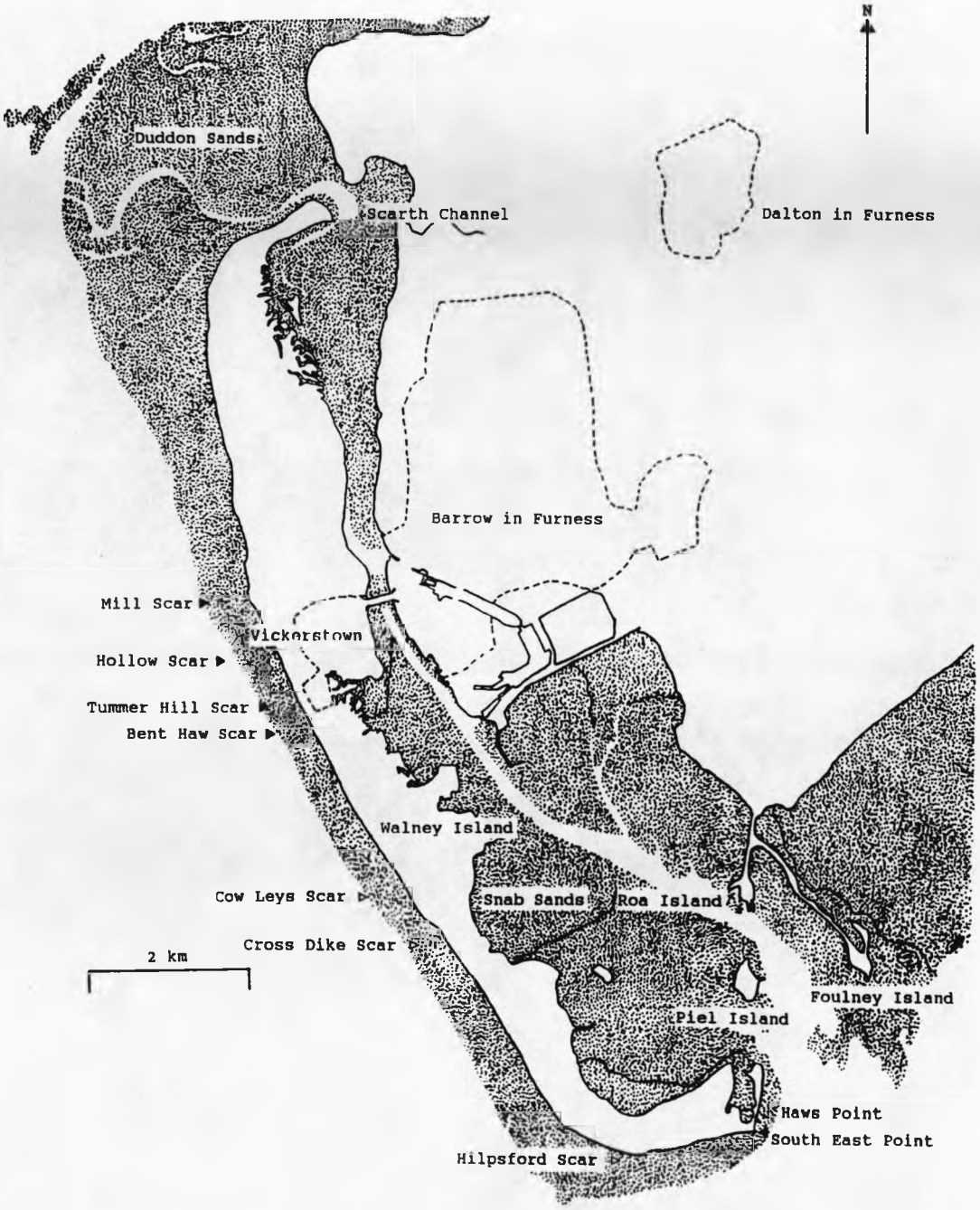


Figure 2.7. Map showing the location of collecting sites on Walney Island, Barrow-in-Furness.

Figure 2.8. Map showing the location of collecting sites on the Isle of Man.

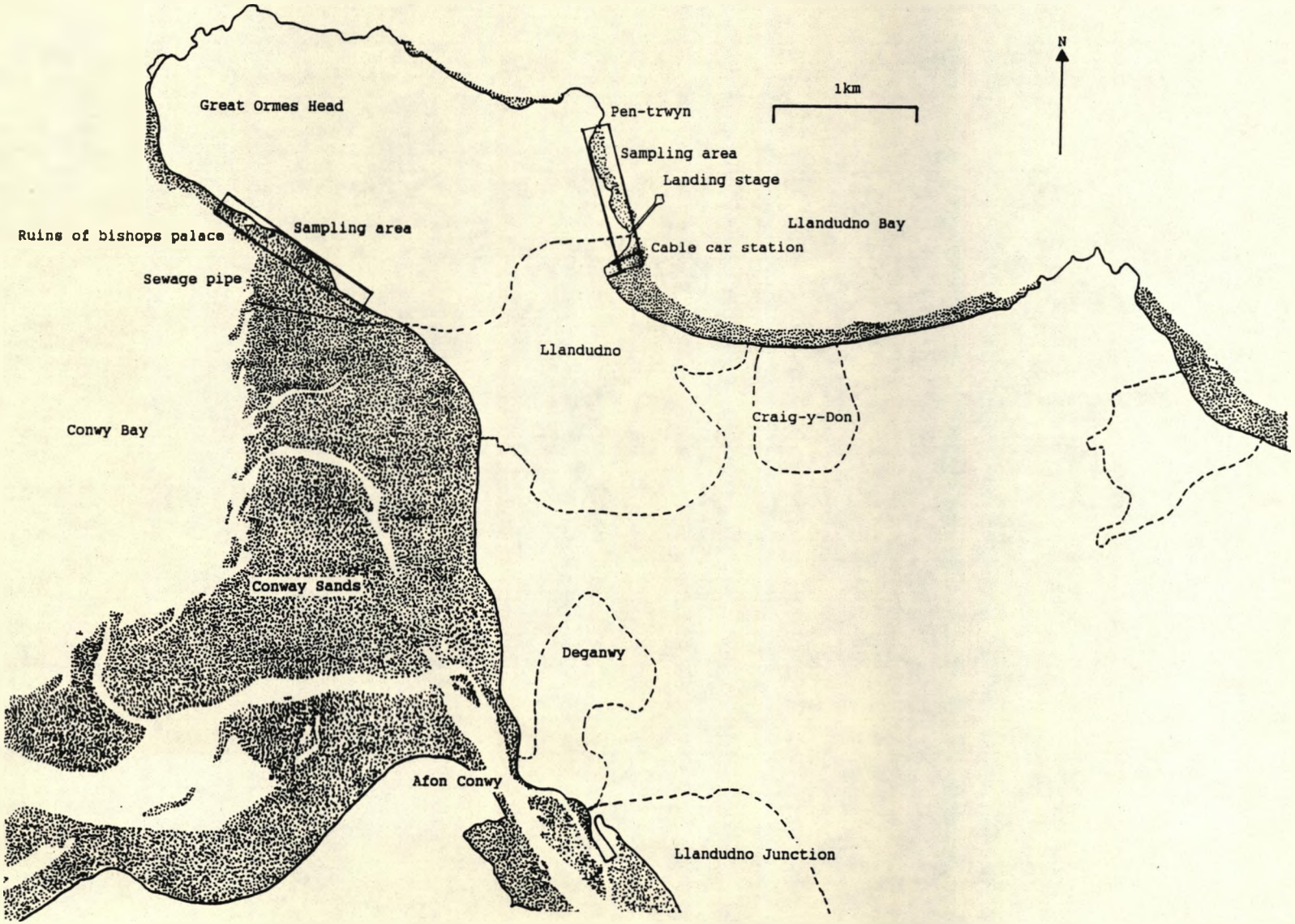


Figure 2.9. Map showing the location of collecting sites at Llandudno, North Wales.

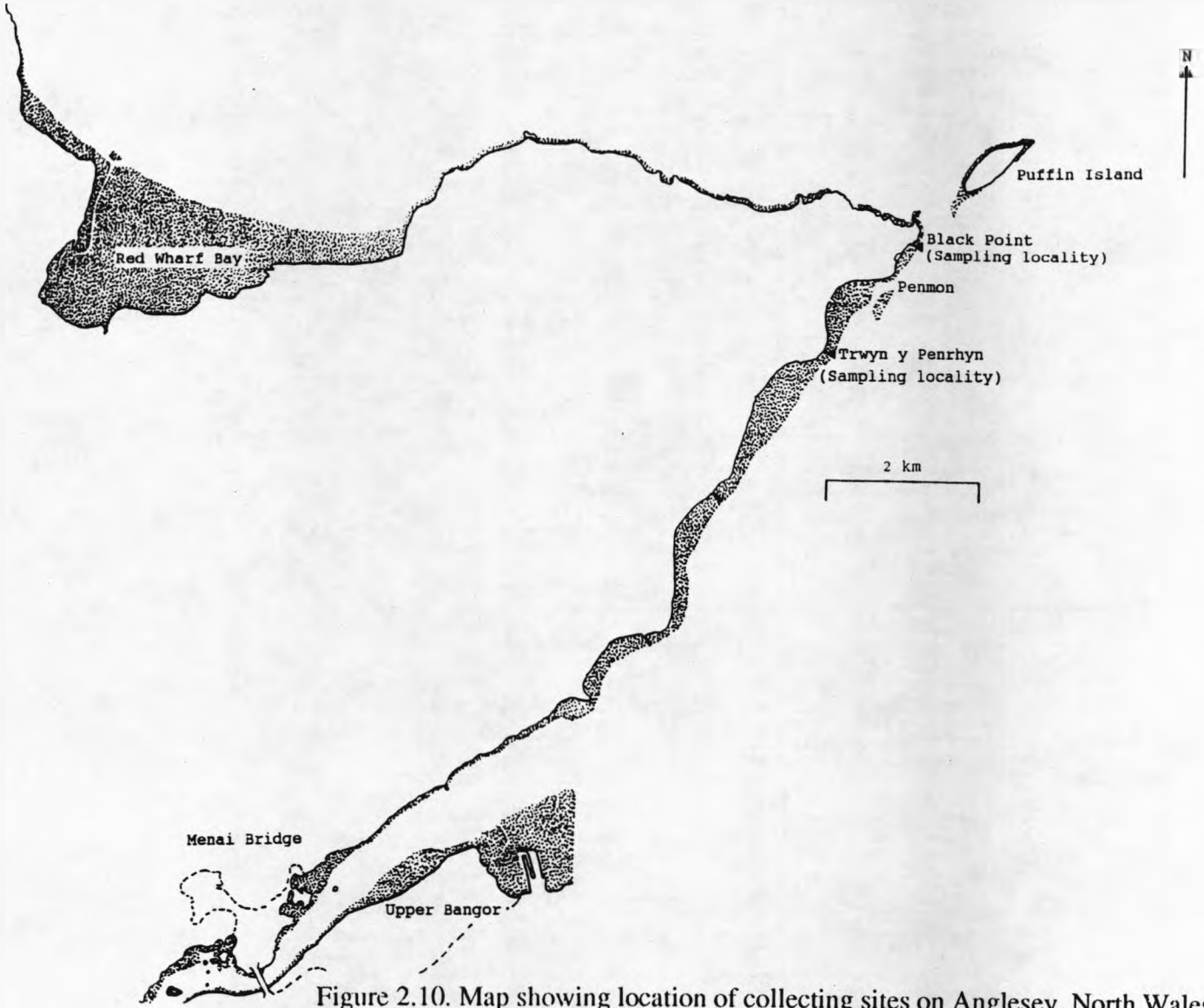


Figure 2.10. Map showing location of collecting sites on Anglesey, North Wales.

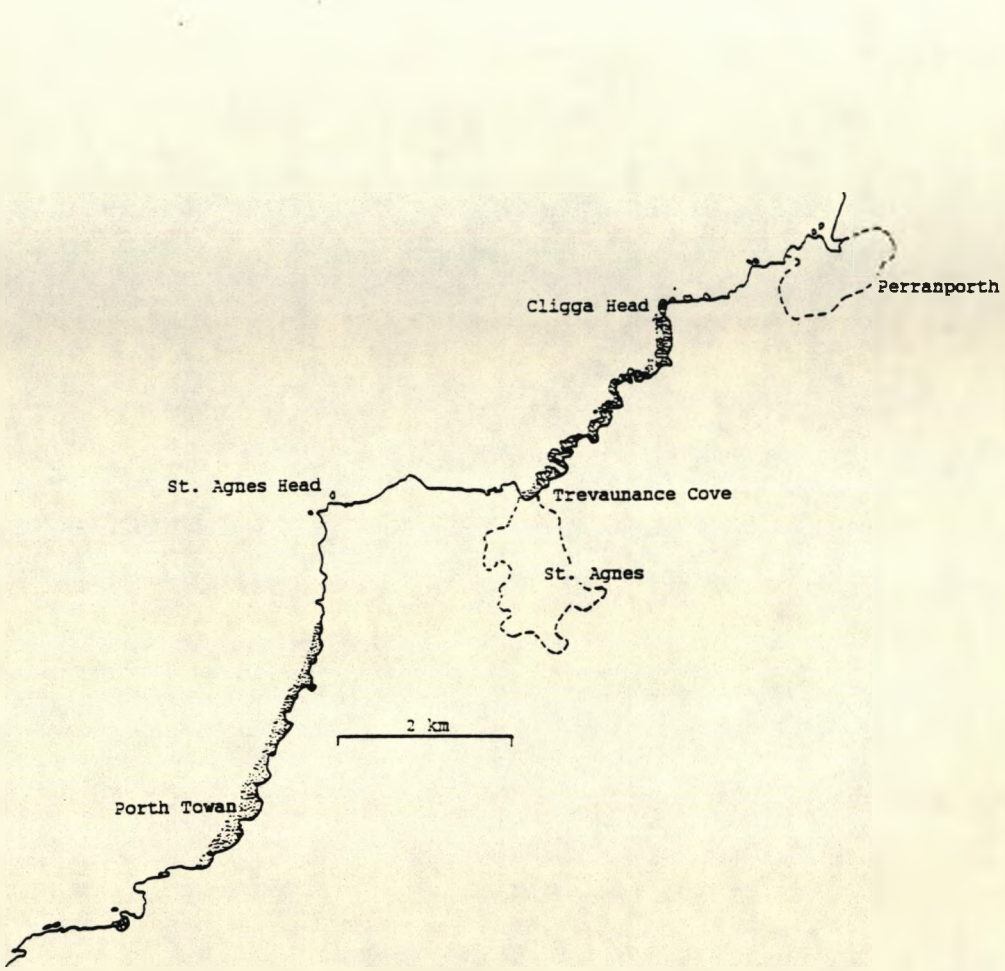


Figure 2.11. Map showing location of the St. Agnes sample site.

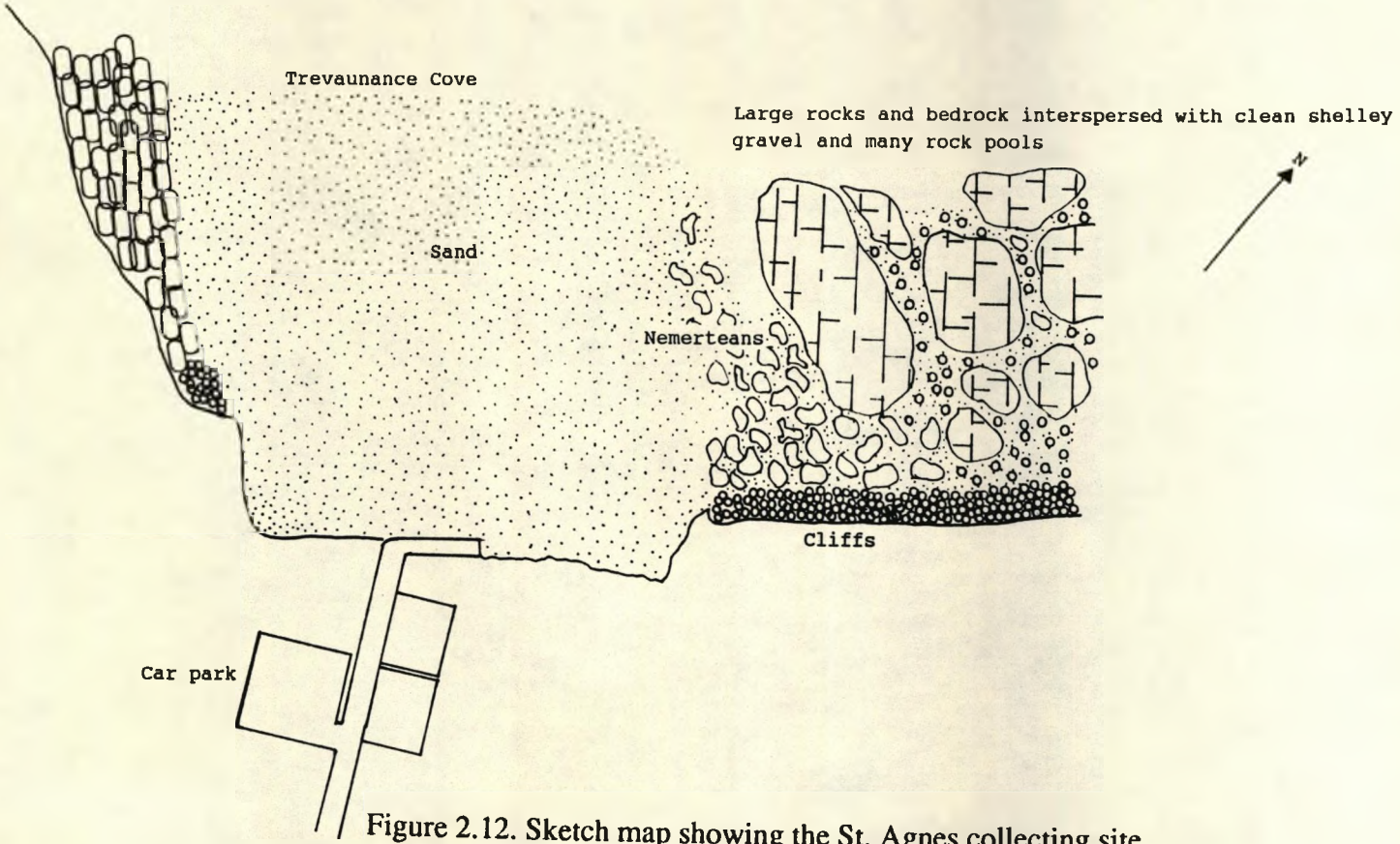


Figure 2.12. Sketch map showing the St. Agnes collecting site.

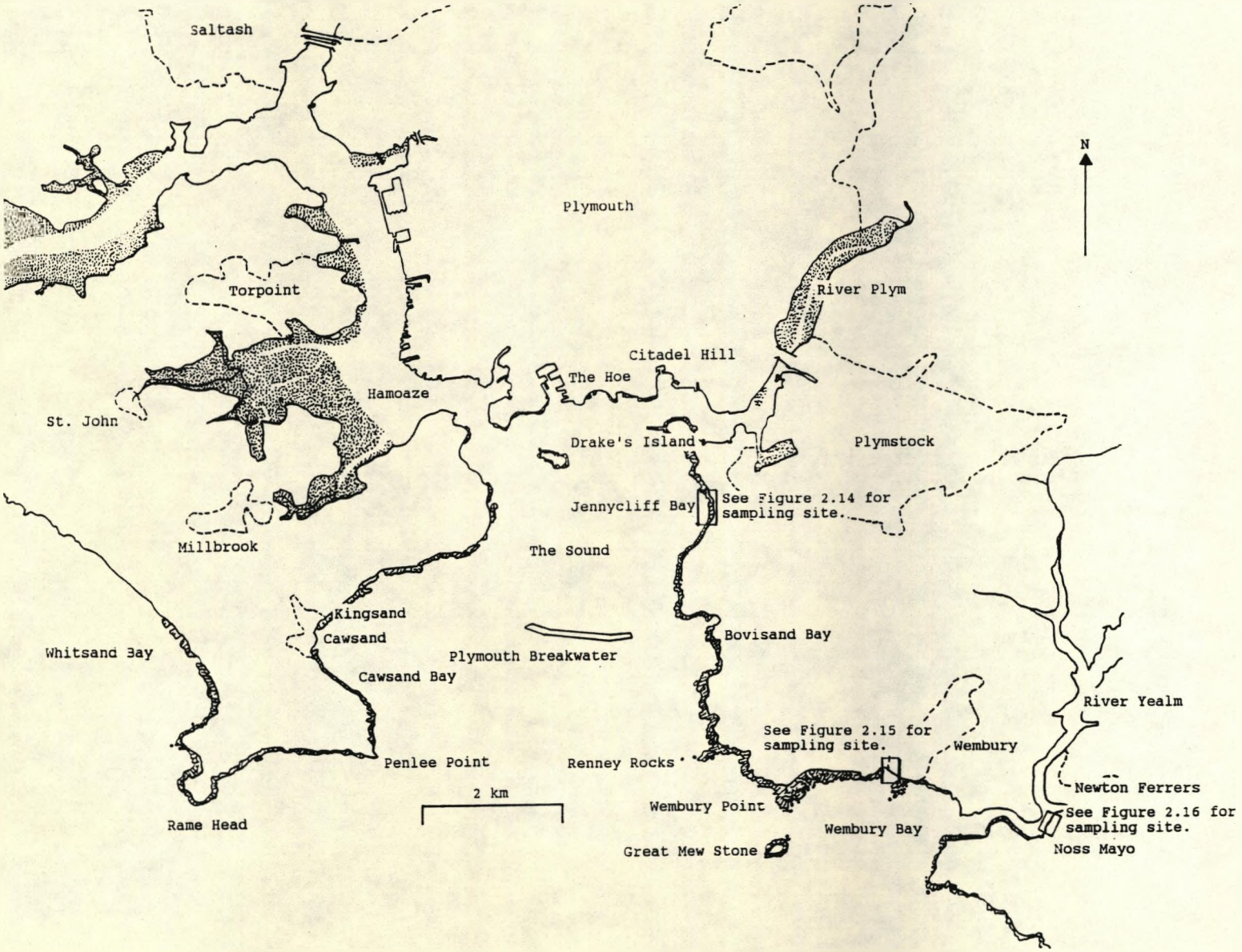


Figure 2.13. Map of Plymouth Sound showing location of collecting sites.

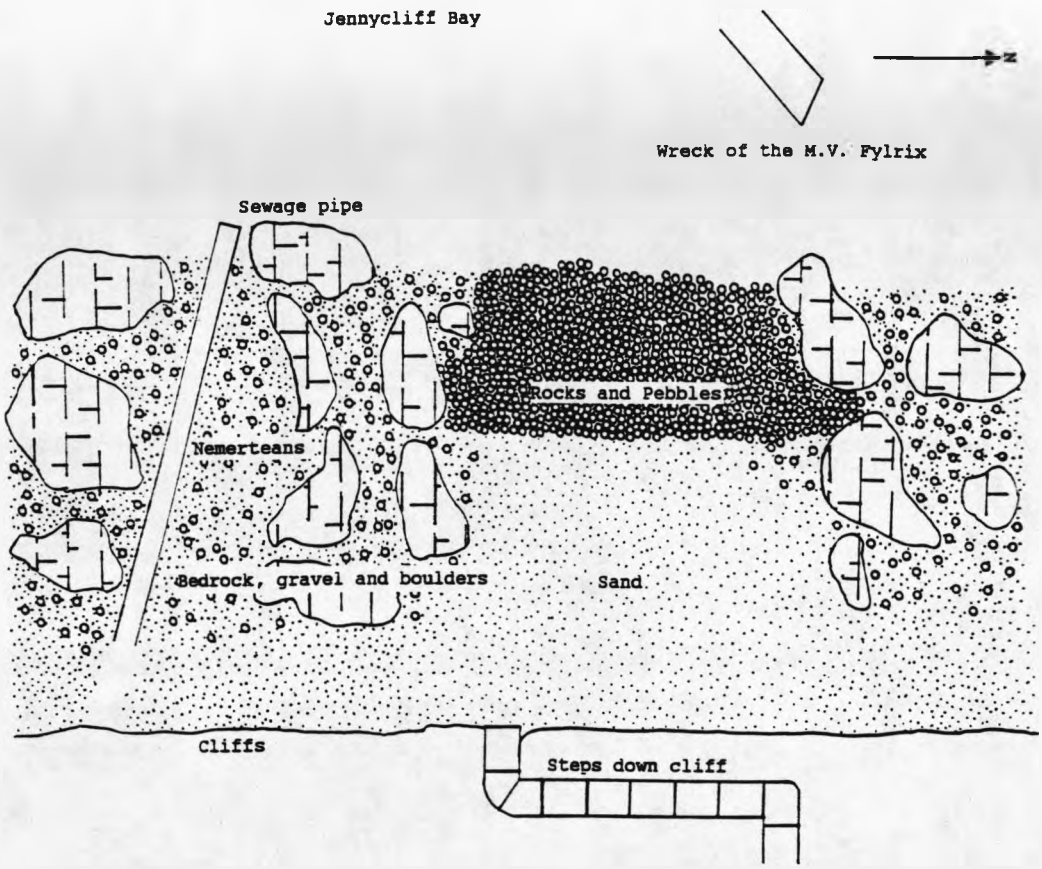


Figure 2.14. Sketch map showing the Jennyclliff collecting site.

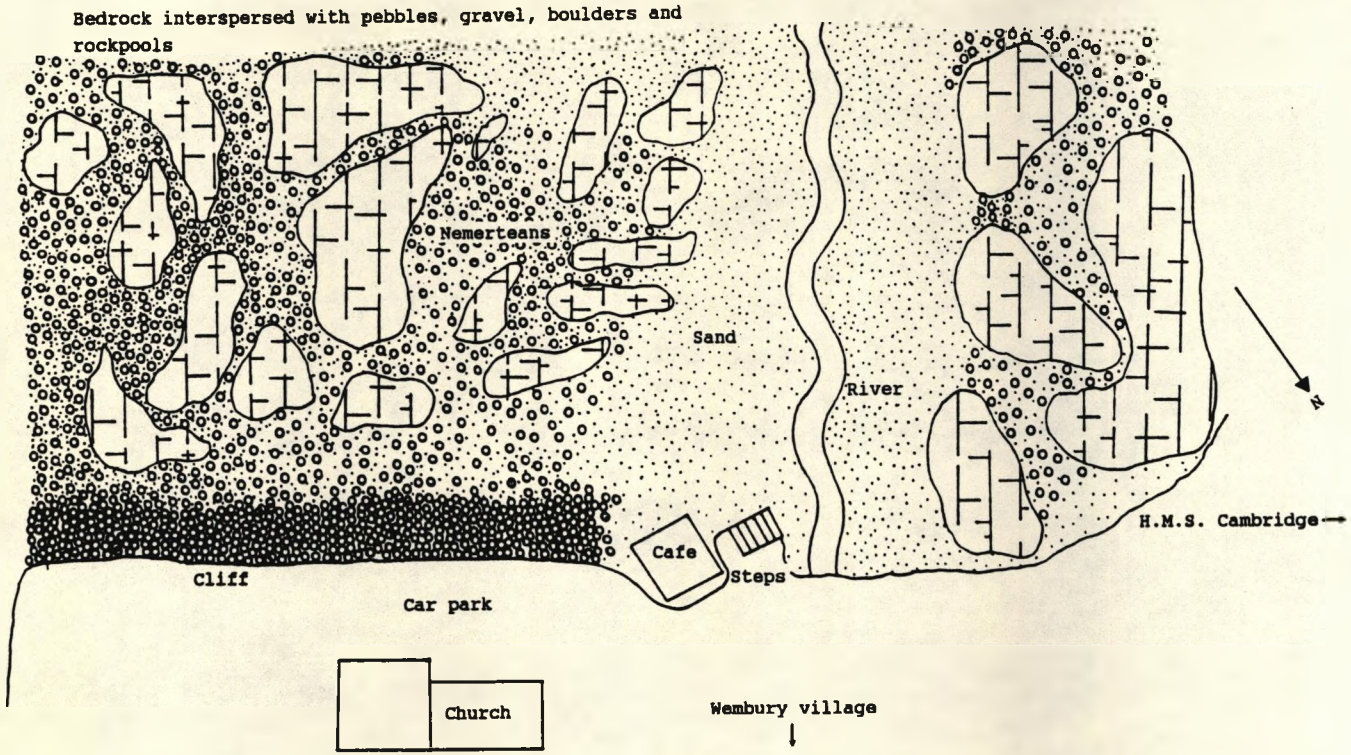


Figure 2.15. Sketch map of the Wembury sample site.

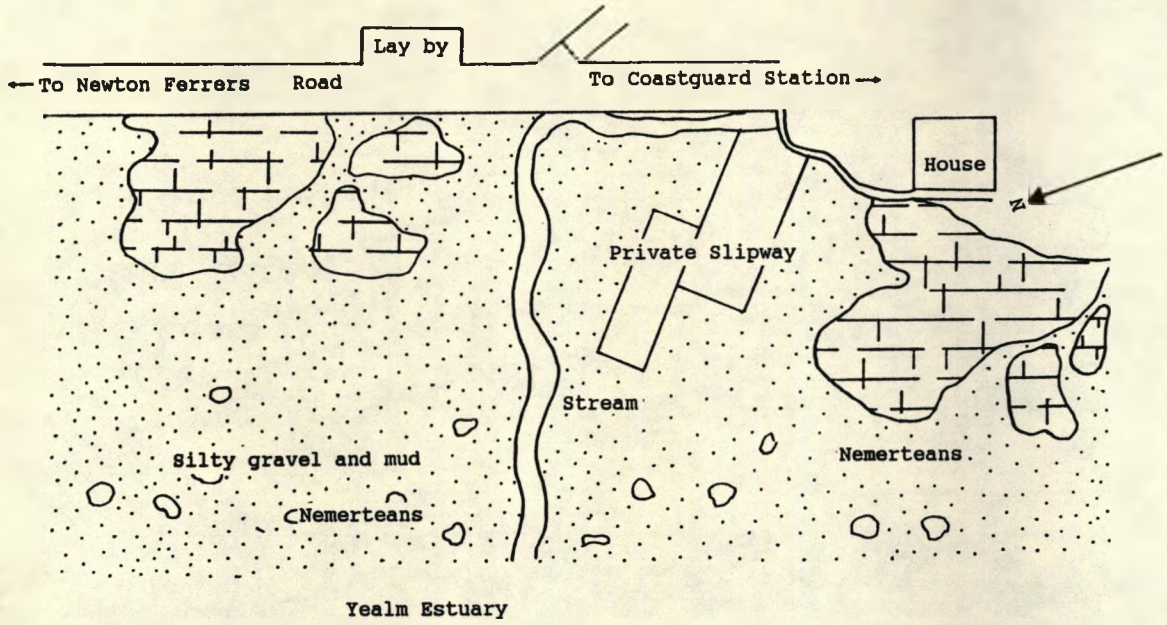


Figure 2.16. Sketch map showing the Yealm sample site.

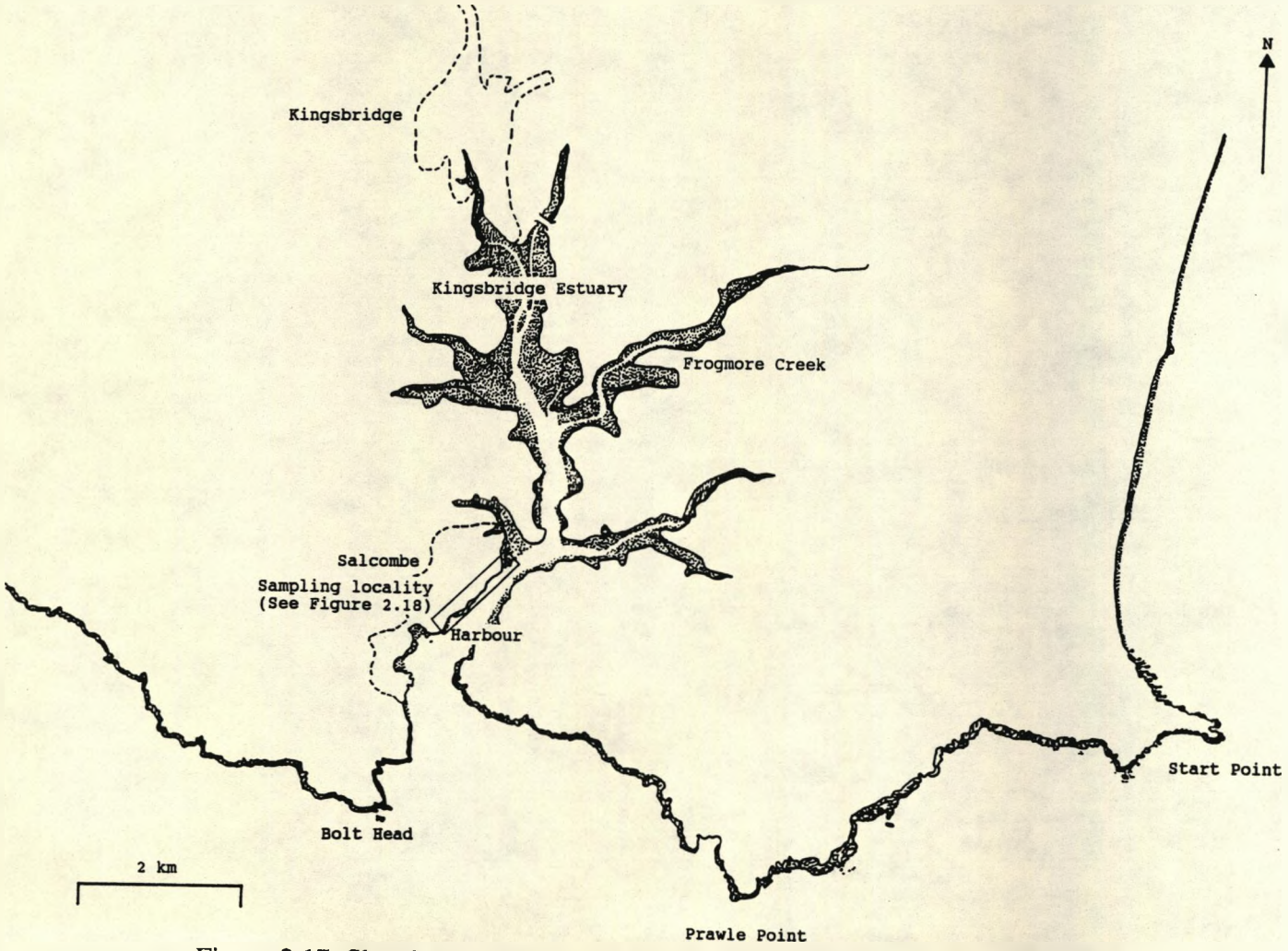


Figure 2.17. Sketch map showing the Kingsbridge estuary and location of the Salcombe sampling locality.

Figure 2.18 Sketch map showing the Salcombe collecting site.

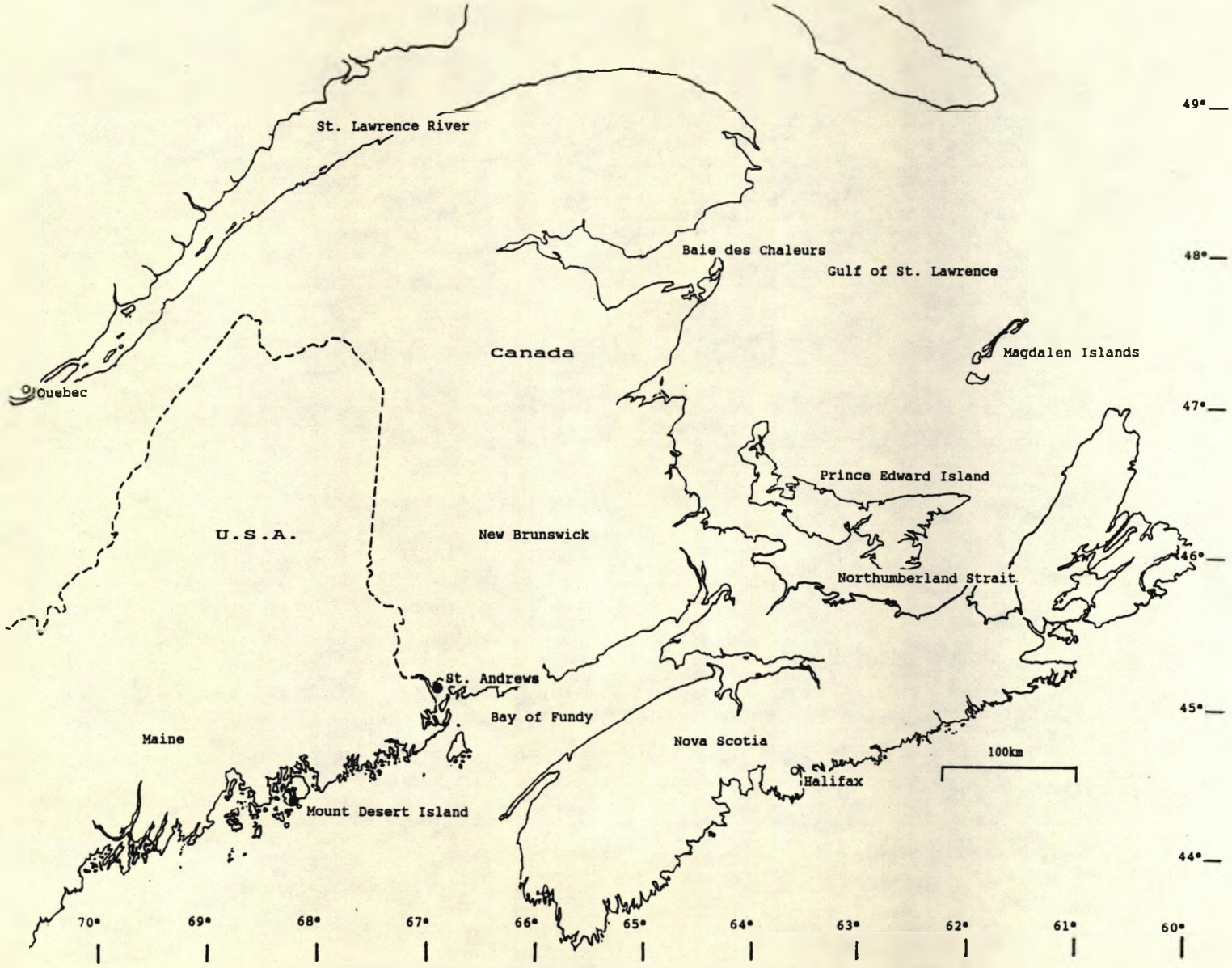


Figure 2.19. Map showing the location of collecting sites in the U.S.A.

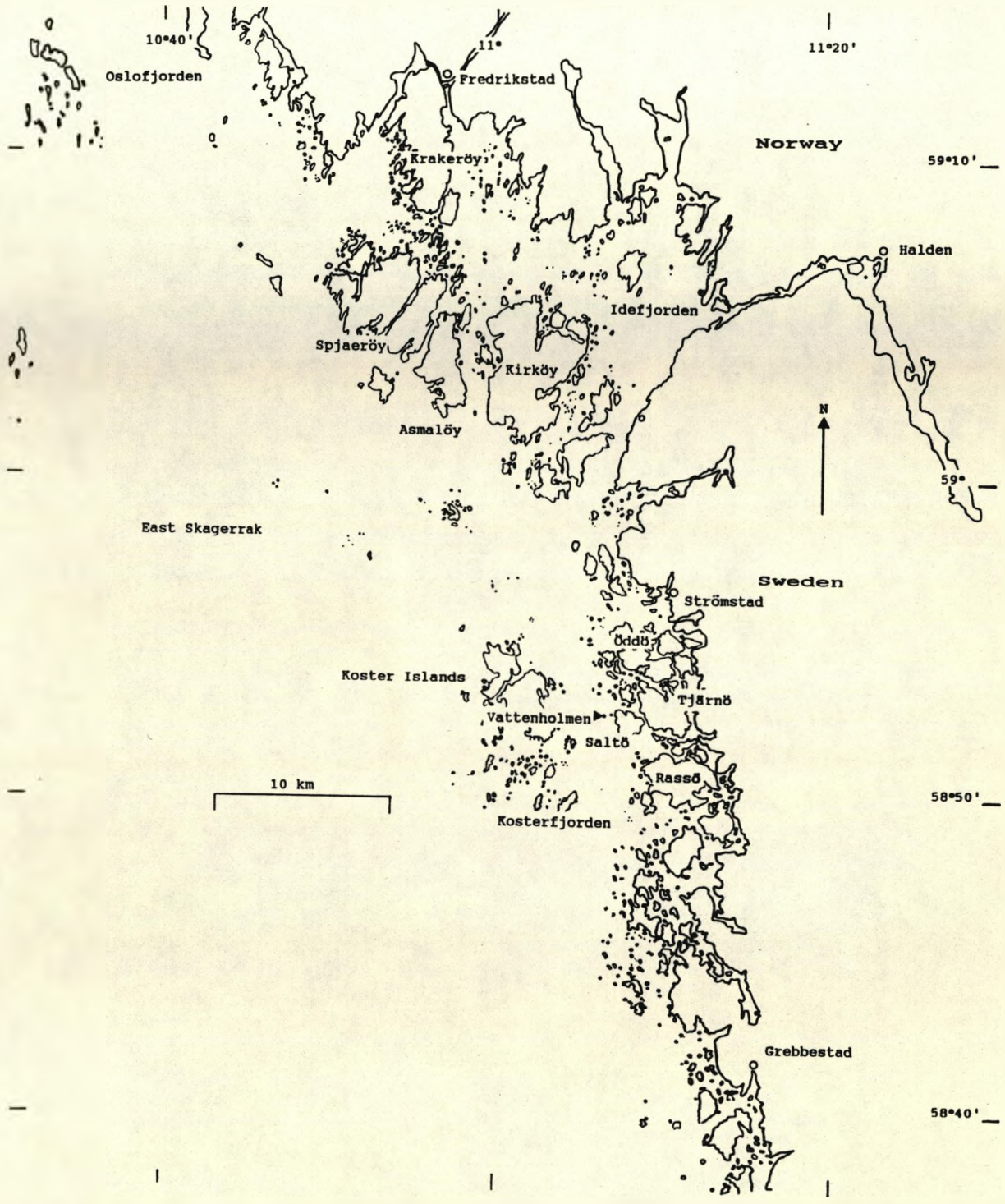


Figure 2.20. Map showing the location of collecting sites in Sweden.

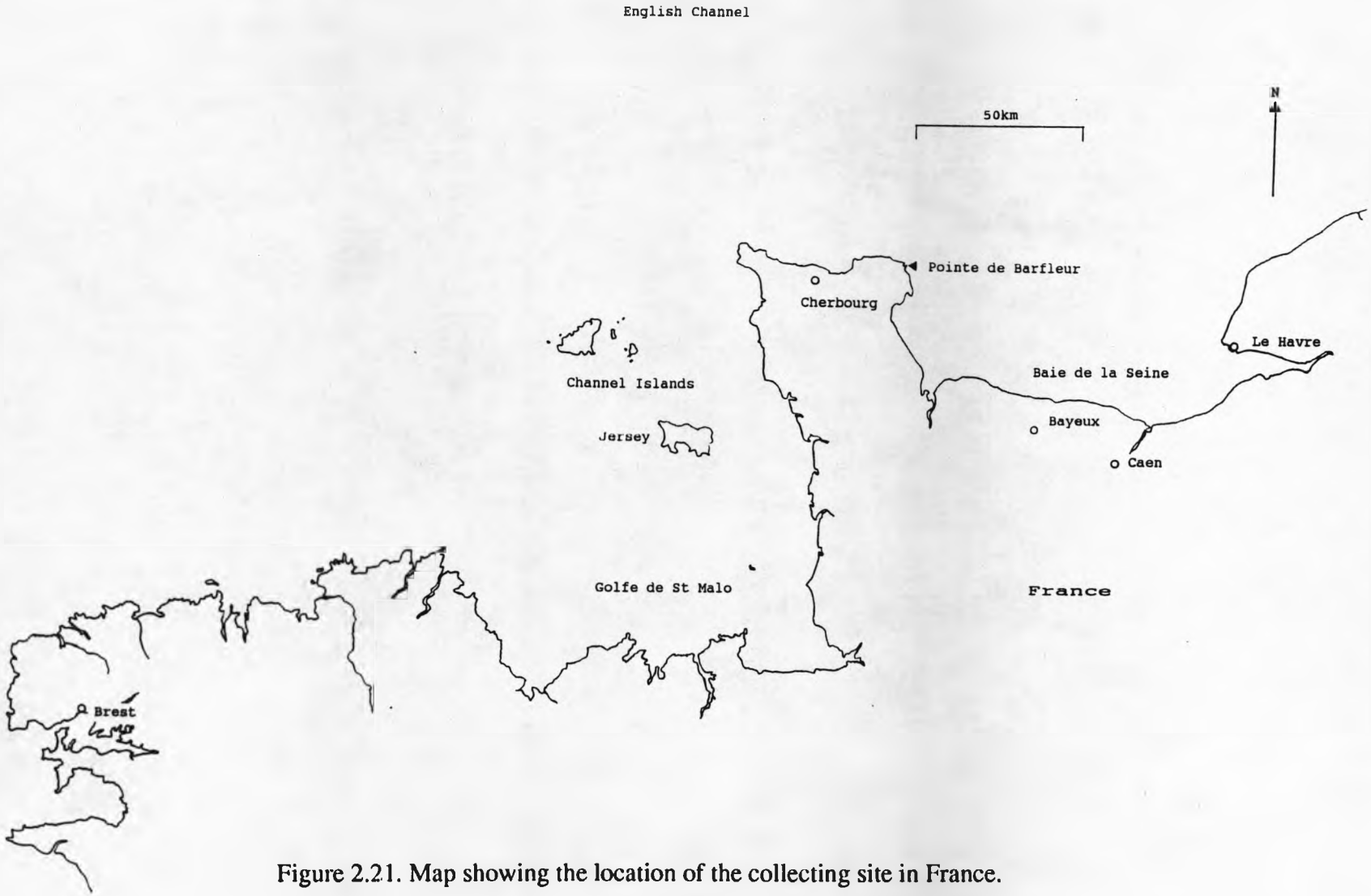


Figure 2.21. Map showing the location of the collecting site in France.

Figure 2.22. Map showing the locality of sampling sites in northern Spain.

2.2 Systematic Cataloguing of Specimen Data

All specimens were assigned a number prior to laboratory investigation. Each specimen was placed in a separate petri dish of sea water labelled with an individual number. This number was recorded on individual records of external morphology and on data sheets from electrophoresis. The anterior part of a given specimen was returned to its relevant petri dish after a piece had been removed for electrophoresis. Subsequent to electrophoresis the animal was fixed and placed in a specimen tube identified with its number. Fixed specimens were then stored prior to histological study.

This system meant that the external description of a specimen could be matched to an individual genotype which could in turn be matched with the fixed specimen in a tube. This could be sectioned to give a description of the internal morphology of the original specimen.

2.3 Description of Nemerteans Prior to Electrophoresis.

Prior to electrophoresis of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*, *Lineus longissimus* and *Amphiporus lactifloreus*, the external characteristics of half the specimens were recorded. Half the number of specimens (10 out of a total of 20 for each electrophoresis run) were described because this took one day. Due to the time constraints of the investigation description of a larger percentage of specimens was considered unnecessary. The external characteristics of any new or unusual species were also recorded.

A standard data sheet was prepared for recording the external morphology of a specimen. This contained the following entries:

Number - The serial number of the specimen (see 2.2).

Date - Date of description.

Date Collected - The date the specimen was collected.

Locality - The name of the locality from which the specimen was obtained.

Description of Habitat - A description of the habitat from which the nemertean was collected. This included details of physical conditions, height on shore, associated species and any other relevant information.

Collector - The name of the person who collected the specimen.

Overall Appearance - A general description of the external characteristics of the specimen seen by the naked eye. e.g. For *Amphiporus allucens* " Deep salmon red nemertean, with yellowish head and post cephalic region. Dark mid-dorsal stripe runs along entire body length in which the proboscis is clearly visible".

Head - A description of the anterior region of the nemertean was given. The following details were recorded: the shape of the anterior region, whether or not it formed a cephalic lobe and if so the shape (lanceolate, spatulate, semi-circular or heart shaped); the presence or absence of ocelli, if present this included a description of the colour, number and distribution of ocelli; the presence or absence of horizontal lateral furrows, longitudinal, transverse or oblique grooves and frontal organs (see Figs. 2.23, 2.24); the colour pattern of the dorsal and ventral surfaces of the head; The position and shape of the cephalic ganglia if visible through the dorsal or ventral surface of the head; the position, size and shape of the proboscis pore (if visible) and the mouth; a sketch diagram of the head was included for each specimen described.

Body Shape - The general body shape of specimens was recorded.

Body Colour - Pigmentation of the dorsal and ventral surface of the entire length of

the body was recorded. This included base colour, presence of pigment granules, presence of any colour patterns and the translucency of the body.

Figure 2.23 External morphology of nemerteans

a) *Tubulanus annulatus* (Palaeonemertea), dorsal view. Shows the cephalic lobe formed by the head and also the characteristic colour pattern of this species (red base colour with white stripes).

b) *Amphiporus allucens* (Hoplonemertea), dorsal view. Shows the cephalic ganglia (a pair of dark patches at the back of the head) and the ocelli scattered on the dorsal surface of the head.

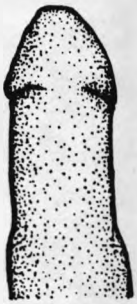
c) *Amphiporus allucens* (Hoplonemertea), ventral view. Shows the cephalic grooves on the ventral surface of the head.

d) *Lineus ruber* (Heteronemertea), dorsal view. Shows the frontal organs (three at the anterior tip of the head) and the cephalic ganglia (pale patches at the back of the head).

a

b

Figure 2.23. External morphology of nemerteans



c

d

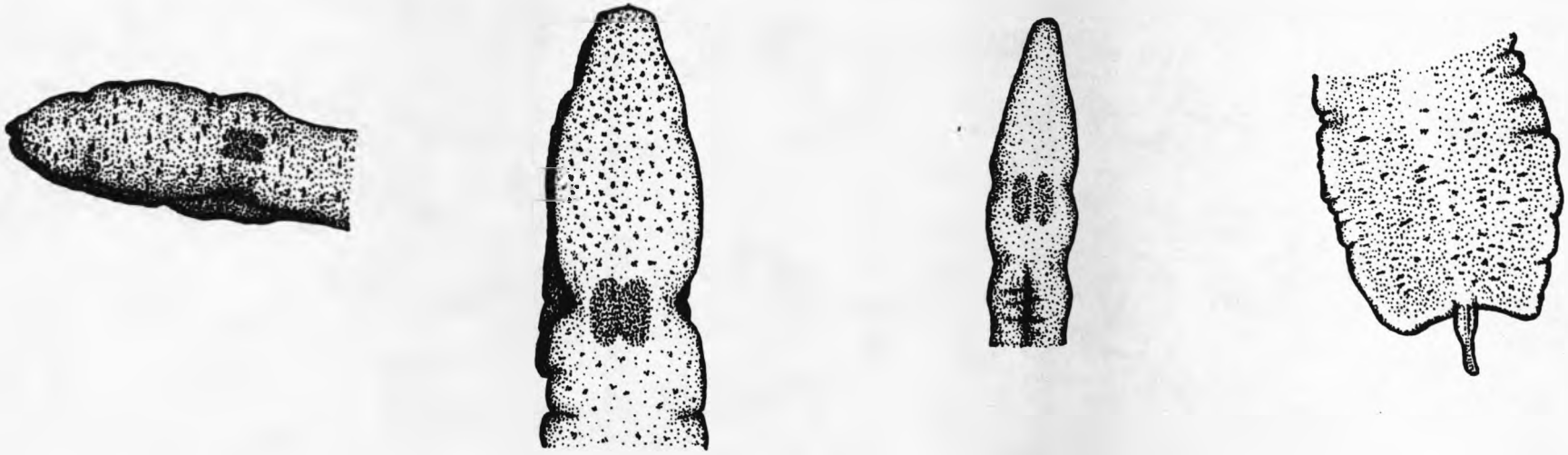
Figure 2.24 External morphology of nemerteans.

a) *Cerebratulus fuscus* (Heteronemertea), lateral view. Shows the lateral cephalic slits and the cerebral ganglia (a pair of dark patches at the back of the head.

b) *Cerebratulus fuscus* (Heteronemertea), dorsal view. Shows the edges of the lateral cephalic slits and the cerebral ganglia (a pair of dark patches at the back of the head).

c) *Cerebratulus fuscus* (Heteronemertea), ventral view. Shows the slit like mid-ventral mouth.

d) *Cerebratulus fuscus* (heteronemertea), dorsal view of the posterior end of the body showing the caudal cirrus.



a b c d

2.24. External morphology of nemerteans.

Length - The maximum length of each specimen was recorded with a set of dial callipers (Camlab Cambridge) to 0.1mm. Length was recorded while the nemertean was crawling freely in a large basin in an undisturbed state. Several measurements were made, the longest was recorded. For very large specimens such as *Lineus longissimus*, length was estimated by attempting to uncoil the worm, stretch it out and measure it with a tape.

Width - The maximum width of each specimen was recorded when the worm was crawling around in a large basin in an undisturbed state.

Nemerteans were described prior to electrophoresis so that electrophoretic results would not bias records of external characteristics by the investigator. External descriptions of specimens were made because the external morphology for the nemertean species under investigation, had not been previously recorded in detail. Also specimens classed as one of the species under investigation, but found to be genetically distinct, could be checked after electrophoresis for any differences in external morphology to the named species.

2.4 Electrophoresis

Horizontal starch gel electrophoresis was carried out in accordance with standard procedures as described by several authors (Smithies, 1955, 1959; Shaw, 1965; Bier, 1967; Scopes, 1968; Brewer, 1970; Gordon, 1975; Sargent & George, 1975; Siciliano & Shaw, 1976; Smith, 1976; Gottlieb, 1977; Harris & Hopkinson, 1976; Ferguson, 1980; Gaal et al., 1980; Richardson *et al.*, 1986; Leary & Brook, 1990; Murphy *et al.*, 1990).

2.4.1 Gel Preparation

The four most commonly used methods of electrophoresis differ in the type of support medium they utilise. These are starch gel, polyacrylamide, agarose gel, and cellulose acetate. Starch gel electrophoresis was used in this investigation since it is cheap compared to other methods, gels are safe to handle and a number of enzymes may be

stained from a single gel (up to 8) (for a more detailed analysis of the advantages and disadvantages of different support media see Murphy *et al.*, [1990]).

A 12.5% starch solution was prepared by heating 47.5g of potato starch (Sigma Chemical Co. S-4501) with 380 ml of gel buffer in a 1000 ml pyrex side arm flask over a bunsen flame. The solution was heated until it had passed from a milky liquid to a viscous opaque fluid then to a clear liquid and finally had commenced boiling.

The liquid was then degassed by attaching a tap aspirator to the side arm of the flask and placing a rubber bung over the top of the flask. Following degassing the molten starch solution was poured into a 10 mm x 150 mm x 180 mm perspex gel mould. This was sandwiched between two plates of glass (180 mm x 210 mm) and allowed to solidify. Gels were prepared 13 - 14 hours prior to electrophoresis.

2.4.2 Tissue preparation

Specimens were kept alive prior to electrophoresis. Specimens were removed from numbered petri dishes and 5-15 mm of the posterior end of the animal was removed with a scalpel. The animal was then placed back in its dish and the tissue placed in a 0.5 cm^{-3} well drilled into a perspex block. Each well was numbered from 1-20.

Tissue was homogenised in the perspex well with 100 µl of 0.06 M Tris-HCl buffer pH 8.0 (6.05g Tris (hydroxymethyl) ammonium-methane (Tris) l^{-1} distilled water, correct to pH 8.0 with 1 M HCl) using a glass rod. Two 3 mm x 12 mm filter paper wicks (Whatman No. 3) were used to absorb the tissue extract from each well through a sheet of single ply tissue paper (inclusion of tissue paper between tissue and wicks prevented gelatinous tissue homogenate from contacting wicks and greatly increased resolution). Note two wicks were required for each specimen since two gels were run for each set of animals.

Wicks were inserted in order (1-20) along a straight slit cut in the gel 2 cm away from the cathode end with a clean scalpel. The gel was then placed in an electrophoresis bath (Shandon Model 600) and the anode and cathode applied (gauze strips approx. 20 cm x 19 cm). The cathode was placed along the filter paper wicks, the anode was placed 10cm

away from the wicks. Anode and cathode were layed as straight as possible to give optimum resolution.

The electrophoresis bath was then placed in a fridge (approx. 5^{o}C) and the gel run at a power and time appropriate to the gel and electrode buffering system being used. After the gels had run for 10-15 minutes, they were switched off, removed from the fridge and the wicks were discarded quickly. The gels and baths were then placed back in the fridge and switched back on. This improved resolution.

2.4.3 Buffering systems

Table 2.1 Buffer systems assessed with a variety of metabolic enzymes for their power to resolve alleles:

I. Ward & Beardmore (1977); continuous pH 8.0. Electrode: 151.45 g Tris, 59.9 g citric acid, 5 l distilled water. Gel: 192.5 ml electrode buffer diluted to 5 l in distilled water. 140 V, 50 mA, 7 hours.

II. Williams *et al.* (1983); continuous pH 7.1. Electrode: 16.35 g Tris, 8.26 g citric acid, 0.46 g disodium salt of ethylene diamine tetra-acetic acid (Na_2EDTA), 1 l distilled water. Gel: 16.35 g Tris, 9.035 g citric acid, 6.9 g Na_2EDTA, 1 l distilled water. Dilute 1:15 in distilled water. 100 V, 50 mA, 7 hours.

III. Shaw & Prasad (1970); continuous pH 6.7 (electrode), 7.0 (gel). Electrode: 29.1 g dibasic potassium phosphate (K_2HPO_4 anyhydrous), 5.7 g citric acid, 1 l distilled water. Gel: 1.08 g K_2HPO_4, 0.259 g citric acid, 1 l distilled water. 100 V, 50 mA, 7 hours.

IV. Shaw & Prasad (1970), Brewer (1970), Selander *et al.* (1971); continuous pH 7.4. Electrode: 12.1 g TRIS, 11.9 g malic acid, 3.72 g Na_2EDTA, 2.03 g $MgCl_2$.6H2O, 1 l distilled water. Gel: 1:9 dilution of electrode buffer. 130 V, 15 mA, 7 hours.

V. Williams *et al.* (1983); continuous pH 7.6 (electrode), 8.0 (gel). Electrode: 11.8g boric acid, 1.2 g lithium hydroxide, 1 l distilled water. Gel: 7.2 g citric acid, 27.9 g TRIS, 5.9 g boric acid, 0.6 g lithium hydroxide, 1 l distilled water. Dilute 1:5 with distilled water. 160 V, 30 mA, 7 hours.

VI. Brewer (1970); discontinuous, pH 8.0. Electrode: 602.90 g trisodium citrate, 5 l distilled water, adjust to pH 8.0 with 1.00 M citric acid. Gel: 9.60 g histidine hydrochloride, 5 l distilled water, adjust to pH 8.0 with 0.10 M sodium hydroxide. 120 V, 25 mA, 7 hours.

VII. Ahmad *et al.* (1977); discontinuous, pH 8.6. Electrode: 18.6 g boric acid, 4.0 g sodium hydroxide (NaOH), 1 l distilled water. Gel: 8.0 g Tris, 1.5 g citric acid, 1 l distilled water. 140 V, 25 mA, 8 hours.

VIII. Poulik (1957); discontinuous, pH 8.3. Electrode: 18.55 g boric acid, 2.4 g NaOH, 1 l distilled water. Gel: 9.21 g Tris, 1.04 g citric acid, 1 l distilled water. 130 V, 30 mA, 8 hours.

Out of these buffers only systems I and II gave sufficient resolution with enough enzyme loci to be useful for this study.

2.4.4 Gel Slicing and Staining

After electrophoresis was complete gels were sliced into 1mm slices using a Shandon slicing table. the top and bottom slices were discarded since these tended to show poor resolution when stained. No problems with electrodecantation (Murphy *et al.*, 1990) were observed.

Gel slices were placed in shallow perspex trays (11cm x 17cm) on to each of which a specific staining solution was poured. Each tray was then covered by a piece of card to keep solutions dark while they developed. Gels were incubated at room temperature. Enzyme loci stained as dark spots or bands on a pale background. Development of stains took 5 minutes to over 1 hour in some cases. Staining bands were recorded by placing the gel on a piece of graph paper and mapping where bands occurred on an identical piece of graph paper.

A number of enzyme stains were assessed with the buffering systems previously described. Table 2.1 lists enzymes found to be unacceptable as they showed little or no activity, poor resolution or results were not consistent enough to be useful for the investigation.

Nine enzyme stains were found to be useful with buffer systems I. and II. Trials were carried out, varying the concentrations of the various components of these enzymes, to find which combination gave the best activity and resolution. The formulae which were subsequently used in this study are as follows:

Table 2.2 Formulae used to stain for enzymes in the present study.

(i) Malate dehydrogenase (MDH) E.C. 1.1.1.37: buffer system I; stain: 100 mg L-malic acid, 970 mg TRIS, 80 mg magnesium chloride, 15 mg nicotinamide adenine dinucleotide (NAD+), 10 mg 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide. (MTT), 1 mg phenazine methosulfate, 25 ml distilled water, 25 ml 2% agar.

(ii) Isocitrate dehydrogenase (ICD) E.C. 1.1.1.42: buffer system II; stain: 80 mg isocitric acid, 285 mg magnesium chloride, 5 mg nicotinamide adenine dinucleotide phosphate (NADP), 5 mg MTT, 5 mg PMS, 25 ml 0.05 M Tris-HCl, pH 8.0.

(iii) Phosphogluconate dehydrogenase (PGD) E.C. 1.1.1.44: buffer system I; stain: 20 mg 6-phosphogluconate, 5 mg NADP, 5 mg MTT, 5 mg PMS, 10 mg magnesium chloride, 25 ml 0.05 M Tris - HCL, pH 8.0.

(iv) Octopine dehydrogenase (ODH) (Walsh & Somero, 1981) E.C. 1.5.1.1: buffer system I; stain: 25 mg octopine, 15 mg NAD+, 7 mg MTT, 2 mg PMS, 25 ml 0.05 M Tris-HCl, pH 8.0, 25 ml 2% agar.

(v) Glutamate-oxaloacetate transaminase (GOT) (Williams *et al.*, 1983) E.C. 2.6.1.1: buffer system II; stain: 0.6 g TRIS, 150 mg aspartic acid, 40 mg a-ketoglutaric acid, 20 mg polyvinylpyrolidone, 15 mg pyridoxal-5-phosphate, 40 ml distilled water. Incubate for 30 minutes at room temperature then add 100 mg fast blue RR in 10 ml of distilled water.

(vi) Phosphoglucomutase (PGM) E.C. 2.7.5.1: buffer system II; stain: 100 mg glucose-1-phosphate, 50 mg magnesium chloride, 10 mg NADP, 5 mg MTT, 5 mg PMS, 100 µl glucose-6-phosphate dehydrogenase (approx. 35 units), 25 ml 0.05M TRIS-HCl pH 7.1, 25 ml 2% agar.

(vii) Aminopeptidase A (APA) (Williams *et al.*, 1983) E.C. 3.4.11.13: buffer system I; stain: 10 mg N-glycyl-L-leucine, 5 mg L-amino acid oxidase, 5 mg o-dianisidine, 10 mg horseradish peroxidase, 1 mg manganous chloride, 25 ml 0.05 M TRIS-HCl pH 8.0, 25 ml 2% agar.

(viii) Fumarate hydratase (FH) (Harris & Hopkinson, 1976) E.C. 4.2.1.2: buffer system II; stain: 60 mg fumaric acid, 20 mg NAD, 20 mg sodium pyruvate, 5 mg MTT, 5 mg PMS, 25 µl malic dehydrogenase (approx. 75 units), 25 ml 0.05 M TRIS-HCl pH 8.0.

(ix) Phosphoglucose isomerase (PGI) (Harris & Hopkinson, 1976) 5.3.1.9: buffer system I; stain: 20 mg fructose-6-phosphate, 5 mg NADP, 5 mg MTT, 5 mg PMS, 25 µl glucose-6-phosphate dehydrogenase (approx. 10 units), 25 ml 0.05 M TRIS-HCl pH 8.0, 25 ml 2% agar.

Table 2.3. Enzyme stains screened and found to be unsuitable for use with nemerteans (E.C. No. - International Union of Biochemistry Nomenclature Commitee [1984] number.)

Enzyme Name & Abbreviation.		E.C. No.	Reference to stain recipe
Glycerol 3P dehydrogenase	(aGPDH)	1.1.1.8.	Harris & Hopkinson, 1976.
Sorbitol dehydrogenase	(SORDH)	1.1.1.14.	Harris & Hopkinson, 1976.
Lactate dehydrogenase	(LDH)	1.1.1.27.	Harris & Hopkinson, 1976.
Malic enzyme	(ME)	1.1.1.40.	Harris & Hopkinson, 1976.
Glucose dehydrogenase	(GDH)	1.1.1.47.	Harris & Hopkinson, 1976.
Octanol dehydrogenase	(OCTDH)	1.1.1.73.	Schaal & Anderson, 1974.
Succinate dehydrogenase	(SDH)	1.3.99.1.	Brewer, 1970.
L Glutamate dehydrogenase	(GLUD)	1.4.1.3.	Harris & Hopkinson, 1976.
D Aspartate oxidase	(DASOX)	1.4.3.1.	Harris & Hopkinson, 1976.
NADH Diaphorase	(DIAPH)	1.6.2.2.	Harris & Hopkinson, 1976.
Catalase	(CAT)	1.11.1.6.	Harris & Hopkinson, 1976.
Superoxide dismutase	(SOD)	1.15.1.1.	Harris & Hopkinson, 1976.
Nucleoside phosphorylase	(NP)	2.4.2.7.	Harris & Hopkinson, 1976.
Adenylate kinase	(AK)	2.7.4.3.	Harris & Hopkinson, 1976.
Aconitase	(ACON)	4.2.1.3.	Harris & Hopkinson, 1976.
Mannose P isomerase	(MPI)	5.3.1.8.	Harris & Hopkinson, 1976.

2.4.5 Analysis of Genetic Data

The position of staining bands on gels was measured to 1 mm accuracy. Mobility of all alleles was calculated relative to the mobility of the most common allele for *Lineus ruber* in all enzyme loci. This was done because of all species studied, *Lineus ruber* showed the lowest levels of heterozygosity across all enzymes. Specimens of *Lineus ruber* were also readily available from Port Erin to use as marker animals. The only exception to this was *Amphiporus lactifloreus* for which enzyme mobility was calculated relative to the mobility of the most common allele for *Amphiporus lactifloreus*. This was done because *Amphiporus lactifloreus* was not being used in taxonomic investigations with the genus *Lineus*. Alleles were labelled alphabetically in ascending order from those showing the greatest mobility to those showing the least (e.g. for *Pgm-1* A = allele with highest mobility, Y = allele with lowest mobility).

Alleles which were very close in mobility (within 0.1) from different species were considered as identical unless specimens of the two or more species involved had been run together on gels and shown to have different enzyme mobilities.

Allele and genotype frequencies were calculated and data analysed using the FORTRAN program BIOSYS - 1 (version 1.7) (Swofford and Selander, 1989). For details on genetic analysis see Chapter 3.

2.5 Fixation of Specimens.

Specimens not completely destroyed in electrophoresis were fixed. Prior to fixation specimens were relaxed in 7% magnesium chloride solution for up to 30 minutes for large specimens.

The specimen was then layed out straight on a flat dry surface (a petri dish or ceramic tile) between the edges of glass slides. It was then irrigated with Bouin's fixative (30 parts saturated picric acid solution, 10 parts 40% formaldehyde solution, 2 parts acetic acid, made up in sea water). Irrigation was continued until the specimen had hardened, to

prevent it from drying out. The specimen was then transferred to a polyethylene specimen tube, full of Bouin's fluid, which was sealed and then labelled with the specimens identity number (see Section 2.2).

Large specimens were cut into several sections after relaxation but before fixation. Each of these sections was placed in a separate specimen tube. From very large specimens a section from the anterior end, one from the middle and one from towards the posterior end would be fixed. The rest of the animal was discarded.

2.6 Sectioning of Specimens.

Specimens were removed from Bouin's and placed in 70% alcohol to remove excess picric acid. This was repeated until no more yellow colour (Bouin's fluid) was imparted to the alcohol. Specimens were then cleared in xylene and placed in molten 56° C m.p paraffin wax, in a vacuum oven for 30 minutes. Specimens were then embedded in 56° C m.p. paraffin wax and sectioned transversely at 6 µm using a Cambridge Rocking Rotary microtome in series. Sections were then floated on warm water (approx. 40 - 45°C) in a section mounting bath and mounted in series on slides coated in glycerin albumin. Slides were labelled using a diamond tipped glass writing pen.

2.7 Staining of sections

Sections were stained using the Mallory triple method (Pantin, 1960). This involves immersion of slides in a sequence of solutions for a given period of time as follows:

(1) Histoclear (National Diagnostics). 5 Minutes.

(2) Histoclear. 5 Minutes.

(3) 100% Ethanol. 1 Minute.

(4) 100% Ethanol. 1 Minute.

(5) 90% Ethanol. 1 Minute.

(6) 70% Ethanol. 1 Minute.

(7) Distilled water. 1.5 Minutes.

(8) Preliminary mordant. Saturated $HgCl_2$ in distilled water with 5%

glacial acetic acid.

(9) Distilled water. 1 Minute.

(10) Mallory I. 1% Acid fuchsin in distilled water. 15 Seconds.

(11) Distilled water. 1 Minute.

(12) Second mordant. 1% Phosphomolybdic acid in distilled water. 1 Minute.

(13) Distilled water. 1 Minute.

(14) Mallory II. 2.5g water soluble aniline blue, 10.0g orange G, 10.0g oxalic acid, 500ml distilled water. 45 Seconds.

(15) Distilled water. 1 Minute.

(16) 70% Ethanol. 1 Minute.

(17) 90% Ethanol. 1 Minute.

(18) 100% Ethanol. 1 Minute.

(19) 100% Ethanol. 1 Minute.

(20) Histoclear. 5 Minutes.

(21) Histoclear. 5 Minutes.

After staining a cover slip was mounted over sections using DPX.

2.8 Note on octopine dehydrogenase E.C. 1.5.1.1.

Octopine dehydrogenase has previously been detected in molluscs, especially in bivalves (e.g. Beaumont *et al.*, 1980; Gäde, 1980; Dando *et al.*, 1981) and in cnidarians (e.g. Walsh & Somero, 1981). Until the present study octopine dehydrogenase has not been detected in any other phyla. It has been suggested that octopine dehydrogenase may have a number of functions in bivalve molluscs. These include the support of vigorous muscular activity and roles in anaerobiosis (Dando *et al.*, 1981). Octopine dehydrogenase activity was detected in the present study in the anoplan heteronemerteans *Lineus longissimus*, *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*, *Micrura fasciolata*, *Micrura purpurea* and *Riseriellus occultus*. Octopine dehydrogenase was not detected in the only enoplan nemertean investigated, *Amphiporus lactifloreus*.

Chapter 3

The Genetic Structure of Populations of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* (Anopla: Heteronemertea) and *Amphiporus lactifloreus* (Enopla: Hoplonemertea) in the North Atlantic.

3.1 Introduction.

Nemerteans are mostly dioecious animals (Iwata, 1960b; Bierne, 1983; Franzén, 1983) with a few exceptions, which are hermaphroditic, occurring among the archinemerteans (Gibson *et al.*, 1990b), freshwater heteronemerteans (Moore & Gibson, 1985) and hoplonemerteans (Franzén, 1983). Reproduction is sexual or asexual, with asexually reproducing species demonstrating greater powers of regeneration than sexually reproducing species (Gibson, 1972; Riser, 1974). Embryonic cleavage is spiral (Iwata, 1960a; Willmer, 1990) and nemertean larvae show direct (Archinemertea, Palaeonemertea, Hoplonemertea) or indirect development (Heteronemertea) (Iwata, 1960a).

Lineus ruber is one of the most common species of British nemertean (Gibson, 1982b). It is reported as having a circumpolar distribution in the northern hemisphere and is recorded from the European coasts of the Atlantic, Mediterranean and North Sea, the Pacific and Atlantic coasts of North America, Madeira, Greenland, Iceland, the Faroes, Siberia and South Africa (Gibson, 1982b).

Lineus ruber generally reproduces sexually in late winter. The exact time varies with geographic locality. In the Gulf of Maine it is reported to reproduce in March/ April (Coe 1899; Riser, 1974), in the Barents Sea, July/August (Schmidt & Jankovskaia, 1938) and in the English Channel, December / January (Oxner 1911; Gontcharoff, 1951; Bierne, 1970). Bierne (1970) noted that under laboratory conditions the seasonality of reproduction of *Lineus ruber* disappears rapidly. It is likely that environmental factors therefore influence the timing and length of the reproductive cycle in this species.

During reproduction in *Lineus ruber* the male and female enclose themselves in a gelatinous mucous cocoon, secreted by the female and firmly attached to a rock, (Gontcharoff, 1951). The male releases sperm of a modified type (Franzén, 1983) compared to the more primitive type produced by other members of the family which shed their gametes directly into the water (e.g. *Lineus bilineatus* [Franzén, 1983]). The sperm fertilise the eggs which are also released into the mucous cocoon. Gontcharoff (1951) observed sperm in the gonoducts and ovaries of *Lineus ruber* which probably act as *receptacula seminis* (Cantell, 1975). Reproduction in *Lineus ruber* is regarded as a

form of pseudocopulation (Riser, 1974; Franzén, 1983). The adults leave the cocoon after reproduction and die 2-3 weeks later (Riser, 1974).

Lineus ruber have an encapsulated larva (Desor larva) of which only about 12 - 13% hatch within the mucous cocoon (Schmidt, 1946) (approximately 10-15 larvae [Gibson, 1972]). The remaining larvae/eggs are devoured by the hatchlings which then leave the cocoon as miniature worms (Schmidt, 1946; Gontcharoff, 1951; Gibson, 1972; Bierne, 1983). The juvenile worms feed immediately on leaving the cocoon and show weak positive or more usually an indifferent response to light which rapidly develops into a strong negative phototaxis (Gontcharoff, 1951).

Another common species of the genus, *Lineus viridis*, shows a similar type of reproduction to *Lineus ruber*. The male and female associate in a gelatinous envelope which is more loosely attached and not as solid as that of *Lineus ruber*. Occasionally two males will associate with the female in the same mucous envelope (Riser, 1974). Riser (1974) reports sperm throughout the epidermis and dermis in the gonoduct region in post-spawning *Lineus viridis*. This species shows a similar form of pseudocopulation to that of *Lineus ruber*. Larvae are of the encapsulated type but development is different to that shown in *Lineus ruber*. In *Lineus viridis* the first larvae to hatch do not devour the rest. As a result 400-500 juvenile worms hatch from each egg mucous string. Also in contrast to *Lineus ruber* juvenile *Lineus viridis* are strongly positively phototactic and do not feed for 2-3 weeks after hatching (Gontcharoff, 1951; Gibson, 1982b).

Lineus sanguineus is regarded by some workers as synonomous with *Lineus pseudo-lacteus* Gontcharoff 1951, *Lineus vegetus* Coe 1931, *Lineus socialis* Leidy 1855 and *Lineus nigricans* Bürger 1892 (R. Gibson, pers. comm., Liverpool John Moores University). At least two of these species, *Lineus vegetus* and *Lineus socialis*, both from the U.S.A., are reported to alternate asexual reproduction in the summer months with a phase of sexual reproduction in the winter (Coe, 1931). *Lineus sanguineus* is reported to reproduce by fragmentation throughout the year (Gontcharoff, 1951). Gontcharoff (1951) observed the development of male and female sex products from October to January. Ovaries developed abnormally, broke through into the gut or were attacked by parasites. Oocyte abortion took place and eggs were reabsorbed by the female. In males spermatogenesis produced a modified type of sperm, these were also observed to

degenerate but on at least one occasion gonoduct formation took place and sperm were released. Active sperm were also observed from this species by McIntosh (1873-1874), who observed egg development in October but made no further reference to sexual reproduction in the animal.

Evidence indicates that *Lineus sanguineus* reproduces asexually for most of the year (Gontcharoff, 1951). Whether it has a short phase of sexual reproduction, and this is eliminated in some areas by parasitic castration, or if oocyte abortion is inherited, is uncertain.

Few data exist on reproduction in *Amphiporus lactifloreus*. McIntosh (1873-1874) reported that this species matured from February - April and that the majority of eggs were deposited from February to March. Reproduction is sexual and McIntosh (1873-1874) provided evidence that fertilisation by male *Amphiporus lactifloreus* was external. Juvenile worms hatched in 12-14 days. They resembled planuloid type larvae and were not free swimming.

Three of these species, *Lineus ruber*, *Lineus viridis* and *Amphiporus lactifloreus*, show sexual reproduction without a larval dispersive phase. Genetic evidence suggests that in species which lack dispersive larval phases, geographically isolated populations show high degrees of genetic differentiation due to poor gene flow between them (Gooch *et al.*, 1972; Snyder & Gooch, 1973; Campbell, 1978; Bulnheim & Scholl, 1981; Parker *et al.*, 1981; Burton, 1983, 1986; Janson & Ward, 1984; Cameron, 1986; Waples, 1987; Ward, 1989). Gene flow is defined as genetically effective migration, i.e., an exchange between conspecific populations of successfully fertilising gametes or individuals which survive to reproduce in the population into which they have migrated (Hedgecock, 1986).

Evidence (Gontcharoff, 1951) suggests that *Lineus sanguineus* reproduces by fragmentation. If this is the case, not only will populations of *Lineus sanguineus* show high degrees of genetic differentiation but it is likely they will also show large deviations from allele frequencies expected under Hardy-Weinberg equilibrium conditions (e.g. Highsmith, 1982; Crisp *et al.*, 1983; Stoddart, 1983, 1984; Hoffmann, 1987; Smith & Potts, 1987; Shaw, 1989; Ward, 1989; Mladenov & Emson, 1990).

In this chapter results of enzyme electrophoresis are used to examine levels of heterozygosity, fits to Hardy-Weinberg expectations and levels of gene flow between

conspecific populations of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus*. These data are intended to show whether the reproductive and developmental strategies of these four species affect genetic population structure in a manner similar to species with comparable life histories from other phyla. Genetic data were also used to indicate whether or not *Lineus sanguineus* reproduces by fragmentation throughout the year.

3.2 Methods and Materials.

3.2.1 Sample Sites

Nemerteans were sampled from sites as detailed in section 2.1.

Table 3.1 Numbers of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* found at sampling sites around the North Atlantic ranging from the U.S.A., through the U.K. and down to France (see 2.1).

Site		Species			
			Lineus		*Amphiporus*
		ruber	*viridis*	*sanguineus*	*lactifloreus*
United States of America					
Mount Desert Island	(USM)	68	25	-	-
Brandy Cove	(USB)	8	10	-	-
United Kingdom					
Oban	(OBN)	90	48	-	70
Whitehaven	(WTN)	-	34	60	100
Barrow	(BRW)	-	13	-	-
Port Erin	(PTE)	13	-	-	-
Bay Ny Carrickey	(PVH)	-	29	-	-
Castletown	(CST)	38	-	-	-
Llandudno	(LDN)	55	59	43	60
Anglesey	(ANG)	88	30	-	-
St. Agnes	(STA)	-	118	-	-
Plymouth Sound	(PMS)	-	20	-	-
Wembury	(WMY)	-	27	-	-
France					
Pointe de Barfleur	(FRA)	7	7	-	-
Total	354	420	103	230	

3.2.2 Starch Gel Electrophoresis

Specimens were described, numbered and subject to electrophoresis as detailed in Sections 2.2, 2.3 and 2.4. Data recorded from starch gel electrophoresis were converted to actual numbers of genotypes occurring in each population for each species and then analysed using the FORTRAN programme BIOSYS-1 (Swofford & Selander, 1989).

3.2.3 Data Analysis

Heterozygosity

This is the basic measure of genetic diversity and is usually expressed as the mean expected heterozygosity per locus (H_E). This is calculated as follows: First the heterozygosity per locus is calculated

$$H_E = 1 - \Sigma xi^2$$

where xi is the frequency of the i'th allele at a locus. For example in a locus with two alleles occurring at a frequency of 0.5

$H_E = 1 - (0.52 + 0.52) = 0.5$. Mean heterozygosity per locus is the sum of H_E over all loci, including monomorphic loci, divided by the total number of loci sampled.

Variance of H_E for r loci is

$$\frac{\Sigma(H_m - H_E)^2}{r(r - 1)}$$

where H_m is the heterozygosity at the mth locus.

Standard error of H_E is the square root of the variance. H_E is the expected heterozygosity per locus. The observed heterozygosity (H_L[obs]) is the number of heterozygous individuals divided by the total number of individuals that are sampled.

In a population that conforms to the Hardy-Weinberg equilibrium with respect to allele frequencies, calculated and observed heterozygosities should not differ significantly (Ferguson, 1980). The mean expected and observed heterozygosities were calculated for all populations sampled in this investigation.

Tests of Conformity to Hardy - Weinberg expectations.

The Hardy-Weinberg equilibrium was discovered by G.H. Hardy and W. Weinberg in 1908. This predicts the genotype frequencies, of a randomly mating sexually reproducing population, from its allele frequencies. The principle of the Hardy-Weinberg equilibrium may be stated as a mathematical relationship:

A locus has two alleles, A and B, which occur at the frequencies p and q. In an outbreeding population two types of male and female gametes (A and B) will be produced. These will form the zygotes AA, AB and BB in the proportions 1:2:1. As the allele frequency for A is p and that for B is q, the frequency of the genotypes AA, AB and BB will be p^2, 2pq and q^2 respectively (Ayala & Kiger, 1980; Ferguson, 1980; Hartl & Clarke, 1989).

Besides random mating and sexual reproduction, a number of other assumptions are made in this model. These are that the organism in question is diploid, it is outbreeding, generations are non overlapping, population size is very large, migration is negligible, mutation is negligible and the genotypes under consideration are not acted on by selection (Hartl & Clarke, 1989).

Due to these assumptions no natural population will be in perfect Hardy-Weinberg equilibrium (Smith, 1970), but a sexually reproducing, outbreeding population will approximate fairly closely to Hardy-Weinberg expectations within the bounds of sampling error. Deviations from genotype frequencies expected under Hardy-Weinberg conditions in a natural population may be attributed to the corruption of one or more of the several assumptions given above. Large deviations from Hardy-Weinberg expectations in natural populations may be caused by non-sexual reproduction, the presence of two or more assortatively mating populations, inbreeding, polyploidization or selection (Lessios, 1992).

The populations of all four species of nemertean were analysed for deviations from the Hardy Weinberg equilibrium. There are three types of test for goodness of fit to Hardy Weinberg expectations. These are (a) goodness of fit χ^2 tests, (b) liklihood ratio tests and (c) exact tests (Lessios, 1992).

Goodness of fit χ^2 tests are based on the formula:

$$\chi^2 = \frac{\Sigma(\text{Observed number - Expected number})^2}{\text{Expected number}}$$

This is calculated for all alleles in a given locus; number refers to the number of specimens showing a given genotype. This formula, which is applicable to a single locus , gives a χ^2 value which is compared to the relevant χ^2 table to find the probability that the observed genotype frequencies fit the Hardy-Weinberg equilibrium. Results are considered significant at the 0.05 probability, i.e. at a probability below 0.05 the null hypothesis that the population genotype frequency conforms to Hardy-Weinberg expectations is rejected.

The major problem with χ^2 goodness of fit tests is that they are severely affected by small sample sizes (Fairbairn & Roff, 1980; Lessios, 1992). This is because for small sample sizes (<100 individuals, according to Speiss [1989]), a finite sample from a population in Hardy-Weinberg equilibrium over represents the number of homozygotes (Levene, 1949; Haldane, 1954; Smith, 1970). Corrections to the calculations for expected genotype frequencies have been formulated, one of the most commonly used of these is Levene's (1949) correction for small sample size. Another way of avoiding problems of low sample size is to pool genotypes. BIOSYS-1 achieves this by pooling genotypes into three classes (i) homozygotes for the most common allele, (ii) heterozygotes for the most common allele and one of other alleles, and (iii) all other genotypes. The resulting χ^2 test uses a single degree of freedom.

Some authors claim that liklihood-ratio tests are more powerful than χ^2 tests, but they have the same disadvantages because of small sample sizes (Lessios, 1992). Details of these tests may be found in Sokal & Rohlf (1981) and Weir (1990) but no further mention of them will be made here since they were not used in this study.

Exact probability tests are based on the principle that an observed sample can be used to reject a hypothesis if the total probability under the hypothesis of a given sample occurring (i.e., of a certain set of genotype frequencies occurring), or a less likely one is small. i.e., the probabilities of all possible samples of the same size as the observed sample are determined with the assumption that the hypothesis is true. The samples are then ordered according to their probabilities. The probability of the observed sample is then

added to this list and it is then added to the probabilities of all the less probable samples. The hypothesis is rejected if the total of these probabilities is less than the critical level (P = 0.05) (Weir, 1990). The exact probability is the probability that an observed sample could be drawn from a population by chance (Lessios, 1992). This was first detailed by Fisher (1935).

Exact tests are used when sample sizes are small so there is a large chance that expected numbers in a χ^2 test would be small. This is because they avoid the problems of χ^2 tests on small sample sizes. If rare alleles are present at a locus, expected numbers can be small even in moderately large sample sizes so once again exact tests are useful (Lessios, 1992).

In this study BIOSYS-1 was used to calculate χ^2 goodness of fit with Levene's (1949) correction for small sample sizes, χ^2 with pooling of genotypes and Fisher's (1935) exact probability for observing genotype frequencies. These calculations were carried out for all populations to look for deviations from Hardy-Weinberg resulting from types of reproduction (i.e., asexual reproduction causes large deviations in Hardy-Weinberg [Crisp *et al.*, 1983; Shaw, 1989; Smith & Potts, 1987; Ward, 1989]) or from inbreeding caused by a lack of dispersal of juvenile animals.

Wright's F-Statistics.

Subdivision within a population causes a deficiency of heterozygotes compared to a single random mating population. This is known as the Wahlund effect (Wahlund, 1928). Wright (1943, 1951, 1965, 1969) measured the deviations of genotype frequency in a subdivided population by the use of three parameters F_{IS}, F_{IT} and F_{ST}. These are known as fixation indices or F-statistics. F_{IT} is the overall inbreeding coefficient of an individual , FIS is the contribution to total inbreeding of non-random mating, F_{ST} is the contribution to total inbreeding due to population subdivision. The three parameters are related;

$$F_{IT} \approx F_{IS} + F_{ST}$$

For details on calculation of F-statistics see Hartl & Clarke (1989), Nei (1987), Weir (1990), Weir & Cockerham (1984) and Wright (1943, 1951, 1965, 1969).

In this study F-statistics were calculated using BIOSYS-1 in order to estimate the amount of deviation from expected genotypic frequencies in the populations of each species

investigated (F_{IT}). The amount of this deviation due to non-random breeding (F_{IS}) and due to population subdivision (F_{ST}) was also calculated. Significance of F_{IS} was calculated using the following formula (Waples, 1987):

$$X^2 = F_{IS}^2 N(K - 1); \qquad \text{d.f.} = \frac{K(K - 1)}{2}$$

where N is the total number of individuals sampled and K is the number of alleles at the locus.

Significance of F_{ST} was calculated using the following formula:

$$X^2 = 2NF_{ST}(K - 1); \qquad \text{d.f.} = (K - 1)(s - 1)$$

where s is the number of populations.

Another parameter that was calculated from F_{st} for each species was the number of migrants per generation between populations (mNe). This can indicate relative gene flow between populations. This is calculated from the formula.

$$\text{mNe} = \{(1/F_{ST}) - 1\}/4 \text{ (Waples, 1987).}$$

Genetic Identity and Genetic Distance.

There are many measures of genetic identity but the most commonly used is Nei's (1972) genetic identity. The genetic identity between two populations is given by:

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

where x_i and y_i are the frequencies of the i'th allele in populations x and y respectively.

The mean genetic identity is the mean over all loci studied, including monomorphic ones, and is calculated as:

$$I = \frac{Ixy}{\sqrt{(I_x I_y)}}$$

where I_{xy}, I_x and I_y are the arithmetic means, over all loci of $\Sigma x_i y_i$, x_i^2 and y_i^2 respectively (Nei, 1972).

The genetic distance (D) is given by the equation D = -ln I.

Genetic distance is supposed to measure the number of codon substitutions per locus that have occurred after divergence between a pair of populations (Swofford &

Olsen, 1990).

BIOSYS-1 was used to calculate Nei's genetic identity and distance between each population within each of the four species in this study. This was to estimate the overall amount of intraspecific genetic divergence between populations.

Clustering

Clustering was performed on genetic identity data using the unweighted pair-group method using arithmetic averages (UPGMA). This is a graphical method of representing genetic identity data in the form of an ultrametric tree. Details of the calculation and construction of dendrograms by UPGMA are given in Sneath & Sokal (1973) and Ferguson (1980). BIOSYS-1 was used to construct dendrograms with UPGMA using Nei's (1978) genetic identity, for the populations of each species in this study. Nei's (1972) genetic identity was not used for this because this measure is non-linear.

3.3 Results

3.3.1 *Lineus ruber*

Thirteen enzyme loci (*Apa-1*, *Fum-1*, *Fum-2*, *Got-1*, *Got-2*, *Icd-1*, *Icd-2*, *Mdh-1*, *Mdh-2*, *Odh-1*, *Pgd-1*, *Pgi-1*, *Pgm-1*) stained consistently throughout the study. Some variation in enzyme activity was noted which could be attributed to a number of factors (difference in season, difference in age of animals, different nutritive states etc.). This variation was not sufficient to adversely affect data collection.

Malate dehydrogenase -1 (*Mdh-1*) showed high activity and two progressively faster migrating satellite bands which occasionally complicated gel interpretation.

A total of eight populations was sampled for *Lineus ruber*. Allele frequencies for all loci in each of these populations are given in table 3.2. Out of thirteen loci sampled, nine were polymorphic at the 0.99 level and five at the 0.95 level, over all populations.

Table 3.3 gives % polymorphic loci and mean observed and expected heterozygosities for each population. Overall levels of observed heterozygosity ranged from 0.008 to 0.044. Note that 0.044 came from the French sample site which had a very low sample size (7), so this result may be inaccurate. In several cases observed and

expected heterozygosities differed significantly (see Tables 3.4. and 3.5).

Mean sample size per locus was not consistent with that given in the methods in several cases because occasionally a stain would not develop. It is more likely that this was for technical rather than genetic reasons (i.e. decomposed chemicals in enzyme specific stains, 'hot' gel, etc.).

Three tests were performed to examine the conformity of observed genotype frequencies to Hardy-Weinberg expectations, χ^2 goodness of fit test, χ^2 goodness of fit with pooling and Fisher's (1935) Exact test. Probabilities of conformity to Hardy-Weinberg expectations with these three tests are given on tables 3.4, 3.5 and 3.6 consecutively. The χ^2 goodness of fit test showed significant deviations from Hardy-Weinberg expectations in populations from Port Erin (*Mdh-1*), Castletown (*Odh-1*, *Pgi-1*) and France (*Mdh-2*). Two of these populations, Port Erin and France had very low sample sizes. Furthermore, some deviations were in genotype frequencies for MDH and interpretation of this enzyme was difficult due to the presence of satellite bands. The χ^2 test with pooling showed reduced levels of significance, with only the Castletown population showing significant deviation for a single locus (*Pgi-1*). The Fisher (1935) Exact test for deviation from Hardy-Weinberg expectations showed no significant deviations from genotype frequency expectations but the Castletown population for the *Pgi-1* locus was just above the P= 0.05 level.

Wright's (1951, 1965) fixation index is given in Table 3.7. This shows whether deviations from Hardy-Weinberg expectations are due to heterozygote excess or heterozygote deficiency. Negative values show heterozygote excess, positive values show a heterozygote deficiency. No populations showed a significant deviation from Hardy-Weinberg expectations under Fisher's (1935) Exact test but those that showed significant deviations under the χ^2 tests all have positive F - values, indicating heterozygote deficiency.

The F-statistics for variable loci across all populations are given in Table 3.8. F_{IS} refers to the deviation of genotype frequencies from Hardy-Weinberg expectations, across all populations and is assumed to result from non-random systems of breeding or selection. Three out of nine loci show highly significant F_{IS} values. Out of these three loci, most of the deviation for *Mdh-1* and *Mdh-2* came from the Port Erin and France populations

respectively. Both of these populations have very small sample sizes. Most of the deviation for *Pgi-1* comes from the Castletown population (N=38) and is likely to represent a genuinely significant result. The mean F_{IS} value is also significant (<0.01), but this reflects the very high *FIS* values at *Mdh-1* and *Mdh-2* due to low sample sizes from Port Erin and France.

F_{ST} values refer to the deviation in genotype frequencies from Hardy-Weinberg expectations due to subdivision across all the populations sampled (e.g. Wahlund effect). Six out of nine populations show highly significant F_{ST} values. Such a high number of significant results is not expected by chance at P<0.01. The overall F_{ST} value is also significant. The number of migrants per generation between populations (mNe) is also given in Table 3.8.

Nei's (1972) overall genetic identity (I) and genetic distance (D), between all populations, is given in Table 3.9. Genetic identity gives a similarity between two populations calculated from allele frequencies. A value of 1.000 indicates total genetic similarity, i.e. the two populations are identical. A value of 0.000 indicates that two populations are genetically unrelated. Genetic identity values (Nei, 1972) for all populations of *Lineus ruber* are very high, ranging from 1.000 (populations identical) to 0.996. Genetic distance (Nei, 1972) is calculated from the identity and is supposed to estimate the number of net codon substitutions that have occurred after population divergence (Swofford & Olsen, 1990). Genetic distance ranges from 0.000 (no genetic divergence) and increases with increasing genetic divergence. Distance values between all populations of *Lineus ruber* range from 0.000 to 0.003, showing little overall genetic divergence across the species' entire sampled range.

Nei's (1978) genetic identity (I) and genetic distance (D) are given in Table 3.10. These were used to perform clustering by UPGMA to produce the dendrogram on Figure 3.1. This demonstrates graphically the high genetic similarity demonstrated by *Lineus ruber* populations.

Table 3.2 Allele frequencies in eight populations sampled for *Lineus ruber*. Alleles designated by letter running from highest mobility (A) to lowest (Y) for each enzyme locus.

Locus	Allele	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
Apa-1	K	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000
	L	1.000	1.000	1.000	1.000	1.000	0.959	0.982	1.000
	P	0.000	0.000	0.000	0.000	0.000	0.027	0.000	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000
Fum-1	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fum-2	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got-1	J	0.022	0.000	0.000	0.000	0.000	0.000	0.014	0.000
	Q	0.978	1.000	1.000	1.000	1.000	1.000	0.986	1.000
Got-2	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Icd-1	F	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000
	J	1.000	1.000	0.986	1.000	0.961	0.994	1.000	1.000
	N	0.000	0.000	0.014	0.000	0.026	0.006	0.000	0.000
Icd-2	F	1.000	1.000	1.000	1.000	1.000	0.989	1.000	1.000
	I	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Mdh-1	H	0.000	0.000	0.000	0.077	0.000	0.000	0.000	0.071
	I	1.000	1.000	0.989	0.846	0.947	1.000	1.000	0.929
	M	0.000	0.000	0.011	0.038	0.053	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000
Mdh-2	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.143
	E	1.000	1.000	0.989	1.000	1.000	1.000	0.991	0.857
	I	0.000	0.000	0.011	0.000	0.000	0.000	0.009	0.000
Odh-1	D	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.063	0.094	0.000	0.132	0.000	0.182	0.071
	J	0.993	0.875	0.839	1.000	0.868	0.994	0.818	0.929
	Q	0.007	0.063	0.000	0.000	0.000	0.006	0.000	0.000
Pgd-1	J	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgi-1	C	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000
	J	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.039	0.000	0.000	0.000
	R	0.993	1.000	1.000	1.000	0.947	1.000	1.000	1.000
Pgm-1	H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071
	I	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000
	L	0.007	0.000	0.000	0.000	0.000	0.000	0.027	0.000
	N	0.985	1.000	0.986	0.962	0.987	0.994	0.973	0.857
	O	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071
	Q	0.000	0.000	0.000	0.038	0.000	0.000	0.000	0.000
	W	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000
	X	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000

Table 3.3 Mean observed and expected heterozygosity (under Hardy-Weinberg expectations) for populations of *Lineus ruber*. Mean sample size per locus and percentage of loci polymorphic (at 0.95 and 0.99 levels) are also given.

Population	Mean Sample size per locus	Percentage polymorphic loci 95%	99%	Mean Heterozygosity Observed	Expected
US Maine	65.1 (1.1)	0.0	30.8	0.008 (0.004)	0.008 (0.004)
US Brandy	8.0 (0.0)	7.7	7.7	0.019 (0.019)	0.019 (0.019)
Oban	78.5 (3.2)	7.7	38.5	0.027 (0.018)	0.030 (0.022)
Port Erin	13.0 (0.0)	7.7	15.4	0.018 (0.013)	0.028 (0.022)
Castletown	38.0 (0.0)	23.1	38.5	0.032 (0.014)	0.041 (0.019)
Anglesey	73.4 (5.9)	0.0	38.5	0.011 (0.006)	0.010 (0.006)
Llandudno	46.7 (3.9)	7.7	38.5	0.030 (0.019)	0.033 (0.023)
France	7.0 (0.0)	30.8	30.8	0.044 (0.025)	0.063 (0.029)

Table 3.4 Probabilities that genotype frequencies observed in all populations of *Lineus ruber* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test. * P<0.05, ** P<0.01.

Locus	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
Apa-1	-	-	-	-	-	0.998	0.923	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	0.880	-	-	-	-	-	1.000	-
Got-2	-	-	-	-	-	-	-	-
Icd-1	-	-	0.932	-	0.998	1.000	-	-
Icd-2	-	-	-	-	-	0.939	-	-
Mdh-1	-	-	0.940	0.000**	0.768	-	-	1.000
Mdh-2	-	-	0.940	-	-	-	1.000	0.000**
Odh-1	1.000	0.994	0.299	-	0.040*	1.000	0.248	1.000
Pgd-1	-	-	-	-	-	-	-	-
Pgi-1	1.000	-	-	-	0.000**	-	-	-
Pgm-1	1.000	-	1.000	1.000	1.000	1.000	0.866	0.993

Table 3.5 Probabilities that genotype frequencies observed in all populations of *Lineus ruber* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test with pooling * P<0.05, **P<0.01.

Locus	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
Apa-1	-	-	-	-	-	0.835	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	-	-	-	-	-	-	-	-
Got-2	-	-	-	-	-	-	-	-
Icd-1	-	-	-	-	0.837	-	-	-
Icd-2	-	-	-	-	-	-	-	-
Mdh-1	-	-	-	0.077	-	-	-	-
Mdh-2	-	-	-	-	-	-	-	-
Odh-1	-	0.994	0.173	-	-	-	-	-
Pgd-1	-	-	-	-	-	-	-	-
Pgi-1	-	-	-	-	0.001**	-	-	-
Pgm-1	0.931	-	0.932	-	-	-	-	0.763

Table 3.6 Probabilities that genotype frequencies observed in all populations of *Lineus ruber* conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies.* P<0.05, ** P<0.01.

Locus	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
Apa-1	-	-	-	-	-	1.000	1.000	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	1.000	-	-	-	-	-	1.000	-
Got-2	-	-	-	-	-	-	-	-
Icd-1	-	-	1.000	-	1.000	1.000	-	-
Icd-2	-	-	-	-	-	1.000	-	-
Mdh-1	-	-	1.000	0.230	1.000	-	-	1.000
Mdh-2	-	-	1.000	-	-	-	1.000	0.077
Odh-1	1.000	1.000	0.231	-	0.099	1.000	0.353	1.000
Pgd-1	-	-	-	-	-	-	-	-
Pgi-1	1.000	-	-	-	0.079	-	-	-
Pgm-1	1.000	-	1.000	1.000	1.000	1.000	1.000	1.000

Table 3.7 Wright's (1951, 1965) fixation index for deviation from Hardy-Weinberg expectations in genotype frequencies for all populations of *Lineus ruber*.

Locus	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
Apa-1	-	-	-	-	-	-	-0.033	-0.019
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	-0.023	-	-	-	-	-	-	--0.014
Got-2	-	-	-	-	-	-	-	-
Icd-1	-	-	-0.014	-	-0.032	-0.006	-	-
Icd-2	-	-	-	-	-	-0.011	-	-
Mdh-1	-	-	-0.011	-0.441	-0.056	-	-	-0.077
Mdh-2	-	-	-0.011	-	-	-	-0.009	1.000
Odh-1	-0.007	-0.103	0.136	-	0.309	-0.006	0.144	-0.077
Pgd-1	-	-	-	-	-	-	-	-
Pgi-1	-0.007	-	-	-	0.478	-	-	-
Pgm-1	-0.011	-	-0.011	-0.040	-0.013	-0.006	-0.028	-0.122

Table 3.8 Summary of F-statistics at all loci for all populations of *Lineus ruber* in the present study. * P<0.05, **P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe
Apa-1	-0.028	-0.005	0.023**	
Got-1	-0.019	-0.004	0.014	
Icd-1	-0.025	-0.006	0.018*	
Icd-2	-0.011	-0.001	0.010*	
Mdh-1	0.199**	0.247	0.060**	
Mdh-2	0.858**	0.874	0.110**	
Odh-1	0.099	0.154	0.061**	
Pgi-1	0.416**	0.436	0.033**	
Pgm-1	-0.076	-0.020	0.052**	
Mean	0.162**	0.211	0.059**	3.99

Table 3.9 Matrix of Nei's (1972) Genetic Identity (I) (above diagonal) and Nei's (1972) Genetic Distance (D) (below diagonal).

Population	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
US Maine	-	0.999	0.999	0.999	0.998	1.000	0.998	0.997
US Brandy	0.001	-	1.000	0.998	0.999	0.999	0.999	0.997
Oban	0.001	0.000	-	0.997	0.999	0.998	0.999	0.997
Port Erin	0.001	0.002	0.003	-	0.998	0.999	0.996	0.997
Castletown	0.002	0.001	0.001	0.002	-	0.998	0.999	0.996
Anglesey	0.000	0.001	0.002	0.001	0.002	-	0.997	0.997
Llandudno	0.002	0.001	0.001	0.004	0.001	0.003	-	0.996
France	0.003	0.003	0.003	0.003	0.004	0.003	0.004	-

Table 3.10 Matrix of Nei's (1978) Genetic Identity (I) (above diagonal) and Nei's (1978) Genetic Distance (D) (below diagonal).

Population	USM	USB	OBN	PTE	CST	ANG	LDN	FRA
US Maine	-	1.000	0.999	0.999	0.999	1.000	0.998	0.999
US Brandy	0.000	-	1.000	0.999	1.000	1.000	1.000	1.000
Oban	0.001	0.000	-	0.998	1.000	0.999	1.000	0.999
Port Erin	0.001	0.001	0.002	-	0.998	0.999	0.997	1.000
Castletown	0.001	0.000	0.000	0.002	-	0.999	1.000	0.999
Anglesey	0.000	0.000	0.001	0.001	0.001	-	0.998	0.999
Llandudno	0.002	0.000	0.000	0.003	0.000	0.002	-	0.999
France	0.001	0.000	0.001	0.000	0.001	0.001	0.001	-

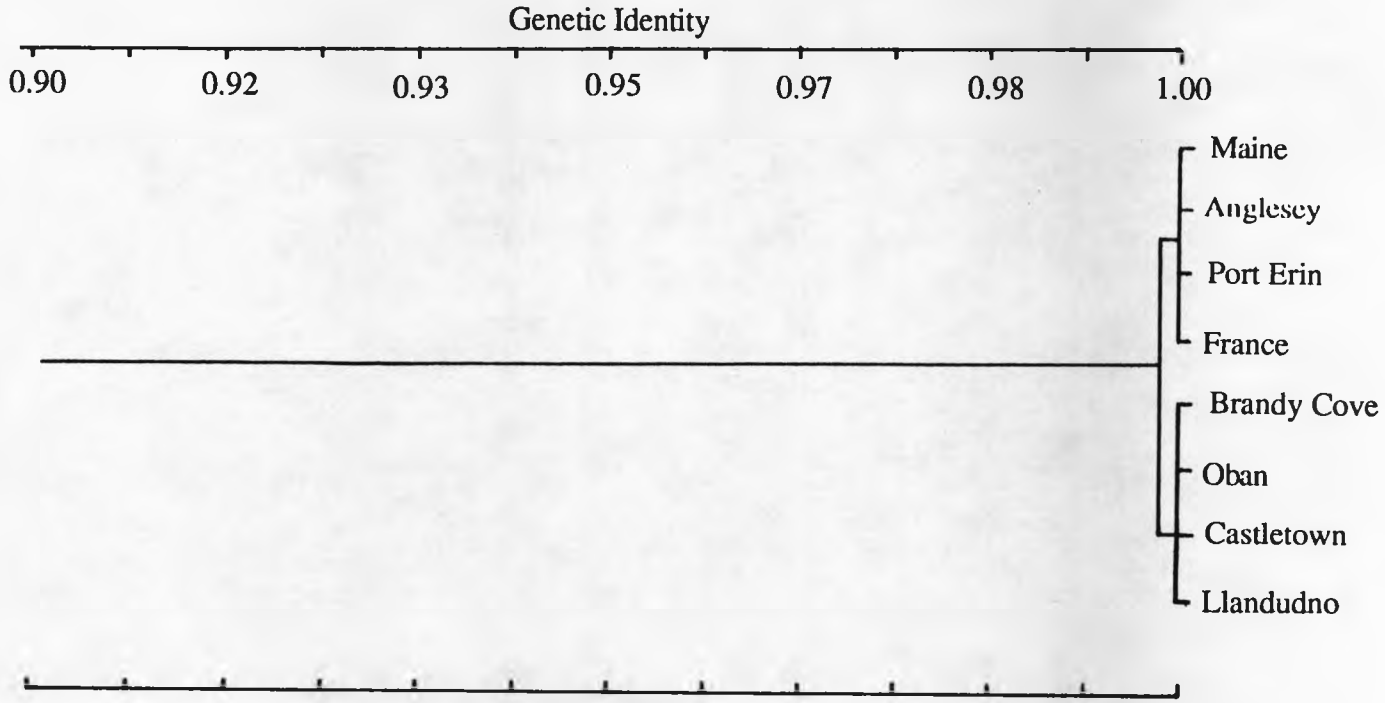


Figure 3.1. UPGMA dendrogram of Nei's (1978) genetic identity (D) between all populations of *Lineus ruber*.

3.3.2 *Lineus viridis*

Eleven enzyme loci (*Apa-1*, *Fum-1*, *Fum-2*, *Got-1*, *Got-2*, *Mdh-1*, *Mdh-2*, *Odh-1*, *Pgd-1*, *Pgi-1*, *Pgm-1*) stained consistently throughout the study. *Icd-1* and *Icd-2* stained for some populations but resolution and activity were too poor for interpretation in others. It most is likely that this was because of a problem with either the starch gels or with the specimens. As in *Lineus ruber*, some variation in enzyme activity was noted between electrophoretic runs. Some difficulty was experienced in interpretation of the *Mdh-1* locus due to high activity and the presence of two progressively more rapidly migrating satellite bands.

A total of twelve populations was sampled for *Lineus viridis*. Allele frequencies for all loci at each of these populations are given in Table 3.10. Out of the eleven loci sampled, eight were polymorphic at the 0.99 level and seven at the 0.95 level, over all populations.

The percentage of loci polymorphic and mean observed and expected heterozygosities are given for each population in Table 3.11. Overall observed heterozygosities ranged from 0.153 to 0.074. These would be higher if *Icd-1* was included in this study as this locus is also highly variable (H_L[obs] Llandudno = 0.5). Observed and expected heterozygosities were higher in *Lineus viridis* than they were in *Lineus ruber*. Note; as for *Lineus ruber*, mean sample size per locus was not always consistent with that given in Table 3.1.

Observed and expected heterozygosities under Hardy-Weinberg equilibrium show marked differences for several populations in Table 3.11. As in *Lineus ruber*, three tests were performed to examine the conformity of observed genotype frequencies to Hardy-Weinberg expectations. Probabilities of conformity to Hardy-Weinberg expectations under these three tests are given in Tables 3.12, 3.13 and 3.14.

The χ^2 goodness of fit test (Table 3.12) showed significant deviations from genotype frequencies expected under Hardy-Weinberg conditions. *Pgm-1* showed highly significant deviation for populations sampled at Llandudno, Anglesey, St. Agnes, Wembury and France. *Pgm-1* is a monomorphic locus which stains with good activity and a high resolution. It is unlikely that these results were due to misinterpretation of allele staining zones. Though sample sizes were low for populations sampled from France and Wembury, sample sizes for other populations were moderate to high (St. Agnes N=118). It

is unlikely that deviation from Hardy-Weinberg expectations in most populations for *Pgm-1* is an artefact of sampling. *Odh-1* also showed significant deviation from genotype frequencies expected under Hardy-Weinberg equilibrium in populations at Whitehaven, Llandudno and St. Agnes. Note that these are the two populations for which the largest sample sizes were recorded. Significant deviation from Hardy-Weinberg expectations was also found in *Mdh-1* for populations at Maine (U.S.A.) and St. Agnes. This could be because of gel misinterpretation.

Pooling genotype frequencies for the χ^2 goodness of fit test reduced the number of populations showing significant deviation from Hardy-Weinberg expectations. *Pgm-1* and *Mdh-1* showed significant deviation from genotype frequencies expected under Hardy-Weinberg in the St. Agnes population. *Odh-1* showed significant deviation in the Whitehaven population. Fisher's Exact test showed very similar results to those shown by the χ^2 goodness of fit test with pooling.

A greater number of populations at a greater number of loci showed deviations in observed genotype frequencies from those expected under Hardy-Weinberg conditions in *Lineus viridis* than in *Lineus ruber*.

Wright's (1951, 1965) fixation index is given in Table 3.15. *Pgm-1* for the St. Agnes population, which showed a significant deviation from Hardy-Weinberg expectations under all tests, gave a negative F value, indicating heterozygote excess at this locus. In the same population *Mdh-1*, which also showed significant deviations from Hardy-Weinberg expectations for all, tests shows a strongly positive F value, indicating a heterozygote deficiency. Note that this enzyme is subject to misinterpretation. All other loci for which F values were calculated showed heterozygote excess, especially *Odh-1*, which was significant in the χ^2 goodness of fit test but not in the other two tests for deviation from Hardy-Weinberg expectations. The only other population which showed significant deviations in genotype frequency from those expected under Hardy-Weinberg expectations was Whitehaven for the *Odh-1* locus. The fixation index for this locus indicated a significant heterozygote deficit.

F-statistics for variable loci across all populations for *Lineus viridis* are given in Table 3.16. Overall F_{IS} values were not significant for *Lineus viridis*. Individually *Got-1*, *Mdh-1* and *Pgm-1* showed highly significant F_{IS} values for *Lineus viridis*. All F_{ST} values

were highly significant for *Lineus viridis*, including the overall F_{ST}. Over the geographic range sampled *Lineus viridis* showed a structured population (i.e., composed of distinct subpopulations). F_{ST} values were more significant in a greater number of loci than in *Lineus ruber*. The number of migrants per generation between populations was consequently lower in *Lineus viridis* (Table 3.16) than in *Lineus ruber* (Table 3.8).

Nei's (1972) overall genetic identity (I) and genetic distance (D), between all populations for *Lineus viridis* are given in Table 3.17. Genetic identity values between populations of *Lineus viridis* were high, ranging from 0.957 to 0.995, but not as high as in *Lineus ruber*. Genetic distances were correspondingly low, ranging from 0.003 to 0.044. As in *Lineus ruber*, there was very little overall genetic divergence across all populations of *Lineus viridis* sampled.

Nei's (1978) genetic identity (I) and genetic distance (D) are given in Table 3.18. These were used to perform clustering by UPGMA to produce the dendrogram in Figure 3.2. The dendrogram shows little relation to geographic location of sample sites. Some sites which are geographically close i.e. Whitehaven and Barrow in Furness (approx. 60 km) and St. Agnes and Wembury (approx. 190 km) are genetically very similar. Llandudno and Anglesey which are only separated by 10 km show a degree of genetic differentiation. Figure 3.3 indicates that Nei's (1972) genetic distance between populations of *Lineus viridis* appears to be unrelated to the geographical distance (by sea) that separates these populations. Populations separated by as much as 6000 km show very low genetic distances. There are two clusters of points due to the wide geographic separation of sites between the western and eastern sides of the Atlantic ocean.

Table 3.10 Allele frequencies in twelve populations sampled for *Lineus viridis*. Alleles designated by letter running from highest mobility (A) to lowest (Y) for each enzyme locus.

Locus	Allele	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
Apa-1	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fum-1	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fum-2	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got-1	D	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000
	E	0.800	0.950	0.979	0.956	1.000	0.983	1.000	0.983
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017
	K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	L	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000
	O	0.200	0.050	0.021	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000
Got-2	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh-1	C	0.000	0.000	0.073	0.000	0.000	0.052	0.000	0.000
	E	0.938	1.000	0.917	1.000	0.962	0.621	0.754	0.983
	H	0.063	0.000	0.010	0.000	0.038	0.328	0.246	0.017
Mdh-2	A	0.938	1.000	1.000	1.000	1.000	1.000	0.992	0.983
	B	0.063	0.000	0.000	0.000	0.000	0.000	0.008	0.017
Odh-1	A	0.053	0.000	0.000	0.000	0.000	0.000	0.000	0.233
	C	0.079	0.063	0.346	0.146	0.115	0.000	0.529	0.033
	E	0.000	0.125	0.135	0.000	0.000	0.000	0.114	0.200
	F	0.316	0.188	0.500	0.292	0.154	0.138	0.171	0.200
	G	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000
	K	0.474	0.188	0.019	0.417	0.423	0.517	0.171	0.267
Odh-1	L	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000
	P	0.079	0.125	0.000	0.146	0.308	0.328	0.014	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067
	T	0.000	0.188	0.000	0.000	0.000	0.000	0.000	0.000
Pgd-1	C	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.958	0.900	1.000	1.000	1.000	1.000	1.000	1.000
	I	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000
Pgi-1	G	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000
	L	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P	1.000	1.000	1.000	1.000	1.000	1.000	0.975	1.000
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgm-1	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000
	K	0.596	1.000	0.927	0.912	1.000	0.845	0.958	0.800
	M	0.404	0.000	0.010	0.088	0.000	0.155	0.042	0.200
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Y	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 3.10 contd.

Locus	Allele	STA	PMS	WMY	FRA
Apa-1	D	1.000	1.000	1.000	0.929
	F	0.000	0.000	0.000	0.071
Fum-1	B	1.000	1.000	1.000	1.000
Fum-2	A	1.000	1.000	1.000	1.000
Got-1	D	0.000	0.000	0.000	0.000
	E	1.000	0.950	0.905	1.000
	F	0.000	0.000	0.000	0.000
	K	0.000	0.025	0.095	0.000
	L	0.000	0.000	0.000	0.000
	O	0.000	0.025	0.000	0.000
	Q	0.000	0.000	0.000	0.000
Got-2	D	1.000	1.000	1.000	1.000
Mdh-1	C	0.008	0.050	0.024	0.000
	E	0.983	0.925	0.952	1.000
	H	0.008	0.025	0.024	0.000
Mdh-2	A	1.000	1.000	1.000	1.000
	B	0.000	0.000	0.000	0.000
Odh-1	A	0.000	0.000	0.000	0.000
	C	0.112	0.000	0.029	0.143
	E	0.009	0.000	0.029	0.000
	F	0.543	0.150	0.353	0.286
	G	0.000	0.000	0.000	0.143
	K	0.310	0.275	0.500	0.071
	L	0.000	0.000	0.000	0.286
	P	0.017	0.550	0.059	0.071
	Q	0.000	0.000	0.000	0.000
	T	0.009	0.025	0.029	0.000
Pgd-1	C	0.000	0.000	0.000	0.000
	F	0.987	1.000	1.000	1.000
	I	0.008	0.000	0.000	0.000
	K	0.004	0.000	0.000	0.000
Pgi-1	G	0.008	0.000	0.048	0.000
	L	0.004	0.000	0.000	0.000
	P	0.983	1.000	0.952	1.000
	S	0.004	0.000	0.000	0.000
Pgm-1	F	0.016	0.000	0.000	0.000
	G	0.026	0.000	0.043	0.000
	K	0.719	0.900	0.717	0.857
	M	0.240	0.050	0.196	0.143
	S	0.000	0.000	0.043	0.000
	Y	0.000	0.050	0.000	0.000

Table 3.11 Mean observed and expected heterozygosity (under Hardy-Weinberg expectations) for populations of *Lineus viridis*. Mean sample size per locus and percentage of loci polymorphic (at 0.95 and 0.99 levels) are also given.

Population	Mean Sample size per locus	Percentage polymorphic loci		Mean Heterozygosity	
		95%	99%	Observed	Expected
US Maine	22.4 (1.4)	45.5	54.5	0.153 (0.063)	0.165 (0.070)
US Brandy	9.8 (0.2)	27.3	27.3	0.118 (0.090)	0.109 (0.081)
Oban	46.0 (2.0)	27.3	36.4	0.074 (0.046)	0.087 (0.056)
Whitehaven	33.1 (0.9)	18.2	27.3	0.068 (0.049)	0.088 (0.065)
Barrow	13.0 (0.0)	9.1	18.2	0.084 (0.077)	0.072 (0.065)
Bay Ny Carrickey	28.8 (0.2)	27.3	36.4	0.119 (0.063)	0.130 (0.069)
Llandudno	53.2 (4.0)	18.2	45.5	0.105 (0.069)	0.107 (0.064)
Anglesey	25.2 (2.0)	18.2	45.5	0.100 (0.072)	0.113 (0.076)
St. Agnes	107.1 (5.6)	18.2	45.5	0.123 (0.079)	0.102 (0.063)
Plymouth S.	18.5 (1.5)	36.4	36.4	0.086 (0.047)	0.095 (0.056)
Wembury	18.3 (1.7)	27.3	45.5	0.116 (0.056)	0.132 (0.065)
Pointe de Barfleur	7.0 (0.0)	27.3	27.3	0.078 (0.065)	0.114 (0.078)

Table 3.12 Probabilities that genotype frequencies observed in all populations of *Lineus viridis* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test (Levene [1949] correction for small sample size). *P<0.05, **P<0.01.

Locus	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
Apa-1	-	-	-	-	-	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	0.239	1.000	0.917	0.997	-	1.000	-	1.000
Got-2	-	-	-	-	-	-	-	-
Mdh-1	0.000**	-	0.365	-	1.000	0.549	0.074	1.000
Mdh-2	0.792	-	-	-	-	-	1.000	1.000
Odh-1	0.377	0.553	0.185	0.018*	0.719	0.386	0.009**	0.073
Pgd-1	0.881	0.996	-	-	-	-	-	-
Pgi-1	0.125	-	-	-	-	-	0.871	-
Pgm-1	-	-	0.969	0.076	-	0.589	0.001**	0.028*

Locus	STA	PMS	WMY	FRA
Apa-1	-	-	-	1.000
Fum-1	-	-	-	-
Fum-2	-	-	-	-
Got-1	-	0.999	0.679	-
Got-2	-	-	-	-
Mdh-1	0.000**	0.994	0.999	-
Mdh-2	-	-	-	-
Odh-1	0.001**	0.802	0.951	0.108
Pgd-1	1.000	-	-	-
Pgi-1	1.000	-	0.873	-
Pgm-1	0.000**	0.981	0.000**	0.000**

Table 3.13 Probabilities that genotype frequencies, observed in all populations of *Lineus viridis*, conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test with pooling. *P<0.05, **P<0.01.

Locus	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
Apa-1	-	-	-	-	-	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	-	-	-	0.827	-	-	-	-
Got-2	-	-	-	-	-	-	-	-
Mdh-1	-	-	0.161	-	-	0.967	-	-
Mdh-2	-	-	-	-	-	-	-	-
Odh-1	0.901	0.604	0.202	0.013*	0.820	0.639	0.072	0.193
Pgd-1	-	0.808	-	-	-	-	-	-
Pgi-1	-	-	-	-	-	-	-	-
Pgm-1	-	-	0.616	-	-	-	-	-

Locus	STA	PMS	WMY	FRA
Apa-1	-	-	-	-
Fum-1	-	-	-	-
Fum-2	-	-	-	-
Got-1	-	0.869	-	-
Got-2	-	-	-	-
Mdh-1	0.000**	0.770	0.873	-
Mdh-2	-	-	-	-
Odh-1	0.114	0.330	0.394	0.371
Pgd-1	0.909	-	-	-
Pgi-1	0.871	-	-	-
Pgm-1	0.023*	0.671	0.188	-

Table 3.14 Probabilities that genotype frequencies observed in all populations of *Lineus viridis* conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies.*P<0.05, **P<0.01.

Locus	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
Apa-1	-	-	-	-	-	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	0.542	1.000	1.000	1.000	-	1.000	-	1.000
Got-2	-	-	-	-	-	-	-	-
Mdh-1	0.064	-	0.271	-	1.000	1.000	0.088	1.000
Mdh-2	1.000	-	-	-	-	-	1.000	1.000
Odh-1	1.000	1.000	0.255	0.031*	1.000	0.719	0.095	0.613
Pgd-1	1.000	1.000	-	-	-	-	-	-
Pgi-1	-	-	-	-	-	-	1.000	-
Pgm-1	0.218	-	1.000	0.214	-	0.518	0.083	0.060

Locus	STA	PMS	WMY	FRA
Apa-1	-	-	-	1.000
Fum-1	-	-	-	-
Fum-2	-	-	-	-
Got-1	-	1.000	1.000	-
Got-2	-	-	-	-
Mdh-1	0.025*	1.000	1.000	-
Mdh-2	-	-	-	-
Odh-1	0.183	0.395	0.627	1.000
Pgd-1	1.000	-	-	-
Pgi-1	1.000	-	1.000	-
Pgm-1	0.024*	1.000	0.299	0.077

Table 3.15 Wright's (1951, 1965) fixation index for deviation from Hardy-Weinberg expectations in genotype frequencies for all populations of *Lineus viridis*.

Locus	USM	USB	OBN	WIN	BRW	PVH	LDN	ANG
Apa-1	-	-	-	-	-	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	-0.250	-0.053	-0.021	-0.036	-	-0.018	-	-0.017
Got-2	-	-	-	-	-	-	-	-
Mdh-1	0.644	-	0.190	-	-0.040	0.044	0.223	-0.017
Mdh-2	-0.067	-	-	-	-	-	-0.009	-0.017
Odh-1	-0.036	-0.185	0.183	0.225	-0.227	0.089	-0.145	-0.014
Pgd-1	-0.043	-0.081						
Pgi-1	-	-	-	-	-	-	-0.026	-
Pgm-1	0.281	-	-0.068	0.269	-	-0.095	0.373	0.375

Table 3.15 contd.

Locus	STA	PMS	WMY	FRA
Apa-1	-	-	-	-0.077
Fum-1	-	-	-	-
Fum-2	-	-	-	-
Got-1	-	-	-0.039	-0.105
Got-2				
Mdh-1	0.494	-0.062	-0.037	-
Mdh-2				
Odh-1	-0.273	0.165	0.050	0.091
Pgd-1	-0.010	-	-	-
Pgi-1	-0.012	-	-0.050	-
Pgm-1	-0.225	-0.081	0.313	1.000

Table 3.16 Summary of F-Statistics at all loci for all populations of *Lineus viridis*. *P<0.05, **P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe
Apa-1	-0.077	-0.006	0.066**	
Got-1	-0.128**	-0.030	0.086**	
Mdh-1	0.138**	0.273	0.156**	
Mdh-2	-0.051	-0.007	0.042**	
Odh-1	-0.010	0.133	0.141**	
Pgd-1	-0.065	-0.009	0.053**	
Pgi-1	-0.035	-0.007	0.028**	
Pgm-1	0.224**	0.310	0.111**	
Mean	0.042	0.166	0.129**	1.68

Table 3.17 Matrix of Nei's (1972) Genetic Identity (I) (above diagonal) and Nei's (1972) Genetic Distance (D) (below diagonal) between populations of *Lineus viridis*.

Population	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
US Maine	-	0.971	0.965	0.985	0.975	0.974	0.961	0.984
US Brandy	0.030	-	0.984	0.991	0.991	0.972	0.978	0.988
Oban	0.036	0.016	-	0.985	0.977	0.957	0.987	0.981
Whitehaven	0.015	0.009	0.015	-	0.997	0.982	0.981	0.990
Barrow	0.025	0.009	0.023	0.003	-	0.987	0.979	0.985
Poyllvaissh	0.026	0.028	0.044	0.018	0.013	-	0.971	0.974
Llandudno	0.040	0.022	0.013	0.019	0.021	0.030	-	0.976
Anglesey	0.016	0.012	0.019	0.010	0.015	0.027	0.025	-
St. Agnes	0.011	0.019	0.013	0.008	0.019	0.030	0.028	0.012
Plymouth S.	0.030	0.015	0.032	0.012	0.006	0.016	0.035	0.023
Wembury	0.008	0.017	0.024	0.005	0.013	0.019	0.031	0.010
Pointe de Barfleur	0.027	0.010	0.013	0.012	0.018	0.035	0.023	0.014

Table 3.17 contd.

Population	STA	PMS	WMY	FRA
US Maine	0.989	0.970	0.992	0.973
US Brandy	0.981	0.985	0.983	0.990
Oban	0.987	0.968	0.976	0.987
Whitehaven	0.992	0.988	0.995	0.988
Barrow	0.981	0.994	0.987	0.983
Poyllvaissh	0.971	0.984	0.981	0.966
Llandudno	0.972	0.966	0.970	0.977
Anglesey	0.988	0.977	0.990	0.986
St. Agnes	-	0.973	0.995	0.986
Plymouth S.	0.027	-	0.979	0.977
Wembury	0.005	0.021	-	0.981
Pointe de Barfleur	0.014	0.023	0.019	-

Table 3.18 Matrix of Nei's (1978) Genetic Identity (I) (above diagonal) and Nei's (1978) Genetic Distance (D) (below diagonal) between populations of *Lineus viridis*.

Population	USM	USB	OBN	WTN	BRW	PVH	LDN	ANG
US Maine	-	0.997	0.968	0.988	0.979	0.978	0.964	0.988
US Brandy	0.024	-	0.989	0.996	0.996	0.977	0.983	0.993
Oban	0.033	0.011	-	0.986	0.979	0.959	0.988	0.983
Whitehaven	0.012	0.004	0.014	-	0.999	0.984	0.983	0.993
Barrow	0.021	0.004	0.021	0.001	-	0.989	0.981	0.988
Poyllvaissh	0.023	0.023	0.042	0.016	0.011	-	0.973	0.977
Llandudno	0.037	0.018	0.012	0.018	0.019	0.028	-	0.978
Anglesey	0.012	0.007	0.017	0.007	0.012	0.024	0.022	-
St Agnes	0.009	0.015	0.012	0.007	0.017	0.028	0.027	0.010
Plymouth S.	0.027	0.010	0.030	0.010	0.003	0.013	0.033	0.020
Wembury	0.004	0.012	0.021	0.002	0.009	0.016	0.028	0.007
Pointe de Barfleur	0.020	0.002	0.008	0.007	0.012	0.029	0.017	0.008

Table 3.18 contd.

Population	STA	PMS	WMY	FRA
US Maine	0.991	0.974	0.996	0.980
US Brandy	0.985	0.990	0.988	0.998
Oban	0.988	0.970	0.979	0.992
Whitehaven	0.993	0.990	0.998	0.993
Barrow	0.983	0.997	0.991	0.989
Poyllvaissh	0.972	0.987	0.984	0.971
Llandudno	0.973	0.968	0.972	0.983
Anglesey	0.990	0.980	0.993	0.992
St. Agnes	-	0.975	0.997	0.991
Plymouth S.	0.026	-	0.982	0.983
Wembury	0.003	0.018	-	0.988
Pointe de Barfleur	0.009	0.017	0.012	-

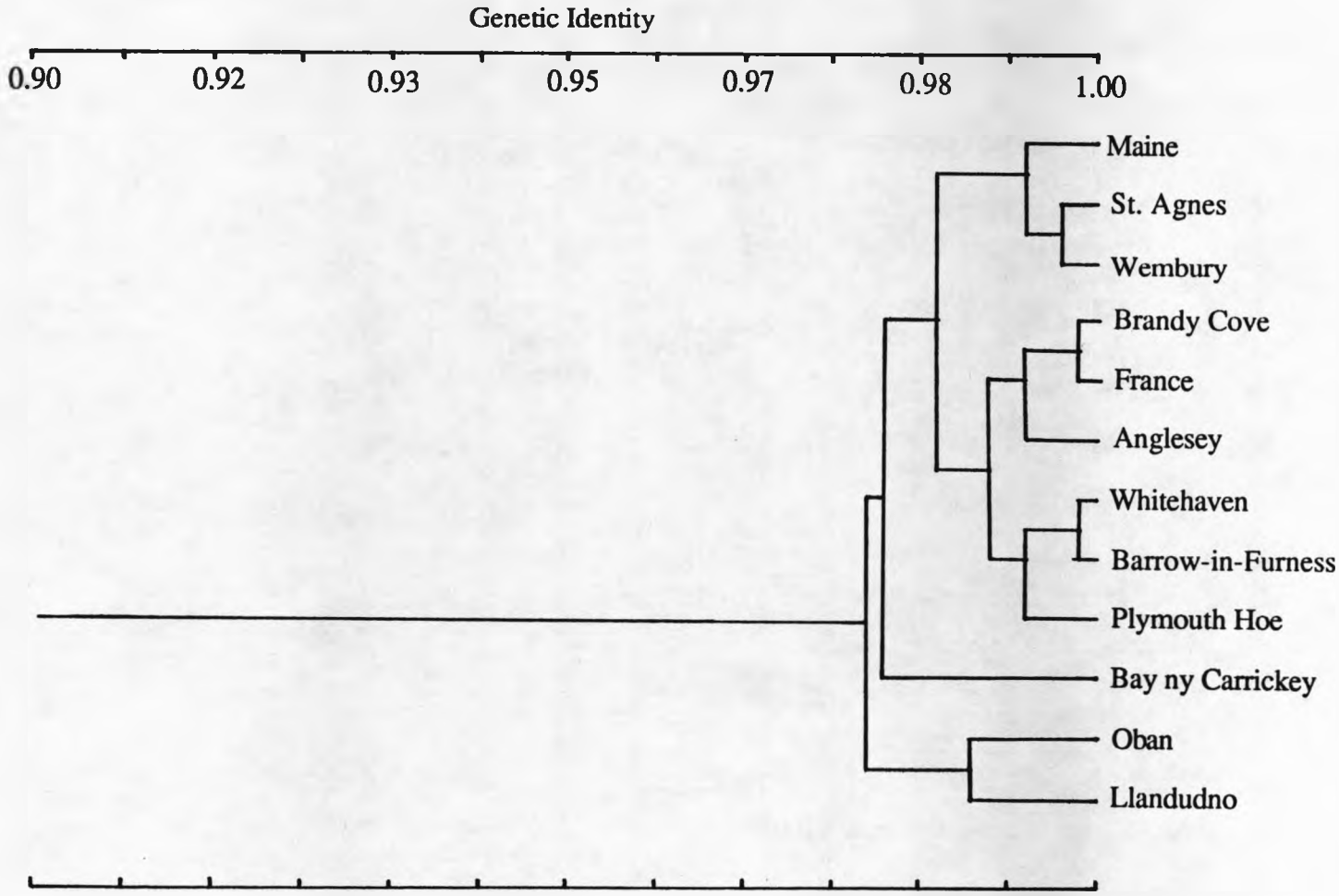
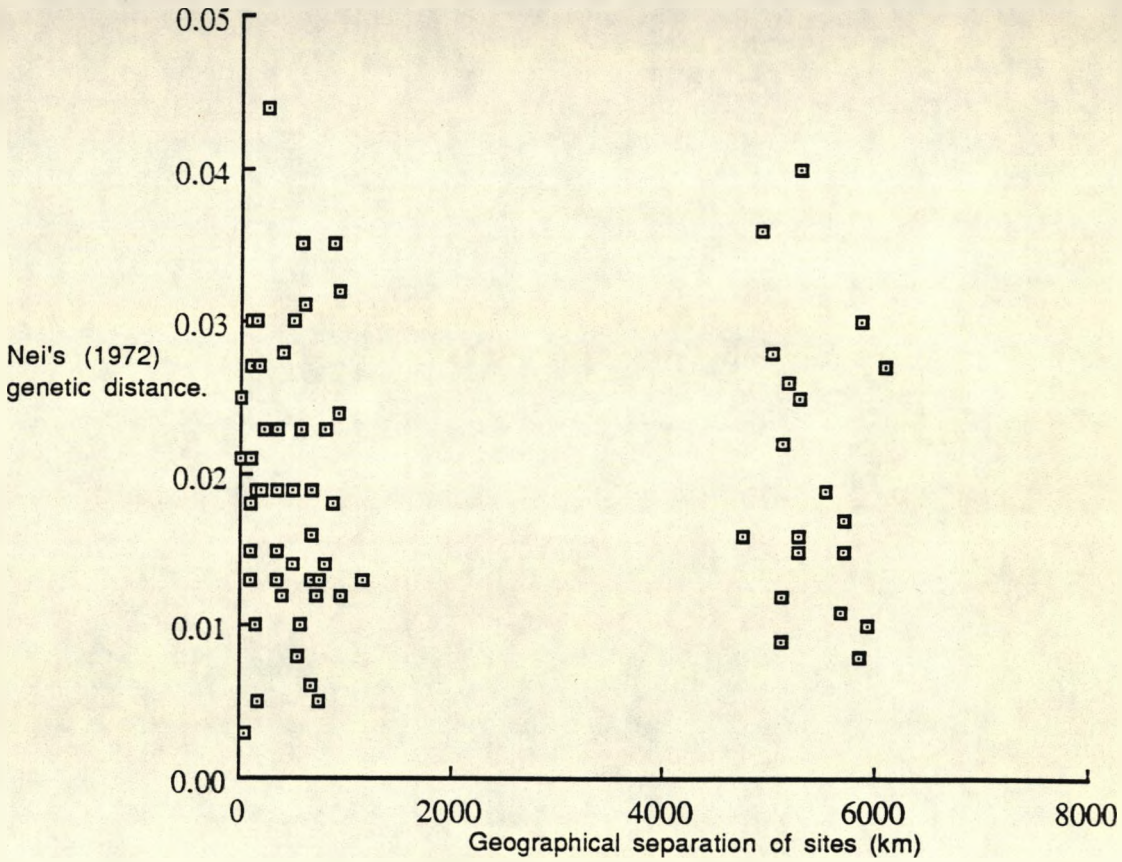


Figure 3.2. UPGMA dendrogram of Nei's (1978) genetic identity (D) between all populations of *Lineus viridis*.

Figure 3.3 Plot of Nei's (1972) genetic distance against geographical separation of sites (km).



3.3.3 *Lineus sanguineus*

Eleven enzyme loci (*Apa-1*, *Got-1*, *Got-2*, *Icd-1*, *Icd-2*, *Mdh-1*, *Mdh-2*, *Odh-1*, *Pgd-1*, *Pgi-1*, *Pgm-1*) stained consistently throughout the study. As in *Lineus ruber* and *Lineus viridis* some variation in enzyme activity was noted between electrophoresis runs. *Mdh-1* showed two progressively faster migrating satellite bands but since this locus was monomorphic there was no effect on gel interpretation.

Lineus sanguineus was rarer than other species of nemerteans in this study on population genetics. For this reason only two populations, Whitehaven and Llandudno provided sufficient specimens for genetic analysis. The Llandudno population contained a number of specimens which were unusual in external morphology and gave aberrant genetic results. These were separated from the sample pending further investigation since it was considered they may represent a cryptic species or a natural hybrid. Out of the eleven loci sampled seven were polymorphic at the 0.99 level, four at the 0.95 level across all populations. Allele frequencies at all loci for both populations of *Lineus sanguineus* sampled are given in Table 3.19.

The percentage of polymorphic loci and mean observed and expected heterozygosities are given for both populations (Table 3.20). Overall observed heterozygosities were in a similar range to those found for *Lineus viridis* but higher than those in *Lineus ruber*.

Three tests were carried out to examine conformity of the observed genotype frequencies to Hardy-Weinberg expectations as in *Lineus ruber* and *Lineus viridis*. Probabilities that observed genotype frequencies conformed to those expected under Hardy-Weinberg conditions for these three tests are given in Tables 3.21, 3.22 and 3.23. Only the χ^2 goodness of fit test showed significant deviations from expected genotype frequencies at a single locus for each population (*Pgi-1*, Whitehaven and *Got-1*, Llandudno). Wrights (1951, 1965) fixation index indicated that for *Pgi-1*, Whitehaven this was due to heterozygote excess and for *Got-1*, Llandudno this was due to a heterozygote deficiency. The Llandudno population also showed a deficiency at the *Pgi-1* locus, but this was not significant. Observed genotype frequencies showed no significant deviation from Hardy-Weinberg expectations using the χ^2 test with pooling or Fisher's (1935) Exact test.

F-statistics for variable loci across both populations for *Lineus sanguineus* are

given in Table 3.25. No individual F_{IS} values and the overall F_{IS} value were significant. Four out of seven individual F_{ST} values and the overall F_{ST} value were significant. *Lineus sanguineus* showed a similar degree of population subdivision to *Lineus ruber* and as a result, similar mNe values. Population subdivision was lower in *Lineus sanguineus* than in *Lineus viridis*. *Lineus viridis* as a result showed lower mNe values than *Lineus sanguineus*.

Nei's (1972) overall genetic identity (I) and genetic distance (D) between the two populations of *Lineus sanguineus* are given in Table 3.26. Genetic identity (I) was very high between the two populations of *Lineus sanguineus*, genetic divergence (D) was very low. Genetic differentiation between *Lineus sanguineus* populations was similar to that found in *Lineus ruber* and *Lineus viridis*. Table 3.27 gives Nei's (1978) genetic identity (I) and genetic distance (D) between the two populations of *Lineus sanguineus* sampled.

Table 3.19 Allele frequencies in two populations sampled for *Lineus sanguineus*. Alleles designated by letter running from the highest mobility (A) to the lowest (Y) for each enzyme locus.

Locus	Allele	WIN	LDN
Apa-1	G	1.000	0.896
	J	0.000	0.083
	P	0.000	0.021
Got-1	N	0.000	0.023
	R	0.125	0.198
	S	0.875	0.756
	T	0.000	0.023
Got-2	C	0.000	0.033
	F	1.000	0.967
Icd-1	E	0.975	1.000
	I	0.025	0.000
Icd-2	E	1.000	1.000
Mdh-1	O	1.000	1.000
Mdh-2	G	1.000	1.000
Odh-1	N	1.000	1.000
Pgd-1	H	0.000	0.143
	L	1.000	0.857
Pgi-1	F	0.675	0.345
	K	0.325	0.634
	M	0.000	0.012
Pgm-1	L	0.975	0.952
	N	0.000	0.012
	R	0.025	0.036

Table 3.20 Mean observed and expected heterozygosity (under Hardy-Weinberg expectations) for populations of *Lineus sanguineus*. Mean sample size per locus and percentage of loci polymorphic (at 0.95 and 0.99 levels) are also given.

Population	Mean Sample size per locus	Percentage polymorphic loci		Mean Heterozygosity Observed	Expected
				95%	99%
Whitehaven	57.7 (2.3)	18.2	36.4	0.079 (0.051)	0.069 (0.042)
Llandudno	35.2 (3.1)	36.4	54.5	0.120 (0.043)	0.134 (0.052)

Table 3.21 Probabilities that genotype frequencies observed in all populations of *Lineus sanguineus* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test. * P<0.05, ** P<0.01.

Locus	WIN	LDN
Apa-1	-	0.955
Got-1	0.941	0.000**
Got-2	-	0.894
Icd-1	0.843	-
Icd-2	-	-
Mdh-1	-	-
Mdh-2	-	-
Odh-1	-	-
Pgd-1	-	0.378
Pgi-1	0.050*	0.268
Pgm-1	0.843	0.991

Table 3.22 Probabilities that genotype frequencies observed in all populations of *Lineus sanguineus* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test with pooling. * P<0.05, ** P<0.01.

Locus	WIN	LDN
Apa-1	-	0.569
Got-1	-	0.235
Got-2	-	-
Icd-1	-	-
Icd-2	-	-
Mdh-1	-	-
Mdh-2	-	-
Odh-1	-	-
Pgd-1	-	-
Pgi-1	-	0.091
Pgm-1	-	0.746

Table 3.23 Probabilities that genotype frequencies observed in all populations of *Lineus sanguineus* conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies. *P<0.05, **P<0.01.

Locus	WTN	LDN
Apa-1	-	1.000
Got-1	1.000	0.230
Got-2	-	1.000
Icd-1	1.000	-
Icd-2	-	-
Mdh-1	-	-
Mdh-2	-	-
Odh-1	-	-
Pgd-1	-	1.000
Pgi-1	0.077	0.098
Pgm-1	1.000	1.000

Table 3.24 Wright's (1951, 1965) fixation index for deviation from Hardy-Weinberg expectations in genotype frequencies for all populations of *Lineus sanguineus*.

Locus	WTN	LDN
Apa-1	-	-0.096
Got-1	0.010	0.222
Got-2	-	-0.034
Icd-1	-0.026	-
Icd-2	-	-
Mdh-1	-	-
Mdh-2	-	-
Odh-1	-	-
Pgd-1	-	-0.167
Pgi-1	-0.254	0.226
Pgm-1	-0.026	-0.040

Table 3.25 Summary of F-statistics at all loci for all populations of *Lineus sanguineus* in the present study. *P<0.05, **P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe
Apa-1	-0.096	-0.046	0.046**	
Got-1	0.145	0.160	0.017*	
Got-2	-0.034	-0.017	0.017	
Icd-1	-0.026	-0.013	0.013	
Pgd-1	-0.167	-0.077	0.077**	
Pgi-1	-0.005	0.094	0.098**	
Pgm-1	-0.035	-0.032	0.003	
Mean	0.007	0.067	0.060**	3.92

Table 3.26 Matrix of Nei's (1972) Genetic Identity (I) (above diagonal) and Nei's (1972) Genetic Distance (D) (below diagonal) for two populations of *Lineus sanguineus*.

Population	WTN	LDN
Whitehaven	-	0.986
Llandudno	0.014	-

Table 3.27 Matrix of Nei's (1978) Genetic Identity (I) (above diagonal) and Nei's (1978) Genetic Distance (D) (below the diagonal).

Population	WTN	LDN
Whitehaven	-	0.988
Llandudno	0.012	-

3.3.4 *Amphiporus lactifloreus*

Nine enzyme loci (*Got-1*, *Got-2*, *Got-3*, *Icd-1*, *Icd-2*, *Mdh-1*, *Mdh-2*, *Pgm-1*) stained consistently throughout the study. As for other species some variation in enzyme activity was noted between electrophoresis runs.

A total of three populations were sampled for *Amphiporus lactifloreus*. Allele frequencies for all loci at each of these populations are given in Table 3.28. Out of nine loci, seven were polymorphic at the 0.99 level and six at the 0.05 level across all populations.

The percentage of polymorphic loci and mean observed and expected heterozygosities are given for each population in Table 3.29. Observed heterozygosities ranged from 0.098 to 0.082, a similar range to those observed for *Lineus viridis* and *Lineus sanguineus* but higher than heterozygosities observed for *Lineus ruber*. Note that as for other species mean sample size per locus was not always consistent with that given on Table 3.1.

Observed heterozygosity and expected heterozygosity for all populations sampled for *Amphiporus lactifloreus*, in Table 3.29, were different. The three tests carried out on the other three species in this study to test conformity of genotype frequencies to Hardy-Weinberg expectations were carried out for *Amphiporus lactifloreus*. Probabilities of conformity to Hardy-Weinberg with these three tests are given in Tables 3.30, 3.31, 3.32. Two populations showed significant deviations in genotype frequencies from Hardy-Weinberg expectations for all three tests. These were Whitehaven (*Pgm-1* locus) and Llandudno (*Mdh-2* locus). Wright's (1951, 1965) fixation index, given in Table 3.33, was positive for both of these loci indicating heterozygote deficiency in each case. *Pgm-1* showed a deficiency of heterozygotes in all the populations sampled but in the Oban and Llandudno populations this deficiency is not significant.

F-statistics for variable loci across all populations are given in Table 3.34. Individually F_{IS} values were highly significant at the *Mdh-2* and *Pgm-1* loci, giving a significant overall F_{IS} value. The overall F_{ST} was also significant with four individual loci, *Got-2*, *Mdh-2*, *Pgi-1* and *Pgm-1* giving highly significant results. The overall F_{ST} value was similar to those obtained for *Lineus ruber* and *Lineus sanguineus* and, as a result the mNe value obtained for *Amphiporus lactifloreus* was similar to those obtained for

Lineus ruber and *Lineus sanguineus*.

Nei's (1972) genetic identity (I) and genetic distance (D) are given in Table 3.35. Genetic identity values between populations of *Amphiporus lactifloreus* were high as for all other species investigated. Genetic distance was low indicating low levels of overall genetic divergence between the three populations of *Amphiporus lactifloreus* sampled.

Nei's (1978) genetic identity (I) and genetic distance (D) are given in Table 3.36. These values were used to perform cluster analysis by UPGMA to produce the dendrogram on Figure 3.4. This demonstrates graphically high levels of similarity between populations of *Amphiporus lactifloreus*.

Table 3.28 Allele frequencies in three populations sampled for *Amphiporus lactifloreus*. Alleles designated by letter, running from highest mobility (A) to lowest (G) for each enzyme locus.

Locus	Allele	OBN	WTN	LDN
Got-1	A	0.021	0.000	0.000
	B	0.000	0.010	0.000
	C	0.000	0.000	0.008
	D	0.979	0.990	0.992
Got-2	A	0.036	0.000	0.000
	B	0.807	0.825	0.917
	C	0.036	0.010	0.083
	D	0.121	0.165	0.000
Got-3	A	1.000	1.000	1.000
Icd-1	A	1.000	1.000	1.000
Icd-2	A	0.000	0.005	0.000
	B	1.000	0.995	1.000
Mdh-1	A	0.000	0.005	0.000
	B	1.000	0.995	1.000
Mdh-2	A	0.000	0.000	0.017
	B	0.864	1.000	0.925
	C	0.136	0.000	0.058
Pgi-1	A	0.000	0.005	0.000
	B	0.993	0.985	0.875
	C	0.007	0.000	0.117
	D	0.000	0.010	0.000
	E	0.000	0.000	0.008
Pgm-1	A	0.000	0.013	0.000
	B	0.000	0.175	0.018
	C	0.600	0.688	0.582
	D	0.000	0.087	0.000
	E	0.329	0.038	0.373
	F	0.064	0.000	0.027
	G	0.007	0.000	0.000

Table 3.29 Mean observed and expected heterozygosity (under Hardy-Weinberg expectations) for populations of *Amphiporus lactifloreus*. Mean sample size per locus and percentage of loci polymorphic (at 0.95 and 0.99 levels) are also given.

Population	Mean Sample size per locus	Percentage polymorphicloci		Mean Heterozygosity	
		95%	99%	Observed	Expected
Oban	65.6 (4.4)	33.3	55.6	0.098 (0.047)	0.129 (0.065)
Whitehaven	91.1 (6.8)	22.2	66.7	0.082 (0.048)	0.095 (0.059)
Llandudno	59.4 (0.6)	44.4	55.6	0.097 (0.051)	0.118 (0.058)

Table 3.30 Probabilities that genotype frequencies observed in all populations of *Amphiporus lactifloreus* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test. *P<0.05, **P<0.01.

Locus	OBN	WTN	LDN
Got-1	0.882	0.943	1.000
Got-2	0.042*	0.928	0.274
Got-3	-	-	-
Icd-1	-	-	-
Icd-2	-	0.928	-
Mdh-1	-	1.000	-
Mdh-2	0.067	-	0.000**
Pgi-1	1.000	0.999	0.975
Pgm-1	0.001	0.016*	0.866

Table 3.31 Probabilities that genotype frequencies observed in all populations of *Amphiporus lactifloreus* conform to Hardy-Weinberg expectations using a χ^2 goodness of fit test with pooling. *P<0.05, **P<0.01.

Locus	OBN	WTN	LDN
Got-1	-	-	-
Got-2	0.253	0.994	-
Got-3	-	-	-
Icd-1	-	-	-
Icd-2	-	-	-
Mdh-1	-	-	-
Mdh-2	-	-	0.000**
Pgi-1	-	0.901	0.887
Pgm-1	0.051	0.018*	0.165

Table 3.32 Probabilities that genotype frequencies observed in all populations of *Amphiporus lactifloreus* conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies. *P<0.05, **P<0.01.

Locus	OBN	WTN	LDN
Got-1	1.000	1.000	1.000
Got-2	0.262	1.000	0.335
Got-3	-	-	-
Icd-1	-	-	-
Icd-2	-	1.000	-
Mdh-1	-	1.000	-
Mdh-2	0.099	-	0.001**
Pgi-1	1.000	1.000	1.000
Pgm-1	0.079	0.027*	0.179

Table 3.33 Wright's (1951, 1965) fixation index for deviation from Hardy-Weinberg expectations in genotype frequencies for all populations of *Amphiporus lactifloreus*.

Locus	OBN	WTN	LDN
Got-1	-0.022	-0.010	-0.008
Got-2	0.181	0.007	0.127
Got-3	-	-	-
Icd-1	-	-	-
Icd-2	-	-0.005	-
Mdh-1	-	-0.005	-
Mdh-2	0.208	-	0.645
Pgi-1	-0.007	-0.012	0.018
Pgm-1	0.296	0.231	0.128

Table 3.34 Summary of F-Statistics at all loci for all populations of Amphiporus lactifloreus in the present study. *P<0.05, **P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe
Got-1	-0.016	-0.009	0.007	
Got-2	0.105	0.133	0.031**	
Icd-2	-0.005	-0.002	0.003	
Mdh-1	-0.005	-0.002	0.003	
Mdh-2	0.372**	0.402	0.047**	
Pgi-1	0.014	0.074	0.062**	
Pgm-1	0.219**	0.266	0.060**	
Mean	0.183**	0.224	0.050**	4.75

Table 3.35 Matrix of Nei's (1972) Genetic Identity (I) (above diagonal) and Nei's (1972) Genetic Distance (D) (below diagonal) between populations of *Amphiporus lactifloreus*.

Population	OBN	WTN	LDN
Oban	-	0.989	0.996
Whitehaven	0.011	-	0.986
Llandudno	0.004	0.015	-

Table 3.36 Matrix of Nei's (1978) Genetic identity (I) (above diagonal) and Nei's (1978) Genetic Distance (D) (below diagonal) between populations of *Amphiporus lactifloreus*.

Population	OBN	WTN	LDN
Oban	-	0.990	0.997
Whitehaven	0.010	-	0.987
Llandudno	0.003	0.013	-

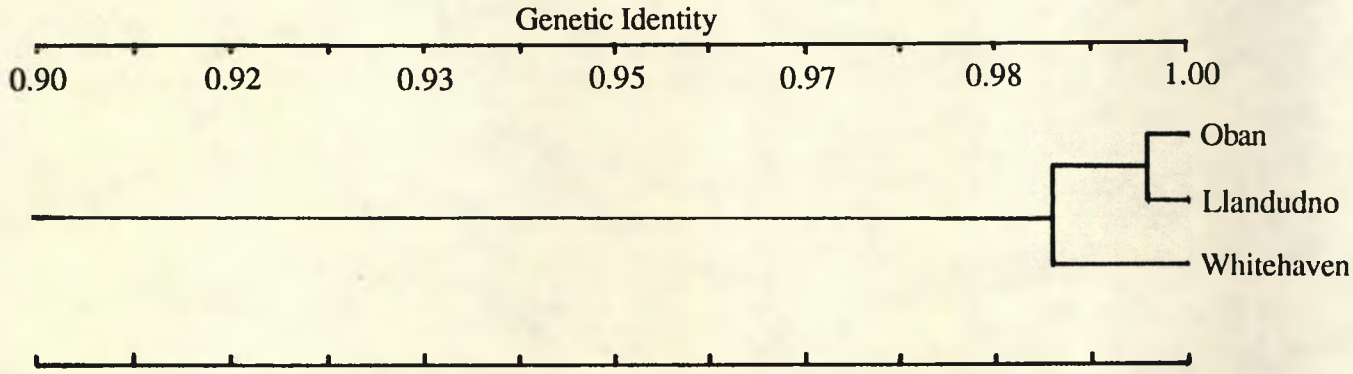


Figure 3.4. UPGMA dendrogram of Nei's (1978) genetic identity (D) between all populations of *Amphiporus lactifloreus*.

3.4 Discussion

Mean observed heterozygosities estimated for *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* (Tables 3.11, 3.20, 3.29) are well within the range of those found in other littoral marine invertebrates and in invertebrate phyla in general (Nevo, 1978; Nevo *et al.* 1984). The mean observed heterozygosity obtained for *Lineus ruber* was unusually low. Studies of heterozygosity should examine large numbers of loci if they are to be reliable (Nei & Roychoudhury, 1974; Nei, 1978, 1987; Gorman & Renzi, 1979). The genome of higher organisms is thought to contain 4,000 - 50,000 structural loci, 60-70% of which are monomorphic (Nei, 1987). Since heterozygosity was calculated over a maximum of 13 loci for all species, it is possible that the very low observed heterozygosity in *Lineus ruber* is a result of interlocus sampling error. A more detailed study of genetic variation in this species, over many more loci, would confirm or refute this hypothesis. Such a detailed study involving many more loci was not possible due to the difficulty of obtaining sufficient activity and resolution of enzymes (see section 2.4.4.) for nemerteans and due to the time constraints of this investigation.

A previous study on isoenzymes in *Lineus ruber*, *Lineus viridis* and *Lineus sanguineus* (Williams *et al.*, 1983) found no genetic variation in any of these three species. Loci stained in this study included PGM and GOT, which were both found to be polymorphic in the present study, even for animals from the same sites as those sampled in Williams *et al.* (1983) (e.g. Anglesey, Llandudno). Since Williams *et al.* (1983) sampled over 100 specimens, results must have been due to enzyme loci appearing monomorphic under the buffering regime used (boric acid/lithium hydroxide for PGM and GOT) or poor tissue preparation prior to electrophoresis; they also did not stain for *Odh-1*, one of the most polymorphic enzyme loci stained in *Lineus ruber* and *Lineus viridis*.

Mean heterozygosities obtained for the four species in the present study were similar to or lower than those reported for two species of the hoplonemertean genus *Oerstedia* by Sundberg & Janson (1988). In this study *Oerstedia striata* was found to have an observed heterozygosity (H_O) = 0.316, whilst two morphs of *Oerstedia dorsalis* had H_O = 0.132 and 0.171 respectively. Manchenko & Balakirev (1982) examined 42 enzyme loci in the nemertean *Lineus torquatus* Coe 1901 and found H_O = 0.139. This is at the

same range of variation to that found in the present study for *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus*.

The cause of variation at the molecular level has been subject to vigorous debate ever since the discovery of widespread protein polymorphism in the majority of organisms. Theories explaining molecular variation have fallen into two groups, neutral theories and selectionist or balance theories. The neutral theory of molecular variation (see Kimura, 1983) states that most intraspecific variability at the level of proteins or DNA is maintained by mutational input and random genetic drift. Phenotypically silent mutants are regarded as neutral or nearly neutral i.e. they have no effect on the survival and reproduction of a species. Selectionist theories refute the importance of random genetic drift and suggest that selection is the main factor maintaining natural polymorphisms (see Ayala, 1977; Milkman, 1978; Clarke, 1979; Wills, 1981).

Neutral theory makes a number of predictions about heterozygosity. Enzymes with high molecular weights are predicted to show higher degrees of polymorphism since they have an increased mutation rate. This correlation has been found in a number of studies (Nei *et al.* 1978; Ward 1978; Turner *et al.* 1979). Correlations have also been found between the quaternary structure of proteins and their heterozygosity. Proteins showing simple quaternary structure (i.e. monomeric proteins) show a higher degree of variation than those with more complicated structures (i.e. tetrameric proteins) (Harris *et al.*, 1977; Nei, 1978).

Neutral theory also predicts a positive correlation in heterozygosity with population size (Kimura, 1983, Nei, 1987; Solé-Cava & Thorpe, 1991). This has been supported by some studies (Nei, 1983; Nei & Graur 1984) but refuted in others (Selander *et al.* 1971; Avise and Selander, 1972). Many other predictions of neutral gene theory have been tested using molecular data (e.g. see Solé-Cava & Thorpe, 1991).

Several studies have analysed the relationship between molecular variation and environmental factors (Ayala & Campbell, 1974; Hedrick *et al.*, 1976; Gillespie, 1978; Nevo, 1978, 1983; Hartl, 1980; Smith & Fujio, 1982; Nevo *et al.* 1984). The discovery of correlations between heterozygosity and environmental factors has been cited as evidence that selection is important in the maintenance of genetic variation rather than random genetic drift.

There is little doubt that selection does affect allele frequencies and therefore heterozygosity in some cases (see Koehn *et al.*, 1976, 1980, 1984; Koehn, 1985; Burton, 1986). For the purposes of this study a null hypothesis that most enzyme polymorphisms are neutral or nearly neutral is assumed (Nei, 1987). Observed enzyme variation in the nemertean species investigated is therefore regarded as a result of mutation and random genetic drift. Testing of this hypothesis would require a detailed study of the occurrence of enzyme polymorphisms within species, the actual function of enzymes coded for by loci and the relation of these to environmental factors.

The statistical tests used in this investigation are based on models calculated from neutral gene theory. This is mainly because selectionist theory requires the building of extremely complex models which are difficult to test practically (Solé-Cava & Thorpe, 1991).

For tests of conformity of genotype frequencies to Hardy-Weinberg expectations for *Lineus ruber* one significant result is expected at $P<0.01$ by chance alone. The χ^2 goodness of fit test gave four significant results for *Lineus ruber*. For sample sizes below 100, χ^2 tests are subject to inaccuracies (Speiss, 1989) (see 3.2.3). Significant results for populations from Port Erin and France are most likely to be type I errors resulting from extremely small sample sizes. Under the χ^2 goodness of fit test with pooling and the Fisher (1935) Exact test these results become insignificant. The conclusion of these tests must be that no significant deviations from Hardy-Weinberg were detected with the current sample sizes.

F_{IS} values for *Lineus ruber* were clearly affected by small sample size. The only significant result which may indicate non-random mating was that for *Pgi-1* reflecting heterozygote deficiency in the Castletown population. This may be a result of inbreeding or an artefact of a moderate sample size (n=38).

F_{ST} in contrast gave highly significant results for almost all loci examined. The mean F_{ST} value was higher than that found in several molluscan species with long lived planktonic larvae over similar geographic distances (Levington & Suchanek, 1978; Johnson & Black, 1984; Benzie & Williams, 1992). This suggests that *Lineus ruber*, a species with no planktonic larval phase, shows lower gene flow between populations than some species with planktonic larval phases. as expected (Scheltema, 1971, 1986, 1989;

Burton, 1983; Hedgecock, 1986; Slatkin, 1987; Waples, 1987; Hunt & Ayre, 1989; Ward, 1989). F_{ST} values obtained for *Lineus ruber* though, were not as high as those obtained for species with planktonic larvae that show degrees of population sub-division (Smith & Potts, 1987; Macaranas *et al.*, 1992; Russo *et al.*, in press;).

Genetic distance values between all populations of *Lineus ruber* were extremely low indicating that this species can maintain panmixia over a distance of at least 6000 km. F_{ST} and genetic distance data indicate that though *Lineus ruber* is divided into a number of populations throughout the range sampled, sufficient gene flow is maintained between populations to prevent substantial genetic divergence developing between populations. The level of gene flow required to prevent divergence between two geographically separated populations is extremely low. Mathematical models suggest that a migration of only one individual per generation is enough to prevent divergence, due to random genetic drift, between two populations (Wright, 1940; Kimura, 1955; Slatkin, 1985; Maynard Smith, 1989).

The loci sampled for *Lineus ruber* show a low level of heterozygosity. Weakly polymorphic loci give little insight into population structure (Burton, 1983) especially at low sample sizes. Results obtained for F_{ST} and genetic distance measures in this case may therefore overestimate gene-flow between populations of *Lineus ruber*.

A larger number of populations and specimens of *Lineus viridis* were sampled than for *Lineus ruber*. Enzyme loci sampled for *Lineus viridis* were also more polymorphic than for *Lineus ruber*. Analysis of genetic data of *Lineus viridis* populations, by the use of tests of conformity to Hardy-Weinberg expectations, F-statistics and genetic distance and identity measures provided a greater resolution of population structure than in *Lineus ruber*.

For tests of conformity of genotype frequencies to Hardy-Weinberg expectations, between one and two significant results were expected by chance alone at the $P<0.01$ level. At the *Odh-1* and *Pgm-1* loci the χ^2 goodness of fit test detected significant deviations from genotype frequencies expected under Hardy-Weinberg equilibrium conditions over several populations. The χ^2 test with pooling and Fisher's (1935) Exact test reduced the significance of most deviations from Hardy-Weinberg but deviations from Hardy-Weinberg expectations for *Odh-1* and *Pgm-1* were still significant in the St. Agnes population. The sample size for this population was large (n=118) and the significance of

deviation is likely to be real. Wright's (1951, 1965) fixation index indicates that these deviations are due to an excess of heterozygotes for *Odh-1* and *Pgm-1* in the St. Agnes population. Populations at Llandudno and Anglesey showed heterozygote deficiency for *Pgm-1* (though insignificant).

The overall F_{IS} value for *Lineus viridis* was not significant but was significant for the *Pgm-1* locus. The St. Agnes population was notably small probably due to a lack of suitable habitats. A high F_{IS} for the *Pgm-1* locus and significant deviations in this locus from genotype frequencies expected under Hardy-Weinberg equilibrium conditions are considered to be a result of inbreeding due to small population size. Excess heterozygosity at the very exposed St. Agnes site could also be a result of selection (overdominance see Lewontin, 1974; Zouros & Foltz, 1987). This is very difficult to prove and highly unlikely since the occurrence of overdominance in natural populations is extremely rare (Cook, 1984).

Highly significant values obtained for F_{ST} at individual loci and for all loci together indicate that *Lineus viridis* is split into sub-populations throughout its range. F_{ST} was higher in *Lineus viridis* than in *Lineus ruber*, a result not expected when the life histories of the two species are compared. *Lineus ruber* has a much lower fecundity than *Lineus viridis* and larvae which show a nil or negative phototaxis. *Lineus viridis*, with a far greater fecundity and number of larvae which have a positive phototaxis, should have a greater potential for larval dispersion than *Lineus ruber*. The differences in F_{ST} and in estimates of mNe between *Lineus ruber* and *Lineus viridis* are not large and comparison may be confounded by the low variation of loci sampled for *Lineus ruber*.

Genetic distance and identity between populations of *Lineus viridis* show a greater level of differentiation than *Lineus ruber*. This may be attributed to the low variation of loci sampled for *Lineus ruber*. Populations of *Lineus viridis* do not show a large degree of genetic divergence over a range of 6000 km. Furthermore divergence between populations is unrelated to geographic distance between populations. Evidence would suggest that over the range sampled *Lineus viridis* forms a number of subpopulations which allow a small degree of random genetic divergence within populations. A low level of gene flow is all that is needed to prevent major genetic divergence between populations (Wright, 1940; Kimura, 1955; Slatkin, 1985; Maynard Smith, 1989).

Lineus sanguineus is reported to reproduce asexually throughout the year (Gontcharoff, 1951). If this is the case large deviations of genotype frequencies from Hardy-Weinberg expectations would be expected with non-random association between loci (Ward, 1989). This has been detected in many species of marine invertebrates which reproduce partially or completely by asexual reproduction (e.g. Crisp *et al.*, 1983; Hoffmann, 1987; Smith & Potts, 1987; Mladenov & Emson, 1990).

Two loci showed deviation from genotype frequencies expected under Hardy-Weinberg equilibrium at the $P<0.05$ level for the χ^2 goodness of fit test. Deviations at these loci (*Pgi-1*, Whitehaven; *Got-1*, Llandudno) from Hardy-Weinberg expectations may be attributed to sampling error derived from a moderate sample size (N=60, Whitehaven; N=43, Llandudno). Other loci demonstrated no significant deviation from genotype frequencies expected under Hardy-Weinberg conditions using the χ^2 goodness of fit test. The χ^2 goodness of fit test with pooling and Fisher's (1935) Exact test indicated that none of the enzyme loci sampled showed deviations from Hardy-Weinberg expectations.

The conformity of genotype frequencies to Hardy-Weinberg expectations, in the two populations of *Lineus sanguineus* sampled, does not support evidence that *Lineus sanguineus* reproduces solely by asexual reproduction (Gontcharoff, 1951), in the United Kingdom. F-statistics cast further doubt on the suggestion that *Lineus sanguineus* only reproduces by fragmentation. Asexual reproduction will cause large values of F_{IS} which are usually significant (e.g. Smith & Potts, 1987). No F_{IS} values calculated for the two populations of *Lineus sanguineus* sampled were significant.

Genotype frequencies found for the two populations of *Lineus sanguineus* could occur for several reasons. The first and most likely of these is that *Lineus sanguineus* does reproduce sexually at least for some of the year. The conclusions reached by Gontcharoff (1951) about incomplete development and reabsorption of female gametes could have come about by laboratory stress of experimental animals or by parasitic castration of females. Another possibility is that Gontcharoff (1951) did not study the same species that was sampled for this investigation and *Lineus sanguineus* is a species complex. A number of genetically 'aberrant' forms which also had a different external colouration to 'normal' *Lineus sanguineus*, were found at Llandudno. Whether these were natural hybrids from

crosses between *Lineus sanguineus* and *Riseriellus occultus* (see Chap. 4) or examples of some cryptic species, has not yet been determined. The possibility that Gontcharoff (1951) studied a different species from that found at Llandudno and Whitehaven cannot therefore be ignored.

F_{ST} values were significant, as was the overall F_{ST} value. The mean F_{ST} value was very similar to that obtained for *Lineus ruber* and is in the same order of magnitude as that obtained for *Lineus viridis*. Like *Lineus ruber* and *Lineus viridis*, *Lineus sanguineus* demonstrated low genetic differentiation between the two populations studied. Nei's (1972) genetic distance and genetic identity were comparable to those found between populations of *Lineus ruber* and *Lineus viridis*. *Lineus sanguineus* exhibits similar levels of population division to *Lineus ruber* and *Lineus viridis*, with moderate levels of gene flow between populations (Wright, 1978).

Sample sizes for the populations of *Amphiporus lactifloreus* were all moderately high. Tests for conformity to genotype frequencies expected under Hardy-Weinberg equilibrium, for two different enzymes (Whitehaven, *Pgm-1*; Llandudno, *Mdh-2*), indicated significant deviations in two populations. Both of these loci showed heterozygote deficiency for all populations (but not significant in most cases) and gave significant F_{IS} values which resulted in a significant mean F_{IS}. Significance of F_{IS} values resulting from heterozygote deficiencies were probably due to inbreeding in populations of *Amphiporus lactifloreus*. Some other form of non-random mating is not out of the question but as yet *Amphiporus lactifloreus* has only been observed reproducing sexually (McIntosh, 1873-1874).

F_{ST} values for the loci studied in *Amphiporus lactifloreus* show a similar pattern to the three *Lineus* species investigated. Most F_{ST} values, including the mean F_{ST} value, are significant indicating that over the range sampled, *Amphiporus lactifloreus* forms a number of populations. Gene flow between these populations is similar to the *Lineus* species studied with the result that there is little overall genetic differentiation between these populations. The slightly higher gene flow between populations of *Amphiporus lactifloreus* may be attributed to sampling error introduced by the low number of loci studied for this species. There is a possibility that the planuloid type larva of *Amphiporus lactifloreus* has a greater potential for dispersal than juveniles of the other species studied.

All four species of nemertean studied in this investigation demonstrated a similar pattern of genetic population structure. Each species, within the ranges sampled, was composed of discrete populations between which there was a low level of gene flow. The levels of gene flow maintained were sufficient to prevent overall genetic divergence between geographically separated populations but they allowed a degree of inbreeding within populations reflected by deviations from genotype frequencies expected under Hardy-Weinberg equilibrium conditions.

All the nemertean species studied apparently lack a true pelagic larva. A lower level of gene flow than that found in species with long lived pelagic larvae (e.g. Scheltema, 1971, 1986, 1989; Burton, 1983; Slatkin, 1987; Waples, 1987; Hedgecock, 1986; Hunt & Ayre, 1989; Ward, 1989) was expected. Scheltema (1971, 1986, 1989) extended the idea that species with long lived larvae have a much greater gene flow between populations than species which have no planktonic larval phase. He hypothesised that pelagic larvae were an adaptation for dispersal and that species with pelagic larvae had a much greater geographic range than those without (Scheltema, 1971, 1986; Scheltema & Williams, 1983). Furthermore it has been suggested that species with pelagic larvae are not as prone to local extinctions as those without and are therefore longer lived in geological time (Jablonski & Lutz, 1983; Scheltema & Williams, 1983; Jablonski, 1986).

It appears that *Lineus ruber* and *Lineus viridis* can maintain gene flow over large geographic distances (6000+ km) by means other than planktonic larvae. Evidence suggests that a degree of gene flow is maintained between the continents of America and Europe since North American populations do not show any evidence of a bottleneck (e.g. reduced heterozygosity) (see Nei *et al.*, 1975).

This is in contrast with studies on other groups of intertidal marine invertebrates which have pelagic larvae but show marked differentiation between populations on the east and west sides of the North Atlantic ocean. For example in the cirripede, *Semibalanus balanoides* allele frequency differences were found for *Pgi* and *Mpi* loci at different localities along the coasts of the North Atlantic (Flowerdew, 1983). These indicated that in the North Atlantic there were two populations of *Semibalanus balanoides* Linnaeus. One of these extended along the coasts of Europe from Spain in the south to Spitzbergen, northern Norway. The other was found along the coast of the United States. through to

Newfoundland and Iceland (Flowerdew, 1983). Genetic studies on the bivalve mollusc *Mytilus edulis* Linnaeus have shown differentiation in allele frequencies for leucine aminopeptidase *(Lap), Ap, Pgi* and *Pgm* loci between populations located on the east and west sides of the North Atlantic ocean (Gosling, 1992).

By what mechanisms could *Lineus ruber* and *Lineus viridis* maintain moderate levels of gene flow between geographically distant populations? It has been shown that wave action can be responsible for the suspension of meiobenthic organisms such as nematodes into the water column (Hagerman & Reiger, 1981; Dobbs & Vozarik, 1983). Such a means of dispersal would be effective along continental coastlines but probably not across ocean basins due to the sinking velocity of the suspended organisms (Hagerman & Reiger, 1981). An obvious means of very long distance dispersal is rafting (Highsmith, 1985; Edgar, 1987). Evidence suggests that organisms may disperse over thousands of kilometres by rafting on drifting algae (Edgar, 1987). Rafting may also occur on sea grasses (Highsmith, 1985), pumice (Jokiel, 1984, 1987, 1989), coralla (DeVantier, 1992), wood (Jackson, 1986) and in more recent times on artificial substrates such as ships bottoms (Bertelson & Ussing, 1936; Crisp, 1958) and drift tar and plastic (Farnham, 1980; Winston, 1982). Suspension of small intertidal organisms into the water column may increase the liklihood that they contact floating pieces of wood and algae (Highsmith, 1985).

The life histories of *Lineus ruber* and *Lineus viridis* involve juvenile worms hatching from mucous egg strings in late winter when there is a high incidence of storms and wave action. This and the tendency of *Lineus viridis* larvae to move towards light increase the probability that some of these animals will become suspended in the water column or be washed out to sea on rafts of buoyant material. The dispersion provided by these means is not as consistent as that provided by teleplanic or even short lived larvae. The stochastic nature of dispersal by rafting possibly explains the lack of a relationship between the geographic distance separating populations and the genetic distance between them (Figure 3.3).

Passive dispersal may increase the fitness of genes by allowing the exploitation of spatially and temporally transient habitats in the littoral environment (Strathmann, 1974). A by product of this type of dispersal is gene flow over long distances. Passive dispersal is

especially important when the limited mobility of adult nemertean worms is considered. It will prevent the extinction of genes by local 'disasters' which may wipe out an entire population at a given locality (e.g. a phytoplankton bloom). The dispersal of newly hatched worms into the water column may have the effect of increasing rates of juvenile mortality.

Another possible explanation of the apparent lack of genetic differentiation between eastern and western North Atlantic populations of *Lineus ruber* and *Lineus viridis* is the introduction of large numbers of individuals of these species from one side of the Atlantic to the other. The lack of genetic differentiation between populations of the cirripede *Elminius modestus* Darwin in Australia, New Zealand and Europe is thought to be due to such large introductions (Dando, 1987). Large introductions of *Lineus ruber* and *Lineus viridis* might have taken place by transport with other organisms (e.g. bivalves for cultivation [Christiansen & Thomsen, 1981]) or as a component of the fouling communities of ships (see Bertelsen & Ussing, 1936; Carlton, 1985).

If dispersal increases fitness of individuals why do the species of nemertean studied not have a planktonic larval phase ? *Lineus ruber* and *Lineus viridis* have encapsulated larvae (Gontcharoff, 1951; Gibson, 1972) and would appear to have lost the pelagic larval stage during the course of evolution. A possible reason for the loss of a planktonic phase in the life history of these species could be the high degree of mortality suffered by planktonic organisms due to predation and in some cases starvation. The few studies on the survival of planktonic larvae in natural environments indicate huge rates of mortality (Korringa, 1941; Bousfield, 1955; Munro *et al.*, 1968). Low food levels may also increase the mortality rates of planktonic larvae (Lucas, 1982). A simple probability model has suggested that encapsulation may reduce the overall mortality of larvae (Pechenik, 1979). Due to the transience of the littoral environment and the low mobility of the adults it may increase fitness in intertidal nemertean species to reproduce as quickly as possible. The small size at maturity of the species studied means that they may produce relatively few larvae so reduction of juvenile mortality may be important (Strathmann & Strathmann, 1982; Grahame & Branch, 1985). Other benefits from encapsulation include protection from environmental stress during the very early stages of life e.g. during settlement and metamorphosis (Cameron, 1986). This may be of great importance to an intertidal organism which lives in a habitat subject to severe environmental fluctuation.

The loss of a planktonic larva could increase the fitness of an organism by affecting reproductive strategy within the species. The reproductive and developmental strategies in the four species of nemertean studied have an effect on the genetic structure of their populations. This effect can be summarised as inbreeding within populations between which a low level of gene flow is maintained as a result of passive dispersal. Sexual reproduction with inbred lines is the best means of producing new genotypes by recombination, and duplicating those rapidly over successive generations (Shields, 1982). This could enhance the adaptation of organisms to local conditions (Clarke *et al.* 1979; Vermeij, 1982 a,b; Strathmann, 1986). Increased sibling competition resulting from inbreeding may also effect adaptive evolutionary processes (Jackson, 1986).

It has been proposed that the spread of sibling larvae can increase the fitness of genes by allowing the sampling of a number of habitats and preventing local extinctions (Thorson, 1946; Scheltema, 1971, 1986, 1989; Crisp, 1974, 1976; Strathmann, 1974; Jablonski & Lutz, 1983; Scheltema & Williams, 1983; Jablonski, 1986). Observational and experimental evidence does not confirm or reject these ideas (Strathmann, 1980) and it is becoming increasingly clear that larval phases are maintained in organisms for reasons other than dispersion. For example, it has been suggested that the length of time planktotrophic larvae remain in the water column is of sufficient length to bridge the time gap between optimum spawning time and optimum settlement time (Todd & Doyle, 1981). Another theory is that larval phases may be selected to exploit an alternative food source away from competition with adult organisms (Istock, 1967) or to track food resources which show temporal variation in availability (Clark *et al.*, 1979).

It is proposed that gene flow between conspecific populations and the geographic range of a species are not simply a function of the length of the planktonic larval phase in all marine invertebrates. Much of the work on which the hypothesis that dispersal is a function of the length of planktonic phase was carried out on relatively few taxa, and concentrated particularly on the Mollusca (Scheltema, 1971, 1986, 1989; Scheltema & Williams 1983). It may be that these taxa suffer constraints on dispersion by means other than by planktonic larvae because of their size or even in the case of molluscs, because of their shell. Studies on other taxa have demonstrated a complete lack of any relationship between developmental mode and geographic range (Bhaud, 1982; Jackson, 1986). The

present study indicates that though the nemertean species investigated have no larval phase, they can maintain sufficient gene flow, over distances of up to 6000 km, to prevent substantial genetic divergence between conspecific populations.

The selection of reproductive and developmental strategies cannot be predicted in organisms by any simple model (Grahame & Branch, 1985). The most advantageous modes of reproduction and development for a species depend on a whole range of environmental and biotic factors. Each species presents a unique case (Todd & Doyle, 1981) which requires very detailed investigation to elucidate the selective pressures that cause an organism to adopt a certain reproductive and developmental strategy. Enough detailed data on marine invertebrate life histories and population genetics do not exist. The construction of models predicting optimum life history strategies for different marine invertebrates in different ecosystems is at present not possible.

Chapter 4

Description of a New Genus and Cryptic Species of Heteronemertean From North Western Spain and North Wales, Identified by Starch-Gel Electrophoresis.

4.1 Introduction

The study of nemertean systematics is plagued by a multitude of inadequately described taxa at and below the family level (Gibson, 1982b, 1985). Many of the older species were established primarily on the basis of external features, many of which are now considered to be taxonomically unreliable (Gibson, 1985; Gibson & Crandall, 1989) and also of little value in determining generic affinities (see Section 1.2). Even where species have been erected after histological investigation, descriptions of their internal anatomy are frequently either incomplete or emphasise characteristics whose taxonomic significance are difficult to assess. This is especially true when problems of intraspecific variability are considered (Friedrich, 1960; Berg, 1972; Sundberg, 1979b, 1980), and it is highly likely that many nemertean "species" are either complexes of different taxa or are synonymous with other forms.

The genus *Lineus* Sowerby, 1806, is the oldest established nemertean taxon. It contains more than 90 nominate species (Gibson, 1982a) and typifies many of the fundamental problems outlined above. During September 1990 nemerteans of this genus were collected at Llandudno, North Wales, and in the Foz Estuary, north-western Spain. The collections included numerous examples of *Lineus longissimus* and *Lineus viridis*, as well as a third group of black to dark green heteronemerteans, in size and general appearance very similar to, but externally distinguishable from, both the *Lineus* species. Whether this third morphotype represented a separate species or was merely a variety of one or the other taxa was uncertain, since previous descriptions of the external features of both *Lineus longissimus* and *Lineus viridis* have been ambiguous. Histological differences between *Lineus* species are often small (Cantell, 1972, 1975) and may simply represent intraspecific variation; further, descriptions of the internal anatomy of species such as *Lineus longissimus* are frequently contradictory (Friedrich, 1935; Cantell, 1975, 1976). Preliminary studies on the systematic relationship between *Lineus longissimus*, *Lineus viridis* and the third morphotype found in Spain and Wales required a more objective

approach, such as an investigation of enzyme variation by electrophoresis. This technique has been used to resolve taxonomic problems in many invertebrate groups (for reviews, see Ferguson, 1980; Thorpe, 1982, 1983; Ayala, 1983), including nemerteans (Cantell & Gidholm, 1977; Williams *et al.*, 1983; Sundberg & Janson, 1988). Enzyme electrophoresis was therefore employed to estimate the genetic distance between sympatric populations of *Lineus longissimus*, *Lineus viridis* and the third unknown heteronemertean morphotype; the results demonstrated that the unknown form was conspecific with neither of the lineids, and indicated that extensive histological investigation of specimens from both locations were needed to resolve the systematic status of this heteronemertean.

4.2 Methods and Materials.

4.2.1 Sample Sites

Nine specimens of *Lineus longissimus* were collected from the Foz Estuary, Spain (approximately 43°34'N, 7°14'W) during September 1990, close to mean low water spring tide level. Ten examples of the third morphotype were also found at Foz, but these occurred much higher up the shore in mud banks or amongst *Spartina* roots. Several additional specimens of the unknown taxon were obtained from the same location during May 1991. Collections made at Llandudno, also during September 1990, yielded 59 examples of *Lineus viridis* and 39 individuals apparently of the same unknown morphotype as those found at Foz. At Llandudno all samples were found on the upper shore beneath stones and rocks lying on damp fine mud or silt. Further examples of the unknown species have subsequently been found (August 1991) at Rhosneigr and Trwyn du Point, Anglesey.

Representative living specimens of each of the three species were examined in detail under a binocular microscope and described, their maximum relaxed lengths and widths recorded (see 2.3).

4.2.2 Starch Gel Electrophoresis

Horizontal starch gel electrophoresis was performed on specimens as described in Section 2.4. Data recorded from starch gel electrophoresis were converted to actual numbers of genotypes occurring in each population for each species and then analysed using the FORTRAN programme BIOSYS-1 (Swofford & Selander, 1989).

4.2.3 Histological Studies

Four specimens of the unknown morphotype, two from each location, were anaesthetised in 7.0% magnesium chloride, fixed in Bouin's fluid (made up in filtered seawater) and sectioned at 6μm in 56°C paraffin wax. Mallory's triple stain (Pantin, 1960) was used as described in section 2.7.

4.3 Electrophoretic Results and Discussion

Allele frequencies for the ten enzyme loci which produced useful results for one or more of the species examined are presented in Table 4.1.

The results show that at nearly all loci there are large differences in allele frequency between sympatric samples of *Lineus longissimus* and the third morphotype from Spain, and between *Lineus viridis* and the unknown type from Llandudno, whereas between specimens of the form occurring both in Spain and North Wales differences in allele frequencies are very small. Over all the loci used, the genetic differentiation between the three species can be reduced to a single figure by using measures of genetic distance or similarity (see 3.2.2). Between *Lineus longissimus* and Spanish specimens of the new morphotype, and between *Lineus viridis* and Welsh examples of the new form, the value of Nei's (1972) genetic identity falls below 0.35 (Table 4.2). This indicates that not only are these likely to be separate species, but that they show genetic identity values lower than those usually found in congeneric comparisons (Ayala, 1975, 1983; Thorpe, 1982, 1983). However, similarly low values of genetic identity have been found between other species

of nemerteans which on morphological grounds are considered to be congeneric (Williams *et al.*, 1983; Sundberg & Janson, 1988).

Distortion of gene frequencies due to inbreeding or the presence of clones is unlikely since there was substantial genetic variation within samples and two of the species showed good fits of allele frequencies to Hardy-Weinberg expectations (but note the statistical weakness of such tests on other than large sample sizes: Lewontin, 1958; Fairbairn & Roff, 1980; Valenzuela, 1985; Lessios, 1992). Fisher's (1935) Exact test gave a significant ($P<0.05$) deviation from genotypes expected under Hardy-Weinberg equilibrium for *Lineus longissimus* at the *Got-1* locus. Fisher's (1935) Exact test is not supposed to be affected by small sample size (Lessios, 1992). It is suggested that the lack of any heterozygotes at the *Got-1* locus for *Lineus longissimus* is because of sampling error due to a sample size of only nine individuals.

Between specimens of the unknown morphotype from Spain and Wales the value of Nei's (1972) genetic identity is above 0.9. This value is within the normal range for conspecific populations and above the range expected for congeneric species, and indicates that the populations of this morphotype from the two geographically separate populations are very likely to be conspecific. The species represented by this morphotype thus apparently shows little genetic differentiation over a moderately large zoogeographical range as indicated in *Lineus ruber* and *Lineus viridis* in the previous chapter. One individual, of the new morphotype, was observed releasing eggs into the surrounding seawater in a manner similar to that described for *Lineus lacteus* McIntosh 1873-1874, by Gontcharoff & Lechenault (1958). Since the new morphotype releases eggs into the surrounding water, rather than fixing them to the substrate in a mucous cocoon (e.g. *Lineus ruber* [Gontcharoff, 1951]), it probably shows an indirect form of larval development as in *Lineus bilineatus* (Cantell, 1972) and some *Micrura* species (Cantell 1972, Iwata, 1960b). If this development involves a pelagic larva (pilidium) high gene flow and low genetic divergence between geographically separated populations of this species may be expected (Scheltema, 1971, 1986, 1989; Burton, 1983; Hedgecock, 1986;

Slatkin, 1987; Waples, 1987; Hunt & Ayre, 1989; Ward, 1989).

Expected and observed values for mean heterozygosity per locus for these species were high (range 9.7-27.8%; Table 4.3), especially for *Lineus viridis* and the unknown form. Heterozygosity estimates for these two taxa are considerably higher than those found for other eukaryotic species (generally 5-15%; see Selander, 1976; Nevo, 1978; Nevo *et al.*, 1984). High values of heterozygosity have also been found previously in the hoplonemertean *Oerstedia striata* (Sundberg & Janson, 1988). These values are at variance with those of Williams *et al.* (1983), who described a complete absence of allelic diversity from *Lineus viridis* (see discussion 3.4). This can partially be explained by the fact that these authors did not stain for *Odh-1* or *Icd-1*, the loci which showed the largest allelic diversity in *Lineus viridis* during the present studies (observed heterozygosity for *Odh-1* = 0.757, for *Icd-1* = 0.707). Loci found to be polymorphic in the present study, but not in that of Williams *et al.* (1983) (e.g. *Pgm-1*, *Got-1*) may have appeared monomorphic under the buffer regime used in the previous study.

Nemertean species show observed heterozygosities which range from very low values (*Lineus ruber*, chapter 3) to values which are higher than most other groups of organisms (Nevo, 1978; Nevo *et al.*, 1984). Such high values of heterozygosity are apparently common in sea anemones and sponges (Solé-Cava & Thorpe, 1990, 1991). The reasons for such high levels of genetic variation are uncertain but have been attributed to e.g. large population size (Kimura, 1983; Nei, 1987; Solé-Cava & Thorpe, 1991) or selection by environmental factors (Ayala & Campbell, 1974; Hedrick *et al.*, 1976; Gillespie, 1978; Nevo, 1978, 1983; Hartl, 1980; Smith and Fujio, 1982; Nevo *et al.*, 1984).

Table 4.1. Allele frequencies at 11 loci for the species of heteronemerteans collected at Llandudno, North Wales, and in the Foz Estuary, north-western Spain. Alleles are arranged in order of decreasing mobility. Enzyme abbreviations are given in general materials and methods.

Locus	Allele	*Lineus viridis*	*Lineus longissimus*	Unknown form	
				Llandudno	Foz
Apa-1	B	0.000	0.000	0.064	0.000
	C	0.000	0.000	0.872	1.000
	D	1.000	0.000	0.000	0.000
	F	0.000	0.000	0.064	0.000
	M	0.000	0.056	0.000	0.000
	O	0.000	0.944	0.000	0.000
Got-1	E	1.000	0.000	0.000	0.000
	K	0.000	0.222	0.000	0.000
	M	0.000	0.778	0.000	0.000
	P	0.000	0.000	1.000	1.000
Got-2	B	0.000	-	1.000	1.000
	D	1.000	-	0.000	0.000
Icd-1	B	0.000	0.000	0.013	0.050
	C	0.000	0.167	0.000	0.000
	D	0.000	0.000	0.821	0.450
	F	0.012	0.000	0.000	0.000
	G	0.000	0.833	0.000	0.000
	H	0.000	0.000	0.154	0.350
	I	0.598	0.000	0.000	0.000
	K	0.000	0.000	0.013	0.150
	L	0.317	0.000	0.000	0.000
	N	0.073	0.000	0.000	0.000
Icd-2	E	0.000	0.000	1.000	1.000
	F	0.000	1.000	0.000	0.000
	H	1.000	0.000	0.000	0.000
Mdh-1	E	0.754	0.000	-	-
	H	0.246	0.000	-	-
	K	0.000	1.000	-	-
Mdh-2	A	0.992	0.000	-	-
	B	0.008	0.000	-	-
	D	0.000	1.000	-	-
Odh-1	C	0.500	0.000	0.000	0.000
	E	0.135	0.000	0.000	0.000
	F	0.162	0.000	0.000	0.000
	I	0.000	0.944	0.000	0.000
	J	0.000	0.000	0.763	0.750
	K	0.189	0.000	0.000	0.000
	O	0.000	0.056	0.118	0.000
	P	0.014	0.000	0.000	0.000
	S	0.000	0.000	0.118	0.250
Pgd-1	D	0.000	-	0.054	0.200
	F	1.000	-	0.000	0.000
	G	0.000	-	0.108	0.000
	K	0.000	-	0.797	0.800

Table 4.1 contd.

Locus	Allele	*Lineus viridis*	*Lineus longissimus*	Unknown form Llandudno	Foz
Pgd-1	N	0.000	-	0.041	0.000
Pgi-1	A	0.000	0.889	0.000	0.000
	B	0.000	0.000	0.053	0.000
	F	0.000	0.111	0.000	0.000
	G	0.025	0.000	0.000	0.000
	K	0.000	0.000	0.184	0.000
	L	0.000	0.000	0.408	0.400
	O	0.000	0.000	0.355	0.600
	P	0.970	0.000	0.000	0.000
Pgm-1	K	0.958	-	0.000	0.000
	M	0.042	-	0.000	0.000
	N	0.000	-	0.029	0.000
	P	0.000	-	0.086	0.200
	R	0.000	-	0.771	0.650
	T	0.000	-	0.014	0.000
	U	0.000	-	0.057	0.000
	V	0.000	-	0.043	0.150

Table 4.2. Pairwise comparisons based on isozyme data from *Lineus longissimus*, *Lineus viridis* and the unknown morphotype. Above diagonal, values for Nei's (1972) genetic distance: below diagonal, values for Nei's (1972) genetic identity ("-" means infinite genetic distance).

	L.longissimus	*L.viridis*	Unknown form Llandudno	Foz
L. longissimus	+	-	6.508	-
L. viridis	0.000	+	-	-
Unknown (Llandudno)	0.010	0.000	+	0.033
Unknown (Foz)	0.000	0.000	0.968	+

Table 4.3. Estimates of genetic variation in populations of *Lineus longissimus*, *Lineus viridis* and the unknown morphotype. H_E and H_O: mean expected and observed heterozygosities per locus respectively; $P_{(0.95)}$ and $P_{(0.99)}$: proportions of polymorphic loci with frequency of most common allele <0.95 or <0.99 respectively. n_e : mean effective number of alleles per locus.

	Lineus longissimus	*Lineus viridis*	Unknown form Llandudno	Foz
H_E	0.136	0.150	0.263	0.274
H_O	0.097	0.174	0.222	0.278
$P_{(0.95)}$	0.625	0.222	0.667	0.556
$P_{(0.99)}$	0.625	0.444	0.667	0.556
n_e	1.6	2.0	3.0	1.9

4.4 A comparison in external features between *Lineus longissimus*, *Lineus viridis* and the third morphotype.

The external features of the unknown morphotype resemble both *Lineus longissimus* and *Lineus viridis*, but close examination of living specimens reveals that all three taxa can be consistently distinguished from each other. Accounts of the external appearance of each of the three taxa are given below.

Lineus longissimus (Gunnerus, 1770)

Living specimens examined in this investigation vary in length from 150 - 590 mm, in maximum width from 0.8 - 1.8 mm. In Salcombe, south-west Britain specimens measuring up to 10 m in length were found beneath mooring blocks in Salcombe Harbour. The body width tends to remain uniform for up to about 70 mm from the tip of the head, but then gradually tapers posteriorly to end in a blunt tail. The body is typically somewhat dorsoventrally compressed.

The rather flattened head (Fig.4.1A) is spatulate but appears short (length approx. twice width); it is characteristically slightly wider than the succeeding body region. The anterior tip of the head usually appears slightly bilobed. The dorsal cephalic surface is dark chocolate brown to black in colour, the anterior margins are white. Five pale and imprecisely defined longitudinal stripes occur on the dorsal cephalic surface, one median flanked on either side by two dorsolateral; the median stripe is typically white whereas the remaining stripes are typically pale brown. The cerebral ganglia may colour the posterior half of the head reddish. The ventral cephalic surface is usually paler than the dorsal and is marked by a single pale median stripe. Each lateral margin of the head bears a distinct horizontal cephalic furrow. There are 10-40 black or silvery-black ocelli on each side of the head, forming an irregular patch near the anterior margin. In one specimen the ocelli were situated along the lower margin of the cephalic furrows. The slit-like mid-ventral mouth is long (5-6 mm) and commences just before or immediately after the posterior end of the

cephalic slits.

The general body colouration is variable. The upper surface may be brown, dark chocolate brown or black; under artificial light it may appear maroon or exhibit a blue or purple iridescence. Five pale longitudinal stripes, continuous with those on the head, extend the full length of the body on the dorsal surface. The ventral surface may be the same colour as or slightly paler than the dorsal.

Lineus viridis (Müller, 1774)

Living specimens vary in length from 35- 109 mm, in width from 0.4-1.5 mm, and exhibit a very variable body shape. Behind the cephalic region the body may be more or less uniform in width for up to about 60% of its length, then gradually narrows posteriorly to end in a finely pointed tail. Alternatively, the body width may increase behind the head for 7-26% of the body length, and then remain uniformly wide up to about 60% of its length before gradually narrowing to the tail. The body is compressed dorsoventrally, becoming progressively more flattened from the anterior to the posterior end.

The dorsoventrally flattened head (Fig.4.1B) is generally spatulate, although its posterior portion is often markedly wider than the anterior to give it a spade-like shape. There is normally a shallow but distinct constriction between the head and the remainder of the body. The cephalic colour is variable, ranging from pale green through dark olive green to a deep bronze green or black. The cerebral ganglia are externally visible in the posterior part of the head, as a pair of more or less distinct red patches. The margins of the head, which bear a single pair of lateral horizontal cephalic furrows, are pale. Two to eight black ocelli form an irregular and often asymmetrical dorsolateral row along either side of the anterior head region. The ocelli may be obscured by pigmentation, especially in darker coloured individuals. The small, mid-ventral and slit-like mouth commences just in front of or close behind the posterior end of the cephalic furrows. The oral margins are generally pale and often appear somewhat swollen.

The general body colour is also very variable. The dorsal surface may be black,

deep bronze green, dark to light olive green or pale green. The colour intensity often shades from darker anteriorly to paler posteriorly. Pale annulations are normally distinguishable along the full length of the body and a pale mid-dorsal line may be present. In sexually mature specimens the gonopores show as an irregular row of white spots on each side of the body, commencing some distance behind the rear of the head. The ventral body surface is usually paler than the dorsal. When touched. the worms characteristically contract without coiling.

The new morphotype

Living specimens are 150-700 mm long and 1.0-1.3 mm wide. Their body width remains uniform for up to half the length, but then decreases posteriorly to terminate in a pointed tail.

The head (Fig.4.1C) is spatulate and continuous with the succeeding body region, with no intervening constriction. In colour the head is very dark brown or green to black, with pale narrow lateral margins bearing a pair of horizontal cephalic slits. Three to eleven black ocelli form a dorsolateral row on each side of the anterior portion of the head. The posterior third of the head is often reddish due to the colour of the cerebral ganglia. The mid-ventral mouth begins far behind the brain lobes, approximately 1 mm beyond the rear end of the cephalic furrows; it is long, slit-like and possesses pale margins.

The body colour is variable. Anteriorly it may be black, greenish-black or dark olive, but the colour is nearly always lighter towards the posterior, shading to a dark or pale olive green or greenish-brown. The ventral surface may be similarly coloured or slightly paler. Under artificial light the body surface often shows a purple iridescence. Towards the posterior end of the body an irregular dorsolateral row of white spots on each side marks the position of the gonopores in sexually mature individuals. When touched, the worm tends to coil up into a tight spiral. The appearance of a complete specimens is shown in Fig.4.2.

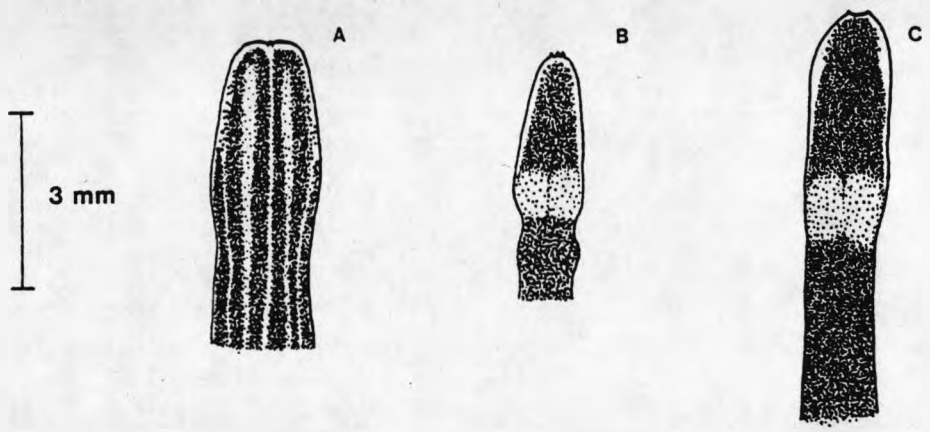


Figure 4.1. Diagram of the heads of (a) *Lineus longissimus*, (b) *Lineus viridis*, (c) *Riseriellus occultus* (new morphotype).

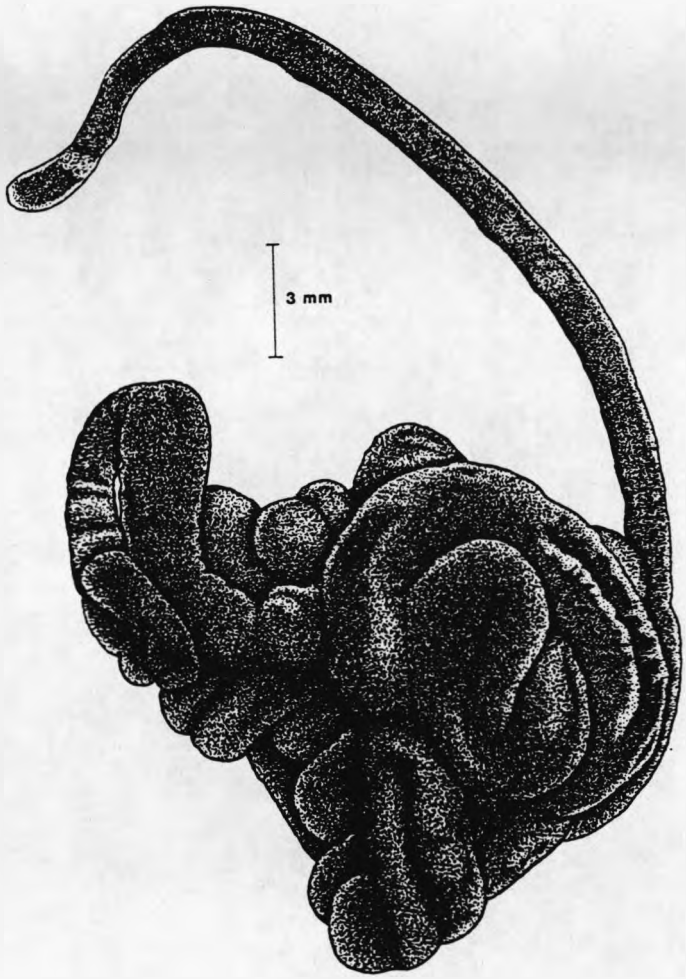


Figure 4.2. Specimen of *Riseriellus occultus* (new morphotype).

4.5 Description of the new taxon.

Genus *Riseriellus* gen. nov.

Type Species: *Riseriellus occultus* sp. nov.

4.5.1 Etymology

The genus is named in honour of Professor Nathan W. Riser as a tribute to his work on the morphology of nemerteans, in particular the Heteronemertea. The specific epithet, the Latin *occultus* (very secret), was chosen to indicate how the new taxon has previously been "hidden" amongst *Lineus* species which externally it resembles.

4.5.2 Diagnosis

Heteronemertea with a single pair of horizontal lateral cephalic slits; proboscis unbranched, containing two (outer circular, inner longitudinal) muscle layers and two muscle crosses; rhynchocoel circular muscles not interwoven with adjacent body wall inner longitudinal muscle fibres; dorsal fibrous core of cerebral ganglia forked only at rear into upper and lower branches; nervous system without neurochordal elements; foregut with neither somatic musculature nor subepithelial gland cell layer; dermis composed of well developed gland cell zone abutting directly against body wall outer longitudinal muscle layer, without distinct connective tissue stratum; body wall musculature without diagonal layer; caudal cirrus absent; intestinal diverticula indistinct; dorsoventral muscles missing from intestinal region; apical organ consisting of three separate sensory pits situated on tip of head; ocelli present, arranged in row on either side of head; blood system comprising single spacious cephalic lacuna, foregut vascular plexus and three longitudinal vessels in intestinal region which are linked by pseudometameric transverse connectives; cephalic glands well developed but confined to dorsal half of head and not posteriorly reaching brain; excretory system situated between brain and mouth, collecting tubules penetrating post-cerebral blood lacunae; sexes separate.

Riseriellus occultus sp. nov.

4.5.3 Type specimens

The holotype, a female consisting of 168 slides of transverse sections, is deposited in the Liverpool Museum, William Brown Street, Liverpool L3 8EN, Registration Number 1992 (LIV); two unsectioned specimens are registered under number 1992 (LIV).

4.5.4 Type locality

Ría de Foz, north-western Spain (43°34'N, 7°14'W), first discovered in September 1984 in seagrass beds (*Zostera noltii* Hornemann) in soft mud, upper shore.

Additional locations: Ría de Foz, in consolidated mud among roots of *Spartina* spp., upper shore, or in muddy sand with *Zostera noltii*; Llandudno, North Wales (53°19'N, 3°49'W), upper shore in damp fine mud or silt under stones and rocks; Rhosneigr, Anglesey (53°15'N, 4°30'W), upper shore in sandy-mud beneath boulders; Trwyn du Point, Anglesey (53°18'N, 4°02'W), upper shore in silty-sand, beneath boulders. In the Foz region recorded densities range from 6-131 worms m^{-2}; dominant associated fauna includes the polychaetes *Capitella capitata* Fabricius, *Streblospio benedicti* Webster, *Pygospio elegans* Claparède, *Heteromastus filiformis* Claparède, *Alkmaria romijni* Horst, *Nereis* (=*Hediste*) *diversicolor* O.F. Müller and *Malacoceros* (=*Scolelepis*) *fuliginosus* Claparède, the molluscs *Hydrobia ulvae* Pennant and *Scrobicularia plana* da Costa, and the crustaceans *Melita palmata* Montagu, *Idotea chelipes* Pallas, *Chaetogammarus marinus* Leach, *Hyale nilssoni* Rathke and *Carcinus maenas* Linnaeus, as well as unidentifiable species of Oligochaeta and Chironomidae. Mean grain sizes of sediments inhabited by the nemerteans are in the range 0.047-0.17 mm, whilst the silt-clay fraction varies from 16.8-73.15% and the organic content from 3.72-9.02%.

4.5.5 Body wall, musculature and parenchyma

The richly glandular epidermis, 25-30 μm in maximum thickness, closely agrees with the generalised heteronemertean form described by Norenburg (1985). Throughout most of the body it is dominated by large numbers of acidophilic rhabditoid glands (Fig. 3) which appear to correspond to the serous cells identified by Norenberg. Rhabditoid density decreases markedly in the anterior ventral cephalic regions, and the cells are completely missing from the epidermis on either side of the median furrow leading to the proboscis pore (Fig. 4); some reduction in their number is also evident in the posterior body regions. Throughout the body the epidermal basement lamina is thin but distinct.

Below the epidermis the subepidermal muscle layers (Figs. 3, 5, 6) are well developed. The outer circular layer, 3-5 μm in maximum thickness, extends to the tip of the head. In contrast, the inner longitudinal fibres, particularly in the anterior ventral cephalic region, are barely distinguishable in front of the proboscis pore, whereas for most of the body length they are 15-30 μm deep (Fig. 5) and proximally abut directly against the well developed dermal gland cell layer.

The dermis is mostly 45-60 μm thick and composed entirely of gland cells (Fig. 5); a laminated connective tissue layer, typical of many heteronemertean taxa, is completely missing. Internally the dermal glands are bordered by the fibres of the main outer longitudinal body wall muscle layer (Fig. 5). Both bacillary and mucus dermal glands (Norenburg, 1985) are abundant, their distal portions in many places extending peripherally to discharge on the body surface. In the cerebral region and further forward the dermal gland cell layer becomes progressively reduced and, in front of the brain, it loses its integrity such that the glands cannot with certainty be distinguished from the cephalic and other subepidermal glands of the head. Behind the mid-foregut region the dermis is only about 20 μm thick and gradually decreases posteriorly.

The main body wall muscles comprise the typical heteronemertean arrangement of outer longitudinal, middle circular and inner longitudinal layers (Fig. 6), respectively 60-135 μm, 20-40 μm and 15-45 μm thick in the foregut region. There is no diagonal

muscle layer. All three muscle layers are considerably reduced in thickness in the posterior portion of the body. The bundles of outer longitudinal muscle fibres are distinguishable from those of the subepidermal longitudinal layer by being enclosed in well developed connective tissue membranes which stain an intense blue colour with Mallory; the subepidermal muscles lack these membranes. Throughout the foregut region large numbers of radially oriented muscle and connective tissue fibrils (Fig. 7) extend peripherally from the wall of the gut, pass through the body wall layers and penetrate the extreme proximal portion of the epidermis. On either side of the mouth these radial muscle strands are reinforced by oblique muscles which have their origin at the outermost surface of the circular muscle coat.

The musculature between the mouth and brain is complex. Close in front of the mouth, where the anterior margins of the buccal chamber bulge forwards, several radial muscle fibrils merge to from thicker transverse bands of horizontal muscles which cross the body below the rhynchocoel. Laterally some of the fibres of these bands extend towards the body margins, passing either above (Fig. 8) or below the lateral nerve cords to terminate among the dermal gland cells. Further forwards, in front of the buccal region, the transverse muscle bundles split up to provide a meshwork of fibres crisscrossing the central part of the body between the lateral nerves and below the rhynchocoel. At this level the main circular muscle layer, which ventrally separates behind the mouth, remains incomplete but laterally gives off isolated fibres which merge with those of the meshwork. Close behind the brain, however, the circular layer for a short distance again forms a complete band of muscle fibres, but then begins to break up as it nears the rear of the cerebral sensory organs, and in the cerebral ring is evident only as isolated fibres with a predominantly circular orientation.

The cephalic musculature consists of a loose meshwork of diagonal and oblique fibres, between which the various glands of the head are distributed (Fig. 9). These muscle fibres originate either in the body wall outer longitudinal layer, some of whose fibres turn obliquely inwards throughout the length of the head, or in the circular muscle layer which

encloses the rhynchodaeum and cephalic blood lacuna. A layer of longitudinal muscle fibres, about 15-20 μm thick, is situated between the blood lacuna and circular muscles, the two muscle coats together forming a muscle cylinder similar to that described for many other heteronemerteans (Figs. 9, 10).

Parenchymatous connective tissues are nowhere extensive; they show their greatest development in the intestinal region adjacent to the blood vessels and between these and the gut wall.

4.5.6 Proboscis apparatus

The ventral, subterminal proboscis pore commences as a longitudinal invagination of the body wall, which internally expands to form an open tubular canal extending back for a short distance before closing off ventrally. For most of the cephalic length the rhynchodaeum appears as a simple tubular duct running below the blood lacuna, lined by a ciliated but non-glandular epithelium 10-12 μm thick, enclosed by longitudinal fibres of the cephalic muscle cylinder (Fig. 10). In the posterior part of the head, however, it begins to expand dorsoventrally, for a while partially protruding into the lumen of the blood channel, until the latter divides posteriorly to form a pair of spacious lacunae located on either side of the rhynchodaeum. The posterior portion of the rhynchodaeum is surrounded by a sphincter-like layer of circular muscles immediately in front of the proboscis insertion; the insertion is located in the brain ring above the ventral cerebral commissure (Fig. 11), muscle fibres leading to it from the body wall outer longitudinal muscle layer passing through a fenestration in the commissure and effectively splitting it into anterior and posterior portions.

The rhynchocoel reaches almost to the posterior tip of the body. Its wall contains separate longitudinal and circular muscle layers (Fig. 12), respectively some 7-8 μm and 15-18 μm in maximum thickness. The circular muscle layer is not interwoven with the fibres of the adjacent body wall inner longitudinal musculature, as in some heteronemertean genera.

The proboscis is unbranched and small; it is less than one-third the length of the body and has a maximum retracted diameter of about 180-200 µm. For most of its length it consists of a glandular epithelium 15-40 µm thick, a delicate nerve layer in which up to 10-12 distinct neural swellings can be distinguished, an outer circular muscle layer 4-6 µm in maximum thickness, an inner longitudinal muscle coat 6-8 µm across and an endothelium 4-5 µm deep (Fig. 13). There are two weakly developed muscle crosses extending from the circular musculature to the endothelial lining. The organisation of the proboscis musculature identifies the nemerteans as members of the family Lineidae, as defined by Gibson (1985). A short anterior region, extending from the proboscis insertion, presents a simpler construction. This region (Fig. 12) comprises an epithelium, 6-10 mm tall, in which there are fewer gland cells than in the main portion of the organ, a longitudinal muscle layer 6-20 µm thick in which two large nerves, 12-15 µm in diameter, can be distinguished, and an endothelium. An unusual feature of the proboscis, not reported for any other nemertean species, is that throughout its length the epithelium is ciliated (Figs. 13, 14); basal bodies of the cilia can just be made out as minute dark spots under oil immersion. Prof. N.W. Riser (pers. comm. Marine Science Institue, Northeastern University, East Point, Nahant, Massachusetts 01908, U.S.A.) reports that in all the lineid species he has examined, the proboscis epithelium contains proboscidial spines (Riser, 1990) but none have been found in the present species.

4.5.7 Blood system

The cephalic blood supply comprises a single spacious thin-walled median lacuna (Fig. 10), extending from near the tip of the head back to just in front of the proboscis insertion. As the rhynchodaeum posteriorly begins to expand dorsoventrally, the cephalic lacuna becomes increasingly constricted mid-dorsally and eventually divides into a pair of spacious lateral channels which flank the posterior portion of the rhynchodaeum. These lacunae become progressively smaller as they approach the brain and enter the cerebral ring. Near the front of the ventral cerebral commissure the lacunae meet ventrally to form a

U-shaped duct (Fig. 15) which continues back beyond the commissure, at one point becoming temporarily subdivided by the ventral muscle fibres leading to the proboscis insertion (Fig. 11). Near the rear of the brain the duct separates medially to form two compressed channels, each of which arches dorsolaterally outwards over, but not bathing, the cerebral sensory organs. The inner ventral portions of these channels again meet medially, so that between the brain and mouth there are essentially three longitudinal blood spaces, the lateral pair on either side of the rhynchocoel being the larger (Fig. 8). Both these and the median ventral channel are irregularly traversed by oblique and radial fibres derived from the median muscle meshwork; all three lacunae sometimes rejoin each other for a short distance, but their subdivision is such that in some sections there may appear to be four or five separate channels. Near the front of the mouth the various blood spaces fuse to form two spacious dorsolateral lacunae which, above the buccal region, begin to split up to form the origin of the well developed foregut vascular plexus (Fig. 16). Throughout the length of the foregut the dorsolateral lacunae flanking the rhynchocoel remain larger than any of the plexal branches.

The mid-dorsal blood vessel arises as a median dorsal branch of the U-shaped duct close to the rear of the ventral cerebral commissure. It immediately penetrates the floor of the rhynchocoel to form the rhynchocoelic villus (Figs. 12, 17), which reaches back for most of the foregut length before emerging to continue to the end of the body.

In the intestinal region there are three spacious longitudinal vessels, irregularly linked by transverse channels arching around the intestinal wall. These vessels possess distinct walls in which occasional muscle fibres can be distinguished.

4.5.8 Nervous system

The brain is well developed; dorsal and ventral lobes are similar in volume but the ventral are more elongate and extend some distance behind the dorsal, ending near the posterior limits of the cerebral sensory organs. Fibrous tissues of the dorsal ganglia are forked only at their rear into upper and lower branches. The dorsal cerebral commissure,

about 15 μm in diameter, is located slightly anterior to the much thicker (90 μm) ventral commissure; an unusual feature of the latter is that it is effectively divided into anterior and posterior portions by muscle fibres leading to the proboscis insertion (Fig. 11). Most of the brain contains no trace of either an inner or outer neurilemma, but delicate connective tissue membranes can be distinguished in the posterior cerebral regions near the sensory organs and origins of the lateral nerve cords; there are no neurochord cells in the ganglionic tissues. The outer surface of the brain lobes is irregularly defined as a consequence of the intermeshed radial and oblique muscle fibres which freely penetrate and pass through the neuroganglionic cerebral components.

The lateral nerve cords lead directly from the rear of the ventral ganglionic lobes; they do not contain neurochords but isolated muscle fibrils (myofibrillae) can be discerned in their fibrous cores (Fig. 7). Throughout their length the lateral nerves possess thin but distinct inner and outer connective tissue neurilemmae.

The peripheral nervous system is well developed and generally similar to that of many other heteronemertean taxa. Large numbers of cephalic nerves lead forwards into the head and can be followed for some distance between the fibres of the cephalic muscle meshwork (Fig. 9). The neural layer extending around the outer surface of the body wall circular muscle coat (Fig. 6) contains a large mid-dorsal nerve, originating from the rear of the dorsal cerebral commissure. From the inner margin of each ventral ganglionic lobe a distinct nerve separates off, runs back some distance among the neuroganglionic tissues and then emerges to continue between the fibres of the median muscle meshwork. Behind the brain the two nerves, which lead to the foregut, are linked by several slender transverse connectives before they move apart to pass on either side of the mouth just below the 'salivary glands' which surround the oral aperture (Fig. 18). Behind the mouth the two nerves begin to branch profusely, forming a nerve network adjacent to the foregut epithelium; there is no post-oral commissure connecting the foregut nerves and the ultimate fate of the network could not be traced.

4.5.9 Alimentary canal

The long, slit-like ventral mouth commences about 1 mm beyond the posterior limit of the cephalic furrows, and is situated some distance behind the brain. It opens into a buccal region whose epithelium is surrounded by large numbers of subepithelial 'salivary glands' of several types (Figs 18, 19); below the ventrolateral buccal borders these glands may extend 90 µm or more below the epithelium. The anterior portion of the buccal region, possibly as a consequence of contraction, bulges forwards, in sections appearing as a pair of large pouches reaching below the post-cerebral blood lacunae. A similar arrangement has been described for several other heteronemertean taxa.

The main foregut (Figs. 6, 16) is lined by a richly glandular, ciliated and folded epithelium, up to 100 µm or more in maximum height. There is no subepithelial gland cell layer, nor somatic musculature, associated with the main foregut region.

The intestine is typically heteronemertean in appearance, its gastrodermal lining being up to 75-80 µm or more thick and containing enormous numbers of acidophilic glands. The lateral margins of the intestine are pouched but do not, as in many taxa, form distinct diverticula. Dorsoventral muscle bundles, typical of the intestinal region in many nemerteans, are completely missing in the present species. The anus opens at the posterior tip of the body.

4.5.10 Sensory organs

Three to eleven black ocelli are arranged in a dorsolateral row on either side of the anterior part of the head. Individual eyes are 30-35 µm in diameter and possess a simple pigment cup construction (Fig. 20). In sections the pigment shows as dense accumulations of minute dark brown granules.

The shallow lateral cephalic furrows extend from the tip of the head back to the rear cerebral region. As they pass the brain they begin to expand internally to form longitudinal chambers whose epithelium lacks gland cells but is densely ciliated. At about the level where upper and lower branches of the dorsal brain lobes separate the inner wall of the

chambers develops longer cilia (20-25 μm) and is associated with a zone of neuroganglionic tissue 25 μm or more deep. The ciliated cerebral canals lead obliquely inwards from this point, extending towards the upper branches of the dorsal cerebral lobes. As the canals reach the brain they meet further neuroganglionic masses and ventral accumulations of glandular tissue. Each canal, about 20 μm in diameter, then turns posteriorly to run along the outer dorsolateral border of the appropriate cerebral sensory organ (Fig. 21). The organs are intimately attached to the brain tissues and have a typically heteronemertean construction. Major and minor canals can clearly be distinguished in the posterior portions of each cerebral canal, just before they turn inwards to end amongst an accumulation of vacuolate glands. Each cerebral organ has a maximum diameter of about 120-150 μm.

4.5.11 Apical organ and cephalic glands

The apical sensory organ consists of three separate ciliated chambers, each about 45 μm in diameter, opening ventrally near the tip of the head (Fig. 4). The median chamber is in front of the ventrolateral pair and opens at the front of the proboscis pore furrow, the other two open on the ventrolateral cephalic surface slightly further back. The epithelium lining the chambers is 5-6 μm thick, densely ciliated and completely lacks gland cells.

Typical basiphilic cephalic gland lobules are well developed and extensive, but are confined to the dorsal half of the head (Fig. 9) and commence behind the level of the proboscis pore. They form a more or less well defined mass of glands interspersed between the fibres of the cephalic muscle meshwork. Posteriorly the glands do not quite reach the anterior borders of the brain.

4.5.12 Excretory system

The thick walled excretory tubules are located between the brain and mouth regions. They are 20-25 μm in diameter and mostly run immediately adjacent to the dorsal walls of the post-cerebral lateral blood lacunae. Up to two or three tubules can be distinguished on

either side of the body. The posterior portions of the collecting tubules usually penetrate and may extend freely into the lumen of the blood lacunae (Figs. 8, 22). A single efferent canal leads from the anterior region of the excretory system on either side of the body to open via a dorsolateral nephridiopore.

4.5.13 Reproductive system

The sexes are separate. Gonads are distributed in dorsolateral rows throughout most of the intestinal region of the body, but are missing from the extreme anterior portion. Testes of specimens collected during late September possess open gonoducts on their dorsolateral body surface. Each testis appears as a somewhat bilaterally compressed pouch lined by an undifferentiated epithelium up to 15 mm thick; in the lumen of many testes small numbers of spermatozoa can be distinguished and it appears that the animals had discharged their gonadal contents shortly before they were found.

Ovaries appear as large pouches containing masses of eggs, most of which are in a similar stage of development. More than 20 such eggs can be counted in any one ovary, and smaller numbers of immature ova can also be found attached to the ovarian wall. Larger eggs, 100 µm or more in diameter contain a nucleus about 40 µm across in which a single nucleolus 10-12 µm wide is distinguishable. Females collected with mature males also possess open gonoducts, and in the laboratory one individual released eggs. The discharge of eggs is very similar to that described for *Lineus lacteus* Rathke 1843 by Gontcharoff & Lechenault (1958); strings of eggs are released from the gonopores but quickly break up so that individual eggs are free. This differs strikingly from the egg-laying shown by *Lineus ruber* and *Lineus viridis*, both of which lay their eggs in mucous sheaths attached to rocks or algal surfaces.

4.5.14 Parasites

Several of the specimens examined contain parasites of various types. In the intestine gregariniform protozoans 45 μm or more long, containing a small nucleus in which two nucleoli are distinguishable, are often partially embedded in the gastrodermal wall. Other, possibly sporozoan, parasites are irregularly distributed in a variety of body tissues, including the brain, muscle layers, and cephalic glands; these parasites are oval in shape 30 μm long by 20 μm wide, and filled with a finely particulate cytoplasm in which a distinctly eccentric acidophilic nucleus 5 μm wide is visible (Figs. 23, 24). The parasites appear to be encapsulated in a thin but distinct membrane. A third type of parasite was found in smaller numbers in the epidermis and dermis; this appears as a large (60 μm or more diameter) more or less spherical cavity containing a membrane-bound amorphous mass of tissue in which no particular structure can be discerned. The appearance of these parasites bears some resemblance to the morula-like masses produced by the plasmodial generation of orthonectids, which are known to occur in a number of different invertebrates including nemerteans (Stunkard, 1982; Vernet, 1990).

4.5.15 Ecology

In the Foz estuary the nemerteans are found in compacted but fairly wet muddy sediments in the upper shore zone, especially among the roots of *Spartina* or among *Zostera* beds, whereas at the same locality *Lineus longissimus* occurs at the mouth of the estuary under stones at low tide level. At Llandudno and on Anglesey *Lineus viridis* and the new species do not exhibit a similar spatial separation, both being found on the upper shore in silty mud beneath boulders.

Figures 3-8. *Riseriellus occultus* gen. et sp. nov.

3. Transverse section between the brain and mouth to show the epidermal rhabditoid glands and body wall layers. Scale bar = 250 μm.

4. Oblique transverse section near the tip of the head, showing two of the apical organ chambers (arrowheads) and absence of epidermal rhabditoid glands from the ventral surface. The ventral median furrow leads back to the proboscis pore. Scale bar = 200 μm.

5. Part of the body wall in transverse section to show the structure of the epidermis, subepidermal muscle layers, dermis and outer portion of the main body wall outer longitudinal muscle layer. Scale bar = 100 μm.

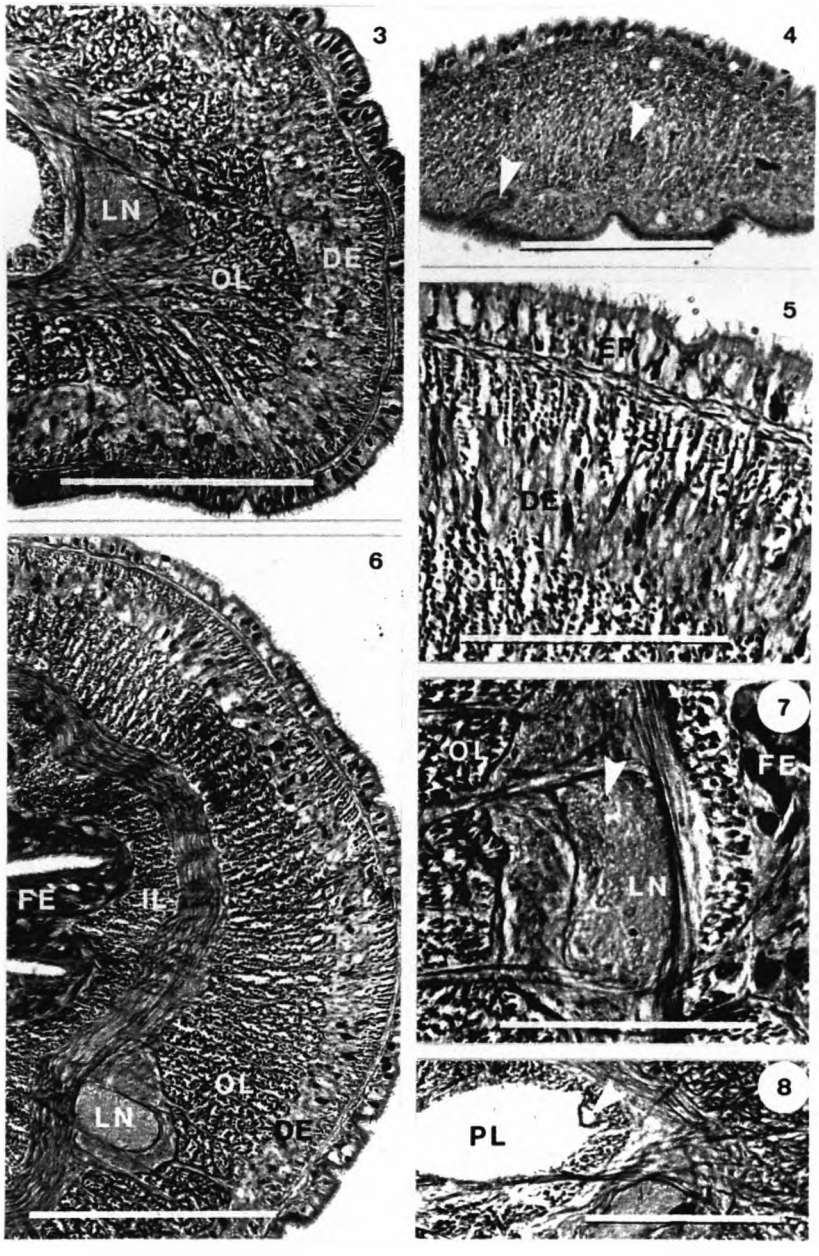
6. Transverse section through the foregut region to show the organisation of the body wall musculature. Scale bar = 250 μm.

7. Transverse section through a lateral nerve cord in the foregut region, showing some of the radial muscle and connective tissue fibres which extend between the foregut wall and proximal portion of the epidermis. A myofibrillar (neuromuscular) strand in the fibrous tissue of the lateral nerve is indicated by an arrowhead. Scale bar = 100 μm.

8. Transverse section through one of the post-cerebral lateral lacunae to show some of the horizontal transverse muscle bands extending peripherally above a lateral nerve cord. The arrowhead points to an excretory tubule running in the lumen of the blood lacuna. Scale bar = 200 μm.

Key to legends for Figures 3-8.

DE = dermis; **EP** = epidermis; **FE** = foregut epithelium; **IL** = body wall inner longitudinal muscle layer; **LN** = lateral nerve cord; **OL** = body wall outer longitudinal muscle layer; **PL** = post-cerebral blood lacuna; **SL** = subepidermal longitudinal muscle layer. All photomicrographs of sections stained with Mallory.


3
LN
OL
DE
4
5
EP
DE
6
FE
IL
OL
LN
DE
7
OL
FE
LN
8
PL

Figures 9 - 15. *Riseriellus occultus* gen. et sp. nov.

9. Transverse section through the cephalic region to show the basiphilic cephalic gland lobules and fibres of the central muscle cylinder. Scale bar = 250 μm.

10. Transverse section through the middle cephalic region to show the single spacious blood lacuna and the rhynchodaeum. Scale bar = 200 μm.

11. Transverse section through the brain region above the ventral cerebral commissure to show fibres leading to the proboscis insertion, indicated by an arrowhead, passing through the commissure. Scale bar = 200 μm.

12. Transverse section to show the construction of the anterior portion of the proboscis, the two distinct muscle layers in the rhynchocoel wall and the rhynchocoelic villus. Arrowheads indicate the two large proboscis nerves. Scale bars = 100 μm.

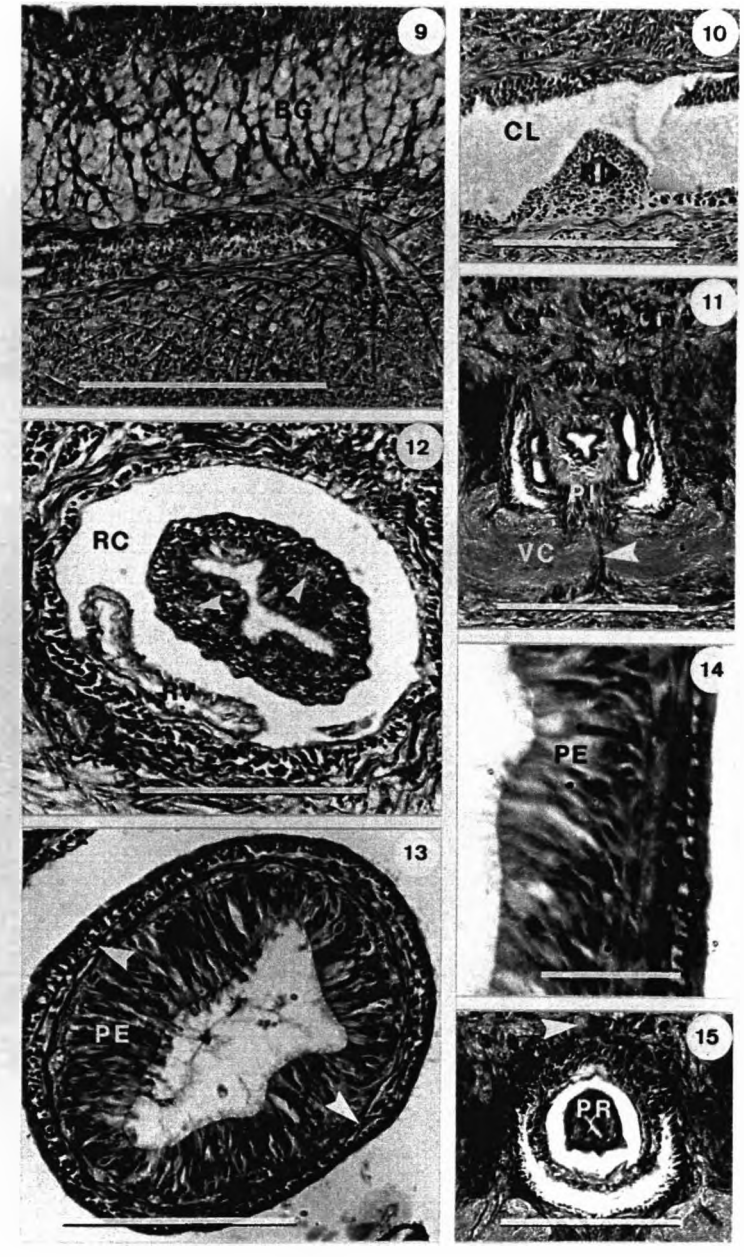
13. Transverse section to show the construction of the main portion of the proboscis. The position of the two muscle crosses are indicated by arrowheads. Scale bar = 100 μm.

14. An oil immersion photomicrograph through part of the proboscis in longitudinal section to show the ciliation of its epithelium. Scale bar = 25 μm.

15. Transverse section through the cerebral region to show the U-shaped blood channel arching below the rhynchocoel. The median dorsal nerve extending back from the dorsal cerebral commissure is indicated by an arrowhead. Scale bar = 200 μm.

Key to legends for Figures 9-15.

BG = basiphilic cephalic gland lobules; **CL** = cephalic lacuna; **PE** = proboscis epithelium; **PI** = proboscis insertion; **PR** = proboscis; **RC** = rhynchocoel; **RD** = rhynchodaeum; **RV** = rhynchocoelic villus; **VC** = ventral cerebral commissure. All photomicrographs of sections stained with Mallory.


9
BG
10
CL
11
PI
VC
12
RC
13
PE
14
PE
15
PR

Figures 16 - 24. *Riseriellus occultus* gen. et sp. nov.

16. Part of the foregut region in transverse section to show branches of the vascular plexus. Scale bar = 250 µm.

17. Transverse section through the posterior region of the rhynchocoelic villus. Scale bar = 50 µm.

18. Transverse section through the mouth region to show the 'salivary glands' situated below the buccal epithelium. Scale bar = 250 µm.

19. Enlargement of the 'salivary glands' to show the different types of constituent cells. Scale bar = 100 µm.

20. Part of the head in transverse section to show one of the pigment cup ocelli. Scale bar = 100 µm.

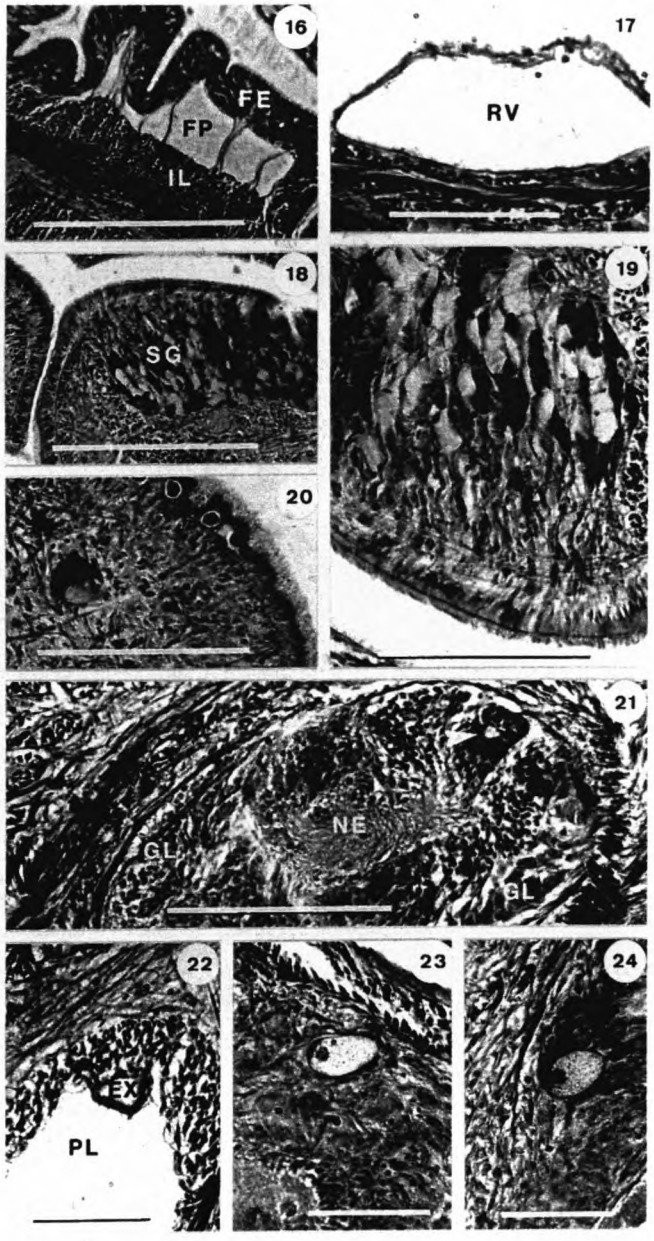
21. Transverse section through the middle portion of a cerebral sensory organ. The ciliated cerebral canal, in which major and minor canals can just be made out, is indicated by an arrowhead. Scale bar = 100 µm.

22. Transverse section through one of the post-cerebral blood lacunae to show an excretory tubule running in its lumen. Scale bar = 50 µm.

23, 24. Transverse sections through parts of the cerebral ganglia to show two of the parasites, possibly sporozoan, described in the text. Scale bars = 50 µm.

Key to legends for Figures 16 - 24.

EX = excretory tubule; **FE** = foregut epithelium; **FP** = branch of foregut vascular plexus; **GL** = glandular components of cerebral sensory organ; **IL** = body wall inner longitudinal muscle layer; **NE** = neural component of cerebral sensory organ; **PL** = post-cerebral blood lacuna; **RV** = rhynchocoelic villus; **SG** = 'salivary glands'. All photomicrographs of sections stained with Mallory.


16
FE
FP
IL
17
RV
18
SG
19
20
21
NE
GL
GL
22
EX
PL
23
24

4.5.16 Systematic Discussion

Gibson (1985), in emphasising the need for complete descriptions of nemerteans, discussed the anatomy of the heteronemertean proboscis and proposed that all those genera with outer circular and inner longitudinal muscle layers should be grouped in the family Lineidae. Like many other authors (e.g. Wijnhoff, 1914), however, Gibson overlooked the significance of the nervous system in the determination of proboscis muscle layer homologies. Norenburg (1993) elegantly demonstrates that the evolution of the proboscis among heteronemerteans is more complex than previously appreciated and both discusses and illustrates several different proboscis morphotypes. His "palaeotype", in which the two muscle layers (outer circular, inner longitudinal) are both subneural, is characteristic of the Lineidae as defined by Gibson, although Norenburg states that it seems more appropriate to consider the morphologically diverse lineids as a suborder, the Lineiformes. The proboscis of the new species possesses a "palaeotype" organisation, and on this basis the species is placed in the Lineidae sensu Gibson (1985) (Lineiformes sensu Norenburg [1993]).

Some 29 heteronemertean genera are known or suspected to possess a "palaeotype" proboscis (Gibson, 1985, 1990b,c; Gibson & Qi Sang, 1991; Iwata, 1993; Riser, 1993), although in a few (e.g., *Tenuilineus* Riser 1993) the proboscis is regarded as transitional to the heterotype because it possesses a short additional outer longitudinal muscle layer just behind the proboscis insertion. Commonly, several morphological features have been used as a means of distinguishing between the numerous heteronemertean genera and these are summarised for the "palaeotype" group in Table 4.4.

The present specimens have a foregut vascular plexus and proboscis containing two muscle crosses, their rhynchocoel circular muscles are quite separate from the adjacent body wall inner longitudinal muscle fibres, they have no dermal connective tissue layer, neurochords or caudal cirrus, and their foregut possesses neither somatic muscles nor subepithelial glands. Two further features of the present taxon have not been described for any other nemertean species; these are the ciliated nature of the proboscis epithelium, and the manner whereby the ventral cerebral commissure is divided by muscle fibres leading to

the proboscis insertion. It is concluded that the combination of morphological characters shown by the nemerteans is such that they cannot be placed in any of the known heteronemertean genera with a "palaeotype" proboscis and the new genus *Riseriellus* is accordingly established for them. The morphological evidence used to establish the new genus and species is supported by the electrophoretic evidence presented.

Table 4.4 Some of the morphological features commonly used for distinguishing between heteronemertean genera, summarised for those forms that are known to, or probably, possess a "palaeotype" proboscis; data from Gibson (1990c), Gibson & Qi Sang (1991), Iwata (1993) and Riser (1993).

Genus	1	2	3	4	5	6	7	8
Amiclineus	0	0	0	c	+	0	0	+a
Antarctolineus	2	0	0	l	+	?	+	+
Apatronemertes	2	0	0	l	0	0	0	+a
Australineus	2	+	0	l	0	0	0	+
Corsoua	2	+	0	c	+	0	+	0
Craticulineus	0	+	+	c	0	0	0	0
Eousia	2	0	0	l	+	+	+	+
Euborlasia	1-2	+	0	c	0	0	+?	+
Flaminga	1	0	0	c?	+	+	0	0
Hinumanemertes	2	0	0	c+l?	0	+	0	+
Kirsteueria	1	0	0	l	0	0	0	+
Lineopsella	0-2	0	0	c	0?	0	0?	+
Lineopselloides	0	0	0	c	0	0	+	+
Lineus	0-2	0-+	0	c/l	0	0	+	0-+
Micrella	2	0	0	?	0	+	0	+
Micrellides	2	0	0	c	0	?	0	+
Micrura	0-2	0-+	0	0/c/l	0	0-+	0	+
Micrurimorpha	2?	0	0	?	0	0	0?	+
Micrurinella	2	0	+	?	0?	?	0?	?
Neolineus	0	0	0	?	0	0	+	+
Paralineopsis	0	0	0	0	0	0	+	0
Paralineus	2	0	0	?	0	0	0	0
Paramicrurinella	0	0	+	c	0	0	0	0
Planolineus	1	0	0	?	0?	?	?	?
Pontolineus	2	0	0	?	+	0?	0	0
Pussylineus	2	0	0	?	+	0	0?	?
Siolineus	2	0	0	l	0	0	0?	+
Tenuilineus	?	0	0	0?	0	0	+	+
Utolineus	0	0	0	c	+	0	+	+
Riseriellus gen.nov.	2	0	0	0	0	0	0	+

Key to Table 4.4

1 = Number of muscle crosses in proboscis.

2 = Dermis with (+) or without (0) distinct connective tissue layer between gland cells and body wall outer longitudinal musculature.

3 = Circular muscles of rhynchocoel wall interwoven between fibres of adjacent body wall inner longitudinal musculature (+) or quite separate (0).

4 = Foregut somatic musculature absent (0) circular (c) or longitudinal (l).

5 = Nervous system with (+) or without (0) subepithelial gland cell layer.

6 = Caudal cirrus present (+) or absent (0).

7 = Foregut with (+) or without (0) subepithelial gland cell layer.

8 = Blood system in foregut region developed into plexus (+) or consisting of only two vessels running in parenchyma (0).

a The foregut vascular plexus in *Amniclineus* (Gibson & Qi Sang, 1991) and *Apatronemertes* (Wilfert & Gibson, 1974) is intimately associated with components of the excretory system, the unusual arrangement in these taxa being interpreted as a consequence of their freshwater habits.

Chapter 5

Genetic Evidence for Cryptic Speciation Within the Heteronemerteans *Lineus ruber* and *Lineus viridis*.

5.1 Introduction

The large number of inadequately described nemertean taxa means that a sound diagnosis of many species, genera and even families remains impossible. Even where the external and internal morphology of species has been described in detail, the systematic value of many characters is not known (Gibson, 1985; Gibson & Crandall, 1989; Chapters 1, 3 this thesis).

The nemertean order Heteronemertea typifies these problems at several taxonomic levels. The order contains over 45 genera, of which over 32 were included in a single family, the Lineidae, because they possessed lateral cephalic slits (Gibson, 1985). The family Lineidae was clearly an artificial grouping. Gibson (1985) attempted to reorganise the Heteronemertea on the basis of a standardised description of the internal morphology of its members into seven new families, but he still retained the 'old' heteronemertean genera. Three of these genera, *Micrura* Ehrenberg 1831, *Cerebratulus* Renier 1804 and *Lineus* Sowerby 1806 contain over 200 species and all three lack a sound diagnosis (Punnett, 1901; Friedrich, 1960; Cantell, 1975; Gibson, 1982a, 1985). Systematic confusion in the Heteronemertea extends right down to the level of species many of which lack complete (if any) descriptions and for which often no type material has been deposited.

The genus *Lineus* contains two such species, *Lineus ruber* and *Lineus viridis*, which are very common on the shores of Britain, northern Europe and North America (see Chapter 3). These two species resemble each other in body size and shape but may be distinguished (not with 100% accuracy) by the external colouration of the body. *Lineus viridis* is various shades of green and *Lineus ruber* is red to red-brown (Gibson, 1982b). These species were synonymised for many years as red and green varieties of *Lineus ruber* (or *Lineus gesserensis*) but differences in larval development (Schmidt, 1946; Gontcharoff, 1951) led to the final separation of the two species by Gontcharoff (1951).

As *Lineus ruber* and *Lineus viridis* are very common intertidal animals and easy to keep alive in captivity, they have been used in numerous physiological, structural and ecological studies on nemerteans. These include studies on; osmoregulation (Lechenault, 1965); neurosecretion (Bierne, 1964, 1966; Willmer, 1970); respiratory and other pigments (Vernet, 1966); digestion (Jennings, 1962; Jennings & Gibson, 1969); uptake of organic compounds through the body surface (Fisher & Cramer, 1967; Fisher & Oaks, 1978); reproduction and development (Barrois, 1877; Hubrecht, 1886; Arnold, 1898; Nusbaum & Oxner, 1913; Schmidt, 1934; Gontcharoff, 1951, 1960; Hyman, 1951; Bierne, 1970a, b, c, 1983; Riser, 1974; Franzén, 1983); regeneration (Nusbaum & Oxner, 1910, 1911a,b, 1912; Bierne, 1962, 1979); the structure of the epidermis (Gontcharoff & Lechenault, 1966; Norenburg, 1985); rhynchocoel and proboscis (Gontcharoff, 1957; Bierne, 1962; Ling, 1971); ocelli (Vernet, 1970, 1992); cerebral organs (Ling, 1969a, b, 1970). *Lineus ruber* was even used to provide support for a theory of vertebrate evolution from nemerteans (Willmer, 1970).

Friedrich (1935) described a new species, *Heterolineus pseudoruber*, which was identical to *Lineus ruber* (which then also incorporated *Lineus viridis*) in appearance (with both green and red forms) and in distribution. The species was separated from *Lineus ruber* on the basis of differences in internal morphology, the new taxon having blood spaces above the gut and dorso-ventral musculature throughout the front part of the body. Friedrich's (1935) description of a cryptic species of *Lineus ruber* has largely fallen into obscurity since the species has never been subsequently recorded and because he erected the genus *Heterolineus* by using the type species from another genus (*Lineus longissimus*) contrary to the rules of the International Commission of Zoological Nomenclature.

With a single exception (Brunberg, 1964), investigations after 1951 (when Gontcharoff assigned specific status to *Lineus viridis*) did not consider the possibility that *Lineus ruber* may include more than one species.

During electrophoresis of specimens of *Lineus ruber* and *Lineus viridis* collected

around Britain and France a small number of individuals from almost all populations sampled gave genetically aberrant results. These individuals appeared identical in external appearance to both *Lineus ruber* and *Lineus viridis* , but they shared very few alleles with these two species and at several monomorphic loci showed fixed differences in enzyme mobility. Subsequent calculation of Nei's (1972) genetic identity between sympatric populations of *Lineus ruber*, *Lineus viridis* and the aberrant type indicated that it was highly likely the latter represented a new cryptic species.

5.2 Methods and Materials

5.2.1 Sample Sites

The number of individuals of each species examined in this investigation from each site are shown in Table 5.1. Details of sampling sites are provided in Section 2.1.

Table 5.1 Numbers of *Lineus ruber*, *Lineus viridis* and the genetically aberrant individuals found at sites in Britain and France. Genetically aberrant specimens were not found among samples from the U.S.A.

Site		Species		
		L.ruber	*L.viridis*	Species ?
United Kingdom				
Oban	(OBN)	90	48	9
Llandudno	(LDN)	55	59	5
Anglesey	(ANG)	88	30	19
Bay Ny Carrickey	(PVH)	-	29	11
Castletown	(CST)	38	-	-
St. Agnes	(STA)	-	11	82
Plymouth Sound	(PMS)	-	20	5
Wembury	(WMY)	-	27	19
France				
Pointe de Barfleur	(FRA)	7	7	3

5.2.2 Starch Gel Electrophoresis

Specimens were described numbered and electrophoresed as detailed in Sections 2.2, 2.3 and 2.4. Data collected from electrophoresis were converted to actual numbers of genotypes occurring in each population for each species and then analysed using the FORTRAN programme Biosys-1 (Swofford and Selander, 1989).

5.2.3 Data Analysis

Mean observed and expected heterozygosity was determined for each population of each species. The Fisher (1935) Exact test for deviations from genotype frequencies expected under Hardy-Weinberg equilibrium was carried out for each population. For three sites where the three species were sympatric (Oban, Llandudno and Anglesey) F_{IS}, F_{IT}, F_{ST} (Wright, 1978) and the number of migrants per generation between populations (mNe) were determined. This was to estimate gene flow between the aberrant type and *Lineus ruber* or *Lineus viridis*. If gene flow occurred between sympatric populations of the aberrant type and either of the two other species - then it would be likely that the aberrant type was not a separate biological species. Nei's (1972) genetic identity (I) and genetic distance (D) were calculated between all populations of each species or type. Cluster analysis was then carried out using UPGMA. BIOSYS-1 (Swofford and Selander, 1989) was used to perform all calculations. For details of data analyses see Section 3.2.3.

5.3 Results

5.3.1 A comparison in external features between *Lineus ruber*, *Lineus viridis* and the genetically aberrant form.

Accounts of the external features of *Lineus ruber*, *Lineus viridis* and the genetically aberrant form are given below. Descriptions are based on all specimens obtained from all the sites investigated between 1989 - 1992.

Lineus ruber Müller 1774

Living specimens vary in length from 28 - 88 mm, in width from 0.4 - 1.5 mm, and exhibit a very variable body shape. Behind the cephalic region the body may be more or less uniform for 16 - 90% of its length, then it gradually narrows posteriorly to end in a finely pointed or blunt tail. Alternatively the body width may increase behind the head

for 6 - 31% of the body length and then remain uniformly wide for up to about 93% of its length before gradually narrowing to the tail. The body width may also increase behind the head for 19 - 58% of the body length and then gradually narrow to the tail. The body is compressed dorsoventrally becoming progressively more flattened from the anterior to the posterior end.

The dorsoventrally flattened head is generally spatulate. There is often a slight constriction between the head and the remainder of the body. Alternatively the constriction may be absent and the head continuous with the rest of the body or the body may be wider than the head. The cephalic colour is variable, ranging through pale red brown, red brown, dark red brown, dark red, dark brown, brown, blackish brown, blackish grey to black. The ventral surface may be the same colour as the dorsal surface or it may be paler ranging through pale red, red, pale red brown, red brown, pink, dull red, red-orange, pale orange to orange-brown. The cephalic ganglia may be visible in the posterior part of the head as a pair of more or less distinct patches which may be pink, red, pink-red, orange-red, grey-brown, brown or dark brown. The margins of the head which bear a single pair of lateral horizontal cephalic furrows, are pale. Zero to seven black ocelli form an irregular and often asymmetrical dorsolateral row along either side of the anterior head region. The ocelli may be obscured by pigmentation, especially in darker coloured individuals. The small, mid-ventral, slit-like mouth commences just in front of, at the same level as or close behind the posterior end of the cephalic furrows. The oral margins are generally pale and may appear swollen.

General body colour is very variable. The dorsal surface may be red, pink-red, dark red, dark red brown, red brown, orange, maroon, dark brown, blackish brown, blackish grey, dark grey brown or black. Specimens examined from the U.S.A. in particular were often very dark in colour. The colour intensity often shades from darker anteriorly to paler posteriorly. Occasionally colour intensity may shade from pale to dark to pale along the body length. Pale annulations may or may not be distinguished along the body length and generally these are not as distinct as in *Lineus viridis*. A pale mid-dorsal

and mid-ventral line may be present. In sexually mature specimens the gonopores show as an irregular row of pale spots in a dorsolateral position on each side of the body, commencing some distance behind the rear of the head. Just behind the head nephridiopores may be visible as a few pale spots in a dorsolateral position on each side of the body. A pale yellowish lateral line may be visible on each side of the body commencing behind the head and running along the entire length of the body. The ventral body surface is usually paler than the dorsal but equally variable, ranging through dark brown, black, dark green (single specimen), dark golden brown, dark yellowish brown, dark chocolate brown, blackish grey, dark grey, dark red, red, reddish pink, dark dull orange, orange, pale orange, buff, pale yellow or white. As with the dorsal surface, colour intensity may shade from darker anteriorly to paler posteriorly. When touched the worms contract without coiling. A photograph of a live specimen, with tail removed for electrophoresis is shown in Figure 5.1.

Lineus viridis Müller 1774

Living specimens vary in length from 33-134 mm, in width from 0.4-1.8 mm and exhibit a very variable body shape. Behind the cephalic region the body may be more or less uniform in width for 15-61% of the body length, then it gradually narrows posteriorly to end in a finely pointed or blunt tail. Alternatively the body width may increase behind the head for 5-37% of the body length and then remain uniform for up to about 66% of its length before gradually narrowing to the tail. The body width may also increase behind the head for 17-44% of the body length and then gradually narrow to the tail. The body is compressed dorsoventrally becoming progressively more flattened from the anterior to the posterior end.

The dorsoventrally flattened head is generally spatulate, although its posterior portion is often markedly wider than the anterior to give it a spade-like shape. There is usually a shallow but distinct constriction between the head and the remainder of the body. The cephalic colour is variable, ranging through pale green, green, dark green, leaf

green, deep bronze green, pale olive green, olive green, dark olive green, brown, olive brown, dark brown, dark grey, blackish grey to black. The ventral surface of the cephalic region is the same colour as the dorsal surface or, more usually, paler. The cerebral ganglia may be visible in the posterior part of the dorsal surface of the head, as a pair of more or less distinct patches which may be pale green, pale red or red. The margins of the head, which bear a single pair of lateral horizontal furrows are pale. Zero to nine black ocelli form an irregular and often asymmetrical dorsolateral row along either side of the anterior head region. The ocelli may be obscured by pigmentation especially in darker coloured individuals. The small, mid-ventral and slit-like mouth commences just in front of, at the same level as, or close behind the posterior end of the cephalic furrows. the oral margins are often pale and may appear swollen.

General body colour is very variable. The dorsal surface may be pale olive green, olive green, dark olive green, emerald green, dark emerald green, dark green, deep bronze green, brownish green, chestnut brown, dark chocolate brown, dark grey-brown, dark grey, blackish grey or black. Colour intensity often shades from darker anteriorly to paler posteriorly. Occasionally colour intensity may be more variable along the body length. Pale annulations are normally distinguishable along the full length of the body. A pale mid-dorsal line and a pale lateral line on each side of the body may or may not be visible. In sexually mature specimens the gonopores show as an irregular row of pale spots on each side of the body, commencing some distance behind the head. Just behind the head nephridiopores may be visible as a few pale spots in a dorsolateral position on each side of the body. The ventral body surface is usually paler than the dorsal but equally variable, ranging through deep bronze green, dark emerald green, emerald green, olive green, pale olive green, mint green, whitish green, pale brown and tan. When touched the worms contract without coiling. A photograph of a live specimen with tail removed for electrophoresis is shown in Figure 5.3.

Genetically aberrant form.

Living specimens vary in length from 34-180 mm, in width from 0.5-1.5 mm and exhibit a very variable body shape. Behind the cephalic region the body may be more or less uniform in width for 25-48% of the body length, then it gradually narrows posteriorly to end in a finely pointed or blunt tail. Alternatively the body width may increase behind the head for 6-37% of the body length and then remain uniform in width for up to about 72% of its length before gradually narrowing to the tail. The body width may also increase behind the head for 31-50% of the body length and then gradually narrow to the tail. The body is compressed dorsoventrally becoming progressively more flattened from the anterior to the posterior end.

The dorsoventrally flattened head is generally spatulate. There may be a more or less distinct constriction between the head and the body and the head may appear narrow. Alternatively the constriction may be absent and the head continuous with the rest of the body or the body may appear wider than the head. The cephalic colour is variable, ranging through pale red, red, deep red, red-brown, brown, greenish brown, dark brownish grey, dark grey, dark green, deep bronze green, blackish green or blackish grey. The ventral surface may be the same colour as, or more usually paler than, the dorsal surface. The cerebral ganglia may be visible in the posterior part of the head, as a pair of more or less distinct patches which may be pale red, red, dark red or pinkish red. The margins of the head, which bear a single pair of lateral horizontal furrows are pale. One to six black ocelli form an irregular and often asymmetrical dorsolateral row along either side of the anterior head region. The ocelli may be obscured by pigmentation, especially in darker coloured individuals. The small mid-ventral and slit-like mouth commences just in front of, at the same level as, or close behind the posterior end of the cephalic furrows. The oral margins of the mouth may be pale and may appear swollen.

General body colour is very variable. The dorsal surface may be red, red-brown, dark red, brown, dirty brown, golden brown, olive brownish green, dark green, deep bronze green, dark grey or blackish grey. Colour intensity often shades from darker

anteriorly to paler posteriorly. Occasionally colour and intensity may be more variable along the body length. Pale annulations may be distinguishable along the full length of the body. A pale lateral line may be visible on each side of the body. In sexually mature specimens the gonopores show as an irregular row of pale spots on each side of the body commencing some distance behind the head. The ventral body surface is usually paler than the dorsal surface but is as equally variable, ranging through red, dull orange, orange-brown, dull buff, greenish orange, brown, dark brown and olive green. When touched the worms contract without coiling. Photographs of red-brown and greenish specimens of the aberrant form are shown in Figures 5.2 and 5.4.

Specimens of *Lineus ruber* and *Lineus viridis* are generally of similar shape but they can often be separated by their external colouration, though note that they can both appear very similar, especially in the case of dark individuals. *Lineus viridis* can grow to a larger size than *Lineus ruber*. The genetically aberrant form can appear almost identical to *Lineus ruber* or *Lineus viridis*, though occasional specimens are larger than either of the two former species. This is demonstrated by Figures 5.1 - 5.4.

Figure 5.1. Dorsal veiw of *Lineus ruber*.

Figure 5.2 'Red' form of genetically aberrant form.

Figure 5.3 Dorsal view of *Lineus viridis*.

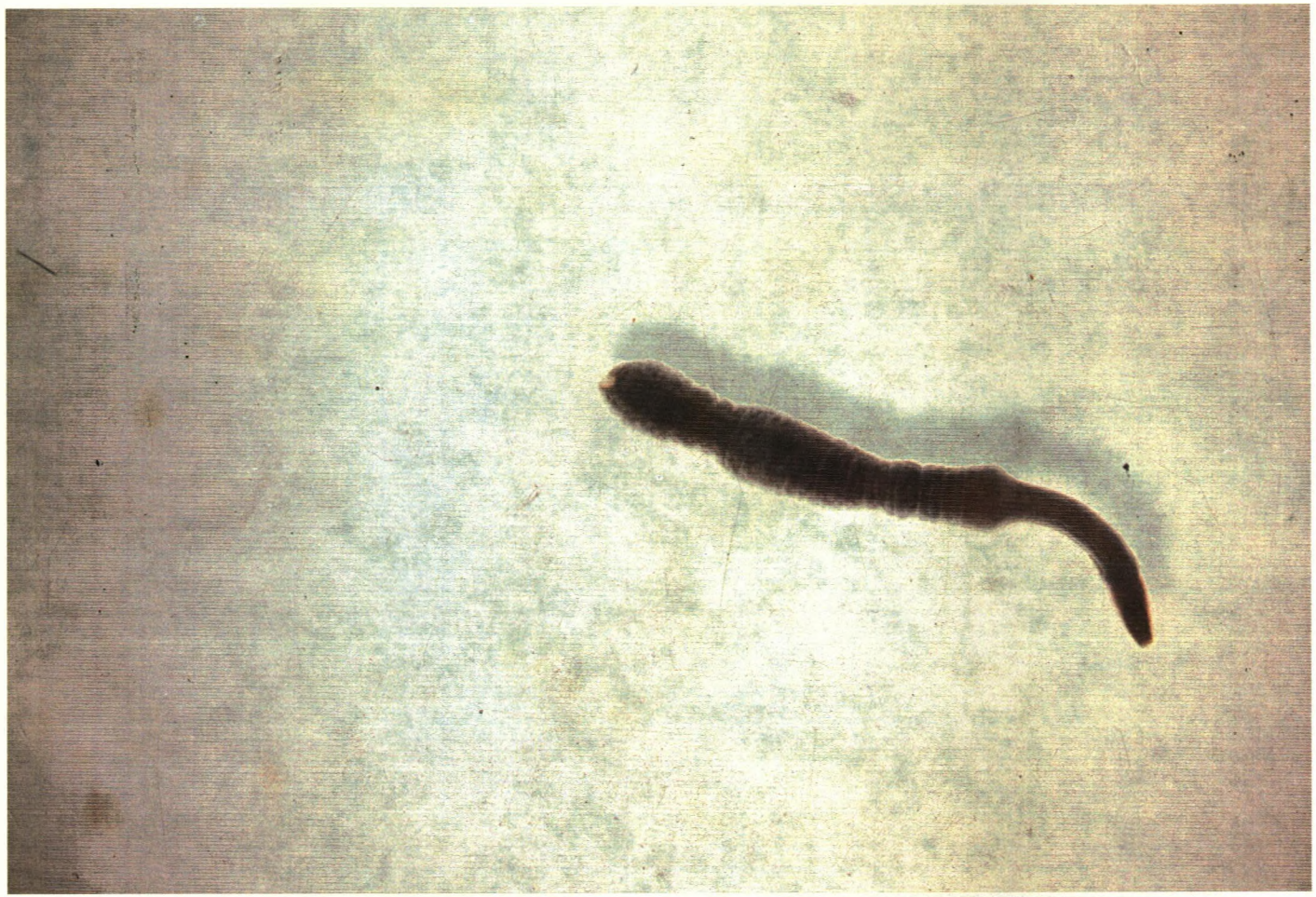


Figure 5.4 'Green' form of genetically aberrant form.

5.3.2 Electrophoretic Results

Allele frequencies for the eleven enzyme loci (thirteen loci for populations at Oban, Llandudno and Anglesey) which produced useful results for *Lineus ruber*, *Lineus viridis* and the aberrant type are presented in Tables 5.2, 5.3 and 5.4. Comparison of sympatric populations of all three types on Tables 5.2, 5.3 and 5.4 show that at nearly all loci there are large differences in allele frequencies.

F-statistics (Wright, 1943, 1951, 1965, 1969) for three sympatric populations of *Lineus ruber* and the aberrant type are given on Table 5.5. F_{IS} values are high for several loci indicating significant degrees of non-random breeding within populations. Wright (1978) suggested that F_{ST} values above 0.25 indicated very great genetic differentiation between populations. The F_{ST} values obtained for sympatric populations of *Lineus ruber* and the genetically aberrant form are above 0.9, indicating virtual fixation of different alleles in the populations compared.

F-statistics for three sympatric populations of *Lineus viridis* and the aberrant form are given in Table 5.6. F_{IS} values are significant indicating non-random breeding within populations. As for populations of *Lineus ruber* and the aberrant form, populations of *Lineus viridis* and the genetically aberrant form give a very large overall value for F_{ST}. This indicates fixation for different alleles at many of the loci detected in this investigation.

Genetic differentiation between populations of *Lineus ruber*, *Lineus viridis* and the aberrant type over all loci can be reduced to a single figure by using Nei's genetic identity (I) or genetic distance (D). Genetic identity and genetic distance between populations of all three types for eleven loci are presented on Table 5.7. Genetic identities between conspecific populations of *Lineus ruber*, *Lineus viridis* and the aberrant type are all very high, generally falling above a value of 0.9. This is within the expected range of I values for conspecific populations (Ayala, 1975, 1983; Thorpe, 1982, 1983; Nei, 1987). I values between populations of *Lineus ruber* and the aberrant type are extremely low lying between 0.094 and 0.000. A value of 0.35 or above is usually associated with

congeneric species, a value of 0.85 or above is usually associated with conspecific populations (Ayala, 1975, 1983; Thorpe, 1982, 1983). Likewise I values between populations of *Lineus viridis* and the aberrant form are also very low, falling between 0.016 and 0.000. I values between populations of *Lineus ruber* and *Lineus viridis* are low, lying between 0.105 and 0.096.

Increasing the number of loci for Nei's (1972) measures of genetic identity and distance will decrease sampling variance (Nei & Roychoudhury, 1974). For three populations, Oban, Llandudno and Anglesey, data was available for thirteen loci for *Lineus ruber*, *Lineus viridis* and the aberrant type. Table 5.8 gives Nei's (1972) genetic identity (I) and genetic distance (D) between these three populations for *Lineus ruber*, *Lineus viridis* and the aberrant type. The genetic identity and genetic distance values between populations of each of the three types are generally the same as those for nine enzyme loci given in Table 5.7.

Genetic similarity between populations of *Lineus ruber*, *Lineus viridis* and the aberrant type is presented graphically in Figures 5.5 and 5.6. These figures show dendrograms constructed by UPGMA cluster analysis for the three types using nine and thirteen loci respectively. Both dendrograms show almost identical patterns. The three types, *Lineus ruber*, *Lineus viridis* and the genetically aberrant form differentiate at very low values of similarity to form three distinct groups which comprise populations of *Lineus ruber*, populations of *Lineus viridis* and populations of the aberrant form. Genetic similarity within each of these three groups is very high.

Fisher's (1935) Exact test for deviation from genotype frequencies expected under Hardy-Weinberg equilibrium (Table 5.9) indicate that for *Lineus ruber* and *Lineus viridis* only two loci for one population (*Lineus viridis*, *Pgm-1*, *Mdh-1*, St Agnes) give significant deviations from Hardy-Weinberg expectations. These deviations may indicate moderate levels of inbreeding (see Chapter 3). The genetically aberrant form gave significant deviations from genotype frequencies expected under Hardy-Weinberg conditions at three different loci for three populations (Anglesey, Bay Ny Carrickey and

Wembury). These were the three populations with the largest sample size for the aberrant form (Table 5.1). The number of significant deviations from expected genotype frequencies under Hardy-Weinberg conditions is higher than that expected by chance. Wright's (1951, 1965) fixation index for these sites show that deviations from expected genotype frequencies were due to heterozygote deficiency.

Observed heterozygosities for populations of the aberrant form were low, ranging from 0.000 to 0.083 (Nevo, 1978; Nevo *et al.* 1984). Observed heterozygosities for this type were generally even lower than those found for *Lineus ruber* (Chapter 3 and the present chapter) and much lower than those recorded for *Lineus viridis*.

Table 5.2 Allele frequencies in the five populations sampled for *Lineus ruber* used in this investigation. Alleles designated by letter according to appendix, running from highest mobility (A) to lowest (Y) for each enzyme locus.

Locus	Allele	OBN	LDN	ANG	CST	FRA
Apa-1	K	0.000	0.018	0.000	0.000	0.000
	L	1.000	0.982	0.959	1.000	1.000
	P	0.000	0.000	0.027	0.000	0.000
	Q	0.000	0.000	0.014	0.000	0.000
Fum-1	A	1.000	1.000	1.000	1.000	1.000
Fum-2	B	1.000	1.000	1.000	1.000	1.000
Got-1	J	0.000	0.014	0.000	0.000	0.000
	Q	1.000	0.986	1.000	1.000	1.000
Got-2	D	1.000	1.000	1.000	1.000	1.000
Icd-1	J	0.986	1.000	0.994	-	-
	M	0.014	0.000	0.006	-	-
Icd-2	F	1.000	1.000	0.989	-	-
	I	0.000	0.000	0.011	-	-
Mdh-1	I	0.989	1.000	1.000	0.947	0.929
	M	0.011	0.000	0.000	0.053	0.000
Mdh-2	C	0.000	0.000	0.000	0.000	0.143
	E	0.989	0.991	1.000	1.000	0.857
	I	0.011	0.009	0.000	0.000	0.000
Odh-1	D	0.067	0.000	0.000	0.000	0.000
	F	0.094	0.182	0.000	0.105	0.071
	J	0.839	0.818	0.994	0.895	0.929
	Q	0.000	0.000	0.006	0.000	0.000
Pgd-1	J	1.000	1.000	1.000	1.000	1.000
Pgi-1	O	0.000	0.000	0.000	0.039	0.000
	R	1.000	1.000	1.000	0.947	1.000
Pgm-1	J	0.007	0.000	0.000	0.000	0.000
	L	0.000	0.027	0.000	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.071
	N	0.986	0.973	0.994	0.987	0.000
	O	0.000	0.000	0.000	0.013	0.000
	S	0.000	0.000	0.000	0.000	0.857
	U	0.000	0.000	0.000	0.000	0.071
	W	0.000	0.000	0.006	0.000	0.000
	X	0.007	0.000	0.000	0.000	0.000

Table 5.3 Allele frequencies in the eight populations of *Lineus viridis* sampled for this investigation. Alleles designated by letter according to the appendix running from highest mobility (A) to the lowest (Y) for each enzyme locus.

Locus	Allele	OBN	LDN	ANG	PVH	STA	PMS	WMY	FRA
Apa-1	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.929
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071
Fum-1	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fum-2	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got-1	E	0.979	1.000	0.983	0.983	1.000	0.950	0.905	1.000
	F	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.000	0.025	0.095	0.000
	L	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000
	O	0.021	0.000	0.000	0.000	0.000	0.025	0.000	0.000
Got-2	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Icd-1	I	0.646	0.422	0.500	-	-	-	-	-
	L	0.344	0.517	0.500	-	-	-	-	-
Icd-2	H	1.000	1.000	1.000	-	-	-	-	-
Mdh-1	C	0.073	0.000	0.017	0.052	0.008	0.050	0.024	0.000
	E	0.917	0.754	0.983	0.621	0.983	0.925	0.952	1.000
	H	0.010	0.246	0.000	0.328	0.008	0.025	0.024	0.000
Mdh-2	A	1.000	0.992	0.983	1.000	1.000	1.000	1.000	1.000
	B	0.000	0.008	0.017	0.000	0.000	0.000	0.000	0.000
Odh-1	A	0.000	0.000	0.233	0.000	0.000	0.000	0.000	0.000
	C	0.346	0.529	0.033	0.000	0.112	0.000	0.028	0.143
	E	0.135	0.114	0.200	0.000	0.009	0.000	0.056	0.000
	F	0.500	0.171	0.200	0.138	0.543	0.150	0.333	0.286
	G	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.143
	K	0.019	0.171	0.267	0.517	0.310	0.275	0.500	0.071
	L	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.286
	P	0.000	0.014	0.000	0.328	0.017	0.550	0.056	0.071
	Q	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000
	T	0.000	0.000	0.000	0.000	0.009	0.025	0.028	0.000
Pgd-1	F	1.000	1.000	1.000	1.000	0.987	1.000	1.000	1.000
	I	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
Pgi-1	G	0.000	0.025	0.000	0.000	0.008	0.000	0.048	0.000
	L	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
	P	1.000	0.975	1.000	1.000	0.983	1.000	0.952	1.000
	S	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
Pgm-1	F	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000
	G	0.063	0.000	0.000	0.000	0.026	0.000	0.043	0.000
	K	0.927	0.958	0.800	0.845	0.719	0.900	0.717	0.929
	M	0.010	0.042	0.200	0.155	0.240	0.050	0.196	0.071
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.043	0.000
	Y	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000

Table 5.4 Allele frequencies in the nine populations sampled for individuals with the aberrant genotype in this investigation. Alleles designated by letter according to the appendix, running from the highest mobility (A) to the lowest (Y) for each enzyme locus.

Locus	Allele	OBN	LDN	ANG	PVH	STA	PMS	WMY	CST	FRA
Apa-1	I	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fum-1	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Fum-2	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got-1	G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Got-2	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Icd-1	M	1.000	1.000	1.000	-	-	-	-	-	-
Icd-2	G	1.000	1.000	1.000	-	-	-	-	-	-
Mdh-1	G	0.000	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.000
	J	1.000	1.000	0.889	0.643	1.000	1.000	0.868	0.000	1.000
	N	0.000	0.000	0.000	0.357	0.000	0.000	0.132	1.000	0.000
Mdh-2	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Odh-1	K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250
	R	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.750
Pgd-1	F	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.182	0.000	0.000	0.000	0.000	0.333
	O	0.833	1.000	1.000	0.818	1.000	1.000	1.000	1.000	0.667
Pgi-1	T	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgm-1	K	0.000	0.000	0.053	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.200	0.000	0.000	0.083	0.167	0.000
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.111	0.000	0.000
	Q	1.000	1.000	0.947	0.800	1.000	1.000	0.722	0.833	1.000
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000

Table 5.5 Summary of F-statistics at 13 loci for three populations where *Lineus ruber* and the genetically aberrant form occur sympatrically. * P<0.05, ** P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe	
Apa-1	-0.028		0.962	0.963**	
Fum-1	-		1.000	1.000**	
Fum-2	-		1.000	1.000**	
Got-1	-0.014		0.991	0.991**	
Got-2	-		1.000	1.000**	
Icd-1	-0.012		0.987	0.987**	
Icd-2	-0.011		0.992	0.993**	
Mdh-1	0.899**		0.993	0.930**	
Mdh-2	-0.010		0.987	0.987**	
Odh-1	0.138**		0.846	0.821**	
Pgd-1	1.000**		1.000	0.912**	
Pgi-1	-		1.000	1.000**	
Pgm-1	0.509**		0.970	0.938**	
Mean	0.426**		0.978	0.961**	0.010

Table 5.6 Summary of F-statistics at 13 loci for three populations where *Lineus viridis* and the genetically aberrant form occur sympatrically. * P<0.05, **P<0.01.

Locus	F_{IS}	F_{IT}	F_{ST}	mNe
Apa-1	-	1.000	1.000**	
Fum-1	-	1.000	1.000**	
Fum-2	-	1.000	1.000**	
Got-1	-0.019	0.975	0.976**	
Got-2	-	1.000	1.000**	
Icd-1	0.085	0.633	0.599**	
Icd-2	-	1.000	1.000**	
Mdh-1	0.409**	0.869	0.779**	
Mdh-2	-0.014	0.983	0.984**	
Odh-1	0.003	0.509	0.507**	
Pgd-1	1.000**	1.000	0.907**	
Pgi-1	-0.026	0.983	0.984**	
Pgm-1	0.378**	0.880	0.806**	
Mean	0.177**	0.895	0.872**	0.037

Table 5.7 Pairwise comparisons based on isozyme data from 11 loci for *Lineus ruber*, *Lineus viridis* and genetically aberrant specimens. Above diagonal, values for Nei's (1972) genetic identity below diagonal values for Nei's genetic distance ("-" means infinite genetic distance). LR = *Lineus ruber*, LV = *Lineus viridis*, ? = aberrant form.

Population	OBNLR	LDNLR	ANGLR	CSTLR	FRALR	OBNLV	LDNLV	ANGLV
OBN (LR)	+	0.999	0.998	0.999	0.915	0.101	0.099	0.100
LDN (LR)	0.001	+	0.997	0.999	0.915	0.106	0.101	0.102
ANG (LR)	0.002	0.003	+	0.999	0.915	0.096	0.097	0.097
CST (LR)	0.001	0.001	0.00	+	0.915	0.102	0.103	0.103
FRA (LR)	0.089	0.089	0.088	0.089	+	0.102	0.103	0.103
OBN (LV)	2.291	2.246	2.348	2.283	2.280	+	0.987	0.981
LDN (LV)	2.309	2.291	2.337	2.305	2.273	0.013	+	0.975
ANG (LV)	2.305	2.284	2.335	2.300	2.276	0.019	0.025	+
PVH (LV)	2.300	2.285	2.325	2.296	2.250	0.044	0.030	0.027
STA (LV)	2.278	2.230	2.340	2.270	2.254	0.013	0.028	0.012
PMS (LV)	2.319	2.302	2.345	2.314	2.297	0.032	0.035	0.023
WMY (LV)	2.281	2.249	2.324	2.275	2.218	0.024	0.030	0.010
FRA (LV)	2.305	2.277	2.343	2.299	2.286	0.012	0.022	0.016
OBN (?)	-	-	-	-	-	4.132	4.121	4.119
LDN (?)	2.381	2.378	2.393	2.379	2.362	-	-	-
ANG (?)	-	-	-	-	-	5.360	5.316	5.494
PVH (?)	4.035	4.031	4.047	4.018	4.015	-	-	-
STA (?)	-	-	-	-	-	-	-	-
PMS (?)	-	-	-	-	-	-	-	-
WMY (?)	-	-	-	9.162	4.969	-	-	-
CST (?)	-	-	-	8.488	-	-	-	-
FRA (?)	3.441	3.438	3.453	3.439	3.422	7.652	5.453	5.009

Table 5.7 contd

Population	PVHLV	STALV	PMSLV	WMYLV	FRALV	OBN?	LDN?	ANG?
OBN (LR)	0.100	0.102	0.098	0.102	0.100	0.000	0.092	0.000
LDN (LR)	0.102	0.107	0.100	0.105	0.103	0.000	0.093	0.000
ANG (LR)	0.098	0.096	0.096	0.098	0.096	0.000	0.091	0.000
CST (LR)	0.101	0.103	0.099	0.103	0.100	0.000	0.093	0.000
FRA (LR)	0.105	0.105	0.101	0.109	0.102	0.000	0.094	0.000
OBN (LV)	0.957	0.987	0.968	0.976	0.988	0.016	0.000	0.005
LDN (LV)	0.971	0.972	0.966	0.970	0.979	0.016	0.000	0.005
ANG (LV)	0.973	0.988	0.977	0.990	0.984	0.016	0.000	0.004
PVH (LV)	+	0.971	0.984	0.981	0.965	0.016	0.000	0.004
STA (LV)	0.030	+	0.973	0.994	0.984	0.016	0.000	0.004
PMS (LV)	0.016	0.027	+	0.979	0.978	0.016	0.000	0.005
WMY (LV)	0.019	0.006	0.02	+	0.979	0.016	0.000	0.004
FRA (LV)	0.035	0.016	0.022	0.021	+	0.016	0.000	0.005
OBN (?)	4.108	4.136	4.128	4.107	4.127	+	0.905	0.996
LDN (?)	-	-	-	-	-	0.099	+	0.907
ANG (?)	5.429	5.606	5.385	5.591	5.353	0.004	0.098	+
PVH (?)	-	-	-	-	-	0.019	0.121	0.015
STA (?)	-	-	-	-	-	0.002	0.095	0.001
PMS (?)	-	-	-	-	-	0.002	0.095	0.001
WMY (?)	-	-	-	7.917	-	0.009	0.105	0.005
CST (?)	-	-	-	-	-	0.104	0.207	0.090
FRA (?)	4.336	4.862	4.988	4.369	6.334	0.014	0.117	0.018

Table 5.7 contd.

Population	PVH?	STA?	PMS?	WMY?	CST?	FRA?
OBN (LR)	0.018	0.000	0.000	0.000	0.000	0.032
LDN (LR)	0.018	0.000	0.000	0.000	0.000	0.032
ANG (LR)	0.017	0.000	0.000	0.000	0.000	0.032
CST (LR)	0.018	0.000	0.000	0.000	0.000	0.032
FRA (LR)	0.018	0.000	0.000	0.007	0.000	0.033
OBN (LV)	0.000	0.000	0.000	0.000	0.000	0.000
LDN (LV)	0.000	0.000	0.000	0.000	0.000	0.004
ANG (LV)	0.000	0.000	0.000	0.000	0.000	0.007
PVH (LV)	0.000	0.000	0.000	0.000	0.000	0.013
STA (LV)	0.000	0.000	0.000	0.000	0.000	0.008
PMS (LV)	0.000	0.000	0.000	0.000	0.000	0.007
WMY (LV)	0.000	0.000	0.000	0.000	0.000	0.013
FRA (LV)	0.000	0.000	0.000	0.000	0.000	0.002
OBN (?)	0.982	0.998	0.998	0.991	0.902	0.986
LDN (?)	0.886	0.909	0.909	0.900	0.813	0.890
ANG (?)	0.985	0.999	0.999	0.995	0.914	0.982
PVH (?)	+	0.982	0.982	0.990	0.957	0.975
STA (?)	0.018	+	1.000	0.994	0.905	0.984
PMS (?)	0.018	0.000	+	0.994	0.905	0.984
WMY (?)	0.010	0.006	0.006	+	0.927	0.976
CST (?)	0.044	0.099	0.099	0.076	+	0.885
FRA (?)	0.025	0.016	0.016	0.024	0.122	+

Table 5.8 Pairwise comparisons based on data for 13 loci for *Lineus ruber*, *Lineus viridis* and genetically aberrant individuals at three populations at which all three species occur sympatrically. Above diagonal values for Nei's (1972) genetic identity (I), below diagonal values for Nei's genetic distance (D) ("-" means infinite genetic distance). LR = *Lineus ruber*, LV = *Lineus viridis*, ? = genetically aberrant specimens.

Population	OBN LR	LDN LR	ANG LR	OBN LV	LDN LV	ANG LV	OBN ?	LDN ?	ANG ?
OBN (LR)	+	0.999	0.998	0.087	0.085	0.085	0.000	0.078	0.000
LDN (LR)	0.001	+	0.997	0.090	0.087	0.087	0.000	0.078	0.000
ANG (LR)	0.002	0.003	+	0.082	0.083	0.083	0.000	0.077	0.000
OBN (LV)	2.446	2.404	2.502	+	0.985	0.982	0.014	0.000	0.004
LDN (LV)	2.462	2.447	2.489	0.015	+	0.978	0.014	0.000	0.004
ANG (LV)	2.461	2.442	2.489	0.019	0.022	+	0.014	0.000	0.004
OBN (?)	-	-	-	4.289	4.275	4.276	+	0.920	0.997
LDN (?)	2.550	2.548	2.560	-	-	-	0.083	+	0.920
ANG (?)	-	-	-	5.516	5.470	5.651	0.003	0.082	+

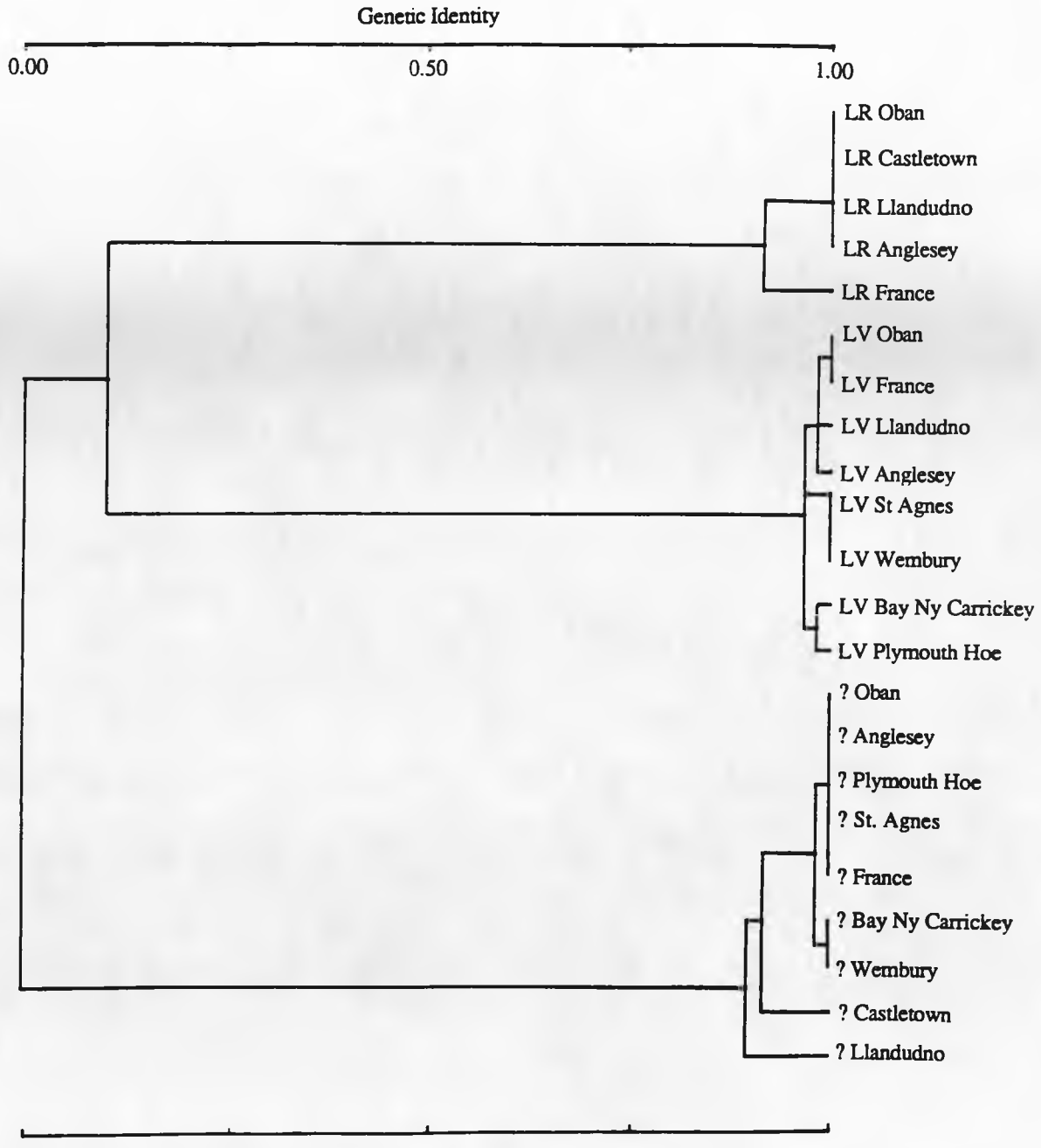


Figure 5.5. UPGMA dendrogram of Nei's (1978) genetic identity (D) between populations of *Lineus ruber*. *Lineus viridis* and the genetically aberrant form calculated using 9 enzyme loci.

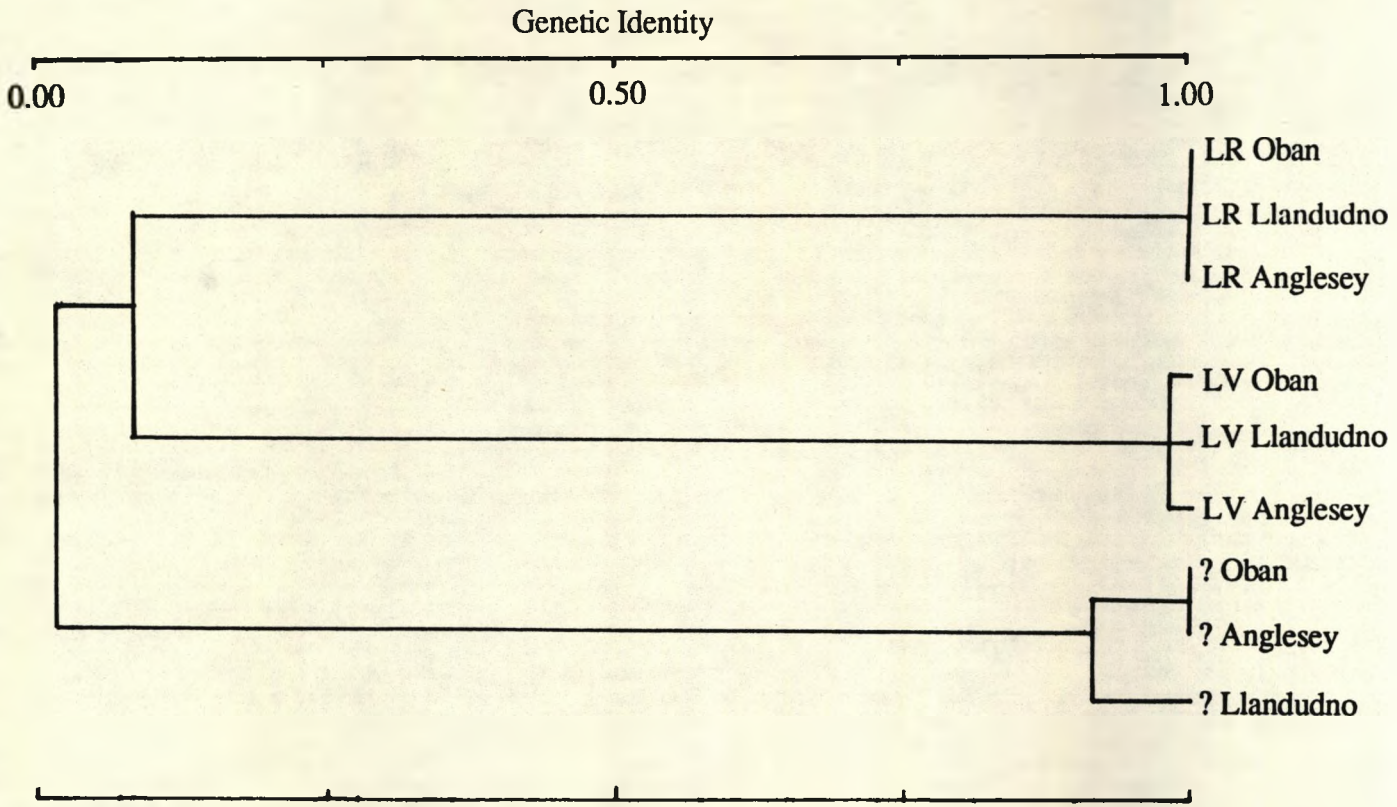


Figure 5.6. UPGMA dendrogram of Nei's (1978) genetic identity (D) between populations of *Lineus ruber*, *Lineus viridis* and the genetically aberrant form calculated using 13 enzyme loci.

Table 5.9 Probabilities that genotype frequencies observed in all populations of *Lineus ruber*, *Lineus.viridis* and the genetically aberrant form, conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies. *P<0.05, **P<0.01.

Locus	OBN LR	LDN LR	ANG LR	CST LR	FRA LR	OBN LV	LDN LV	ANG LV
Apa-1	-	1.000	1.000	-	-	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	-	1.000	-	-	-	1.000	-	1.000
Got-2	-	-	-	-	-	-	-	-
Mdh-1	1.000	-	-	1.000	1.000	0.271	0.088	1.000
Mdh-2	1.000	1.000	-	-	0.077	-	1.000	1.000
Odh-1	0.231	0.353	1.000	0.336	1.000	0.255	0.095	0.613
Pgd-1	-	-	-	-	-	-	-	-
Pgi-1	-	-	-	0.079	-	-	1.000	-
Pgm-1	1.000	1.000	1.000	1.000	1.000	1.000	0.083	0.060

Table 5.9 contd.

Locus	PVH LV	STA LV	PMS LV	WMY LV	FRA LV	OBN ?	LDN ?	ANG ?
Apa-1	-	-	-	-	1.000	-	-	-
Fum-1	-	-	-	-	-	-	-	-
Fum-2	-	-	-	-	-	-	-	-
Got-1	1.000	-	1.000	1.000	-	-	-	-
Got-2	-	-	-	-	-	-	-	-
Mdh-1	1.000	0.025	1.000	1.000	-	-	-	0.003
Mdh-2	-	-	-	-	-	-	-	-
Odh-1	0.719	0.183	0.395	0.654	1.000	-	-	-
Pgd-1	-	1.000	-	-	-	0.091	-	-
Pgi-1	-	1.000	-	1.000	-	-	-	-
Pgm-1	0.518	0.024	1.000	0.299	1.000	-	-	0.027

Table 5.9 contd.

Locus	PVH?	STA?	PMS?	WMY?
Apa-1	-	-	-	-
Fum-1	-	-	-	-
Fum-2	-	-	-	-
Got-1	-	-	-	-
Got-2	-	-	-	-
Mdh-1	0.441	-	-	1.000
Mdh-2	-	-	-	-
Odh-1	-	-	-	-
Pgd-1	0.008	-	-	-
Pgi-1	-	-	-	-
Pgm-1	0.297	-	-	0.004

5.10 Mean observed and expected heterozygosity (under Hardy-Weinberg expectations) for populations of *Lineus ruber*, *Lineus viridis* and the genetically aberrant form used in this investigation. Mean sample size per locus and percentage of polymorphic loci (at 0.95 and 0.99 levels) are also given.

Population	Mean Sample size per locus	Percentage polymorphic loci 95%	99%	Mean Heterozygosity Observed	Expected
L.ruber					
Oban	80.0	9.1	36.4	0.029	0.032
Llandudno	45.2	9.1	45.5	0.036	0.040
Anglesey	70.7	0.0	27.3	0.009	0.009
Castletown	38.0	27.3	36.4	0.031	0.038
Pointe de Barfleur	7.0	36.4	36.4	0.052	0.075
L. viridis					
Oban	46.0	27.3	36.4	0.074	0.087
Llandudno	53.2	18.2	45.5	0.105	0.107
Anglesey	25.2	18.2	45.5	0.100	0.113
Bay Ny Carrickey	28.8	27.3	36.4	0.119	0.130
St. Agnes	107.1	18.2	45.5	0.123	0.102
Plymouth S.	18.5	36.4	36.4	0.086	0.095
Wembury	18.4	27.3	45.5	0.118	0.133
Pointe de Barfleur	7.0	27.3	27.3	0.091	0.103
Aberrant type					
Oban	8.0	9.1	9.1	0.000	0.028
Llandudno	3.8	0.0	0.0	0.000	0.000
Anglesey	14.7	18.2	18.2	0.000	0.028
Bay Ny Carrickey	10.0	27.3	27.3	0.083	0.104
St. Agnes	1.8	0.0	0.0	0.000	0.000
Plymouth S.	5.0	0.0	0.0	0.000	0.000
Wembury	17.4	18.2	18.2	0.039	0.064
Castletown	2.1	9.1	9.1	0.030	0.030
Pointe de Barfleur	2.3	18.2	18.2	0.045	0.094

5.4 Discussion

The comparison of the external morphology of *Lineus ruber*, *Lineus viridis* and the genetically aberrant specimens indicated that though the aberrant form could grow to a larger size than *Lineus ruber* or *Lineus viridis*, it was almost indistinguishable from them. Variation in the external colouration of the aberrant type was sufficient to cover all colour variations in *Lineus ruber* and *Lineus viridis*. The shape of the body of the aberrant form was variable but again covered almost all variations found in *Lineus ruber* and *Lineus viridis*. The aberrant forms were found in exactly the same habitat as *Lineus ruber* and *Lineus viridis*. The genetically aberrant forms could not therefore be reliably separated from *Lineus ruber* or *Lineus viridis* by external morphology or by habitat differentiation.

Comparison of allele frequencies between the aberrant form and *Lineus ruber* and *Lineus viridis* showed that for most loci sampled there were few common alleles between all three types. F-statistics (Wright, 1943, 1951, 1965, 1969) demonstrated fixation of different alleles for almost all loci between sympatric populations of *Lineus ruber* and the aberrant type and between populations of *Lineus viridis* and the aberrant type (Wright, 1978). Since sympatric, conspecific populations should interbreed freely, there should be no significant difference in allele frequencies between any loci. Fixation of different alleles for any given locus in sympatric populations indicates a barrier to gene flow between populations (Thorpe, 1982). Since almost all loci are fixed for different alleles than those of *Lineus ruber* and *Lineus viridis* it is likely that the aberrant genetic form therefore represents a separate biological species (Thorpe, 1982). Biological species in this case are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr, 1957). Reproductive isolation in this case is brought about by mechanisms that are biological properties of individuals which prevent the interbreeding of populations that are actually or potentially sympatric (Mayr, 1963).

Values for genetic identity between the aberrant type and *Lineus ruber* and *Lineus*

viridis are well below critical values associated with conspecific populations (I=0.85 [Thorpe, 1982]) and are lower than values usually found in congeneric comparisons. This is compatible with the suggestion that the aberrant type represents a separate species and confirms the findings of the F-statistics.

Distortion of gene frequencies may have occurred with the aberrant type since it shows deviations of genotype frequencies from those expected under Hardy-Weinberg equilibrium. This may partially explain the extremely low genetic identities found between the aberrant type and the two *Lineus* species. Since sample sizes of the aberrant type were so low it is not possible to come to any conclusions about causes of the heterozygote deficiency responsible for deviations of genotype frequencies from Hardy-Weinberg expectations. Possible explanations could include some form of non-random breeding (i.e. asexual reproduction), very small population size or even the presence of more than one species in the aberrant type. The rarity of the aberrant type in samples compared to *Lineus ruber* and *Lineus viridis* may support, to some extent, small population size as a cause of inbreeding. Neutral gene theory predicts low heterozygosity with a small populations size (Kimura, 1983), the aberrant form generally showed lower levels of genetic variation than the other two species of *Lineus* sampled for this investigation. This low heterozygosity may have been the result of sampling error caused by very low sample sizes (Nei, 1978).

Electrophoretic evidence suggests that a cryptic species that cannot be distinguished by external morphology from *Lineus ruber* or *Lineus viridis* is found sympatrically with the two *Lineus* species along the coasts of Britain and France. In some localities (i.e. Wembury) red brown '*Lineus ruber*' are in fact all specimens of the cryptic species. The cryptic species has not been detected in the U.S.A. in the samples taken for this investigation. The identity of specimens of *Lineus ruber* and *Lineus viridis* used in numerous physiological, structural, reproductive and ecological investigations over the past 150 years (see 5.1), must be in some doubt. Results obtained in such investigations and attributed to *Lineus ruber* and *Lineus viridis* may refer to the cryptic species found in

this investigation.

The low genetic identities found between the new type and the two *Lineus* species are not usually found in congeneric comparisons (Thorpe, 1982). Taxonomic placement of this form must wait until histological investigations have provided a complete description of the internal morphology of the new species. The internal morphology will be compared with other heteronemertean taxa and in conjunction with electrophoretic data be used to identify the taxonomic status of the new cryptic species. The low genetic identities between the morphologically very similar species examined in this investigation may be a result of convergent evolution. Alternatively mechanisms of genetic divergence in these species may be faster than expected from other organisms, possibly due to the reproductive and dispersive strategies employed by these species (e.g. for *Lineus ruber* and *Lineus viridis* - sexual reproduction with inbreeding, see 3.4).

Chapter Six

Genetic Relationships Between Some Members of the Genera *Lineus* Sowerby 1806 and *Micrura* Ehrenberg 1831 (Heteronemertea: Lineidae)

6.1 Introduction

Gibson (1981) pointed out that intraspecific differences found by different workers indicated that many species in the genus *Lineus* Sowerby 1806, could not be satisfactorily diagnosed. Furthermore, he suggested that "the 'species' described may in fact represent a complex of externally similar but taxonomically distinct forms". The discovery of *Riseriellus occultus* (Rogers *et al.* 1993; Chapter 4, this thesis) and evidence for a cryptic species which may be confused with *Lineus ruber* or *Lineus viridis* (Chapter 5, this thesis), support Gibson's (1981) hypothesis.

Gibson (1981) also pointed out that the genus *Lineus* "remains without a definitive diagnosis because far too few of the 90 or more species have been carefully enough investigated [Friedrich, 1960; Cantell, 1975]. Doubtless many of the named species belong to other genera". Similar conclusions have been reached by other authors not only for the genus *Lineus* Sowerby 1806, but also for other genera in the family Lineidae such as *Micrura* Ehrenberg 1831 and *Cerebratulus* Renier 1804 (Punnett, 1901; Friedrich, 1960; Cantell, 1975; Gibson, 1982a, 1985).

The genera of the family Lineidae are distinguished on the basis of differences in internal anatomy. The genera *Cerebratulus* and *Micrura* have two or three proboscis muscle layers, one or two proboscis muscle crosses and a distinct caudal cirrus (Gibson, 1982a). The two genera can only be distinguished on the presence (*Cerebratulus*) or absence (*Micrura*) of neurochord cells in the nervous system (Gibson, 1982a). Members of the genus *Lineus* also lack neurochord cells in the nervous system but unlike members of the genus *Micrura* they are not reported to possess a caudal cirrus (Gibson, 1982a). Also the ganglionic cell layer of the brain is reported as usually being separated from the body wall muscles by a connective tissue neurilemma in *Lineus* but not in *Micrura* (Gibson, 1990c). Many of the morphological characters used to separate these three heteronemertean genera have only been determined for few species and do not provide a reliable generic diagnosis (see Punnett, 1901; Friedrich, 1960; Cantell, 1975; Gibson,

1982a, 1985).

Thorpe (1982), using published data, plotted the probability of populations, within species, between congeneric species and between different genera, falling in to certain values of genetic identity (I). He concluded that there was a clear relationship between taxonomic divergence and genetic identity (I) and distance (D). The critical I value (Nei's, [1972] genetic identity) for distinguishing between genera was 0.35, for congeneric species it was between 0.35 and 0.85 and for conspecific populations it was above 0.85 (see Section 1.2, this thesis).

If levels of genetic separation between congeneric species in nemerteans complies with those found in many other taxa (e.g. Thorpe, 1982, 1983; Ayala, 1983; Nei, 1987) then Nei's (1972) genetic identity (I) between members of the genus *Lineus* would be expected to fall between 0.35 and 0.85. I values between conspecific populations of members of the genus *Lineus* will be expected to fall above 0.85 and I values between species from the genus *Lineus* and species from other genera of the family Lineidae (Gibson, 1985) should fall below 0.35.

In this chapter Nei's (1972) genetic identity and distance (D) were calculated between populations of several *Lineus* species (*Lineus longissimus*, *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*), including the cryptic species indicated in chapter 5. As an outgroup two species from another genus in the family Lineidae, *Micrura* (*Micrura fasciolata* and *Micrura purpurea*) were included in these calculations. The results indicated that I values between species of the genus *Lineus* were below those usually expected for congeneric species. Furthermore *Lineus viridis* appeared to be more closely related to *Micrura fasciolata* and *Micrura purpurea* than to the other *Lineus* species investigated. Implications for the taxonomy of the family Lineidae (sensu Gibson, 1985) are discussed.

6.2 Methods and Materials

6.2.1 Specimens and Sample Sites

Species used in this investigation, the sites from which they were obtained and the numbers of specimens examined are given in Table 6.1. Details of sampling sites are provided in Section 2.1.

Table 6.1 Species used in the investigation of genetic relatedness between species of the genera *Lineus* and *Micrura*. Species investigated, populations sampled and the number of individuals from each population electrophoresed are given. Cryptic species refers to the aberrant type described in Chapter 5.

Species	Population		No. of Individuals
Lineus longissimus	Port Erin	(PTE)	8
Lineus ruber	Oban	(OBN)	90
Lineus ruber	Llandudno	(LDN)	55
Lineus ruber	Anglesey	(ANG)	88
Lineus viridis	Oban	(OBN)	48
Lineus viridis	Whitehaven	(WTN)	34
Lineus viridis	Llandudno	(LDN)	59
Lineus sanguineus	Whitehaven	(WTN)	60
Lineus sanguineus	Llandudno	(LDN)	43
Cryptic species	Bay Ny Carrickey	(PVH)	11
Cryptic species	Anglesey	(ANG)	19
Cryptic species	Wembury	(WMY)	19
Micrura fasciolata	Tjärnö,Sweden	(SWE)	30
Micrura purpurea	Tjärnö,Sweden	(SWE)	10

6.2.2 Starch Gel Electrophoresis

Specimens were described, numbered and electrophoresed as detailed in Sections 2.2, 2.3 and 2.4. Data collected from electrophoresis were converted to actual numbers of genotypes occurring in each population for each species and then analysed using the FORTRAN programme BIOSYS-1 (Swofford & Selander, 1989).

6.2.3 Data Analysis

Data from only nine enzyme loci were analysed, since these were the only loci which stained consistently in all species investigated in this study. For these nine loci Nei's (1972) genetic identity (I) and genetic distance (D) were calculated between all populations of each species or type. Cluster analysis was carried out between all populations using UPGMA (Sneath & Sokal, 1973). The Fisher (1935) Exact test for

deviations from genotype frequencies expected under Hardy-Weinberg equilibrium was carried out for all populations. BIOSYS-1 was used to perform all calculations. For details of data analysis see Section 3.2.3. Note that for the *Pgm-1* locus in *Micrura fasciolata* 5 rare alleles were pooled into the allele E for convenience of calculation.

6.3 Results

Allele frequencies for the nine loci which produced comparable results for the species in this investigation are given in Table 6.2. Conspecific populations show similar allele frequencies (e.g Oban, Llandudno and Anglesey for *Lineus ruber*; Oban, Whitehaven and Llandudno for *Lineus viridis*). Populations of congeneric species show large differences in allele frequencies at nearly all loci.

Genetic differentiation between populations of all species was reduced to a single figure using Nei's (1972) genetic identity (I) and genetic distance (D). I and D for comparisons between all populations of all species are presented in Table 6.3. Values for Nei's (1972) genetic identity are extremely high between conspecific populations of *Lineus ruber*, *Lineus viridis* and *Lineus sanguineus*, falling in the range of 0.999-0.958. This is well within the range expected for I values between conspecific populations (Thorpe, 1982, 1983; Ayala, 1983; Nei, 1987). I values for conspecific populations of the suspected cryptic species described in Chapter 5 are below those obtained for conspecific populations of *Lineus ruber*, *Lineus viridis* and *Lineus sanguineus*, with a range of 0.890-0.992. These I values are still well within those expected between conspecific populations.

Values for Nei's (1972) genetic identity (I) between populations of species of the genus *Lineus* range between 0.000-0.237 (see Table 6.3) are lower than those usually obtained for congeneric species (generally in the range of 0.35-0.85). Between populations of *Lineus longissimus*, *Lineus viridis*, *Lineus ruber* and *Lineus sanguineus* I values range from 0.000-0.237. Only between *Lineus ruber* and *Lineus longissimus* are I

values above 0.04, between populations of all other members of the genus *Lineus* examined, I falls below this value. I values between *Micrura fasciolata* and *Micrura purpurea* (0.104) are also low for congeneric species (Thorpe, 1982, 1983).

I values between *Lineus viridis* and the two *Micrura* species examined (Table 6.3) are markedly higher than those between *Lineus viridis* and the other members of the genus *Lineus* with which it was compared. I values between *Lineus viridis* and *Micrura purpurea* are the highest obtained between two different species in this investigation (0.238) but are still low for congeneric species (Thorpe, 1982, 1983).

Genetic similarity, calculated from Nei's (1978) genetic distance, between populations of all species studied in this investigation are presented graphically in Figure 6.1. This figure shows a dendrogram constructed by UPGMA cluster analysis using genetic similarity values between all populations over nine enzyme loci. The dendrogram shows graphically the same findings as pairwise comparisons using Nei's (1972) genetic identity (I) and distance (D).

Lineus sanguineus and the cryptic species described in Chapter 5 show little genetic similarity to any of the other species investigated. *Lineus longissimus* and *Lineus ruber* form one group which splits at a similarity level lower than that expected for congeneric species. *Lineus viridis*, *Micrura fasciolata* and *Micrura purpurea* form a separate group which again splits at a similarity level lower than expected for congeneric species. *Lineus viridis* appears to be genetically more similar to the *Micrura* species than to other *Lineus* species electrophoresed. Conspecific populations all show a high genetic similarity.

Fisher's (1935) Exact test for deviation from genotype frequencies expected under Hardy-Weinberg equilibrium for all populations are presented in Table 6.4. No significant deviations of genotype frequencies from those expected under Hardy-Weinberg equilibrium were found for *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*, *Lineus longissimus* and *Micrura fasciolata*. Significant deviation from genotype frequencies expected under Hardy-Weinberg conditions were found for the

cryptic species described in Chapter 5 *Mdh-1*, *Pgm-1*), for populations at Anglesey and Wembury and also for *Micrura purpurea* from Sweden (*Pgi-1*, *Pgm-1*). Wright's (1951, 1965) fixation index for these populations show that deviations from expected genotype frequencies were all due to heterozygote deficiency.

Table 6.2 Allele frequencies in all populations sampled for *Lineus longissimus* (LL), *Lineus ruber* (LR), *Lineus viridis* (LV), *Lineus sanguineus* (LS), *Micrura fasciolata* (MF), *Micrura purpurea* (MP) and the cryptic species (CS), described in Chapter 5 in this investigation.

Species/Popn		LR	LR	LR	LV	LV	LV	CS	CS
Locus	Allele	OBN	LDN	ANG	OBN	WTN	LDN	ANG	PVH
Apa-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
	J	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
	L	1.000	0.982	0.959	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P	0.000	0.000	0.027	0.000	0.000	0.000	0.000	0.000
	Q	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.000
Got-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.979	0.956	1.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
	I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.014	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000
	Q	1.000	0.986	1.000	0.000	0.029	0.000	0.000	0.000
	R	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Icd-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.646	0.529	0.422	0.000	0.000
	J	0.986	1.000	0.994	0.000	0.000	0.000	0.000	0.000
	L	0.000	0.000	0.000	0.344	0.309	0.517	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
	N	0.014	0.000	0.006	0.010	0.103	0.052	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.059	0.000	0.000	0.000
Icd-2	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 6.2 contd

Locus	Allele	OBN	LDN	ANG	OBN	WTN	LDN	ANG	PVH
Icd-2	F	1.000	1.000	0.989	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
	H	0.000	0.000	0.000	1.000	0.735	1.000	0.000	0.000
	I	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.000	0.265	0.000	0.000	0.000
Mdh-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.073	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.917	1.000	0.754	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.111	0.000
	H	0.000	0.000	0.000	0.010	0.000	0.246	0.000	0.000
	I	0.989	1.000	1.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.000	0.000	0.000	0.889	0.643
	K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	M	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.357
	O	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mdh-2	A	0.000	0.000	0.000	1.000	1.000	0.992	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
	E	0.989	0.991	1.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mdh-2	I	0.011	0.009	0.000	0.000	0.000	0.000	0.000	0.000
Odh-1	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.237	0.113	0.500	0.000	0.000
	D	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.092	0.000	0.135	0.000	0.000
	F	0.094	0.182	0.000	0.500	0.226	0.162	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.839	0.818	0.994	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.171	0.435	0.189	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P	0.000	0.000	0.000	0.000	0.226	0.014	0.000	0.000
	R	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
Pgi-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P	0.000	0.000	0.000	1.000	1.000	0.975	0.000	0.000

Table 6.2 contd

Locus	Allele	OBN	LDN	ANG	OBN	WTN	LDN	ANG	PVH
Pgi-1	Q	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	R	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
	T	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000
Pgm-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.927	0.912	0.958	1.000	0.000
	L	0.000	0.027	0.000	0.000	0.000	0.000	0.000	0.000
	M	0.000	0.000	0.000	0.010	0.088	0.042	0.000	0.000
	N	0.986	0.973	0.994	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.800
	R	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	X	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 6.2 contd

Species		CS	LS	LS	LL	MF	MP
Locus	Allele	WMY	WTN	LDN	PTE	SWE	SWE
Apa-1	A	0.000	0.000	0.000	0.000	0.017	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.600
	E	0.000	0.000	0.000	0.000	0.933	0.000
	F	0.000	0.000	0.000	0.000	0.033	0.000
	G	0.000	1.000	0.875	0.000	0.000	0.350
	H	0.000	0.000	0.000	0.000	0.017	0.000
	I	1.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.104	0.188	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.000	0.050
	L	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.813	0.000	0.000
	P	0.000	0.000	0.021	0.000	0.000	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.000
Got-1	A	0.000	0.000	0.000	0.000	0.060	0.000
	B	0.000	0.000	0.000	0.000	0.640	0.000
	C	0.000	0.000	0.000	0.000	0.220	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.060	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000
	G	1.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.020	0.000
	J	0.000	0.000	0.000	0.000	0.000	0.300
	K	0.000	0.000	0.000	0.813	0.000	0.000
	M	0.000	0.000	0.000	0.188	0.000	0.000

Table 6.2 contd

Locus	Allele	WMY	WIN	LDN	PTE	SWE	SWE
Got-1	N	0.000	0.000	0.023	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.000	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.000
	R	0.000	0.125	0.198	0.000	0.000	0.700
	S	0.000	0.875	0.756	0.000	0.000	0.000
	T	0.000	0.000	0.023	0.000	0.000	0.000
Icd-1	A	0.000	0.000	0.000	0.000	0.983	0.000
	B	0.000	0.000	0.000	0.000	0.017	0.000
	C	0.000	0.000	0.000	0.750	0.000	0.150
	E	0.000	0.975	1.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.000	0.000	0.250	0.000	0.000
	I	0.000	0.025	0.000	0.000	0.000	0.850
	J	0.000	0.000	0.000	0.000	0.000	0.000
	L	0.000	0.000	0.000	0.000	0.000	0.000
	M	1.000	0.000	0.000	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.000	0.000	0.000
Icd-2	B	0.000	0.000	0.000	0.000	0.000	1.000
	D	0.000	0.000	0.000	0.000	1.000	0.000
	E	0.000	1.000	1.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	1.000	0.000	0.000
	G	1.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.000	0.000	0.000
Mdh-1	A	0.000	0.000	0.000	0.000	0.050	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.500
	C	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.063	0.867	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.083	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.500
	H	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.868	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.938	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.000	0.000
	N	0.132	0.000	0.000	0.000	0.000	0.000
	O	0.000	1.000	1.000	0.000	0.000	0.000
Mdh-2	A	0.000	0.000	0.000	0.000	1.000	0.000
	B	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.000	0.000	0.000	1.000
	D	1.000	0.000	0.000	1.000	0.000	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	1.000	1.000	0.000	0.000	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.000
Odh-1	B	0.000	0.000	0.000	0.000	0.054	0.000
	C	0.000	0.000	0.000	0.000	0.357	0.000
	D	0.000	0.000	0.000	0.000	0.000	0.000

Table 6.2 contd.

Locus	Allele	WMY	WTN	LDN	PTE	SWE	SWE
Odh-1	E	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.554	0.350
	I	0.000	0.000	0.000	0.938	0.000	0.000
	J	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.000	0.036	0.000
	N	0.000	1.000	1.000	0.000	0.000	0.000
	O	0.000	0.000	0.000	0.063	0.000	0.000
	P	0.000	0.000	0.000	0.000	0.000	0.000
	R	1.000	0.000	0.000	0.000	0.000	0.000
Pgi-1	A	0.000	0.000	0.000	0.563	0.000	0.000
	D	0.000	0.000	0.000	0.000	0.017	0.000
	F	0.000	0.675	0.354	0.438	0.017	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.017	0.000
	I	0.000	0.000	0.000	0.000	0.000	0.263
	J	0.000	0.000	0.000	0.000	0.933	0.474
	K	0.000	0.325	0.634	0.000	0.000	0.000
	M	0.000	0.000	0.012	0.000	0.000	0.000
	N	0.000	0.000	0.000	0.000	0.017	0.000
	P	0.000	0.000	0.000	0.000	0.000	0.000
	Q	0.000	0.000	0.000	0.000	0.000	0.263
	R	0.000	0.000	0.000	0.000	0.000	0.000
	T	1.000	0.000	0.000	0.000	0.000	0.000
Pgm-1	A	0.000	0.000	0.000	0.000	0.182	0.000
	B	0.000	0.000	0.000	0.000	0.273	0.000
	C	0.000	0.000	0.000	0.000	0.136	0.000
	D	0.000	0.000	0.000	0.000	0.159	0.000
	E	0.000	0.000	0.000	0.000	0.250	0.000
	G	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.000	0.000	0.000	0.550
	J	0.000	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.143	0.000	0.450
	L	0.000	0.975	0.952	0.000	0.000	0.000
	M	0.000	0.000	0.000	0.000	0.000	0.000
	N	0.000	0.000	0.012	0.857	0.000	0.000
	O	0.083	0.000	0.000	0.000	0.000	0.000
	P	0.111	0.000	0.000	0.000	0.000	0.000
	Q	0.722	0.000	0.000	0.000	0.000	0.000
	R	0.000	0.025	0.036	0.000	0.000	0.000
	S	0.083	0.000	0.000	0.000	0.000	0.000
	W	0.000	0.000	0.000	0.000	0.000	0.000
	X	0.000	0.000	0.000	0.000	0.000	0.000

Table 6.3 Pairwise comparisons based on isozyme data from 9 loci for *Lineus longissimus* (LL), *Lineus ruber* (LR), *Lineus viridis* (LV), *Lineus sanguineus* (LS), *Micrura fasciolata* (MF), *Micrura purpurea* (MP) and the cryptic species (CS) described in Chapter 5. Above diagonal, values for Nei's (1972) genetic identity (I) below diagonal values for Nei's (1972) genetic distance (D) ("-" means infinite genetic distance).

Population	OBNLR	LDNLR	ANGLR	OBNLV	WTNLV	LDNLV	ANGCS	PVHCS
OBN (LR)	+	0.999	0.998	0.006	0.007	0.002	0.000	0.000
LDN (LR)	0.001	+	0.996	0.011	0.009	0.004	0.000	0.000
ANG (LR)	0.002	0.004	+	0.000	0.004	0.000	0.000	0.000
OBN (LV)	5.137	4.483	11.848	+	0.972	0.976	0.114	0.000
WTN (LV)	5.006	4.709	5.575	0.028	+	0.958	0.116	0.000
LDN (LV)	6.200	5.589	10.226	0.025	0.043	+	0.120	0.000
ANG (CS)	-	-	-	2.174	2.157	2.122	+	0.890
PVH (CS)	-	-	-	-	-	-	0.116	+
WMY (CS)	-	-	-	-	-	-	0.096	0.009
WTN (LS)	-	5.756	-	6.192	6.357	6.597	-	-
LDN (LS)	6.551	5.385	6.510	-	-	-	-	-
PTE (LL)	1.441	1.444	1.457	4.008	3.992	3.957	1.930	2.029
SWE (MF)	-	-	-	1.814	1.813	1.721	-	-
SWE (MP)	-	7.252	-	1.434	1.471	1.534	2.642	-

Table 6.3 contd.

Population	WMYCS	WTNLS	LDNLS	PTELL	SWEMF	SWEMP
OBN (LR)	0.000	0.000	0.001	0.237	0.000	0.000
LDN (LR)	0.000	0.003	0.005	0.236	0.000	0.001
ANG (LR)	0.000	0.000	0.001	0.233	0.000	0.000
OBN (LV)	0.000	0.002	0.000	0.018	0.163	0.238
WTN (LV)	0.000	0.002	0.000	0.018	0.163	0.230
LDN (LV)	0.000	0.001	0.000	0.019	0.179	0.216
ANG (CS)	0.908	0.000	0.000	0.145	0.000	0.071
PVH (CS)	0.992	0.000	0.000	0.131	0.000	0.000
WMY (CS)	+	0.000	0.000	0.131	0.000	0.000
WTN (LS)	-	+	0.985	0.039	0.002	0.067
LDN (LS)	-	0.015	+	0.025	0.001	0.066
PTE (LL)	2.035	3.251	3.695	+	0.009	0.028
SWE (MF)	-	6.485	7.105	4.708	+	0.104
SWE (MP)	-	2.706	2.711	3.581	2.268	+

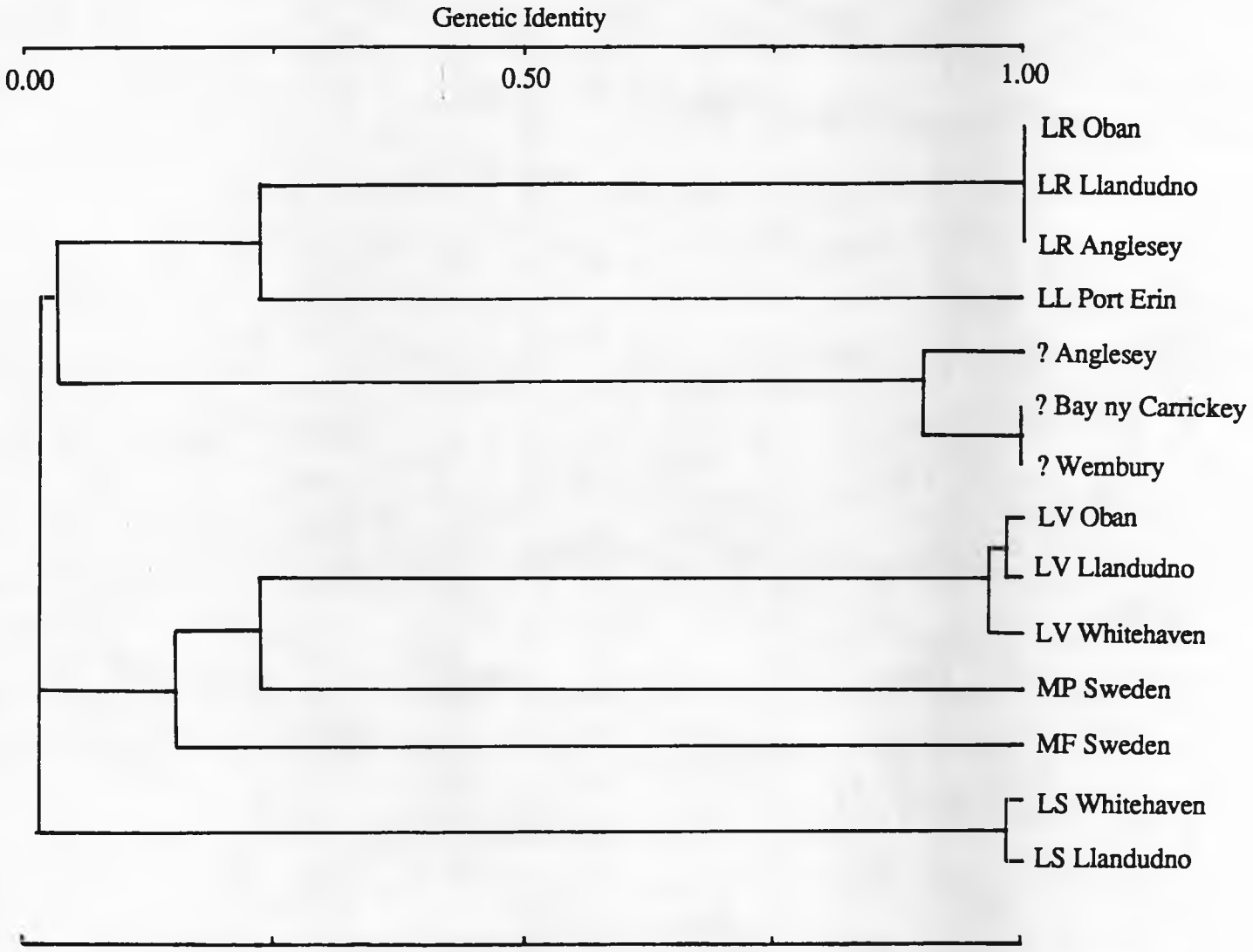


Figure 6.1. UPGMA dendrogram of Nei's (1978) genetic identity (D) between populations of *Lineus ruber*, *Lineus longissimus*, *Lineus viridis*, *Lineus sanguineus*, a cryptic species (Chapter 5), *Micrura purpurea* and *Micrura fasciolata*.

Table 6.4 probabilities that genotype frequencies observed in all populations of *Lineus longissimus* (LL), *Lineus ruber* (LR), *Lineus viridis* (LV), *Lineus sanguineus* (LS), *Micrura fasciolata* (MF), *Micrura purpurea* (MP) and the cryptic species (CS) described in Chapter 5, conform to Hardy-Weinberg expectations using Fisher's (1935) Exact test for observing genotype frequencies. *P<0.05, **P<0.01.

Locus	OBNLR	LDNLR	ANGLR	OBNLV	WTNLV	LDNLV	ANGCS	PVHCS
Apa-1	-	1.000	1.000	-	-	-	-	-
Got-1	-	1.000	-	1.000	1.000	-	-	-
Icd-1	1.000	-	1.000	1.000	0.317	0.438	-	-
Icd-2	-	-	1.000	-	1.000	-	-	-
Mdh-1	1.000	-	-	0.271	-	0.088	0.003**	0.441
Mdh-2	1.000	1.000	-	-	-	1.000	-	-
Odh-1	0.231	0.353	1.000	0.516	0.469	0.198	-	-
Pgi-1	-	-	-	-	-	1.000	-	-
Pgm-1	1.000	1.000	1.000	1.000	0.214	0.083	-	0.297

Table 6.4 contd.

Locus	WMYCS	WTNLS	LDNLS	PTELL	SWEMF	SWEMP
Apa-1	-	-	0.298	1.000	1.000	0.573
Got-1	-	1.000	0.230	1.000	0.663	0.333
Icd-1	-	1.000	-	0.143	1.000	1.000
Icd-2	-	-	-	-	-	-
Mdh-1	1.000	-	-	1.000	0.058	1.000
Mdh-2	-	-	-	-	-	-
Odh-1	-	-	-	1.000	0.121	0.220
Pgi-1	-	0.077	0.098	0.481	1.000	0.000**
Pgm-1	0.004**	1.000	1.000	1.000	1.000	0.015*

6.4 Discussion

Nine loci only were used for this investigation because of the difficulties involved in the electrophoresis of nemerteans. Few enzyme loci gave sufficient activity and resolution to provide useful data. Enzyme loci that stained well in some species either gave uninterpretable results (e.g. Mdh in *Riseriellus occultus*), or did not stain at all in others. The nine enzyme loci scored for this investigation were the only ones which gave sufficient activity and resolution and were common to all species in the present study.

When only a few loci are used to study genetic distances between populations, sampling error is large and the estimate of genetic distance may deviate from the real value by a large amount (Nei & Roychoudhury, 1974; Nei, 1987). This is especially the case in this study because of the high genetic distances between the species investigated. Nei (1987) pointed out that if D is too large (D>1) then its variance becomes very large, even if a substantial number of loci are studied. The high frequency of backward and parallel mutations in isozymes when D>1 also makes measures of genetic distance unreliable (Nei, 1987). In some interspecific comparisons in this study D exceeded 11 (e.g. between *Lineus ruber* and *Lineus viridis*) or was too high to calculate.

Fisher's (1935) Exact test for deviations of observed genotype frequencies from those expected under Hardy-Weinberg gave significant results for two species, *Micrura purpurea* and the cryptic species described in Chapter 5. Deviations of observed genotype frequencies in these two species from those expected under Hardy-Weinberg conditions also effect the sampling error of genetic distance (Nei & Roychoudhury, 1974). This may cause further deviation of observed D values for comparisons with these species from real D values.

Though the number of enzyme loci used in this study were low the data indicate a number of things. Genetic identity between conspecific populations was high (e.g. Oban, Llandudno and Anglesey for *Lineus ruber*; Oban, Whitehaven and Llandudno for *Lineus*

viridis) as expected from data on other taxa (Thorpe, 1982, 1983; Ayala, 1983; Nei, 1987). Genetic identities between species from within the genera *Lineus* and *Micrura* were very low for intrageneric comparisons and were similar to those found between confamilial genera in other organisms (Thorpe, 1982, 1983). Similarly low levels of genetic identity have been found in a previous study of three members of the genus *Lineus* (Williams *et al.* 1983) and also between species of *Oerstedia* (Sundberg & Janson, 1988).

The high levels of genetic differentiation between species which are morphologically similar and are therefore considered to be congeneric by conventional taxonomic methods could arise in two ways. It is possible that nemerteans do not show the same relationship between taxonomic separation and genetic identity or distance as in other taxa. There are a few instances of animal groups undergoing rapid speciation, e.g., passerine birds, in which rapid morphological adaptation to unexploited niches is thought to have permitted little biochemical evolution with the result that species have unusually high genetic identities (Corbin, *et al.*, 1974; Baker, 1975; Martin & Selander, 1975; Handford & Nottebohm, 1976; Smith & Zimmerman, 1976; Barrowclough & Corbin, 1978; Corbin *et al.* 1979; Avise et al. 1980; Avise & Aquadro, 1982; Thorpe, 1982). It is not entirely impossible that nemerteans show rapid biochemical evolution with little morphological change, but it is considered unlikely since at the molecular level rates of evolution are generally considered to be relatively constant (Wilson *et al.*, 1977; Thorpe, 1982; Nei, 1987).

In most cases where unexpectedly low genetic identities have been found between taxa, doubt has been cast on the systematics of the group concerned rather than on the molecular evolutionary clock (Thorpe, 1982). In microbial organisms morphologically very similar strains can show large genetic distance values e.g. *Escherichia coli* (Whittam *et al.*, 1983). Solé-Cava *et al.* (1991a) found extremely low genetic identities between supposedly conspecific populations of the sponges *Clathrina clathrus* and *Clathrina cerebrum*. Genetic identity values between allopatric samples of these species were again

similar to those found in confamilial genera in other taxa. Both bacteria and sponges have relatively few morphological characters which are useful in taxonomy. A similar situation arises in nemerteans where, though there is a fair number of internal morphological characters, the descriptions of these are incomplete for many taxa and the taxonomic significance of the characters is unknown or at best very subjective. It is more likely that low genetic identities between allegedly congeneric species of *Lineus* and *Micrura* result from fundamental problems in heteronemertean systematics rather than unusual forms of speciation.

A more detailed examination of genetic identity data and the dendrogram derived by UPGMA cluster analysis suggests that *Lineus sanguineus* and the cryptic species found in populations of *Lineus ruber* and *Lineus viridis* are both genetically unrelated to any of the other species examined in this investigation. Nei's (1972) genetic identity (I) between *Lineus sanguineus* and other members of the genus *Lineus*, including the type species *Lineus longissimus*, fall below levels of I which may be expected to occur by chance alone (estimated at about 0.05 [Thorpe, 1982]). The two populations of this species form a separate branch on the dendrogram at a similarity level near zero. *Lineus sanguineus* is known to possess a slender diagonal muscle layer in the body wall which is apparently not present in *Lineus ruber*, *Lineus viridis* or *Lineus longissimus* (Gibson, 1982b). This additional layer of body wall musculature may be of major taxonomic significance and indeed would be considered to be so in many other phyla.

Lineus longissimus and *Lineus ruber* form one group on the dendrogram whilst *Micrura fasciolata*, *Micrura purpurea* and *Lineus viridis* form a separate group. In both these groups, genetic identities fall below values normally associated with congeneric species. If nemerteans show levels of genetic separation in relation to taxonomic separation similar to those found in other taxa (see Thorpe, 1982, 1983) then these two groups may represent separate families, with each species probably best placed in a separate genus. Errors in genetic distance calculations discussed previously must be considered, but the very large and relatively consistent levels of genetic divergence found

between the species studied supports the hypothesis that there are fundamental flaws in the current systematics of these groups.

Confusion between the genera *Lineus* and *Micrura* is not surprising since neither have been adequately defined (Friedrich, 1960; Cantell, 1975; Gibson, 1981, 1982a, 1985). Gibson (1990b) summarised some of the major anatomical characteristics of heteronemertean genera included in the family Lineidae. The eight major characteristics he recorded were:

1: Number of muscle crosses in the proboscis.

2: Dermis with (+) or without (0) a connective tissue layer between the gland cells and body wall outer longitudinal musculature.

3: Rhynchocoel wall circular muscles interwoven with adjacent body wall inner longitudinal muscle fibres (+) or quite separate (0).

4: Nature of foregut somatic musculature: 0=absent, c=circular, l=longitudinal.

5: Presence (+) or absence (0) of neurochord cells in the nervous system.

6: Presence (+) or absence (0) of a caudal cirrus.

7: Foregut with (+) or without (0) a subepithelial gland cell layer.

8: Blood system in foregut region developed into a vascular plexus (+) or consisting of a pair of vessels only.

For the genera *Lineus* and *Micrura* the following characteristics were given:

Genus	1	2	3	4	5	6	7	8
Lineus	0-2	0-+	0	c/l	0	0	+	0-+
Micrura	0-2	0-+	0	0/c/l	0	0-+	0	+

The only difference between the two genera on the basis of these characters is the presence or absence of a subepithelial gland cell layer in the foregut region. It is unlikely that this characteristic has even been recorded for most species of *Lineus* or *Micrura* and,moreover, this characteristic is of unknown systematic value. The presence of a caudal cirrus is often used as a diagnostic feature of the genus *Micrura* (Gibson, 1981), but such a structure, which has no obvious advantage to the fitness of a nemertean in the

wild, may well easily be lost in the course of evolution. The genera *Lineus* and *Micrura* are so poorly defined that they are inseparable on on the basis of the morphological characters given above. Some characters may distinguish the two genera, e.g., the cephalic slits opening into large posterior bays in *Micrura* but not in *Lineus* or the occurrence of an outer neurilemma in the nervous system of *Lineus* but not in *Micrura* Friedrich, 1960). These characters though are recorded for very few species. The two groups are almost certainly, on both the genetic evidence of this investigation and from morphological evidence, polyphyletic.

It would appear from this investigation that there are major and fundamental taxonomic problems within the order Heteronemertea. If the findings of this investigation are correct then the family Lineidae sensu Gibson 1985, is polyphyletic. The oldest and largest genera of the Lineidae lack any firm diagnosis, are clearly polphyletic, and the present system of classification at this taxonomic level urgently requires complete revision (see Sundberg, 1991).

Chapter 7

A New Species of *Callinera* (Nemertea, Anopla, Palaeonemertea) From the Isle of Man.

7.1 Introduction

Palaeonemerteans are a group of benthic marine worms typically found in muddy or sandy sediments (Gibson, 1982a). Of the four families at present recognised (Carinomidae, Hubrechtidae, Statolitonemertidae, Tubulanidae) (see Gibson, 1982a; Korotkevich, 1982), the Tubulanidae, containing the genera *Callinera*, *Carinesta*, *Carinina* and *Tubulanus*, "maintains the most primitive characters" (Hylbom, 1957: 575). In the Tubulanidae the brain and lateral nerve cords are situated either in the epidermis (Carinina) or between the epidermal basement membrane and the outer circular muscle layer of the body wall (*Callinera*, *Carinesta*, *Tubulanus*). The body wall musculature comprises outer and inner circular and middle longitudinal layers, and cerebral sensory organs are either extremely simple or completely missing.

During 1989 and 1991 specimens of a previously unknown nemertean species were found in mud and muddy sand at Derbyhaven (south-east Isle of Man, Fig. 2.8). Histological studies reveal that they belong in the genus *Callinera*, a taxon not hitherto recorded from the British Isles (Gibson, 1982b), but that they constitute a new species for which the name *Callinera monensis* sp. nov. is proposed.

7.2 Materials and Methods

Two specimens of *Callinera monensis* sp. nov. were found during October 1989 at Derbyhaven, Isle of Man (approx. 54°04'N, 4°37'W). Both were discovered intertidally, one in mud near low water neap level, the other at about mid-shore in fairly anoxic mud. A third individual was obtained from a depth of 8-10 cm in muddy sand at the same location during March 1991. One specimen was fixed in Bouin's fluid, sectioned transversely at 6 mm in 56°C m.p. paraffin wax and stained by the Mallory triple method (see Section 2.7). The anatomy of this individual has been compared with that of sectioned specimens of *Callinera buergeri* and *Callinera buergeri f. grandis* loaned by the Naturhistoriska Riksmuseet, Stockholm, courtesy of Dr L. Sandberg.

7.3 Results

7.3.1 Diagnosis

Callinera Bergendal, 1900

Callinera buergeri Bergendal, 1900

Hylbom (1957: 552) gave the following diagnosis for the genus: "Nervous system situated between the basement membrane and the outer circular muscle layer. No cerebral sense organs. In the posterior end of the proboscis sheath a peculiar, well-developed muscle sac. Basement membrane thin."

***Callinera monensis* sp. nov. (Figs 7.1-19)**

Type locality

Derbyhaven, Isle of Man; intertidal in beach and harbour mud (October 1989) or muddy sand (March, 1991).

Type material

Holotype sexually immature, complete specimens sectioned transversely (70 slides), deposited in the Liverpool Museum, William Brown Street, Liverpool L3 8EN, Registration Number 1990.141 (LIV).

Etymology

The specific epithet, referring to the type locality, is based on the Latin name *Isla Monensis* for the Isle of Man.

7.3.2 Description

External features

The three individuals were virtually identical in their external appearance, 31.5-35 mm long and 1.5 mm in maximum width. Each possessed a moderately stout body, approximately cylindrical in form, with a distinctly pointed head, 1.5-2.0 mm long, demarcated from the rest of the body by a slight constriction. The anterior portion of the body was the widest, gradually narrowing posteriorly to end in a blunt tail. The small mid-ventral mouth was located immediately behind the head.

The dorsal body surface was greyish-white, the ventral surface white; under magnification minute white pigment granules were distinguishable on or below the epidermal surface against a pale grey background. The head somewhat flattened dorsoventrally, was more translucent than the remainder of the body.

Body wall, musculature and parenchyma.

The richly glandular epidermis is about 60-75 μm in maximum height in the cephalic and foregut regions of the body (Fig.7.1), but throughout the intestinal region is generally less than 35-40 μm high. Large numbers of rhabditoid cells, many filled with coarsely granular acidophilic contents but more often having apparently discharged their secretions and appearing vacuolate (Fig. 7.2), are predominantly restricted to the distal half of the epidermis; in contrast the remaining gland cell types mostly occupy the proximal zone. Throughout the length of the body delicate radial lamellae, presumably derived from the underlying epidermal basal lamina, penetrate the epidermis but do not extend for more than about half its height. The organisation of the epidermis thus closely agrees with that illustrated by Norenburg (1985: fig.1) for a generalised archi- and palaeonemertean type. The appearance of the epidermis in specimens of *C. buergeri* and *C. buergeri f. grandis* examined closely corresponds with that of the present form, although in *f. grandis* the epidermis is much thicker and in places has a height of 125-150

μm or more.

The dermis (Figs. 7.1,2) is typically less than 4-5 μm thick except where the body wall is locally contracted, when it may be 12-15 μm deep.

The body wall musculature possesses a complex arrangement. The longitudinal layer is particularly strongly developed and for much of the body length is 230-275 μm in maximum thickness (Fig. 7.1); in *C. buergeri* and *C. buergeri f. grandis* this layer is at most only 45-50 μm and 125-130 μm thick, respectively. In contrast the outer and inner circular muscle layers of the present species are weaker and only 20-25 μm thick. The inner circular layer extends from the oral region back into the anterior intestinal portion of the body, whereas the outer circular layer reaches the full body length. The first traces of the inner circular musculature appear above the mouth, where fibres of the outer circular layer lead inwards from the dorsal margin, pass between the longitudinal muscle fibres and then spread outwards to run around the outer margin of the spacious lateral blood vessels. Behind the mouth these fibres join ventrally below the foregut, but dorsally they retain their link with the outer circular muscles and do not form a complete inner circular layer until they near the posterior portion of the rhynchodaeum (Fig. 7.3). Throughout the remaining foregut and anterior intestinal regions the inner circular muscles enclose the rhynchocoel, blood vessels and gut, their fibres dorsally merging with the circular muscles of the rhynchocoel wall (Fig. 7.1). Occasional muscle fibres extend between the longitudinal fibres to link the outer and inner circular layers above the rhynchocoel. Behind the junction between foregut and intestine the lateral blood vessels pass through the inner circular muscle layer to continue for the remaining body length adjacent to the main body wall longitudinal muscle layer (Fig. 7.2). In the foregut region there is also an inner longitudinal muscle layer. Where the lateral blood vessels move outside the inner circular muscle layer, the inner longitudinal muscle fibres form a U-shaped coat, dorsally incomplete, extending on either side of and below the rhynchocoel (Fig. 7.2), but where the blood vessels run internal to the inner circular muscles the inner longitudinal musculature is separated into three blocks of fibres. These comprise small bundles

extending on either side of the rhynchocoel and a large median muscle plate reaching forwards between the rhynchocoel and foregut (Fig. 7.1). The longitudinal muscle plate, 60-100 μm or more thick, continues forwards to the oral region, in front of the proboscis insertion. Longitudinal muscle fibres enclosing the posterior portion of the rhynchodaeum merge with those of the muscle plate (Fig. 7.3). In front of the mouth the longitudinal muscle layer surrounding the rhynchodaeum is 30 μm or more thick (Fig. 7.4) and continues forwards, past the brain, to the tip of the head. Pre-cerebrally the rhynchodaeum and its longitudinal muscles, almost completely enclosed by the cephalic blood supply (Fig. 7.5), are suspended by delicate dorsoventral connective tissue strands.

Throughout the cephalic region the body wall outer circular and longitudinal muscle layers, both of which reach the tip of the head, are thinner than in other parts of the body. Radially orientated strands lead off peripherally from the circular muscle layer, pass through the cephalic neural sheath which encircles the head below the epidermal basement membrane (Fig. 7.6) and penetrate the proximal portions of the epidermis.

No dorsoventral muscle bundles could be distinguished in the intestinal region of the body, and the parenchymatous connective tissues are sparingly developed.

Although broadly similar in their overall arrangement, certain differences can be distinguished between the muscle systems of *C. buergeri*, *C. buergeri f. grandis* and the Isle of Man species. In *f. grandis* large numbers of irregularly shaped and variably sized subepidermal glands, filled with coarsely granular cytoplasm, are situated between the fibres of the principle body wall longitudinal muscle layer throughout much of the body length; neither *C. buergeri* nor the present form possess comparable intramuscular glands. The development of the longitudinal muscle plate between the gut and rhynchocoel also differs between the taxa; in *f. grandis* the plate is mostly only 30-40 μm deep, whilst in *C. buergeri* it is very weakly formed and rarely exceeds a thickness of more than a few fibres. Additionally, the distinct dorsolateral bundles of inner longitudinal muscle fibres flanking the rhynchocoel of the present form are completely

missing from *C. buergeri*, but in *f. grandis* consist of a small number of isolated fibres enclosed by longitudinal wedges of parenchyma. Further differences can be found in the posterior extent of the inner circular musculature; in both Scandinavian taxa traces of this layer can be discerned far back in the hind part of the body, in the vicinity of the powerful rhynchocoel muscle sac which is a characteristic feature of the genus, whereas in the present form the layer ends much further forwards.

Proboscis apparatus.

The proboscis pore opens at the tip of the head. It leads into an exceptionally long, tubular rhynchodaeum whose epithelium is essentially organised into 4 longitudinal blocks of tissue, each 30 μm or more in maximum thickness (Figs. 7.3-5), enclosed by a thin but distinct connective tissue membrane. The epithelium is at first neither ciliated nor glandular, but near the brain and for its remaining length contains isolated gland cells (Figs. 7.4, 7). An unusual feature of the rhynchodaeum is that it is ventrolaterally innervated by a pair of thick neural tracts (Fig. 7.8) leading from the anteroventral portions of the cerebral ganglia. The fate of these nerves after they enter the rhynchodaeal wall is uncertain; traces of nerves running in the epithelium further back could be distinguished and it seems probable that they represent the origin of the proboscis neural supply. This suggestion is supported by the far post-cerebral position of the proboscis insertion and absence of other nerve tracts leading to this organ. Towards its rear the rhynchodaeum narrows and is enclosed by a sphincter-like layer of circular muscles 20-25 μm thick (Fig. 7.9). The overall length of the rhynchodaeum is about 1.3 mm, its posterior limit being well behind the mouth and far behind the brain. A similar post-cerebral insertion of the proboscis was first reported for *C. buergeri*, Bergendal (1900a: 317) noting that "die über der Mundöffnung gelegene Insertion des Rüssels" (the proboscis insertion is situated behind the mouth). The organisation of the rhynchodaeum in both Scandinavian taxa closely agrees with that described for the present form, although the origin of the presumed proboscis nerves, distinguishable in *f. grandis*, could

not be confirmed in *C. buergeri*.

The extreme anterior portion of the rhynchocoel bulges forwards on either side of the proboscis insertion to form caecum-like protrusions extending into the lateral blood vessels (Fig. 7.10); it is possible these have developed as a consequence of fixation. Behind the proboscis insertion the rhynchocoel wall is first thin (Fig. 7.11) and composed of a layer of connective tissue externally clad by a weakly developed layer of circular muscles. In the posterior foregut region, however, the circular muscles become more strongly developed and are 12-15 μm or more thick (Figs. 7.1,2). No evidence of longitudinal muscle fibres could be distinguished in any part of the rhynchocoel wall; in the Scandinavian forms isolated muscle strands can be found running along the inner border of the rhynchocoel, but there is no distinct longitudinal muscle layer as is characteristic of most nemerteans. The rhynchocoel reaches almost to the posterior tip of the body, its hind portion being developed into an immensely thick (up to 600 μm or more) muscular sac (Fig. 7.12) similar to those seen in the sections of both Scandinavian taxa examined during the present study and previously illustrated for *C. buergeri* (for example, see Bergendal 1900a: Fig. 7.2; 1900b: pl. I, Figs. 7.2, 3, 14; 1901, Fig. XIX). The sac is located on the ventral side of the rhynchocoel and presses down against the intestinal wall.

The proboscis is comparatively slender, with a maximum retracted diameter of 200-230 μm, but it possesses a complex construction in which several histologically distinct regions can be recognised. A short anterior portion, leading from the proboscis insertion, comprises a thin and rather flattened epithelium with no distinguishable gland cells, a longitudinal muscle layer organised into four distinct blocks of fibres (Fig. 7.11, 7.13A) 80-90 μm in maximum thickness, a thick connective tissue zone 8-10 μm across, and a delicate lining endothelium. Two large nerves are situated between the epithelium and underlying musculature on opposite sides of the proboscis (Fig. 7.13A). This part of the proboscis soon merges into a short second region, in which the epithelium is 8-15 μm thick and composed largely of basiphilic gland cells with a vacuolate appearance. In

the third region the epithelium is up to 30 μm or more thick and along one side contains dense distal accumulations of strongly acidophilic granular bodies, 12-15 μm long and 3-4 μm in maximum width, which somewhat resemble the rhabditous barbs described from the proboscis of a number of archi- and heteronemertean taxa (for example, see Iwata, 1967; Jennings & Gibson, 1969). The longitudinal musculature of this region initially retains its separation into four discrete fibre bundles and is at most 7-8 μm thick and intimately intermingled with connective tissue, but further back, as the granular acidophilic bodies begin to disappear from the epithelium, the muscle fibres become organised into a single asymmetrically developed layer, 20-25 μm thick on one side of the organ but virtually indistinguishable on the other. The fourth proboscis region, accounting for approximately two-thirds of its overall length, is also asymmetrically developed (Fig. 7.13B). This portion consists of a richly glandular epithelium, 20-95 μm tall, in which basiphilic glands predominate on one side, acidophilic on the other. Throughout much of this region the distal margins of the acidophilic half bear large numbers of spherical or ovoid, coarsely granular, structures 2-3 μm in maximum diameter (Fig. 7.13B). The epithelium is in turn underlain by a distinct outer connective tissue layer, a longitudinal muscle coat, an inner connective tissue zone and a flattened endothelium. The longitudinal muscles are most strongly developed below the acidophilic half of the epithelium; elsewhere they are rarely more than one or two fibres thick. In the fifth and final portion of the proboscis, forming a relatively short posterior region, the epithelium is reduced to a maximum height of about 15 μm, completely lacks the granular spheres and has its few basiphilic glands restricted to a narrow band along one side. Both connective tissue layers are reduced to thin membranes, and the longitudinal musculature forms a distinct layer, 5-15 μm thick, on all sides of the proboscis. Only in this portion of the proboscis can any evidence of circular muscle fibres be found; a thin, often indistinct, layer of fibres extends between the epithelium and outer connective tissue zone. The proboscis retractor muscle is connected to the rhynchocoel wall at the front of the muscular sac.

The proboscides of the two Scandinavian taxa generally resemble that of the Isle of Man form both in containing several histologically distinct regions and in exhibiting asymmetrical development; they differ significantly, however, in that they possess an additional region between the anterior and basiphilic portions which is not found in the present species. In this extra region the longitudinal muscles form a complete layer rather than being organised into four distinct bundles, and a circular muscle coat up to 15 μm or more thick is situated between the epithelial basement membrane and the longitudinal musculature.

Alimentary Canal

The small ventral slit-like mouth opens a short distance behind the brain. The spacious foregut is lined by a folded, richly glandular and densely ciliated epithelium, thicker ventrally (75-90 μm) than dorsally (40-60 μm) (Fig. 7.1). In its anterior region the foregut is dominated by large numbers of strongly acidophilic gland cells which occupy much of the proximal two-thirds of the epithelial height; basiphilic glands are fewer in number and mainly restricted to the distal epithelial margins (Figs. 7.9-11). There is a progressive reduction in the density of the acidophils posteriorly and they become largely replaced by basiphils, but towards the rear of the foregut there is a general decrease in the abundance of all gland types.

The junction between the foregut and intestine is marked by the sudden appearance of pyriform gastrodermal glands, filled with strongly acidophilic and finely particulate cytoplasm; in the same region the epithelial cilia are longer but much less densely distributed. Approximately 20-25% of the way along the intestine the appearance of the gland cells begins to change; the glands become progressively less densely packed, lose their acidophilic nature and contain a more homogenous cytoplasm. Towards the rear of the gut there are few or no gland cells in the dorsal intestinal wall (Fig. 7.12), and glands are missing entirely from the short rectal region. The anus opens at the posterior tip of the body. Throughout its length the intestine possesses shallow lateral pouches but

lacks distinct diverticula.

The organisation of the gut in the Scandinavian taxa shows no significant differences from that found in the present form.

Blood system.

The cephalic blood supply essentially consists of a single, thin walled spacious lacuna in the middle of which the anterior portion of the rhynchodaeum and associated longitudinal muscle fibres are suspended by dorsoventral connective tissue strands (Fig. 7.5). In several places, however, extensions of the body wall longitudinal muscle layer alongside the connective tissue strands subdivide the cephalic lacuna into large lateral and smaller dorsal and ventral channels (Fig. 7.14); a similar condition pertains in *C. buergeri*, but in *f. grandis* a separation into 4 distinct thicker walled vessels is much more pronounced and for most of the cephalic length they remain quite separate. In the Isle of Man form the lacuna near the front of the brain separates mid-dorsally, whilst the ventral portion becomes further subdivided by connective tissue and muscle fibrils to provide a distinct median duct (Figs 7.7, 8). As it passes the brain the blood system thus comprises a pair of large lateral lacunae and a smaller mid-ventral channel. The ventral branch is dorsally separated from the lateral lacunae by fibres of the body wall outer circular muscle layer, but is otherwise almost totally enclosed by the longitudinal muscle fibres which pass through the hemispherical loop of the ventral cerebral commissure (Fig. 7.4). Towards the rear of the brain the ventral channel divides and rejoins the lateral lacunae.

Behind the brain the two voluminous lateral lacunae continue back on either side of the rhynchodaeum and above the dorsolateral margins of the foregut (Fig. 7.3), to run internal to the body wall inner circular muscle layer (Fig. 7.1). At the proboscis insertion the anterior portion of the rhynchocoel bulges into the lacunae (Fig. 7.10), behind which the lateral channels become smaller and less spacious (Fig. 7.11).They continue back on either side of the rhynchocoel into the anterior intestinal region and then extend outwards, pass through the inner circular muscle layer, and continue for the remaining body length

adjacent to the inner border of the principle body wall longitudinal muscle layer (Fig. 7.12). Throughout the anterior half of the body the blood channels are thin-walled, but in the posterior half, where they are much smaller, they develop thicker walls in which a layer of circular muscle fibres can be distinguished. The lateral vessels join posteriorly via a sub-intestinal connective just in front of the anus. There are no pseudometameric transverse connectives between the lateral vessels in the intestinal region, as found in many anoplan taxa, nor is there a mid-dorsal vessel. The organisation of the blood system in the intestinal region of both Scandinavian forms broadly agrees with that described for the present species; in neither, however, does the rhynchocoel anteriorly protrude into the lateral vessels at the proboscis insertion.

Nervous system.

The brain and lateral nerve cords are situated between the dermis and body wall outer circular muscle layer.

The cerebral ganglia are positioned ventrolaterally, dorsal and ventral lobes being similar in size. A thin but distinct connective tissue inner neurilemma encloses the fibrous components of the brain (Fig. 7.4), whereas the outer neurilemma investing the neuroganglionic tissues is less apparent and peripherally indistinguishable from the dermal connective tissues. Neuroganglionic cells are missing from the inner brain margins, the inner neurilemma here abutting directly against the body wall circular muscle fibres.

The ventral cerebral commissure, 30 μm thick, forms a distinct loop across the body (Fig. 7.4). In contrast, the dorsal commissure is extremely long, thinner (10 μm) and loops anterodorsally to cross the body some distance in front of the brain. Bergendal's (1900a: 314) statement that in *C. buergeri* "die dorsal [Commissur] ist nicht sehr lang. Außer der gewöhnlichen existiert eine zweite, von den hinteren Theilen der dorsalen Ganglien ausgehende dorsale Commissur" (The dorsal commissure is not very long. In addition to the usual one there exists a second, from the hind part of the dorsal

ganglia behind the dorsal commissure) is confirmed in sections of this species examined during the present investigations, and the same situation prevails in *f. grandis*. In contrast, no evidence of a second dorsal commissure could be found in the Isle of Man species. There are neither neurochord cells in the brain nor neurochords in the lateral nerves. The longitudinal nerves extend to the posterior tip of the body close to the lateral margins (Fig. 7.1), meeting immediately in front of the anus by a sub-intestinal commissure. Occasional longitudinal muscle fibres (myofibrillae) are distinguishable in the fibre cores of the lateral nerves. Neuroganglionic tissues associated with the nerves are confined to a peripheral zone adjacent to the dermis (Fig. 7.15) and are frequently subdivided by concentric layers of connective tissue arranged more or less parallel to the longer axis of each nerve.

The peripheral nervous system, particularly in the head, is extremely well developed. In front of the brain large numbers of cephalic nerves leads forwards from each of the cerebral lobes and the dorsal commissure to form a distinct peripheral layer, completely encircling the head, between the dermis and the body wall outer circular muscle layer (Figs. 7.5, 6). This layer, up to about 25 μm thick, is more or less regularly split into discrete nerves by radially oriented connective tissue and muscle fibres; at least 50 such nerves can be counted in any one section. The cephalic nerve ring reaches to the tip of the head. The situation is similar in *C. buergeri*, but *f. grandis* differs in possessing additional bundles of large nerves, some 60 μm or more in diameter, running in the longitudinal musculature under the neural sheath above and below the rhynchodaeum in the posterior half of the head.

Other peripheral nerves include a small but distinct median dorsal nerve, arising from the rear of the dorsal commissure. This nerve extends the full length of the body immediately below the dermis. *Callinera buergeri* similarly has a single mid-dorsal nerve, but in *f. grandis* a second, deeper, nerve extends back adjacent to the dorsal wall of the rhynchodaeum and rhynchocoel; radial fibrils intermittently link the mid-dorsal and deeper dorsal nerves, both of which can be followed well back into the intestinal region,

in this taxon.

The foregut neural supply has its origin in a pair of nerves emanating from the rear of the ventral cerebral lobes where these lead into the lateral nerve cords. The two foregut nerve roots lead obliquely inwards between the longitudinal muscle fibres adjacent to the ventral branch of the cephalic blood lacuna, meeting just behind where this rejoins the lateral lacunae to form a single large median ventral nerve, 70-75 μm in diameter (Fig. 7.16). This extends posteriorly for only a short distance and then forks to form two large nerves (Fig. 7.17) which gradually move apart as they approach the mouth. As they pass the mouth each nerve branches into two (Fig. 7.18), becomes smaller and then moves immediately adjacent to the foregut basement membrane. There is no post-oral commissure and the ultimate fate of the foregut nerves could not be traced. The cerebral origin of the foregut nerves is similar in both Scandinavian taxa. In *f. grandis*, however, the nerves, each 50 μm or more in diameter, remain distinct but are briefly joined transversely by a short commissure about 15 μm thick. Behind the mouth about 5-6 small but easily distinguishable nerves can be followed for much of the foregut length. A different condition prevails in *C. buergeri*, where a short single median ventral nerve is formed by fusion of the two foregut nerve roots, as in the Isle of Man form, but after this has posteriorly forked the two nerves so formed do not further branch, rapidly become smaller and cannot be traced beyond about mid-oral level.

Sensory organs.

As in *C. buergeri* (Bergendal 1900a, b, 1901), the present species possesses neither ocelli nor cerebral sensory organs; it also has no frontal (apical) sensory structure, and cephalic glands are entirely missing.

Bergendal (1900a: 314) briefly mentioned a pair of lateral sensory organs situated in the epidermis of *C. buergeri* " auflen von den Seitenstämmen gelegne...unmittelbar vor den Ausmündungsöffnungen der Nephridien", illustrating their appearance and position in this 1900b (fig. XIV, pl.I, fig. 1) article. In sections of this species examined during

the present study these lateral organs were easily distinguished from the adjacent epidermis by their dense ciliation and absence of gland cells. Comparable structures could not be found in *f. grandis*, nor in the Isle of Man species.

In the present form a pair of short lateral furrows is evident in the epidermis adjacent to the rear of the brain (Fig. 7.19). The cilia lining these furrows are approximately as long as, and more sparsely distributed than, those covering the remainder of the body surface, and the wall of the furrows contains very few gland cells in comparison with that of the adjacent epidermis. No innervation to the furrows could be discerned, however, and whether or not they are sensory cannot be confirmed. Neither *C. buergeri* nor *f. grandis* have these furrows.

Excretory system.

The excretory system is located entirely within the anterior intestinal portion of the body. Soon after the lateral blood lacunae move outside the inner circular muscle layer, which occurs beyond the junction between foregut and intestine, a single nephridial tubule 25-30 μm in diameter appears in each lacuna. The tubules extend posteriorly for a short distance and then emerge from the blood channels, leading to thick-walled ciliated collecting ducts, 60-70 μm or more in diameter, which continue backwards close above the blood lacunae (Fig. 7.2) for about 1.7 mm. Near their posterior limits each collecting canal narrows before leading dorsolaterally to open at a small nephridiopore on the body surface. The position, extent and organisation of the excretory systems in *C. buergeri* and *f. grandis* are similar, although in sections of *C. buergeri* examined no evidence of tubules running inside the blood vessel lumen could be found.

Reproductive system.

The holotype is an immature male. The testes are situated on either side of the gut, above the lateral blood vessels, in the extreme posterior portion of the body behind the end of the rhynchocoel muscle sac. Most of the testes appear as hollow, spherical

pouches 35-40 μm in diameter, enclosed by thin but distinct connective tissue membranes, but a few are larger, ovoid, up to 90-100 μm in maximum dimension and contain what appear to be sperm tails. No evidence of gonoducts or gonopores was found. The position of the reproductive system is the same as in specimens of *C. buergeri* examined, originally described and illustrated by Bergendal (1900a: 320, fig. 3).

7.3.4. Abbreviations used in figures.

AE	acidophilic portion of proboscis epithelium
BG	basiphilic gland of proboscis epithelium
BR	brain lobe
DE	dermis
DL	dorsal branch of cephalic lacuna
DN	mid-dorsal nerve
EF	epithelium of cephalic furrow
EP	epidermis
EX	excretory duct
FG	foregut
IC	body wall inner circular muscle layer
IL	body wall inner longitudinal muscle layer
IN	intestine
LC	lateral branch of cephalic lacuna
LL	lateral blood lacuna
LN	lateral nerve cord
LP	longitudinal muscle plate
MN	median foregut nerve
NS	cephalic neural sheath
OC	body wall outer circular muscle layer
OL	body wall outer longitudinal muscle layer
PC	proboscis connective tissue layer
PD	proboscis endothelium
PE	proboscis epithelium
PL	proboscis longitudinal muscle layer
PN	proboscis nerve
PR	proboscis
RC	rhynchocoel
RD	rhynchodaeum
RE	rhynchodaeal epithelium
RL	rhynchodaeal longitudinal muscles
RM	rhynchocoel muscle sac
SC	sphincter-like layer of circular muscle fibres and connective tissue
VC	ventral cerebral commissure
VL	ventral branch of cephalic lacuna

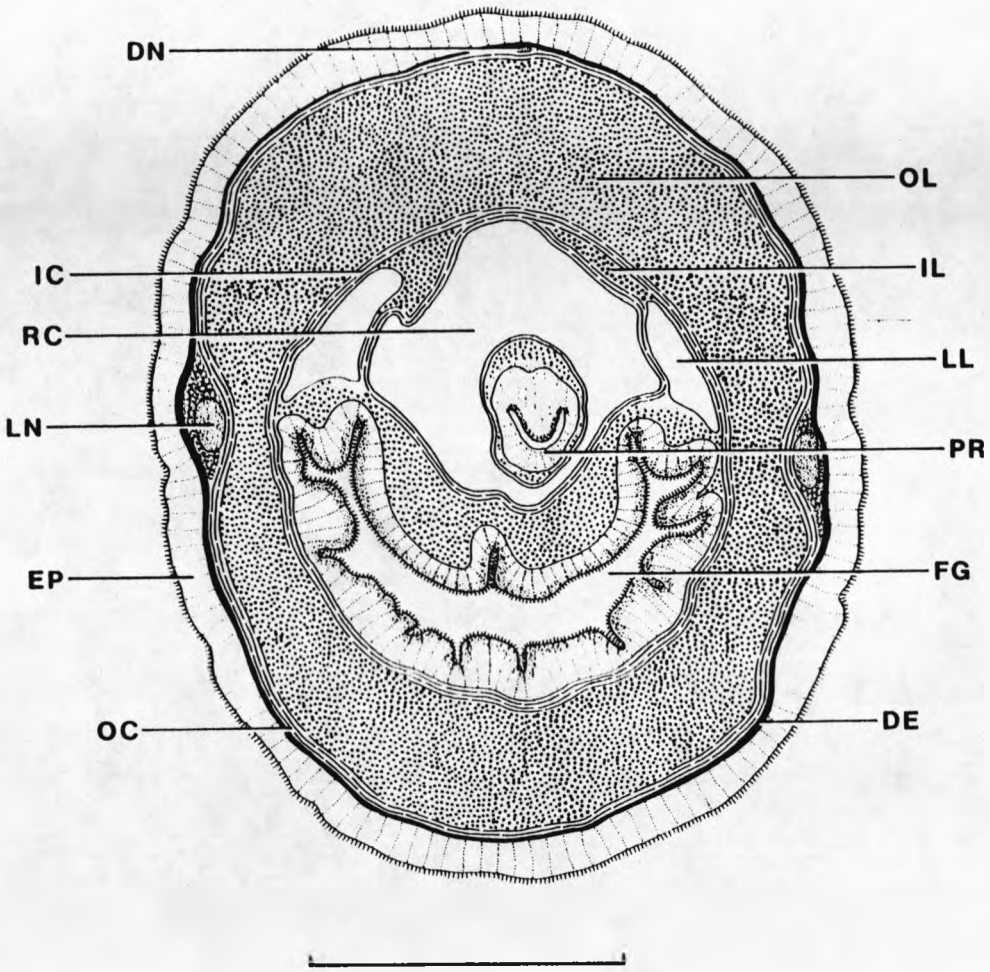


Figure 7.1. *Callinera monensis* sp. nov. Transverse section through the foregut region to show the general organisation of the various body structures. Scale: 500 μm.

Figures 7.2-7. *Callinera monensis* sp. nov. (Mallory).

-2. Transverse section through the anterior intestinal region to show a main excretory collecting duct, a lateral blood lacuna running outside the inner circular muscle layer, and the inner longitudinal muscle fibres extending alongside and below the rhynchocoel.

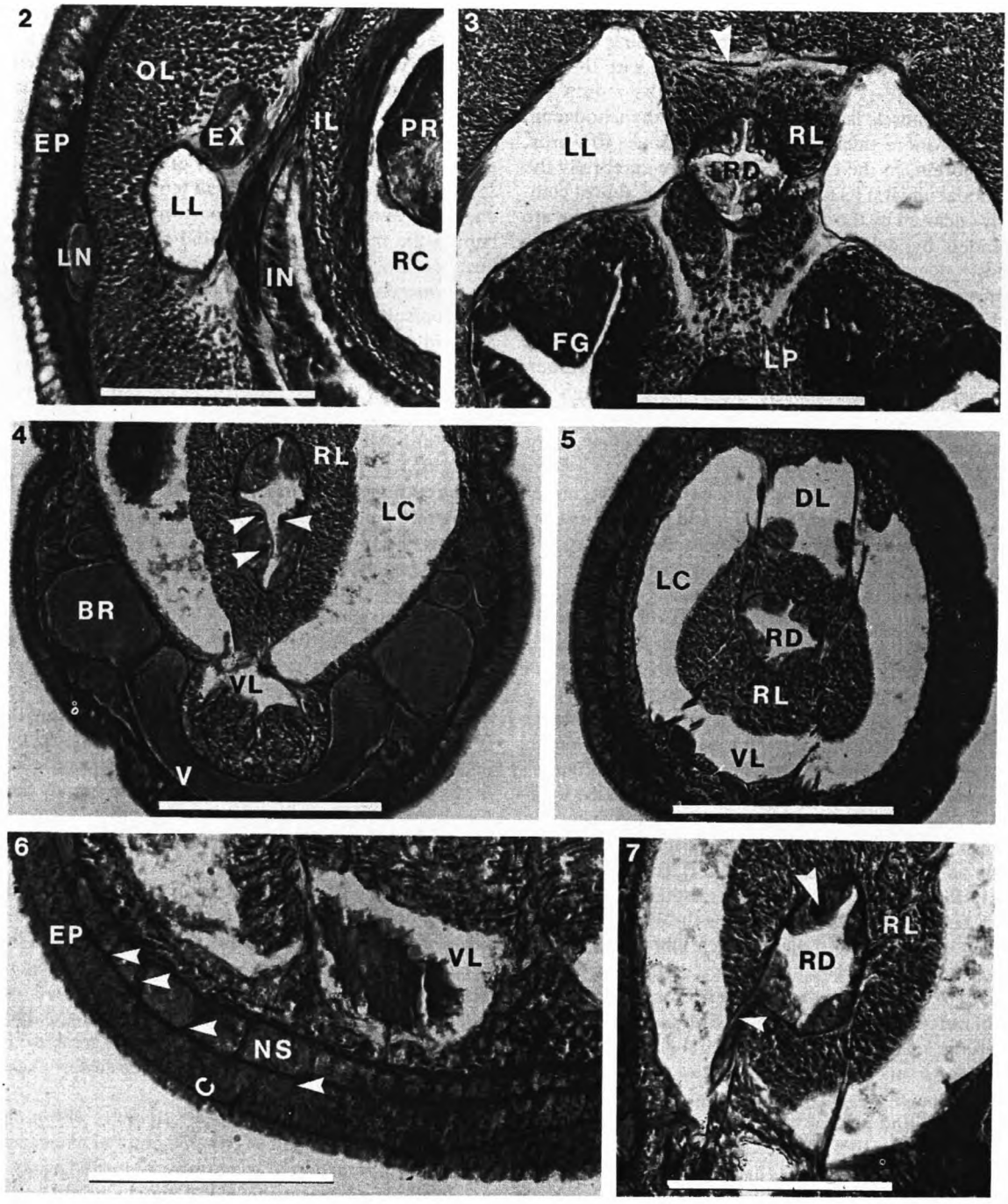
-3. Transverse section through the anterior foregut region to show the rhynchodaeum with its associated longitudinal musculature, the longitudinal muscle plate above the foregut, and the inner circular muscle fibres (indicated by an arrowhead) extending across between the lateral lacunae to dorsally complete this muscle layer.

-4. Transverse section through the brain region to show the ventral cerebral commissure and ventral branch of the cephalic blood lacuna, and the rhynchodaeum enclosed by a well developed layer of longitudinal muscle fibres. Gland cells in the rhynchodaeal epithelium are indicated by arrowheads.

-5. Transverse section through the head a short distance in front of the brain to show the organisation of the cephalic blood supply and the way in which the rhynchodaeum and its longitudinal muscle layer are suspended by dorsoventral connective tissue strands.

-6. Part of the head in transverse section to show the well-developed cephalic neural sheath extending between the dermis and body wall outer circular muscle layer. Radial muscle fibres passing through the neural layer into the proximal portion of the epidermis are indicated by arrowheads.

-7. Transverse section through the rhynchodaeum in the cerebral region to show a gland cell, indicated by the large arrowhead, and part of one of the rhynchodaeal nerve roots (small arrowheads). Scales 7.2-5 250 μm; 7.6 100 μm; 7.7 200 μm.


2
OL
EP
EX
IL
PR
LL
LN
IN
RC
3
LL
RL
RD
FG
LP
4
RL
LC
BR
VL
V
5
DL
LC
RD
RL
VL
6
EP
VL
NS
7
RL
RD

Figures 7.8-12. *Callinera monensis* sp. nov. (Mallory).

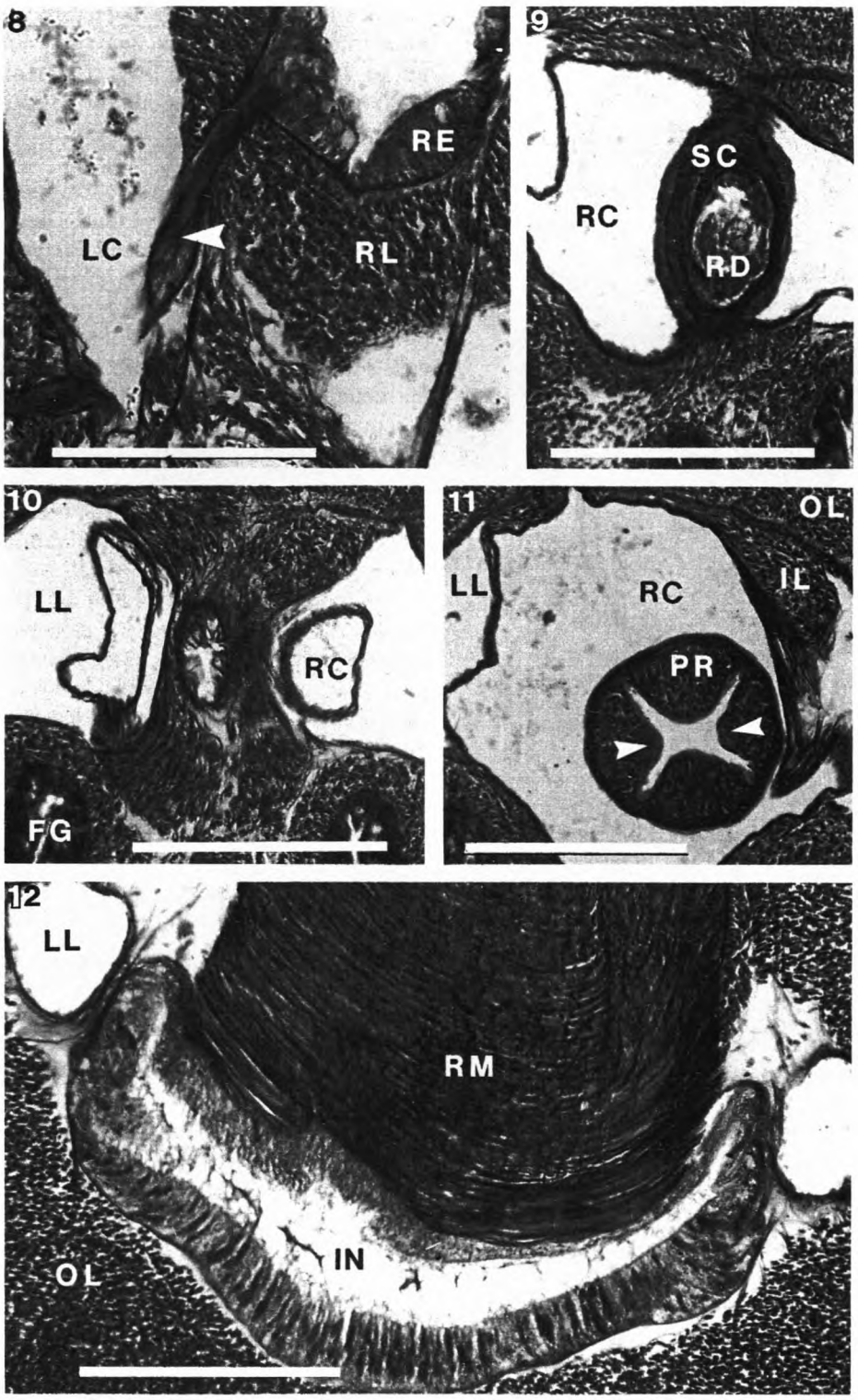
-8. Enlargement of part of the rhynchodaeal wall shown in Fig. 7.7; the rhynchodaeal nerve root is indicated by the arrowhead.

-9. Transverse section through the posterior region of the rhynchodaeum to show its sphincter-like layer of circular muscle fibres.

-10. Transverse section through the rhynchodaeum close in front of the proboscis insertion, showing how the anterior portion of the rhynchocoel bulges forwards on either side into the lateral blood lacunae.

-11. Transverse section through the anterior portion of the rhynchocoel to show its thin wall composed of connective tissue and weakly developed circular muscle fibres. The two nerves in the extreme anterior region of the proboscis are indicated by arrowheads; compare with Fig. 13A.

-12. Transverse section through the hind part of the body to show the powerful muscular sac associated with the posterior portion of the rhynchocoel. Scales: 7.8 100 μm; 7.9-12 250 μm.


8
RE
LC
RL
9
SC
RC
RD
10
LL
RC
FG
11
OL
LL
RC
IL
PR
12
LL
RM
IN
OL

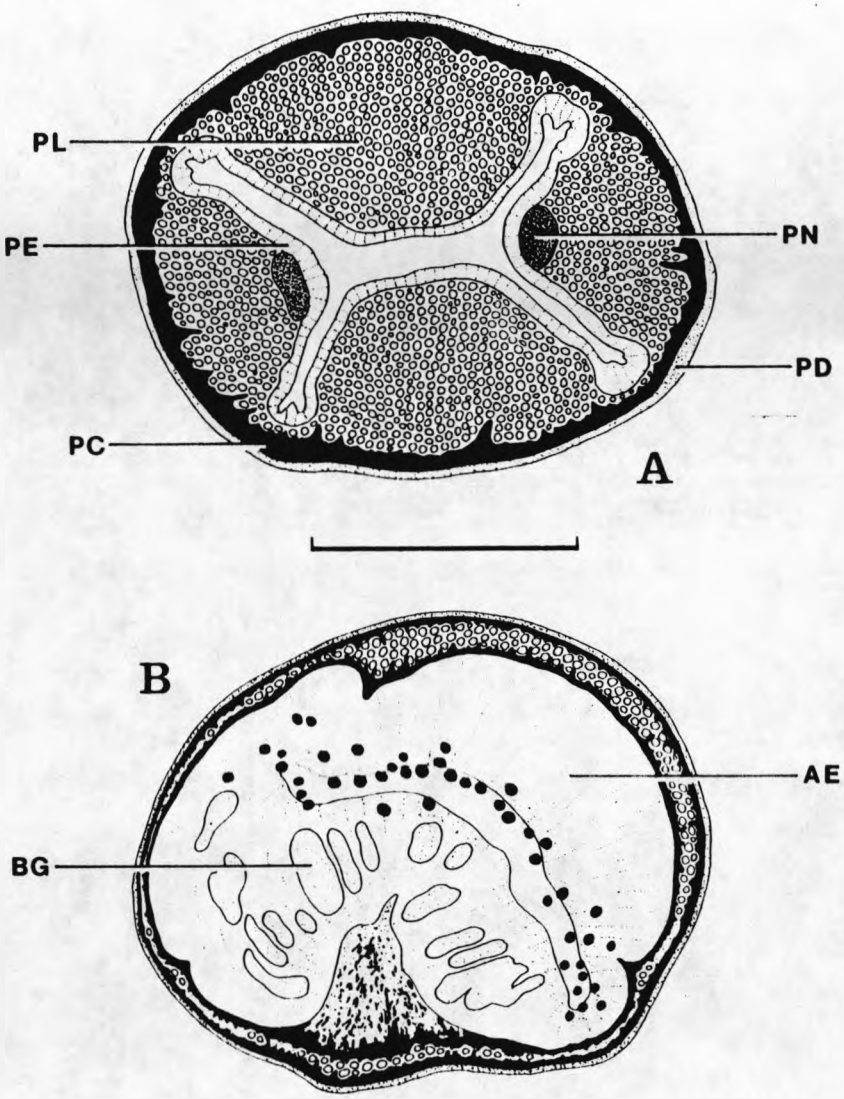


Figure 7.13. *Callinera monensis* sp. nov. Transverse sections to show the structure of the extreme anterior (A) and main fourth (B) regions of the proboscis. Scale: 100 μm.

Figures 7.14-19. *Callinera monensis* sp. nov. (Mallory).

-14. Transverse section through the head to show the large lateral and smaller dorsal and ventral branches of the cephalic lacuna.

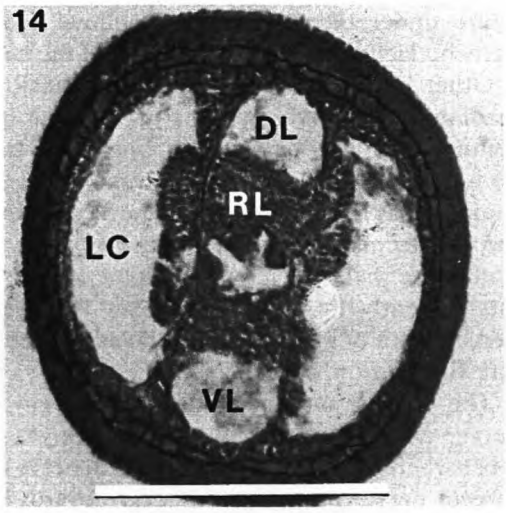
-15. Transverse section through a lateral nerve cord; note the absence of neuroganglionic tissues from the inner margin of the fibrous core and the concentric layers of connective tissue in the outer ganglionic zone.

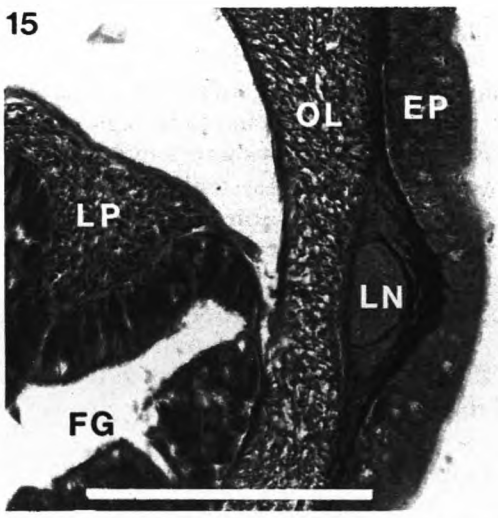
-16. Transverse section close behind the brain to show the large median ventral nerve resulting from the fusion of the two foregut nerve roots.

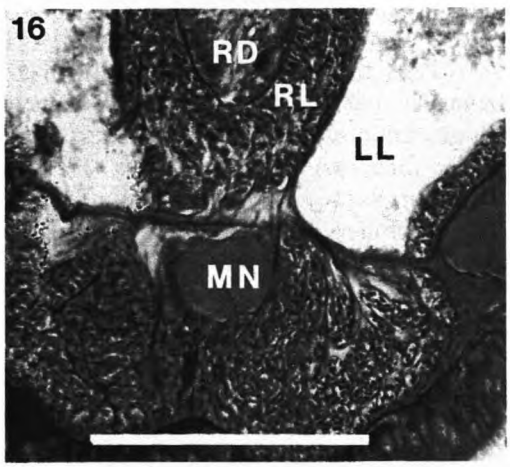
-17. Transverse section immediately in front of the mouth; the median foregut nerve has now branched to form a pair of nerves, indicated by arrowheads.

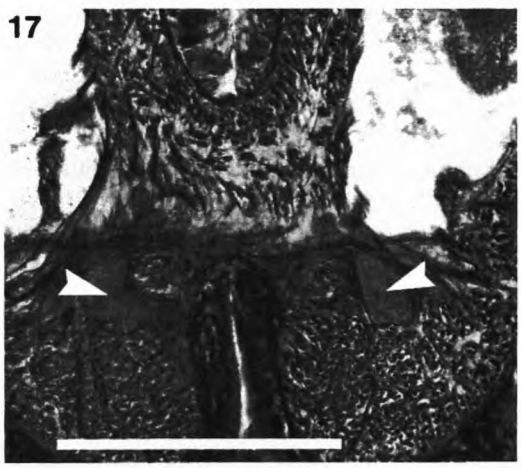
-18. Transverse section through the oral region to show the further subdivision of the foregut nerves, indicated by arrowheads.

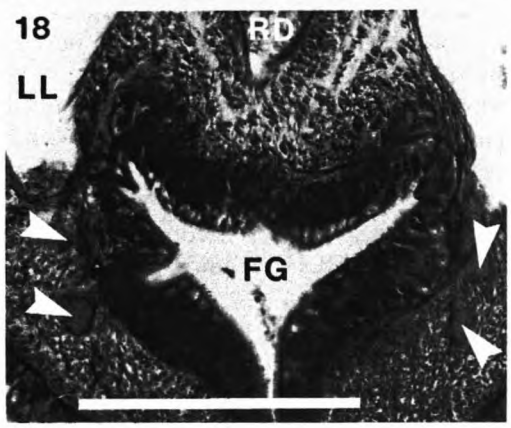
-19. Transverse section through the body wall in the rear cerebral region to show one of the epidermal furrows; note the absence of gland cells from the furrow epithelium and longer epithelial cilia. Scales: 7.14,7.18 250 μm; 7.15-17 200 μm; 7.19 100 mm.

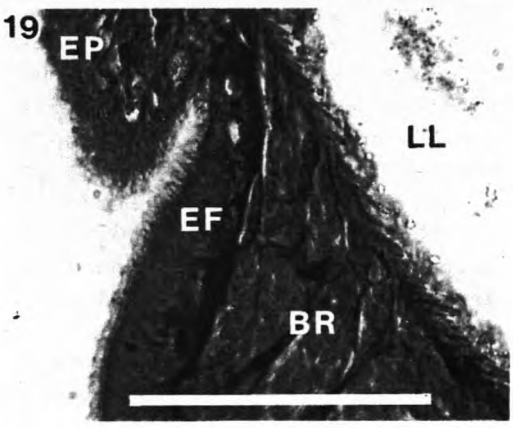

14
DL
RL
LC
VL


15
OL
EP
LP
LN
FG


16
RD
RL
LL
MN


17


18
RD
LL
FG


19
EP
LL
EF
BR

7.4 Systematic discussion.

The anoplan nemerteans comprise three orders, the Archinemertea, Palaeonemertea and Heteronemertea distinguished by the arrangement of their body wall muscle layers and position of their central nervous system (Iwata, 1960a). The present nemerteans possess outer circular, middle longitudinal and inner circular muscle layers in their body wall, with an incomplete inner longitudinal layer restricted to the foregut region, have the nervous system throughout the length of their body situated between the dermis and outer circular muscle layer, and have no cerebral sensory organs; these features identify them as members of the palaeonemertean family Tubulanidae.

Amongst the four known genera of tubulanids only *Callinera* possesses a large muscular sac developed on the hind end of its rhynchocoel, and Hylbom (1957) regarded this structure as the diagnostic feature of the genus. The Derbyhaven nemerteans, which likewise have a powerful posterior rhynchocoelic muscle sac, also share many other anatomical characters with *Callinera*, particularly an unusually long rhynchodaeum terminating in a post cerebral proboscis insertion, the organisation of the blood and excretory systems, and the far posterior position of the gonads. Indeed the general arrangement of the body in the present species closely corresponds with that seen in *C. buergeri* and *f. grandis* and there is no doubt that the three taxa belong in the same genus. *Callinera* is at present a monospecific genus, *C. buergeri* having been reported from muddy sediments on the coasts of Sweden (Bergendal 1900a-d, 1901, 1902a-c, 1903; Hylbom, 1957), Norway (Punnett, 1903) and Denmark (Brunberg, 1964). In his original description, Bergendal (1900a: 313) noted that *C. buergeri* was 2-5cm long and 0.5-1 mm thick but he later (Bergendal, 1902a : 2) described two larger specimens and he regarded these larger individuals as a special form to which he subsequently (Bergendal, 1903) gave the name *C. buergeri f. grandis*. Hylbom (1957: 533) noted that his material could "easily be separated [into two groups] according to the size of the specimens: (1) length 9-15 mm, width 0.3-0.5 mm (2) (*f. grandis*), length 25-30 mm, width 0.5-0.75

mm". Hylbom was unable to determine whether the larger form constituted a second species or merely represented older individuals of *C. buergeri*, but Brunberg (1964: 83) described *C. buergeri* as "10-25 mm long, 0.5-1.5 mm wide" without referring to *f. grandis*. The Derbyhaven nemerteans correspond in size (31.5-35.0 mm long) to *C. buergeri f. grandis*, but possess anatomical features, particularly in the organisation of the proboscis and nervous system, which differ significantly from those of both Scandinavian taxa and cannot be explained as merely due to either intraspecific variation or artifacts caused by fixation; it is accordingly concluded that although the nemerteans from the Isle of Man undoubtedly belong to the genus *Callinera*, they constitute a new species for which the name *Callinera monensis* sp. nov. is proposed. Further, it is concluded that differences distinguishable between *C. buergeri* and *C. buergeri f. grandis* are also of major taxonomic importance and that *f. grandis* should be afforded separate specific status as *C. grandis* Bergendal, 1903. A summary of the major distinguishing features of these three species is provided in table 7.1.

Table 7.1 Summary of the major anatomical features which can be used to distinguish between *Callinera buergeri* (C.b), *C. grandis* (C.g) (formerly *C. buergeri f. grandis*) and *C. monensis* sp. nov.(C.m).

Species	C.b	C.g	C.m
Body Wall			
Epidermis close to nephridiopores with (+) or without (0) lateral sensory organs.	+	0	0
Outer longitudinal muscle layer with (+) or without (0) subepidermal gland cells.	0	+	0
Proboscis			
With (+) or without (0) second region possessing circular muscle layer.	+	+	0
Blood system			
Cephalic blood supply essentially comprising a single thin-walled lacuna irregularly subdivided into four longitudinal channels (+) or consisting of four thicker-walled vessels which are mostly quite separate (0).	+	0	+
Nervous system			
With one (1) or two (2) dorsal cerebral commissures.	2	2	1
Foregut nerves fuse to form short single median ventral nerve before branching in front of mouth (1) or remaining as two distinct nerves transversely linked by a commissure (2).	1	2	1
Cephalic peripheral nerve ring with (+) or without (0) additional dorsal and ventral nerves running in body wall longitudinal muscle layer.	0	+	0

Chapter 8
General Discussion.

8.1 Methodology, problems and practicalities.

Estimation of the average heterozygosity within a population is affected by both the number of individuals sampled at each locus and by the number of loci examined (Nei & Roychoudhury, 1974; Nei, 1978; Gorman & Renzi, 1979; Nei, 1987). Estimation of genetic distance between two populations is also affected by these two parameters but particularly by the number of loci examined (Nei & Roychoudhury, 1974; Nei, 1987). When estimating heterozygosity, intralocus variance is reduced by increasing the number of individuals sampled (N) whilst the interlocus variance is reduced by increasing the number of loci examined (Nei & Roychoudhury, 1974; Nei, 1987). When estimating genetic distance sampling variance may be reduced by increasing the number of loci examined as single locus genetic distance varies considerably with locus (Nei & Roychoudhury, 1974).

Tests for significance of deviations of genotype frequencies from those expected under Hardy-Weinberg equilibrium are also affected by small sample size. The power of χ^2 goodness of fit tests is severely affected by small expected allele frequencies which occur due to small sample size, or due to properties of natural polymorphisms which often include alleles at low frequencies (Lewontin, 1958; Elston & Forthofer, 1977; Fairbairn & Roff, 1980; Pamilo & Varvio-Aho, 1984; Valenzuela 1985; Lessios, 1992). Several suggestions have been made as to appropriate sample sizes for use with χ^2 goodness of fit tests and these have fallen between approximately 100-200 individuals (Fairbairn & Roff, 1980; Speiss, 1989). F-statistics (Wright, 1943, 1951, 1965, 1969) are also affected by sample size (Weir & Cockerham, 1984).

The number of individuals sampled (N) in this study exceeded 100 in only a single population for one species (St. Agnes, *Lineus viridis*). Generally N fell between 20 and 100 individuals, though in a few cases fewer than 10 individuals of a species were found at a particular site. Such low sample sizes will have increased sampling variance in estimates of heterozygosity and genetic distance and will have affected tests for deviations of observed heterozygosity from those expected under Hardy-Weinberg

equilibrium and possibly F-statistics. Though a larger sample size was desirable the number of individuals sampled for each species was limited by the number of specimens that could be collected from the localities studied. As pointed out above in the case of estimates of heterozygosity or genetic distance numbers of individuals are not as important as the number of loci investigated (Nei & Roychoudhury, 1974; Nei, 1978; Gorman & Renzi, 1979; Nei, 1987).

In an attempt to reduce the effects of low sample size on tests for significance of deviations of observed genotype frequencies from those expected under Hardy-Weinberg equilibrium, exact tests were used in all cases. These are generally less affected than χ^2 tests of goodness of fit (Lessios, 1992). F-statistics present a special case with regard to sample size. Since gamete formation in a population is random then a large degree of variation in F-statistics is inherent. Correction of variation by increasing sample size has little affect on the variance of F-statistics (Weir & Cockerham, 1984) unless sample sizes are extremely small (see Section 3.3.1).

A more important contributor to variance in estimates of heterozygosity and genetic distance is the number of loci sampled (Nei & Roychoudhury, 1974; Nei, 1978; Gorman & Renzi, 1979; Nei, 1987). The maximum number of loci sampled in this investigation was thirteen. This was largely due to difficulties in obtaining acceptable levels of enzyme activity and resolution in loci for the nemertean species investigated. A few other loci may have been obtainable by further trials with different enzymes stains and buffer systems. In view of the large number of specimens screened by electrophoresis in this investigation (approximately 1500 individuals) running more than two buffer systems would probably have been impracticable.

To gain any significant reductions in sampling variance the number of loci investigated would have to be increased by a large degree (Nei & Roychoudhury, 1974; Nei, 1987). It would not have been practical to stain such large numbers of enzyme loci in the present investigation. Furthermore, in the case of genetic distance, the very high values obtained for interspecific comparisons in the present study are associated with a

large variance even if a substantial number of loci are studied (Nei, 1987).

Approximately one in three enzyme alleles are detectable by electrophoresis (Selander, 1976). Some staining bands detected during electrophoresis represent enzyme "phenotypes" and may comprise more than one allele (Hubby & Lewontin, 1966). Under given conditions of pH, temperature and gel composition and concentration some alleles may have the same mobility. This has the affect of "hiding" heterozygosity during electrophoretic studies (Murphy *et al.*, 1990). Highly polymorphic enzyme loci, such as *Odh-1* for *Lineus viridis* are more likely to harbour cryptic alleles than monomorphic enzyme loci (Murphy *et al.* 1990). Though this phenomenon means that measures of heterozygosity for the nemertean species studied will not be absolute, they are still comparable with the majority of electrophoretic studies on other organisms. Furthermore, differences detected between populations and species during this investigation are real and could only be increased by the resolution of further alleles (Coyne, 1982; Murphy *et al.* 1990).

Measures of heterozygosity and genetic distance assume that the populations being studied are in Hardy-Weinberg equilibrium (Nei & Roychoudhury, 1974; Nei, 1987). No natural population perfectly fulfils all the assumptions of the Hardy-Weinberg equilibrium. In natural populations generations are often overlapping, migration often occurs and mating may be non-random. It is assumed for the purposes of this investigation that variances generated in estimations of heterozygosity and genetic distance due to the corruption of one or more of the assumptions of the Hardy-Weinberg equilibrium, are negligible for species in which no significant deviation from Hardy-Weinberg expectations was detected. Even in populations where genotype frequencies showed significant differences to expected frequencies the variance generated probably had little effect on results, especially where genetic distances between populations compared were large.

Other factors may also have affected estimations of genetic distance between populations. These include the non-linear relationship of genetic distance with time when

the time of divergence between two species has been very long (Nei, 1987). This is because when D is very large its variance becomes very large and the high frequency of backward and parallel mutations in isozymes make the molecular evolutionary clock unreliable (Nei, 1987). This is particularly relevant to Chapter 6 in this thesis.

The evolutionary history of the organisms under study may also affect genetic distance and heterozygosity. When populations go through a bottleneck, heterozygosity will be temporarily decreased and genetic distance between populations may be temporarily increased (Nei *et al.* 1975; Nei, 1987). Whether or not the populations of nemertean species studied in this investigation have undergone bottlenecks in the recent past is difficult to ascertain. A likely cause of such a bottleneck could be extinctions of populations during periods of glaciation. Some of the species studied may have colonised the North Atlantic from the North Pacific ocean (see Dunton, 1992). In such a case populations may have undergone a form of bottleneck known as the 'founder effect', where a population is established by a small group of individuals (see Holgate, 1966; Nei *et al.* 1975; Chakraborty & Nei, 1977; Neel & Thompson, 1978).

One of the assumptions underlying the Hardy-Weinberg principle is that natural selection does not affect the gene under investigation (Hartl & Clark, 1989). The assumption of neutral or non-Darwinian molecular evolution (see Kimura, 1983; Nei, 1987) is fundamental to many of the statistical tests and estimates of genetic parameters in the present study. Selectionist theories (see Ayala, 1977; Milkman, 1978; Clarke, 1979; Wills, 1981; Nei, 1987) require the building of extremely complex models which are difficult to test practically (Solé-Cava & Thorpe, 1991). Whilst there is evidence of selection occurring at the molecular level (Koehn, 1975; Koehn *et al.* 1976, 1980, 1984; Burton, 1986), neutral gene theory is capable of explaining most observations on molecular evolution and variation (Kimura, 1983; Nei, 1987). For the purposes of this investigation enzyme loci are considered as neutral or nearly neutral. It is acknowledged that selection does occur at the molecular level but mutation and random genetic drift are generally more important in determining molecular variation and evolution. Detailed proof

that rules out neutral explanations for observed genotype frequencies in a locus for any organism is required if it is claimed that an enzyme locus is responding to some form of selection.

8.2 Findings on the genetics and taxonomy of nemerteans.

Table 8.1 summarises the highest mean observed heterozygosity for each species of nemertean studied during the course of this investigation. Mean observed heterozygosity ranged from 0.044 in *Lineus ruber* to 0.278 in *Riseriellus occultus*. Levels of heterozygosity found in *Lineus ruber* were low for marine invertebrates and invertebrate phyla in general (Nevo, 1978; Nevo *et al.*, 1984). Levels of heterozygosity found in *Riseriellus occultus* and *Micrura fasciolata* were high, Nevo *et al.* (1984) list just six species out 1111 studied with observed heterozygosities above 0.30 (excluding parthenogenetic forms). Levels of heterozygosity observed in *Riseriellus occultus* and *Micrura fasciolata* were comparable to high values observed in cnidarians and sponges (Solé-Cava & Thorpe, 1991).

Table 8.1 Highest values of mean observed heterozygosity estimated for all species of nemerteans studied in the present investigation. The percentage of polymorphic loci at the 95% and 99% levels are given.

Species	Percentage polymorphic loci		Mean observed
	95%	99%	Heterozygosity
Lineus ruber	30.8	30.8	0.044
Cryptic species (Chap. 5)	27.3	27.3	0.083
Lineus longissimus	62.5	62.5	0.097
Amphiporus lactifloreus	33.3	55.6	0.098
Lineus sanguineus	36.4	54.5	0.120
Lineus viridis	22.2	44.4	0.174
Micrura purpurea	80.0	80.0	0.210
Micrura fasciolata	70.0	80.0	0.257
Riseriellus occultus	55.6	55.6	0.278

Values for heterozygosity obtained previously for *Lineus ruber*, *Lineus sanguineus* and *Lineus viridis* (Williams et al., 1983), do not agree with those found in the present study. This was probably due to differences in methodology between the present study and that of Williams *et al.* (1983) (see 3.4). Values of heterozygosity

obtained for species in the present study fall in a similar range to those obtained for *Oerstedia dorsalis*, *Oerstedia striata* (0.132-0.316) (Sundberg & Janson, 1988) and for *Lineus torquatus* (0.139) (Manchenko & Balakirev, 1982; Balakirev & Manchenko, 1984). It appears that nemerteans have levels of genetic variation which range from values which are low for eukaryotic organisms (Nevo, 1978; Nevo *et al.* 1984) to values which are very high and comparable with a few groups of lower marine invertebrates (Solé-Cava & Thorpe, 1991). The majority of nemertean species studied to date have heterozygosities which fall in the normal range for invertebrate phyla (Nevo, 1978; Nevo *et al.*, 1984).

Studies on the population genetics of *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and *Amphiporus lactifloreus* indicate that a moderate level of gene-flow (see Wright, 1978) was maintained between conspecific populations over remarkable distances (over 6000 km in the case of *Lineus ruber* and *Lineus viridis*). *Lineus ruber* and *Lineus viridis* are not reported as having pelagic larval dispersive phases (Gontcharoff, 1951) and gene-flow is thought to be maintained by passive dispersal, possibly by rafting (see Jokiel, 1984, 1987, 1989; Highsmith, 1985; Jackson, 1986; Edgar, 1987; DeVantier, 1992). This is contrary to the theory that gene-flow and geographic range in marine organisms is directly related to their mode of development (Scheltema, 1971, 1986, 1989; Crisp, 1978; Scheltema & Williams, 1983; Waples, 1987). Much of the work that supported the hypothesis that long larval phases resulted in high levels of gene-flow between populations and large geographic ranges was carried out on relatively few taxa (molluscs, sessile Crustacea and fish) and studies on other taxa have revealed no such relationships (Bhaud, 1983; Jackson, 1986)

The present study adds to the growing body of evidence that there is not a simple relationship between gene-flow, geographic range and the type of development a marine organism exhibits. In fact evidence suggests that larval phases are maintained in organisms for reasons other than dispersion (Istock, 1967; Clark *et al.*, 1979; Todd & Doyle, 1981). Questions about the effects of reproductive and developmental modes on

the population dynamics of marine organisms are very important when considering the impact of man on marine ecosystems. Studies of gene-flow between conspecific populations of nemertean species with different modes of reproduction and development could make important contributions to this area of marine biology. Work has already commenced on the population genetics of the Antarctic heteronemertean *Parborlasia corrugatus*, which reproduces sexually and has a long larval dispersive phase (Peck, L. pers. comm.Marine Life Sciences Division, British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 OET, United Kingdom). This will provide a useful comparison to the present study, which concentrates on nemertean species without larval dispersive phases. Further work is planned on gene-flow between populations of pelagic nemertean species.

Cryptic speciation has now been detected in many marine invertebrate phyla. These include: the Porifera (Solé-Cava & Thorpe, 1986, 1987a; Solé-Cava *et al.*, 1991a,b, 1992); the Cnidaria (Carter & Thorpe, 1981; Bucklin & Hedgecock, 1982; Haylor et al., 1984; Shaw *et al.*, 1987; Thorpe, *et al.*, 1992); Nemertea (Sundberg & Janson, 1988); Bryozoa (Thorpe *et al.*, 1978a,b); Annelida (Grassle & Grassle, 1976); Mollusca (Murphy, 1978; Mastro *et al.*, 1982; Sarver *et al.*, 1992); Arthropoda (Hedgecock, 1979; Dando & Southward, 1980; McKinnon *et al.*, 1992); and Echinodermata (Manwell & Baker, 1963). Cryptic species appear to be particularly common in animal groups in which there are taxonomic difficulties, generally arising from a lack of useful systematic characters. It is therefore of no surprise that the present study revealed the existence of two cryptic species of nemerteans, *Riseriellus occultus* and the as yet undescribed species cryptic with *Lineus ruber* and *Lineus viridis* (see Chap. 5).

In the case of *Riseriellus occultus* starch gel electrophoresis provided initial evidence of a separate species that had a similar external appearance to *Lineus longissimus* and *Lineus viridis*. Subsequent detailed morphological description confirmed the systematic status of this type and showed that it was morphologically quite distinct

from the other two species both externally and internally (Rogers *et al.*, in press). The second cryptic species revealed by electrophoresis is indistinguishable from *Lineus ruber* and *Lineus viridis* on external characteristics. A description of the internal morphology of this species will be forthcoming.

Electrophoresis of the hoplonemertean, *Amphiporus lactifloreus* revealed no cryptic speciation in the populations surveyed. This species has just been designated as the type species for the genus *Amphiporus* (International Commission of Zoological Nomenclature, 1992).

The occurrence of two cryptic species associated with populations of four of the commonest, and probably most well studied, nemertean species in Europe (*Lineus ruber*, *Lineus viridis*, *Lineus sanguineus*, *Lineus longissimus*) indicates that there is probably a high incidence of cryptic speciation within the phylum Nemertea. It is recommended that molecular techniques, such as starch-gel electrophoresis, should be routinely employed at the species level in nemertean systematics. This is partly because most nemertean species lack a sound diagnosis and also because the systematic value of many internal and external characteristics in nemerteans is unknown (Gibson, 1985; Gibson & Crandall, 1989). The use of molecular techniques provides a more objective approach to nemertean taxonomy. Electrophoresis not only detects cryptic species but is also useful in determining whether or not nominate species, such as *Amphiporus lactifloreus*, are monophyletic.

New species which have been discovered or old species which have been confirmed as 'good' by electrophoresis should be completely described as recommended by Gibson (1985). This is because many of the present problems in nemertean taxonomy are a consequence of inadequate description at the species level (Gibson, 1985). Note that in some groups of nemerteans e.g., the genus *Carcinonemertes*, differences in internal morphology between separate species may not be detectable by conventional methods of histological investigation (Gibson, R., pers. comm., Liverpool John Moores University). In this case electrophoresis may be the only way to separate species.

In view of the current findings on levels of cryptic speciation in nemerteans (see Chapters 4 and 5) and on the number of new or poorly described taxa found during the present study (see Chapters 2 and 7) it is likely that present estimates of the number of species in the phylum are far too low. This may also be the case for several other phyla of marine invertebrates in which there are systematic problems.

Chapter 6 indicates that problems in nemertean taxonomy extend from the species level right up to the family level and possibly beyond. Even considering sampling errors in the data Nei's (1972) genetic identity between congeneric species of the genera *Lineus* and *Micrura* were well below those expected from observations on other taxa (Thorpe, 1982, 1983). Isozyme data from the species examined could only be explained by fundamental mistakes in the systematics of the family Lineidae and probably in the order Heteronemertea (see Sundberg, 1991).

Isozyme data could establish very little genetic relationship between the species studied. This is because the species investigated are separated genetically at a level to great to be accurately estimated by starch gel electrophoresis. Genetic distance has been directly related to the time of divergence between species. A value of 1D = 18 million years has been generally adopted for a large range of proteins (Thorpe, 1989) from various efforts to calibrate the molecular evolutionary clock (see Yang *et al.*, 1974; Sarich, 1977; Wilson *et al.*, 1977). Using this value the species investigated in Chapter 6 would appear to have diverged between 25 to over 200 million years ago. The phylogenetics of species which show such levels of divergence can only be effectively investigated by the use of DNA or RNA sequencing. (Hillis & Moritz, 1990).

Future work on the systematics of the family Lineidae, and probably on other higher taxa in nemerteans, will require a combined approach between conventional description and molecular techniques. DNA or RNA sequencing should be used to provide an estimate of genetic distance between nemertean taxa. Once natural groupings of the species, genera, families and orders have been established by sequencing, complete descriptions of the taxa investigated can be compared with genetic data in order

to estimate the systematic importance of anatomical features in nemertean taxonomy. Ultrastructural studies may also prove useful in such a study.

Such an approach is necessary since biological classification should be consistent with the inferred phylogeny of any group of organisms (Mayr, 1982; Thorpe, 1982). The systematic organisation of nemerteans should reflect as far as possible the evolutionary history of the group. For example, times of divergence between species in different genera should be greater than between species within those genera. At the present time this is clearly not the case (see Chap. 6). This is because the systematic value of characters used in nemertean taxonomy is often at present unknown with the result that characters are used inappropriately to diagnose nemertean taxa at the levels of species, genera, families and possibly orders (see Gibson, 1988) and even classes (Sundberg, 1991).

References

Abildgaard, P.C. 1806. Article on *Planaria dorsalis*. In: O.F. Müller (Ed.) *Zoologica Danica. Vol. 4.*

Ahmad, M., Skibinski, D.O.F. and Beardmore, J.A. 1977. An estimate of the amount of genetic variation in the common mussel *Mytilus edulis*. *Biochemical Genetics. 15*: 833-846.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. 1983. *Molecular Biology of the Cell.* Garland Publishing Incorporated, New York. 1146pp.

Andriashev, A.P. 1966. The problem of the life community associated with the Antarctic fast ice. *Symposium on Antarctic oceanography. Scott Polar Institute (Cambridge), Santiago, Chile. September 13-16.*

Arnold, G. 1898. Zur Entwicklungsgeschicte des *Lineus gesserensis* O.F. Müller (*L. obscurus* Barrois). *Trudy Imperatorskago Saint-Peterburgskago obshchestva estestvoispytatelei. 28*: 21-30.

Avise, J.C. and Selander, R.K. 1972. Evolutionary genetics of cave dwelling fishes of the genus *Astyanax*. *Evolution. 26*: 1-19.

Avise, J.C., Patton, J.C., Aquadro, C.F. 1980. Evolutionary genetics of birds. I. Relationships among North American thrushes and allies. *Auk. 97*: 135-147.

Ayala, F.J. 1975. Genetic differentiation during the speciation process. In: Dobzhansky, T., Hecht, M.K. and Steere, W.C. (Eds.). *Evolutionary Biology*. Plenum Press, New York. *8*: 1-78

Ayala, F.J. 1977. Protein evolution in different species: Is it a random process? In: Kimura, M. (Ed.) *Molecular Evolution and Polymorphism. Proceedings of the Second Taniguchi International Symposium on Biophysics, National Institute of Genetics, Mishima, Japan.* 73-102.

Ayala, F.J. 1983. Enzymes as taxonomic characters. In: Oxford, G.S. and Rollinson, D. (Eds.). *Protein polymorphism: adaptive and taxonomic significance.* Academic Press, London. pp. 3-26.

Ayala, F.J. and Campbell, C.A. 1974. Frequency dependant selection. *Annual Review of Ecology and Systematics. 5*: 115-138.

Ayala, F.J. and Kiger, J.A. 1980. *Modern Genetics*. The Benjamin/Cummings Publishing Company, Incorporated. 923pp.

Baker, M.C. 1975. Song dialects and genetic differences in white-crowned sparrows (*Zonotrichia leucophrys*). *Evolution. 29*: 226- 241.

Balakirev, E.S. and Manchenko, G.P. 1984. Vnutrividovaya geneticheskaya izmenchivost belkov u nemertinui *Lineus torquatus*. *Genetika. 20*: 2070-2071.

Barrois, J. 1877. Mémoire sur l'embryologie des Némertes. *Annales des sciences naturelles. Paris. Series 6, Zoology. 6 (no.3)*: 1-232.

Barrowclough, G.F. and Corbin, K.W. 1978. Genetic variation and differentiation in the Parulidae. *Auk. 95*: 691-702.

Bartsch, I. 1973. Zur Nahrungsaufnahme von *Tetrastemma melanocephalum* (Nemertini). *Helgolander Wissenschaftliche Meeresuntersuchungen. 25*: 326-331.

Beaumont, A.R., Day, T.R. and Gäde, G. 1980. Genetic variation at the octopine dehydrogenase locus in the adductor muscle of *Cerastoderma edule* (L.) and six other bivalve species. *Marine Biology Letters. 1*: 137-148.

Beaumont, A.R. and Wei, H.C. 1991. Morphological and genetic variation in the Antarctic limpet *Nacella concinna* (Strebel, 1908). *Journal of Molluscan Studies. 57*: 443-450.

Benzie, J.A.H. and Williams, S.T. 1992. No genetic differentiation of giant clam (*Tridacna gigas*) populations in the Great Barrier Reef, Australia. *Marine Biology. 113*: 373-377.

Berg, G. 1972. Taxonomy of *Amphiporus lactifloreus* (Johnston, 1828) and *Amphiporus dissimulans* Riches 1893 (Nemertini, Hoplonemertini). *Astarte. 5*: 19-26.

Bergendal, D. 1900a. Über ein Paar sehr eigenthümliche nordische Nemertinen.

Zoologischer Anzeiger. 23: 313-328.

Bergendal, D. 1900b. Studier öfver Nemertiner. I. *Callinera bürgeri* Bergendal, en representant för ett afvikande slägte bland Paläonemertinerna. *Acta Universitatis lundensis. 36*: 1-47.

Bergendal, D. 1900c. Till Kännedomen om de nordiska Nemertinerna. I. Förteckning öfver vid Sveriges vestkust iakttagna Nemertiner. *Öfversigt af K. Vetenskapsakademiens Förhandlinger. 57*: 581- 602.

Bergendal, D. 1900d. Bör ordningen Palaeonemertini Hubrecht uppdelas i tvänne ordninger Protonemertini och Mesonemertini? *Öfversigt af K Vetenskapsakademiens Förhandlinger. 57*: 721-742.

Bergendal, D. 1901. Studier öfver Nemertiner. I. *Callinera bürgeri* Bergendal, en representant för ett afvikande slägte bland Paläonemertinerna. *Acta Universitatis lundensis. 37*: 49-116.

Bergendal, D. 1902a. Ueber die Nemertinengattung *Callinera* Bgdl. *International Congress of Zoology. Berlin 1901*: 1-11.

Bergendal, D. 1902b. Zur Kenntniss der nordischen Nemertinen. *Bergens museums Årsberetning. 4*: 1-22.

Bergendal, D. 1902c. Zur Kenntniss der nordischen Nemertinen. 2. Eine der construierten Urnemertine entsprechende Palaeonemetine aus dem Meere der schwedischen Westküste. *Zoologischer Anzeiger. 25*: 421-432.

Bergendal, D. 1903. Till kännedomen om de nordiska Nemertinerna. 4. Förteckning öfver vid Sveriges vestkust iakttagna Nemertiner. *Arkiv för Zoologi. 1*: 85-156.

Bertelson, E. and Ussing, H. 1936. Marine tropical animals carried to the Copenhagen Sydhavn on a ship from the Bermudas. *Dansk Naturhistorisk Forening i Kjobenharn Videnskabelige Medeleser. 100*: 237-245.

Bhaud, M. 1982. Relations entre stratégies de reproduction et aire de répartition chez annélides polychètes. *Oceanologica Acta. 5, No.4*: : 465-472.

Bier, M. (Ed.) 1967. *Electrophoresis, theory, methods and applications.* Volume 2.

Academic Press, New York.

Bierne, J. 1962. La régénération de la trompe chez les Némertes. *Bulletin Biologique de la France et de la Belgique. 96*: 481- 504.

Bierne, J. 1964. Maturation sexuelle anticipée par décapitation de la femelle chez l'Hétéronémerte *Lineus ruber* Müller. *Compte rendu hebdomadaire des séances de l'Académie des sciences. Paris. 259*: 4841-4843.

Bierne, J. 1966. Localisation dans les ganglions cérébroïdes du centre régulateur de la maturation sexuelle chez la femelle de *Lineus ruber* Müller. (Hétéronémertes). *Compte rendu hebdomadaire des séances de l'Académie des sciences. Paris. 262*: 1572-1575.

Bierne, J. 1970a. Influence des facteurs hormonaux gonado-inhibiteur et androgène sur la differenciation sexuelle des parabionts hétérosexués chez un némertien. *Annals of Biology. 9*: 395-400.

Bierne, J. 1970b. Recherches sur la différenciation sexuelle au cours de l'ontogenèse et de la régéneration chez le némertien *Lineus ruber* (Müller). *Annales des sciences naturelles. Zoologie. 12*: 181-298.

Bierne, J. 1970c. Aspects expérimentaux de la différenciation sexuelle chez *Lineus ruber* (Hétéronémertes). *Bulletin de la Société Zoologique de France. 95*: 529-543.

Bierne, J. 1979. Viable animals obtained by grafting pieces from several nemertean adults. *Transplantation. 29 (No. 1)*: 74-75.

Bierne, J. 1983. Nemertina. In: Adiyodi, K.G. and Adiyodi, R.G. (Eds.) *Reproductive Biology of the Invertebrates. Volume I: Oogenesis, Oviposition, and Oosorption.* John Wiley & Sons Ltd.

Borlase, W. 1758. *The Natural History of Cornwall.* Jackson, Oxford. 326pp.

Bousfield, E.L. 1955. Ecological control of the appearance of barnacles on the Miramichi Estuary. *Bulletin National Museum of Canada. Biology Series. 137*: 1-65.

Brewer, G.J. 1970. *An Introduction to Isozyme Technique.* Academic Press, New York.

Brierly, A.S. 1992. *Aspects of genetic diversity and population structure in squid.* Ph.D.

Thesis. University of Liverpool. 154pp.

Brunberg, L. 1964. On the nemertean fauna of Danish waters. *Ophelia. 1 (1)*: 77-111.

Brusca, R.C. and Brusca, G.J. 1990. *Invertebrates*. Sinauer Associates, Incorporated. Sunderland, Massachusetts. 922pp.

Bucklin, A. 1985. Biochemical genetic variation, growth and regeneration of the sea anemone, *Metridium*, of British shores. *Journal of the Marine Biological Association of the United Kingdom. 65*: 141-157.

Bucklin, A. and Hedgecock, D. 1982. Biochemical genetic evidence for a third species of *Metridium* (Coelenterata: Actiniaria). *Marine Biology. 66*: 1-7.

Bulnheim, H.-P. and Scholl, A. 1981. Genetic variation between geographic populations of the amphipods *Gammarus zaddachi* and *G. salinus*. *Marine Biology. 64*: 105-115.

Bürger, O. 1892. Zur Systematik der Nemertinen fauna des Golfs von Neapel. *Nachricten König. Gesellscaft der wissenschaften und der Georg-Auguste Universitat, Gottingen. 5*: 137-178.

Bürger, O. 1895. Die Nemertinen des Golfes von Neapel und der Angrenzenden Meeres-Abschnitte. *Fauna und Flora des Golfes von Neapel. 22*: 1-743.

Burton, R.S. 1983. Protein polymorphisms and genetic differentiation of marine invertebrate populations. *Marine Biology Letters. 4*: 193-206.

Burton, R.S. 1986. Evolutionary consequences of restricted gene flow among natural populations of the copepod, *Tigriopus californicus*. *Bulletin of Marine Science. 39 (2)*: 526-535.

Cameron, R.A. 1986. Introduction to the invertebrate larval biology workshop: A brief background. *Bulletin of Marine Science. 39 (2)*: 145-161.

Campbell, C.A. 1978. Genetic divergence between populations of *Thais lamellosa* (Gmelin). In: Battaglia, B. and Beardmore, J.A. (Eds.) *Marine Organisms: Genetics, Ecology and Evolution*. pp. 157-170.

Cantell, C.E. 1972. Studies on the morphology, taxonomy, and larval development of

heteronemertines (Nemertina). *Acta Universitatis Uppsala. 218*: 1-7.

Cantell, C.E. 1975. Anatomy, taxonomy and biology of some Scandinavian heteronemertines of the genera *Lineus*, *Micrura* and *Cerebratulus*. *Sarsia*. 58: 89-122.

Cantell, C.E. 1976. Complementary description of the morphology of *Lineus longissimus* (Gunnerus, 1770) with some remarks on the cutis layer in heteronemertines. *Zoologica Scripta. 5*: 117-120.

Cantell, C.E. and Gidholm, L. 1977. Isozyme studies of two related nemerteans, *Micrura fasciolata* Ehrenberg 1831, and *Micrura baltica* Cantell 1975. *Zoon. 5*: 87-89.

Carlton, J.T., 1985. Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. *Oceanography and Marine Biology Annual Review. 23:* 313-317.

Carter, M.A. and Thorpe, J.P. 1981. Reproductive, genetic and ecological evidence that *Actinia equina* var. *mesembryanthemum* and var. *fragacea* are not conspecific. *Journal of the Marine Biological Association of the United Kingdom. 61*: 71-93.

Cavalli-Sforza, L.L. and Edwards, A.W.F. 1967. Phylogenetic analysis: Models and estimation procedures. *Evolution. 21*: 550-570.

Chakraborty, R. and Nei, M. 1977. Bottleneck effects on average heterozygosity and genetic distance with the stepwise mutation model. *Evolution. 31*: 347-356.

Christiansen, J. and Thomsen, J.C., 1981. *Styela clava* Herdman, 1882, a species new to the Danish fauna (Tunicata Ascidiacea). *Steenstrupia. 7 (2):* 15-24.

Clarke, B. 1979. The evolution of genetic diversity. *Proceedings of the Royal Society of London, B. 205*: 453-474.

Clarke, K.B., Busacca. M. and Stirts, H. 1979. Nutritional aspects of development of the saccoglossan *Elysia cauze*. In: Stancyk, S.E. (Ed.) *Reproductive ecology of marine invertebrates*. University of South Carolina Press. 11-24.

Coe, W.R. 1899. Notes on the times of breeding of some common New England nemerteans. *Science. 9*: 167-169.

Coe, W.R. 1901. Papers from the Harriman Alaska Expedition, 20. The Nemerteans. *Proceedings of the Washington Academy of Science. 3*: 1-110.

Coe, W.R. 1902. The nemertean parasites of crabs. *American Naturalist. 36*: 431-450.

Coe, W.R. 1931. A new species of nemertean (*Lineus vegetus*) with asexual reproduction. *Zoologischer Anzeiger. 94*: 54-60.

Coe, W.R. 1940. Revision of the nemertine fauna of the Pacific coasts of North, Central, and northern South America. *Allan Hancock Pacific Expeditions. 2 (13)*: 247-323.

Coe, W.R. 1943. Biology of the nemerteans of the Atlantic coasts of North America. *Transactions of the Conneticut Academy of Arts and Science. 35*: 129-328.

Cook, L.M. 1984. The Problem. In (Ed.) G.S. Mani. *Evolutionary Dynamics of Genetic Diversity*. Springer-Verlag, Heidelberg. 1- 12.

Corbin, K.W., Sibley, C.G. and Ferguson, A. 1979. Genic changes associated with the establishment of sympatry in orioles of the genus *Icterus*. *Evolution. 33*: 624-633.

Corbin, K.W., Sibley, C.G., Ferguson, A., Wilson, A.C. Brush, A.H. and Ahlquist, J.E. 1974. Genetic polymorphism in New Guinea starlings of the genus *Aplonis*. *Condor. 76*: 307-318.

Corrêa, D.D. 1961. Nemerteans from Florida and Virgin Islands. *Bulletin of Marine Science of the Gulf and Caribbean. 11*: 1-44.

Coyne, J. 1982. Gel electrophoresis and cryptic protein variation. In: Rattazzi, M., Scandalios, J. and Whitt, G. (Eds.). *Isozymes: Current Topics in Biological and Medical Research. Vol. 6*: 1-32. Liss, New York.

Crisp, D.J. 1958. The spread of *Elminius modestus* in North West Europe. *Journal of the Marine Biological Association of the United Kingdom. 37*: 483-520.

Crisp, D.J. 1974. Factors influencing settlement of marine invertebrate larvae. In: Grant,

P. and Mackie, A.M. (Eds.). *Chemoreception in Marine Organisms*. Academic Press, New York and London. 295pp.

Crisp, D.J. 1976. The role of the pelagic larva. In: Spencer-Davies, P. (Ed.) *Perspectives in Experimental Biology. Volume I, Zoology*. Pergamon Press. Oxford. pp. 145-155.

Crisp, D.J., Burfitt, A., Rodrigues, K. and Budd, M.D. 1983. *Lasaea rubra*: An apomictic bivalve. *Marine Biology Letters. 4*: 127- 136.

Crothers, J.H. 1973. On variation in *Nucella lapillus* (L.): Shell shape in populations from Pembrokeshire, South Wales. *Proceedings of the Malacological Society of London. 40*: 319- 327.

Crothers, J.H. 1985. Two different patterns of shell-shape variation in the dog-whelk *Nucella lapillus* (L.). *Biological Journal of the Linnean Society. 25*: 339-353.

Crowe, J.H., Crowe, L.M., Roe, P. and Wickham, D.E. 1982. Uptake of DOM by nemertean worms: Association of worms with arthrodial membranes. *American Zoologist. 22*: 671-682.

Dalyell, J.G. 1853. *The Powers of the Creator Displayed in the Creation. Vol. 2*. Van Voorst, London. 327pp.

Dando, P.R., 1987. Biochemical genetics of barnacles and their taxonomy. In: Southward, A.J. (Ed.) *Crustacean Issues 5. Barnacle Biology*. A.A. Balkema, Rotterdam, Netherlands.

Dando, P.R. and Southward, A.J. 1980. A new species of *Chthalamus* (Crustacea: Cirripedia) characterised by enzyme electrophoresis and shell morphology: with a revision of other species of *Chthalamus* from the western shores of the Atlantic ocean. *Journal of the Marine Biological Association of the United Kingdom. 60*: 787-831.

Dando, P.R., Storey, K.B., Hochachka, P.W. and Storey, J.M. 1981. Multiple dehydrogenases in marine molluscs: Electrophoretic analysis of alanopine dehydrogenase, strombine dehydrogenase, octopine dehydrogenase and lactate

dehydrogenase. *Marine Biology Letters. 2*: 249-257.

Dayton, P.K., Robilliard, G.A., Paine, R.T. and Dayton, L.B. 1974. Biological accommodation in the benthic community at McMurdo Sound, Antarctica. *Ecological Monographs. 44 (1)*: 105-128.

Dearborn, J.H. 1965. *Ecological and faunistic investigations of the marine benthos at McMurdo Sound, Antarctica.* Ph.D. Thesis. Stanford University. Stanford, California. 238pp.

Delle Chiaje, S. 1841. *Descrizione et Notomia Degli Animali Invertebrati Della Sicilia Citeriore. 3*: 125-130. Naples.

DeVantier, L.M. 1992. Rafting of tropical marine organisms on bouyant coralla. *Marine Ecology Progress Series. 86*: 301-302.

Dobbs, F.C. and Vozarik, J.M. 1983. Immediate effects of a storm on coastal infauna. *Marine Ecology Progress Series. 11*. 273-279.

Dunton, K. 1992. Arctic biogeography: The paradox of the marine benthic fauna and flora. *Trends in Ecology and Evolution. 7 (No.6)*: 183-189.

Edgar, G.J. 1987. Dispersal of faunal and floral propagules associated with drifting *Macrocystis pyrifera* plants. *Marine Biology. 95*: 599-610.

Ehrenberg, C.G. 1831. Phytozoa turbellaria. In: Hemprich, F.G. and Ehrenberg, C.G. (Eds.) *Symbolae Physicae, seu Icones et Descriptiones Corporum Naturalium Novorum aut Minus Cognitorum*, Berolini.

Elston, R.C. and Forthofer, R. 1977. Testing for Hardy-Weinberg equilibrium in small samples. *Biometrics. 33*: 536-542.

Fairbairn, D.J. and Roff, D.A. 1980. Testing genetic models of isozyme variability without breeding data: Can we depend on the χ^2. *Canadian Journal of Fisheries and Aquatic Sciences. 37*: 1149-1159.

Farnham, W.F. 1980. Studies on aliens in the marine flora of Southern England. In: Price, J.H., Irvine, D.E.G. and Farnham, W.F. (Eds.) *The Shore Environment. Vol.2. Ecosystems.* Academic Press, London.

Ferguson, A. 1980. *Biochemical Systematics and Evolution*. Blackie, Glasgow. 194pp.

Fincham, A.A. 1968. *Ecology of some inshore and intertidal Amphipoda*. Ph.D. thesis. University of Liverpool.

Fisher, R.A. 1935. The logic of inductive inference. *Journal of the Royal Statistical Society. 98*: 39-54.

Fisher, F.M. and Cramer, N.M. 1967. New observations on the feeding mechanism in *Lineus ruber* (Rhynchocoela). *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 132*: 464.

Fisher, F.M. and Oaks, J.A. 1978. Evidence for a nonintestinal nutritional mechanism in the rhynchocoelan, *Lineus ruber. Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 154*: 213-225.

Fitch, W.M. 1976. Molecular Evolutionary Clocks. In: Ayala, F.J. (Ed.) *Molecular Evolution*. Sinauer Associates, Sunderland, Mass. 160-178.

Fleming, L.C. and Gibson, G. 1981. A new genus and species of monostiliferous hoplonemerteans, ectohabitant on lobsters. *Journal of Experimental Marine Biology and Ecology. 52*: 79-93.

Flowerdew, M.W., 1983. Electrophoretic investigation of populations of the cirripede *Balanus balanoides (L.)* around the North Atlantic seaboard. *Crustaceana. 45(3):* 260-278.

Franzén, Å. 1983. Nemertina. In Adiyodi, K.G. and Adiyodi, R.G. (Eds.). *Reproductive Biology of Invertebrates. Vol. II. Spermatogenesis and Sperm Function*. John Wiley & Sons Ltd.

Friedrich, H. 1935. Studien zur Morphologie, Systematik und Ökologie der Nemertinen der Kieler Bucht. *Archiv fur Naturgeschicte, N.S. 4*: 293-375.

Friedrich, H. 1960. Bemerkungen uber die Gattung *Micrura* Ehrenberg 1831 und zur Klassification der Heteronemertinen nebst vorlaufigem Bestimmungsslussel. *Veroffentlichungen des Instituts fur Meeresforschung in Bremerhaven. 7*: 48-62.

Gaal, D., Medgyesi, G.A. and Vereczkei, A. 1980. *Electrophoresis in the Separation of Biological Macromolecules*. John Wiley and Sons, Toronto.

Gäde, G. 1980. Biological role of octopine formation in marine molluscs. *Marine Biology Letters. 1*: 121-135.

Gardner, J.P.A. 1992. *Mytilus galloprovincialis* (Lmk) (Bivalvia, Mollusca): The taxonomic staus of the mediterranean mussel. *Ophelia. 35 (3)*: 219-243.

Gibson, R. 1972. *Nemerteans*. In Cain, A.J. (Ed.) Hutchinson University Library. Hutchinson & Co. (Publishers) Limited. London. 224pp.

Gibson, R. 1981. Nemerteans of the Great Barrier Reef 3. Anopla Heteronemertea (Lineidae). *Zoological Journal of the Linnean Society. 71*: 171-235.

Gibson, R. 1982a. Nemertea. In: Parker, S.P. (Ed.) *Synopsis and classification of living organisms. McGraw-Hill. New York. 1*: 823-846.

Gibson, R. 1982b. *British Nemerteans*. In: Kermack, D.M. and Barnes, R.S.K. (Eds.) Synopses of the British Fauna. Cambridge University Press. 212pp.

Gibson, R. 1983. Antarctic nemerteans: The anatomy, distribution, and biology of *Parborlasia corrugatus* (McIntosh, 1876) (Heteronemertea, Lineidae). *Biology of the Antarctic Seas XIV Antarctic Research Series. 39*: 289-316.

Gibson, R. 1985. The need for a standard approach to taxonomic descriptions of nemerteans. *American Zoologist. 25*: 5-14.

Gibson, R. 1988. Evolutionary relationships between mono- and polystiliferous hoplonemerteans: *Nipponnemertes* (Cratenemertidae), a "missing link" genus? *Hydrobiologia. 156*: 61-74.

Gibson, R. 1990a. Classification of the Nemertea Enopla: response to Sundberg's critique and cladistic analysis. *Zoologica Scripta. 19 (2)*: 141-142.

Gibson, R. 1990b. The macrobenthic nemertean fauna of Hong Kong. In: Morton, B. (Ed.). *Proceedings of the Second International Marine Biological Workshop: The Marine Flora and Fauna of Hong Kong and southern China. 1*: 33-212. University Press, Hong Kong.

Gibson, R. 1990c. The macrobenthic nemertean fauna of the Albany region, Western Australia. In: Wells, F.E., Walker, D.I., Kirkman, H. and Lethbridge, R. (Eds.). *Proceedings of the Third International Marine Biological Workshop: The Marine Flora and Fauna of Albany, Western Australia. 1*: 89-194. Western Australian Museum, Perth.

Gibson, R. and Crandall, F.B. 1989. The genus *Amphiporus* Ehrenberg (Nemertea, Enopla, Monostiliferoidea). *Zoologica Scripta. 18 (4)*: 453-470.

Gibson, R. and Moore, J. 1989. Functional requirements for the invasion of land and freshwater habitats by nemertean worms. In: Brief Reviews. Colonizing new worlds. *Journal of Zoology, London. 219*: 517-526.

Gibson, R., Sánchez, M. and Méndez, M. 1990a. A new species of *Procephalothrix* (Nemertea, Anopla, Archinemertea) from Chile. *Journal of Natural History. 24*: 277-287.

Gibson, R. and Sang, Q. 1991. A new freshwater heteronemertean from the Zhujiang (Pearl River), People's Republic of China. *Hydrobiologia. 220*: 167-178.

Gibson, R., Wickham, D.E. and Kuris, A.M. 1990b. A new genus and species of monostiliferoidean nemertean (Nemertea:Enopla) found on an egg mass of the anomuran decapod *Paralithodes camtschatica. Zoological Journal of the Linnean Society. 98*: 185-198.

Gillespie, J.H. 1978. A general model to account for enzyme variation in natural populations. V. The SAS-CFF model. *Theoretical Population Biology. 14*: 1-45.

Gooch, J.L., Smith, B.S. and Knupp, D. 1972. Regional survey of gene frequencies in the mud snail *Nassarius obsoletus. Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 142*: 36-48.

Gontcharoff, M. 1951. Biologie de la Régénération et de la Reproduction chez quelques Lineidae de France. *Annales des Sciences Naturelles, Zoologie. Series 11. 13*: 149-235.

Gontcharoff, 1957. Étude des rhabdites de la trompe de *Lineus ruber* (Némertien) au microscope électronique. *Compte rendu hebdomadaire des séances de l'Académie des sciences. Paris. 244*: 1539-1541.

Gontcharoff, M. 1960. Le développement post-embryonnaire et la croissance chez *Lineus ruber* et *Lineus viridis* (Nemertes Lineidae). *Annales des Sciences Naturelles, Zoologie. Series 12. 2*: 225-279.

Gontcharoff, M. and Lechenault, H. 1958. Sur le déterminisme de la ponte chez *Lineus lacteus. Compte rendu hebdomadaire des séances de l'Academie des sciences. Paris. 246*: 1630-1632.

Gontcharoff, M. and Lechenault, H. 1966. Ultrastructure et histochemie des glandes sous épidermiques chez *Lineus ruber* et *Lineus viridis. Histochemie. 6*: 320-335.

Gordon, A.H. 1975. *Electrophoresis of Proteins in Polyacrylamide and Starch Gels.* Amsterdam, North Holland.

Gorman, G.C. and Renzi, J. 1979. Genetic distance and heterozygosity estimates in electrophoretic studies: Effects of sample size. *Copeia 1979*: 242-249.

Gosling, E.M., 1992. Genetics of *Nytilus*. In: Gosling, E.M. (Ed.) *The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Developments in Aquaculture and Fisheries Science, Volume 25*. pp. 309-382. Elsevier Science Publishers B.V. The Netherlands.

Goss-Custard, J.D., Jones and Newbury. 1977. The ecology of the Wash, I, distribution and diet of wading birds. *Journal of Applied Ecology. 14*: 701-719.

Gottlieb, L.D. 1977. Electrophoretic evidence and plant systematics. *Annals of the Missouri Botanical Garden. 64*: 161-180.

Grahame, J and Branch, G.M. 1985. Reproductive patterns of marine invertebrates. *Oceanography and Marine Biology Annual Review. 23*: 373-398.

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

Grassle, J.F. and Maciolek, N.J. 1992. Deep-sea species richness: Regional and local diversity estimates from quantitative bottom samples. *American Naturalist. 139 (2)*: 313-341.

Gunnerus, J.E. 1770. Nogle smaa rare og Meestendeelen nye norske søedyr. *In Skrifter Kiobenhavnske Selskab*. Kopenhagen. Bd 10: 166pp.

Hagerman, G.M. and Reiger, R.M. 1981. Dispersal of benthic meiofauna by wave and current action in Bogue Sound, North Carolina, U.S.A. *Pubblicazioni della Stazione Zoologica di Napoli, I. Marine Ecology. 2 (3)*: 245-270.

Haldane, M.B.S. 1954. An exact test for randomness of mating. *Journal of Genetics. 52*: 631-635.

Handford, P. and Nottebohm, F. 1976. Allozymic and morphological variation in population samples of rufous-collared sparrow *Zonotrichia capensis* in relation to vocal dialects. *Evolution. 30*: 802-817.

Harris, H. and Hopkinson, D.A. 1976 et seq.. *Handbook of Enzyme Electrophoresis in Human Genetics*. Amsterdam, North Holland.

Harris, H., Hopkinson, D.A. and Edwards, Y.H. 1977. Polymorphism and the subunit structure of enzymes: A contribution to the neutralist-selectionist controvesy. *Proceeding of the National Academy of Science. U.S.A. 74*: 698-701.

Hartl, D.L. 1980. *Principles of Population Genetics*. Sinauer Associates Incorporated. Sunderland. Mass.

Hartl, D.L. and Clark, A.G. 1989. *Principles of Population Genetics*. Second Edition. Sinauer Associates Incorporated, Sunderland, Mass. 682pp.

Haylor, G.S., Thorpe, J.P. and Carter, M.A. 1984. Genetic and ecological differentiation between sympatric colour morphs of the common intertidal sea anemone *Actinia equina*. *Marine Ecology Progress Series. 16*: 281-289.

Hedgecock, D. 1979. Biochemical genetic variation and evidence of speciation in *Cthalamus* of the tropical eastern Pacific. *Marine Biology. 54*: 207-214.

Hedgecock, D. 1986. Is gene flow from pelagic larval dispersal important in the

adaptation and evolution of marine invertebrates? *Bulletin of Marine Science. 39 (2)*: 550-564.

Hedrick, P.W., Ginevan, M.E. and Ewing, E.P. 1976. Genetic polymorphism in heterogenous environments. *Annual Review of Ecology and Systematics. 7*: 1-32.

Heine, J.N., McClintock, J.B., Slattery, M. and Weston, J. 1991. Energetic composition, biomass, and chemical defense in the common antarctic nemertean *Parborlasia corrugatus* McIntosh. *Journal of Experimental Marine Biology and Ecology. 153*: 15-25.

Highsmith, R.C. 1982. Reproduction by fragmentation in corals. *Marine Ecology Progress Series. 7*: 207-226.

Highsmith, R.C. 1985. Floating and algal rafting as potential dispersal mechanisms in brooding invertebrates. *Marine Ecology Progress Series. 25*: 169-179.

Hillis, D.M. and Moritz, C. An overview of applications of molecular systematics. In: Hillis, D.M. and Moritz, C. (Eds.) *Molecular systematics*. Sinauer Associates, Incorporated. Sunderland, Mass. 588pp.

Hoffmann, R.J. 1987. Short-term stability of genetic structure in populations of the sea anemone *Metridium senile*. Marine Biology. 93: 499-507.

Holgate, P. 1966. A mathematical study of the founder principle of evolutionary genetics. *Journal of Applied Probability. 3*: 115-128.

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

Hubrecht, A.A.W. 1886. Contributions to the embryology of the Nemertea. *Quaterly Journal of Microscopical Science. 26*: 417- 448.

Humes, A.G. 1942. The morphology, taxonomy, and bionomics of the nemertean genus *Carcinonemertes. Illonois Biological Monographs. 18*: 1-105.

Hunt, A. and Ayre, D.J. 1989. Population structure in the sexually reproducing sea

anemone *Oulactis muscosa*. *Marine Biology. 102*: 537-544.

Hylbom, R. 1957. Studies on palaeonemerteans of the Gullmar Fiord area (west coast of Sweden). *Arkiv för Zoologi. 10*: 539-582.

Hyman, L.H. 1951. *The Invertebrates: Volume 2. Platyhelminthes and Rhynchocoela. The acoelomate Bilateria.* McGraw-Hill Book Company, Incorporated. London. 550pp.

International Commission of Zoological Nomenclature. 1992.Opinion 1675. *Amphiporus* Ehrenberg, 1831 (Nemertea): *Planaria lactiflorea* Johnston, 1828 designated as the type species. In: Tubbs, P. (Ed.) *Bulletin of Zoological Nomenclature. 49(2)*: 157.

Istock, C.A. 1967. The evolution of complex life cycle phenomena: an ecological perspective. *Evolution. 21*: 592-605.

Iwata, F. 1960a. Studies on the comparative embryology of nemerteans with special reference to their interrelationships. *Publications from the Akkeshi Marine Biological Station. 10*: 1-51.

Iwata, F. 1960b. The life history of the nemertea. *The Bulletin of the Marine Biological Station of Asamushi Tohoku University. 10. No.3*: 95-97.

Iwata, F. 1967. *Uchidana parasita* nov. gen. et nov. sp., a new parasitic nemertean from Japan with peculiar morphological characters. *Zoologischer Anzeiger. 178*: 122-136.

Iwata, F. 1993, in press. *Paralineopsis taki* Gen. et sp. nov. A littoral heteronemertean from Japan, provided with special proboscidean and circulatory organs of significance to nemertean systematics. *Hydrobiologia.*

Jablonski, D. 1986. Larval ecology and macroevoltuion in marine invertebrates. *Bulletin of Marine Science. 39*: 565-587.

Jablonski, D. and Lutz, R.A. 1983. Larval ecology of marine benthic invertebrates: Paleobiological implications. *Biological Review. 58*: 21-89.

Jackson, J.B.C. 1986. Modes of dispersal of clonal benthic invertebrates: Consequences

for species, distributions and genetic structure of local populations. *Bulletin of Marine Science. 39(2)*: 588-606.

Janson, K.and Ward, R.D. 1984. Microgeographic variation in allozyme and shell characters in *Littorina saxatilis* Olivi (Prosobranchia: Littorinidae). *Biological Journal of the Linnean Society. 22*: 289-307.

Jennings, J.B. 1962. A histochemical study of digestion and digestive enzymes in the rhynchocoelan *Lineus ruber* (O.F. Müller). *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 122*: 63-72.

Jennings, J.B. and Gibson, R. 1969. Observations on the nutrition of seven species of rhynchocoelan worms. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 136*: 405-433.

Johannesson, K. and Johannesson, B. 1990. Genetic variation within *Littorina saxatilis* (Olivi) and *Littorina neglecta* Bean; is *L. neglecta* a good species. Hydrobiologia. 193: 89-97.

Johnson, M.S. and Black, R. 1984. The Wahlund effect and the geographical scale of variation in the intertidal limpet *Siphonaria* sp. *Marine Biology. 79*: 295-302.

Johnston, G. 1827-1828. Contributions to the British fauna. *Journal of Zoology. 3*: 486-491.

Johnston, G. 1833. Illustrations in British zoology. *Magazine of Natural History, London. 6*: 232-235.

Johnston, G. 1837. Miscellanea Zoologica. II. A description of some planarian worms. *Magazine of Zoology and Botany. 1*: 529-538.

Jokiel, P.L. 1984. Long distance dispersal of reef corals by rafting. *Coral Reefs. 3*: 113-116.

Jokiel, P.L. 1987. Ecology, biogeography and evolution of corals in Hawaii. *Trends in Ecology and Evolution. 2*: 179-182.

Jokiel, P.L. 1989. Rafting of reef corals and other organisms at Kwajalein Atoll. *Marine Biology. 101*: 483-493.

Joubin, L. 1894. *Les Nemertiens*. In: Blanchard, R. and de Guerne, J. Societe d'Editions Scientifiques, Paris. 235pp.

Keferstein, W. 1862. Untersuchungen uber die Nemertinen. *Zeitschrift fur Wissenschaftliche Zoologie. Bd. 12*: 51-90.

Kem, W.R. 1973. Biochemistry of Nemertine Toxins. In: Martin, D.F. and Padilla, G.M. (Eds) *Marine Pharmacognosy. Action of Marine Biotoxins at the Cellular Level. Cell Biology: A Series of Monographs*. Academic Press Incorporated, New York and London.

Kimura, M. 1955. Solution of a process of random genetic drift with a continuous model. *Proceedings of the National Academy of Science. U.S.A. 41*: 144-150.

Kimura, M. 1983. *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge. 367pp.

Kirsteuer, E. 1967. New marine nemerteans from Nossi Be, Madagascar. *Zoologischer Anzeiger. 178*:110-122.

Kirsteuer, E. 1971. The interstitial nemertean fauna of marine sand. *Smithsonian Contributions to Zoology. 76*: 17-19.

Knox, G.A. 1970. Antarctic marine ecosystems. In: Holgate, M.W. (Ed.). *Antarctic ecology*. Academic, New York. 1: 69-96.

Koehn, R.K., 1985. Adaptive aspects of biochemical and physiological variability. In: Gibbs, P.E. (Ed.) *Proceedings of the 19th European Marine Biology Symposium*. Cambridge University Press, Cambridge. 425-441.

Koehn, R.K., Hall, J.G., Innes, D.J. and Zera, A.J. 1984. Genetic differentiation of *Mytilus edulis* in eastern North America. *Marine Biology. 79*: 117-126.

Koehn, R.K., Milkman. R. and Mitton, J.B. 1976. Population gentics of marine pelecypods. IV. Selection, migration and genetic differentiation in the Blue Mussel, *Mytilus edulis*. Evolution. 30: 2-32.

Koehn, R.K., Newell, R.I.E. and Immermann, F. 1980. Maintenance of an aminopeptidase allele frequency cline by natural selection. *Proceedings of the*

National Academy of Science. U.S.A. 77: 5385-5389.

Kölliker, A. 1845. Über drei neue Gattungen von Würmern. *Veröffentlichungen der Schweizerischen Gesellschaft für Geschichte der Medizin und der Naturwissenschaften*. 86-98.

Korotkevich, V.S. 1982. New and rare nemerteans (Nemertini) from the southern shelf of the Sakhalin Island. *Issledovaniya Faunui Morei. 29(37)*:12-26.

Korringa, P. 1941. Experiments and observations on swarming, pelagic life and settling in the European flat oyster, *Ostrea edulis* L. *Archives Néerlandaises de Zoologie. 5*: 1-249.

Koukouras, A. and Russo, A. 1991. Midlittoral soft substratum macrofaunal assemblages in the North Aegean Sea. *Marine Ecology. Pubblicazioni della Stazione Zoologica di Napoli I. 12 (4)*:293-316.

Kuris, A.M. 1978. Life-cycle, distribution and abundance of *Carcinonemertes epialti*, a nemertean egg predator of the shore crab, *Hemigrapsus oregonensis*, in relation to host size, reproduction and molt cycle. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 154*: 121-137.

Kuris, A.M., Blau, S.F., Paul, A.J., Shields, J.D. and Wickham, D.E. 1991. Infestation by brood symbionts and their impact on egg mortality of the red king crab, *Paralithodes camtschatica*, in Alaska: geographic and temporal variation. *Canadian Journal of Fisheries and Aquatic Sciences. 48 (4)*: 559-568.

Kuris, A.M. and Wickham, D.E. 1987. Effect of nemertean egg predators on crustaceans. *Bulletin of Marine Science. 41 (2)*: 151-164.

Leary, R.F. and Booke, H.E. 1990. Starch gel electrophoresis and species distinctions. In: Schreck, C.B. and Moyle, P.B. (Eds.). *Methods for Fish Biology*. American Fisheries Society, Bethesda. Maryland.

Lechenault, H. 1965. Neurosécrétion et osmorégulation chez les Lineidae (Hétéronémertes). *Compte rendu hebdomadaire des séances de l'Académie des sciences. Paris. 261*: 4868-4871.

Leidy, J. 1851. Corrections and additions to former papers on helminthology published in the Proceedings of the Academy. *Proceedings of the Academy of Natural Sciences of Philadelphia. 5*: 124-126.

Leidy, J. 1855. Contributions towards a knowledge of the marine invertebrate fauna of the coasts of Rhode Island and New Jersey. *Journal of the Academy of Natural Sciences of Philadelphia. 3*: 135-152.

Lessios, H.A. 1992. Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Marine Biology. 112*: 517-523.

Leuckart, F.S. 1828. *Breves animalium quorundam maxima ex parte marinorum descriptiones*. Heidelbergae.

Levene, H. 1949. On a matching problem arising in genetics. *The Annals of Mathematical Statistics. 20*: 91-94.

Levington, J.S. and Suchanek, T.H. 1978. Geographic variation, niche breadth and genetic differentiation at different geographic scales in the mussels *Mytilus californianus* and *M. edulis*. *Marine Biology. 49*: 363-375.

Lewontin, R.C. 1958. A general method for investigating the equilibrium of gene frequencies in a population. *Genetics. 43*: 419-434.

Lewontin, R.C. 1974. *Genetic basis of evolutionary change*. Columbia University Press, New York.

Ling, E.A. 1969a. The structure and function of the cephalic organ of the nemertine *Lineus ruber*. *Tissue Cell. 1*: 503-524.

Ling, E.A. 1969b. *The structure and function of the cephalic organs of the nemertines (Lineus ruber and Amphiporus lactifloreus)*. Ph.D. thesis. University of Cambridge. 202pp.

Ling, E.A. 1971. The proboscis apparatus of the nemertine *Lineus ruber*. *Philosophical Transactions of the Royal Society of London. Series B. 262*: 1-22.

Lucas, J.S. 1982. Quantitative studies of feeding and nutrition during larval development of the coral reef asteroid, *Acanthaster planci* (L.). *Journal of Experimental*

Marine Biology and Ecology. 65: 173-193.

Macaranas, J.M., Ablan, C.A., Plante, M.J.R., Benzie, J.A.H. and Williams, S.T. 1992. Genetic structure of giant clam (*Tridacna derasa*) populations from reefs in the Indo-Pacific. *Marine Biology. 113*: 231-238.

Manchenko, G.P. and Balakirev, E.S. 1982. Levels of genetic variability in marine invertebrates. In: Kafanov (Ed.) *Biology of Shelf Zones of the World Ocean. Vladivostok. Vol. 2*: 91-93.

Manchenko, G.P. and Balakirev, E.S. 1984. Allozymic variation in *Anthopleura orientalis* from Peter the Great Bay of the Sea of Japan. *Genetika. 20*: 2072-2074.

Manwell, C. and Baker, C.M.A. 1963. A sibling species of sea cucumber discovered by starch gel electrophoresis. *Comparative Biochemistry and Physiology. 10*: 39-53.

Martin, R.F. and Selander, R.K. 1975. Morphological and biochemical evidence of hybridization between cave and barn swallows. *Condor. 77*: 362-364.

Mastro, E., Chow, V. and Hedgecock, D. 1982. *Littorina scutulata* and *Littorina plena*: sibling species status of two prosobranch gastropod species confirmed by electrophoresis. *Veliger. 24*: 239-246.

Maynard Smith, J. 1989. *Evolutionary Genetics*. Oxford University Press, Oxford. 325pp.

Mayr, E. 1957. Species concepts and definitions, in The Species Problem. *American Association for the Advancement of Science. Publication No. 50*. Washington D.C. 1-22.

Mayr, E. 1963. *Animal Species and Evolution*. Cambridge: Harvard University Press.

Mayr, E. 1982. *The Growth of Biological Thought. Diversity, Evolution and Inheritance*. Belknap Press of Harvard University Press. Cambridge, Mass. 974 pp.

McDermott, J.J. 1976. Predation of the razor clam *Ensis directus* by the nemertean worm

Cerebratulus lacteus. *Chesapeake Science*. *17 (4)*: 299-301.

McDermott, J.J. 1984. The feeding biology of *Nipponnemertes pulcher* (Johnston) (Hoplonemertea), some ecological implications. *Ophelia*. *23 (1)*: 1-21.

McIntosh, W.C. 1873-74. *A monograph of the British annelids*. *Part 1*. The nemerteans. Ray Society. London. 214pp.

McIntosh, W.C. 1876. Descriptions of some new species of Annelida from Kerguelen's Island. *Annals and Magazine of Natural History*. *Series 4*. *17*: 318-323.

McKinnon, A.D., Kimmerer, W.J. and Benzie, J.A.H. 1992. Sympatric sibling species within the genus *Acartia* (Copepoda: Calanoida): A case study from Westernport and Port Phillip Bays, Australia. *Journal of Crustacean Biology*. *12 (2)*: 239-259.

Milkman, R. 1978. The maintenance of polymorphisms by natural selection. In: Battaglia, B. and Beardmore, J.A. (Eds.) *Marine organisms, Genetics, Ecology and Evolution*. Plenum Press, New York. 3-22.

Mladenov, P.V. and Emson, R.H. 1990. Genetic structure of populations of two closely related brittle stars with contrasting sexual and asexual life histories, with observations on the genetic structure of a second asexual species. *Marine Biology*. *104*: 265-274.

Montagu, G. 1804. Description of several marine animals found on the south coast of Devonshire. *Transactions of the Linnean Society of London*. *7*: 61-85.

Moore , J. and Gibson, R. 1981. The *Geonemertes* problem (Nemertea). *Journal of Zoology*. *194*: 175-207.

Moore, J. and Gibson, R. 1985. The evolution and comparative physiology of terrestrial and freshwater nemerteans. *Biological Review*. *60*: 257-312.

Müller, O.F. 1774. *Vermium Terrestrium et Fluviatilium, seu Animalium Infusoriorum, Helminthicorum, et Testaceorum, non Marinorum, Succincta Historia*. Havniae et Lipsiae, Copenhagen. *1(2)*: 57- 72.

Munksgaard, C. 1990. Electrophoretic separation of morphologically similar species of

the genus *Rissoa* (Gastropoda: Prosobranchia). *Ophelia. 31 (2)*: 97-104.

Munro, J.L., Jones, A.C. and Dimitriou, D. 1968. Abundance and distribution of the pink shrimp (*Panaeus duodarum*) on the Tortugas Shelf of Florida, August, 1962-October, 1964. *Fishery Bulletin of the Fish and Wildlife Service. United States Department of the Interior. 67*: 165-181.

Murphy, P.G. 1978. *Collisella austrodigitalis* sp. nov.: a sibling species of limpet (Acmaeidae) discovered by electrophoresis. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 155*: 193-206.

Murphy, R.W., Sites, J.W., Buth, D.G. and Haufler, C.H. 1990. Proteins I: Isozyme electrophoresis. In: Hillis, D.M. and Moritz, C. (Eds.). *Molecular Systematics*. Sinauer Associates Incorporated, Sunderland, Mass. 45-146.

Neel, J.V. and Thompson, E.A. 1978. Founder effect and the number of private polymorphisms observed in Amerindian tribes. *Proceedings of the National Academy of Science of the U.S.A. 75*: 1904-1908.

Nei, M. 1972. Genetic distance between populations. *American Naturalist. 106*: 283-292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics. 89*: 583- 590.

Nei, M. 1983. Genetic polymorphism and the role of mutation in evolution. In: Nei, M. and Koehn, R. (Eds.) *Evolution of Genes and Proteins*. Sinauer Associates, Sunderland, Mass. 165-190.

Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press. New York. 512pp.

Nei, M., Fuerst, P.A. and Chakraborty, R. 1978. Subunit molecular weight and genetic variability of proteins in natural populations. *Proceedings of the National Academy of Science. U.S.A. 75*: 3359-3362.

Nei, M. and Graur, D. 1984. Extent of protein polymorphism and the neutral mutation theory. *Evolutionary Biology. 17*: 73-118.

Nei, M., Maruyama, T. and Chakraborty, R. 1975. The bottleneck effect and genetic variability in populations. *Evolution. 29, No. 1*: 1-10.

Nei, M. and Roychoudhury, A.K. 1974. Sampling variances of heterozygosity and genetic distance. *Genetics. 76*: 379-390.

Nevo, E. 1978. Genetic variation in natural populations: patterns and theory. *Theoretical Population Biology. 13*: 121-177.

Nevo, E. 1983. Adaptive significance of protein variation. In: Oxford, G.S. and Rollinson, D. (Eds.) *Protein Polymorphism: Adaptive and Taxonomic Significance*. Academic Press, London. 239-282.

Nevo, E., Beiles, A. and Ben-Schlomo, R. 1984. The evolutionary significance of genetic diversity: ecological demographic and life-history correlates. In: Mani, G.S. (Ed.) *Evolutionary Dynamics of Genetic Diversity*. Heidelberg: Springer-Verlag. 13- 212.

Nordhausen, W. 1988. Impact of the nemertean *Lineus viridis* on its polychaete prey on an intertidal sand flat. *Hydrobiologia. 156*: 39-46.

Norenburg, J.L. 1985. Structure of the nemertine integument with consideration of its ecological and phylogenetic significance. *American Zoologist. 25*: 37-51.

Norenburg, J.L. 1988. Remarks on marine interstitial nemertines and key to the species. *Hydrobiologia. 156*: 87-92.

Norenburg, J.L. 1993, in press. *Riserius pugetensis* n.g., n.sp., a mesopsammic heteronemertine with a problematic phylogeny. *Hydrobiologia.*

Nusbaum, J. and Oxner, M. 1910. Studien über die Regeneration der Nemertinen, I. Regeneration bei *Lineus ruber* (Müll). *Archiv für Entwicklungsmechanik der Organismen. 30*: 74-132.

Nusbaum, J. and Oxner, M. 1911a. Die Bildung des ganzen neuen Darmkanals durch Wanderzellen mesodermalen Ursprunges bei der Kopfrestitution des *Lineus lacteus* (Grube) (Nemertine). *Zoologischer Anzeiger. 37*: 302-315.

Nusbaum, J. and Oxner, M. 1911b. Weitere Studien über die Regeneration der

Nemertinen. I. Regeneration bei *Lineus ruber* Müll. *Archiv für Entwicklungsmechanik der Organismen. 32*: 349-396.

Nusbaum, J. and Oxner, M. 1912. Fortgesetzte Studien über Regeneration der Nemertinen. II. Regeneration des *Lineus lacteus* Rathke. *Archiv für Entwicklungsmechanik der Organismen. 35*: 236-308.

Nusbaum, J. and Oxner, M. 1913. Die Embryonalentwicklung des *Lineus ruber* Müll. Ein Beitrag zur Entwicklungsgeschicte der Nemertinen. *Zeitschrift für wissenschaftliche Zoologie. 107*: 78-197.

Oken, L. 1815. *Lehrbuch der Naturgeschicte. 3. Theil. Zoologie.* Leipzig and Jena. 365pp.

Örsted, A.S. 1843. Forsog til en ny classification af Planarierne (Planariea Duges) grundet paa mikroskopisk-anatomiske Undersogelser. *Naturhistorisk Tidskrift udgived af Henrik Krøyer. Kjøbenhavn. 4*: 519-581.

Otto, R.S. 1981. Eastern Bering Sea crab fisheries. In: Hood, D.W. and Calder, J.A. (Eds.). *The Eastern Bering Sea Shelf: oceanography and resources.* University of Washington Press, Seattle, WA. 1037-1066.

Oxner, M. 1908. Sur de nouvelles espèces de Némertes de Roscoff et quelques remarques sur la coloration vitale. *Bulletin L'Insitut Océanographique Monaco. No. 127*: 1-16.

Oxner, M. 1911. Analyse biologique d'une serie d'expériences concernant l'avenement de la maturité sexuelle, la régénération et l'inanition chez les némertiens *Lineus ruber* (Müll). et *L. lacteus* (Rathke). Compte rendu de l'Académie de sciences, Paris. 153: 1168-1171.

Pamilo, P. and Varvio-Aho, S. Testing genotype frequencies and heterozygosities. *Marine Biology. 79.* 99-100.

Pantin, C.F.A. 1960. *Notes on microscopical technique for zoologists.* Cambridge University Press. Cambridge.

Pantin, C.F.A. 1969. The genus *Geonemertes. Bulletin of the British Museum of*

Natural history, (Zoology). 18: 263-310.

Parker, E.D., Burbanck, W.D., Burbanck, M.P. and Anderson, W.W. 1981. Genetic differentiation and speciation in the estuarine isopods *Cyathura polita* and *Cyathura burbancki*. *Estuaries. 4*: 213-219.

Pearse, J.S. and Giese, A.C. 1966. The organic constitution of several benthonic invertebrates from McMurdo Sound, Antacrtica. *Comparative Biochemistry and Physiology. 18*: 47-57.

Pechenik, J.A. 1979. Role of encapsulation in invertebrate life histories. *American Naturalist. 114*: 859-870.

Powell, J.R. 1975. Protein variation in natural populations of animals. *Evolutionary Biology. 8*: 79-119.

Poulik, M.D. 1957. Starch gel electrophoresis in a discontinuous system of buffers. *Nature. 180*: 1477-1479.

Punnett, R.C. 1901. Lineus. In: Herdman, W.A. (Ed.) *Liverpool Marine Biology Committee Memoirs on Typical British Marine Plants and Animals. 7*: 37pp.

Punnett, R.C. 1903. On the nemerteans of Norway. *Bergens museums Årsberetning. 2*: 1-35.

Quatrefages, A. De. 1846. Etudes sur les types inferieurs de l'embranchement des anneles. Memoire sur la famille des Nemertiens. *Annales des Sciences Naturelles. 6 (3)*: 173-303.

Rathke, J. 1799. Jagttagelser henhorende til Indvoldeormenes og Bloddyrenes Naturhistorie. *Skrivter af Naturhistoire Selskabet, Kiobenhavn. 5*: 61-153.

Renier, S.A. 1804. Propetto della Classe dei Vermi, nominati e ordinati secundo il Sistema di Bosc. An uncompleted work issued in G. Meneghini's introduction to *Osservazione postume di Zoologica Adriatica del Professore Stefano Andrea Renier*. Venice, 1847. 57-66.

Richardson, B.J., Baverstock, P.R. and Adams, M. 1986. *Allozyme Electrophoresis - A Handbook for Animal Systematics and Population Studies*. Academic Press,

Sydney.

Riser, N.P. 1974. Nemertinea. In Giese, A.C. and Pearse, J.S. (Eds.). *Reproduction of Marine Invertebrates. Vol. I. Acoelomate and Pseudocoelomate Metazoans.* Academic Press, New York and London.

Riser, N.W. 1993, in press. Observations on the morphology of some North American nemertines. *Hydrobiologia.*

Roe, P. 1970. The nutrition of *Paranemertes peregrina* (Rhynchocoela: Hoplonemertea). I. Studies on food and feeding behaviour. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 139*: 80-91.

Roe, P. 1976. Life history and predator-prey interactions of the nemertean *Paranemertes peregrina* Coe. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 150*: 80-106.

Roe, P. 1979. A comparison of aspects of the biology of *Paranemertes peregrina* (Nemertea) from Bodega harbor, California, and Washington State. *Pacific Science. 33 (3)*: 281-287.

Roe, P., Crowe, J.H., Crowe, L.M. and Wickham, D.E. 1981. Uptake of amino acids by juveniles of *Carcinonemertes errans* (nemertea). *Comparative Biochemistry and Physiology. 69A*: 423-427.

Roe, P. 1984. Laboratory studies of feeding and mating in species of *Carcinonemertes* (Nemertea: Hoplonemertea). *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 167*: 426-436.

Rogers, A.D., Gibson, R. and Thorpe, J.P. 1992. A new species of *Callinera* (Nemertea, Anopla, Palaeonemertea) from the Isle of Man. *Zoologica Scripta. 21 No.2*: 119-128.

Rogers, A.D., Junoy, J., Gibson, R. and Thorpe, J.P. in press. Enzyme electrophoresis, genetic identity and description of a new genus and species of heteronemertean (Nemertea, Anopla) from north-western Spain and North Wales. *Hydrobiologia.*

Rogers, J.S. 1972. Measures of genetic similarity and genetic distance. *Studies In Genetics VII. University of Texas Publication. 7213*: 145-153.

Rowell, T.W. and Woo, P. 1990. Predation by the nemertean worm, *Cerebratulus lacteus* Verrill, on the soft-shell clam, *Mya arenaria* Linnaeus, 1758, and its apparent role in the destruction of a clam flat. *Journal of Shellfish Research. 9 (2)*: 291-297.

Russo, C.A.M., Solé-Cava, A.M. and Thorpe, J.P. in press. Population structure and genetic variation in two tropical sea anemones (Cnidaria, Actiniidae) with differing reproductive strategies.

Sargent, J.R. and George, S.G. 1975. *Methods in Zone Electrophoresis*. B.D.H. Chemicals. Poole, Dorset, England.

Sarich, V.M. 1977. Rates, sample sizes and the neutrality hypothesis for electrophoresis in evolutionary studies. *Nature. 265*: 24-28.

Sarver, S.K., Landrum, M.C. and Foltz, D.W. 1992. Genetics and taxonomy of ribbed mussels (*Geukensia* spp.) *Marine Biology. 113*: 385-390.

Schaal, B.A. and Anderson W.W. 1974. An outline of techniques for starch gel electrophoresis of enzymes from the American oyster *Crassostrea virginica. Co. Mar. Sci. Center Tech. Rep. Ser. 74*: 3-19.

Scheltema, R.S. 1971. Larval dispersal as a means of genetic exchange between geographically separated populations of shoal-water benthic marine gastropods. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 140*: 284-322.

Scheltema, R.S. 1986. On dispersal and planktonic larvae of benthic invertebrates: An eclectic overview and summary of problems. *Bulletin of Marine Science. 39 (2)*: 290-322.

Scheltema, R.S. 1989. Planktonic and non-planktonic development among prosobranch gastropods and its relationship to the geographic range of species. In: Ryland, J.S. and Tyler, P.A. (Eds.) *Reproduction, Genetics and Distributions of*

Marine Organisms. 23rd European Marine Biology Symposium. Universuty of Wales, Swansea. Olsen & Olsen, Denmark. 183-188.

Scheltema, R.S. and Williams, I.P. 1983. Long-distance dispersal of planktonic larvae and the biogeography and evolution of some Polynesian and Western Pacific mollusks. *Bulletin of Marine Science. 33*: 545-565.

Schepotieff, A. 1913. Ueber die Bedeutung der Wassermann'schen Reaction fur die biologische Forschung. *Zoologischer Anzeiger. 41*: 49-54.

Schmidt, G.A. 1934. Ein zweiter Entwicklungstypus von *Lineus gesserensis-ruber* O.F. Müll. (Nemertini). *Zoologischer Jahresbericht. 58*; 607-660.

Schmidt, G.A., 1946. Genetic and ecological relations of littoral nemerteans of the genus *Lineus. Comptes Rendus (Doklady) de l'Académie des Sciences de l'URSS. 51, No.5*: 405-407.

Schmidt, G.A. and Jankovskaia, L.A. 1938. Biologie de la réproduction de *Lineus gesserensis-ruber* de Roscoff et du Golfe de Kola. *Archives de Zoologie Expérimentale et Générale. 79*: 487-513.

Schultze, M.S. 1851. *Beitrage zur Naturgeschichte der Turbellarian.* C. A. Koch, Griefswald.

Schultze, M.S. 1853. Zoologische Skizzen. *Zeitschrift fur Wissenschaftliche Zoologie. Bd 4*: 178-195.

Scopes, R.K. 1968. Methods for starch gel electrophoresis of sarcoplasmic proteins. *Biochemical Journal. 107*: 139-150.

Selander, R.K. 1976. Genic variation in natural populations. In: Ayala, F.J. (Ed.). *Molecular Evolution.* Sinauer Associates Incorporated, Sunderland, Mass. 21-45.

Selander, R.K., Yang, S.Y., Johnson, W.E. and Gentry, J.B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*, I. Variation in the old-field mouse (*Peromyscus polionotus*). *Studies in Genetics IV: 49-90. University of Texas publication 7103.* Austin, Texas: University of Texas.

Semper, C. 1863. Reisebericht. *Zeitschrift fur Wissenschaftliche Zoologie. Bd 13*: 558-570.

Shaw, C.R. 1965. Electrophoretic variation in enzymes. *Science. 144*: 936-943.

Shaw, C.R. and Prasad, R. 1970. Starch gel electrophoresis of enzymes: A compilation of recipes. *Biochemical Genetics. 4*: 297-320.

Shaw, P.W. 1989. Seasonal patterns and possible long-term effectiveness of sexual reproduction in three species of Sagartiid sea anemones. In: Ryland, J.S. and Tyler, P.A. (Eds.) *Reproduction, Genetics and Distributions of Marine Organisms. 23rd European Marine Biology Symposium, 1988*. Olsen & Olsen, Denmark. 189-200.

Shaw, P.W., Beardmore, J.A. and Ryland, J.S. 1987. *Sagartia troglodytes* (Anthozoa: Actiniaria) consists of two species. *Marine Ecology Progress Series. 41*: 21-28.

Shields, W.M. 1982. *Philopatry, inbreeding, and the evolution of sex*. State University. New York Press, Albany. 245pp.

Shields, J.D., Wickham, D.E. and Kuris, A.M. 1989. *Carcinonemertes regicides* n. sp. (Nemertea) a symbiotic egg predator from the red king crab, *Paralithodes camtschatica* (Decapoda: Anomura), in Alaska. *Canadian Journal of Zoology. 67*: 923-930.

Siciliano, M.J. and Shaw, C.R. 1976. Seperation and Vizualisation of enzymes on gels. In: Smith, I. (Ed.). *Chromatographic and Electrophoretic Techniques, 2. Zone Electrophoresis*. William Heineman Medical Books, London.

Skibinski, D.O.F., Beardmore, J.A. and Cross, T.F. 1983. Aspects of the population genetics of *Mytilus* (Mytilidae; Mollusca) in the British Isles. *Biological Journal of the Linnean Society. 19*: 137-183.

Skibinski, D.O.F., Cross, T.F. and Ahmad, M. 1980. Electrophoretic investigation of systematic relationships in the marine mussels *Modiolus modiolus* L., *Mytilus edulis* L., and *Mytilus galloprovincialis* Lmk. (Mytilidae; Mollusca). *Biological Journal of the Linnean Society. 13*: 65-73.

Slatkin, M. 1985. Gene flow in natural populations. *Annual Review of Ecology and Systematics. 16*: 393-430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science. 236*: 787-792.

Smith, C.A.B. 1970. A note on testing the Hardy-Weinberg law. *Annals of Human Genetics. 33*: 377-383.

Smith, C.A.B. 1977. A note on genetic distance. *Annals of Human Genetics. 40*: 463-479.

Smith, I. 1976. Starch gel electrophoresis. In: Smith, I. (Ed.). *Chromatographic and Electrophoretic Techniques, 2. Zone Electrophoresis*. William Heineman Medical Books, London.

Smith, P.J. and Fujio, Y. 1982. Genetic variation in marine teleosts: High variability in habitat specialists and low variability in habitat generalists. *Marine Biology. 69*: 7-20.

Smith, B.L. and Potts, D.C. 1987. Clonal and solitary anemones (*Anthopleura*) of western North America: population genetics and systematics. *Marine Biology. 94*: 537-546.

Smith, J.K. and Zimmerman, E.G. 1976. Biochemical genetics and evolution of North American blackbirds, family Icteridae. *Comparative Biochemistry and Physiology. 53B*: 319-324.

Smithies, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochemical Journal. 61*: 629-641.

Smithies, O. 1959. An improved procedure for starch gel electrophoresis: Further variations in the serum proteins of normal individuals. *Biochemical Journal. 71*: 585-587.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. W.H. Freeman and Company. San Francisco. 573pp.

Snyder, T.P. and Gooch, J.L. 1973. Genetic differentiation in *Littorina saxatilis* (Gastropoda). *Marine Biology. 22*: 177-182.

Sokal, R.R. and Rohlf, F.J. 1981. *Biometry. The principles and practice of statistics in biological research.* 2nd Edition. W.H. Freeman & Co. New York.

Solé-Cava, A.M., Boury-Esnault, N., Vacelet, J. and Thorpe, J.P. 1992. Biochemical genetic divergence and systematics in sponges of the genera *Coticium* and *Oscarella* (Demospongiae: Homoscleromorpha) in the Mediterranean sea. *Marine Biology. 113*: 299-304.

Solé-Cava, A.M., Klautau, M., Boury-Esnault, N., Borojecic, R. and Thorpe, J.P. 1991a. Genetic evidence for cryptic speciation in allopatric populations of two cosmopolitan species of the calcareous sponge genus *Clathrina. Marine Biology. 111*: 381- 386.

Solé-Cava, A.M. and Thorpe, J.P. 1986. Genetic differentiation between morphotypes of the marine sponge *Suberites ficus* (Demospongiae: Hadromerida). *Marine Biology. 93*: 247-253.

Solé-Cava, A.M. and Thorpe, J.P. 1987a. The uses of electrophoresis in sponge taxonomy. In: Vacelet, J. and Boury-Esnault, N. (Eds.). *Taxonomy of Porifera.* Springer Verlag, Berlin. 243-258.

Solé-Cava, A.M. and Thorpe, J.P. 1987b. Further genetic evidence for the reproductive isolation of the green sea anemone *Actinia prasina* Gosse from the common intertidal beadlet anemone *Actinia equina* (L.). *Marine Ecology Progress Series. 38*: 225- 229.

Solé-Cava, A.M. and Thorpe, J.P. 1990. High levels of genetic variation in marine sponges. In: Rutzler, K. (Ed.). *New Perspectives In Sponge Biology*: Washington: Smithsonian Institution Press. 332-337.

Solé-Cava, A.M. and Thorpe, J.P. 1991. High levels of genetic variation in natural populations of marine lower invertebrates. *Biological Journal of the Linnean Society. 44*: 65-80.

Solé-Cava, A.M., Thorpe, J.P. and Kaye, J.G. 1985. Reproductive isolation with little genetic divergence between *Urticina* (= *Tealia*) *felina* and *U. eques* (Anthozoa: Actiniaria). *Marine Biology. 85*: 279-284.

Solé-Cava, A.M., Thorpe, J.P. and Manconi, R. 1991b. A new Mediterranean species of *Axinella* detected by biochemical genetic methods. In: Reitner, J. and Keupp, H. Fossil and Recent Sponges. Springer-Verlag, Berlin. 315-321.

Southern, R. 1913. Nemertinea. *Proceedings of the Royal Irish Academy. 31 No.55*: 1-20.

Sowerby, J. 1806. *The British Miscellany: or Coloured Figures of New, Rare, or Little Known Animal Subjects; many not before Ascertained to be Inhabitants of the British Isles*. Taylor, London. 136pp.

Speiss, E.B. 1989. *Genes in Populations*. 2nd Edition. John Wiley & Sons, New York.

Stephenson, J. 1911. The nemertines of Millport and its vicinity. *Transactions of the Royal Society of Edinburgh. 48*: 1-29.

Stoddart, J.A. 1983. Asexual production of planulae in the coral *Pocillopora damicornis*. *Marine Biology. 76*: 279-284.

Stoddart, J.A. 1984. Genetical structure within populations of the coral *Pocillipora damicornis*. *Marine Biology. 81*: 19-30.

Strathmann, R.R. 1974. The spread of sibling larvae of sedentary marine invertebrates. *American Naturalist. 108*: 29-44.

Strathmann, R.R. 1980. Why do larvae swim so long? *Paleobiology. 6*: 373-376.

Strathmann, R.R. 1986. What controls the type of larval development? Summary statement for the evolution session. *Bulletin of Marine Science. 39(2)*: 616-622.

Strathmann, R.R. and Strathmann, M.F. 1982. The relation between adult size and brooding in marine invertebrates. *American Naturalist. 119*: 91-101.

Stricker, S.A. 1983. S.E.M. and polarization microscopy of nemertean stylets. *Journal of Morphology. 175*: 153-169.

Stricker, S.A. 1984. Styletogenesis in nemertean worms: The ultrastructure of organelles

involved in intracellular calcification. *Journal of Morphology. 179*: 119-134.

Stricker, S.A. and Cloney, R.A. 1982. Stylet formation in nemerteans. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 162*: 387-403.

Stunkard, H.W. 1982. Mesozoa. In: Parker, S.P. (Ed.) *Synopsis and Classification of Living Organisms. 1*: 853-855. McGraw-Hill, New York.

Sundberg, P. 1979a. *Tubulanus annulatus*, an aposematic nemertean? *Biological Journal of the Linnean Society. 12*: 177-179.

Sundberg, P. 1979b. Statistical analysis of variation in characters in *Tetrastemma laminariae* (Nemertini), with a redescription of the species. *Journal of Zoology. 189*: 39-56.

Sundberg, P. 1980. Intraspecific variation in *Tetrastemma laminariae* (Nemertini): An examination of one of its possible causes. *Journal of Zoology. 192*: 137-141.

Sundberg, P. 1988. A new monostiliferous hoplonemertean (Nemertea), *Oerstedia striata* sp.n., from the west coast of Sweden. *Zoologica Scripta. 17*: 135-139.

Sundberg, P. 1989. Phylogeny and cladistic classification of terrestrial nemerteans: The genera *Pantinonemertes* Moore and Gibson and *Geonemertes* Semper. *Zoological Journal of the Linnean Society. 95*: 363-372.

Sundberg, P. 1990. Gibson's reclassification of the enoplan nemerteans (Enopla, Nemertea): a critique and cladistic analysis. *Zoologica Scripta. 19*: 133-140.

Sundberg, P. 1991. A proposal for renaming the higher taxonomic categories in the phylum Nemertea. *Journal of Natural History. 25 (1)*: 45-48.

Sundberg, P. and Janson, K. 1988. Polymorphism in *Oerstedia dorsalis* (Abilgaard, 1806) revisited. Electrophoretic evidence for a species complex. *Hydrobiologia. 156*: 93-98.

Swofford, D.L. and Olsen, G.J. 1990. Phylogeny Reconstruction. In: Hillis, D.M. and Moritz, C. (Eds.) *Molecular Systematics*. Sinauer Associates Incorporated, Sunderland, Mass.. 411-501.

Swofford, D.L. and Selander, R.B. 1989. *Biosys-1. A computer program for the analysis of allelic variation in population genetics and biochemical systematics. Release 1.7.* Illinois Natural History Survey.

Thorpe, J.P. 1982. The molecular clock hypothesis: Biochemical evolution, genetic differentiation and systematics. *Annual Review of Ecology and Systematics. 13*: 139-168.

Thorpe, J.P. 1983. Enzyme variation, genetic distance and evolutionary divergence in relation to levels of taxonomic separation. In: Oxford, G.S. and Rollinson, D. (Eds.) *Protein polymorphism: adaptive and taxonomic significance.* Academic Press, London. pp. 131-152.

Thorpe, J.P., Beardmore, J.A. and Ryland, J.S. 1978a. Genetic evidence for cryptic speciation in the marine bryozoan *Alcyonidium gelatinosum. Marine Biology. 49*: 27-32.

Thorpe, J.P. and Mundy, S.P. 1980. Biochemical genetics and taxonomy in *Plumatella emarginata* and *P. repens* (Bryozoa: Phylactolaemata). *Freshwater Biology. 10*: 361-366.

Thorpe, J.P., Ryland, J.S. and Beardmore, J.A. 1978b. Genetic variation and biochemical systematics in the marine bryozoan *Alcyonidium mytili.* Marine Biology. 49: 343-350.

Thrope, J.P., Ryland, J.S., Cornelius, P.F.S. and Beardmore, J.A. 1992. Genetic divergence between branched and unbranched forms of the thecate hydroid *Aglaophenia pluma. Journal of the Marine Biological Association of the United Knigdom. 72 (4)*: 887-895.

Thorson, G. 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the Sound (Öresund). *Meddelser fra Kommissionen for Danmarks Fiskeri og Havundersøgelser. 4*: 1- 523.

Todd, C.D. and Doyle, R.W. 1981. Reproductive strategies of marine benthic

invertebrates: A settlement-timing hypothesis. *Marine Ecology Progress Series. 4*: 75-83.

Turbeville, J.M., Field, K.G. and Raff, R.A. 1992. Phylogenetic position of phylum Nemertini, inferred from 18S rRNA sequences: Molecular data as a test of morphological character homology. *Molecular Biology and Evolution. 9 (2)*: 235-249.

Turbeville, J.M. and Ruppert, E.E. 1985. Comparative ultrastructure and the evolution of nemertines. *American Zoologist. 25*: 53-71.

Turner, J.R.G., Johnson, M.S. and Eanes, W.F. 1979. Contrasted modes of evolution in the same genome: Allozymes and adaptive change in *Heliconius*. *Proceedings of the National Academy of Science. U.S.A. 76*: 1924-1928.

Uschakow, P. 1928. Beschreibung einiger neuen Nemertinenarten von Barents-Meere, Weissen Meere und Norwaja-Semlja. *Zoologischer ahresbericht. Zoologische Station zu Neapel. 54*: 407-424.

Valenzuela, C.Y. 1985. Algebraic and epistemological restrictions in studies on Hardy-Weinberg equilibrium. *American Naturalist. 125*: 744-746.

Vermeij, G.J. 1982a. Environmental change and the evolutionary history of the periwinkle (*Littorina littorea*) in North America. *Evolution. 36*: 561-580.

Vermeij, G.J. 1982b. Phenotypic evolution in a poorly dispersing snail after arrival of a predator. *Nature. 299*: 349-350.

Vernet, G. 1966. Les pigments de *Lineus ruber* (O.F. Müller) (Hétéronémertes Lineidae). *Compte rendu hebdomadaire des séances de l'Académie des sciences. Paris. 263*: 191-194.

Vernet, G. 1970. Ultrastructure des photorécepteurs de *Lineus ruber* (O.F. Müller) (Hétéronémertes Lineidae). I. Ultrastructure de l'oeil normal. *Zeitschrift für Zellforschung und mikroskopische Anatomie. 104*: 494-506.

Vernet, G. 1990. *Rhopalura linei*, Orthonectide parasite de l'Hétéronémerte *Lineus ruber*. *Cahiers de Biologie Marine. 31*: 251-255.

Vernet, G. 1992. Localisation du réflexe lucifuge dans les ganglions cérébroïdes dorsaux de la némerte marine, *Lineus ruber* (O.F. Müller): étude expérimentale. *Canadian Journal of Zoology. 70 (No. 4)*: 750-752.

Wahlund, S. 1928. The combination of populations and the appearance of correlation examined from the standpoint of the study of heredity. *Hereditas. 11*: 65-106.

Walsh, P.J. and Somero, G.N. 1981. Temperature adaptation in sea anemones. Physiological and biochemical variability in geographically separate populations of *Metridium senile*. *Marine Biology. 62*: 25-34.

Waples, R.S. 1987. A multispecies approach to the analysis of gene flow in marine shore fishes. *Evolution. 41*: 385-400.

Ward, R.D. 1978. Subunit size of enzymes and genetic heterozygosity in vertebrates. *Biochemical Genetics. 16*: 799-810.

Ward, R.D. 1989. Molecular population genetics of marine organisms. In: Ryland, J.S. and Tyler, P.A. (Eds.) *Reproduction, Genetics and Distributions of Marine Organisms. 23rd European Marine Biology Symposium University of Wales, Swansea,1988*. Olsen & Olsen, Denmark. 235-250.

Ward, R.D. and Beardmore, J.A. 1977. Protein variation in the plaice *Pleuronectes platessa*. *Genetical Research. 30*: 45-62.

Ward, R.D. and Warwick, T. 1980. Genetic differentiation in the molluscan species *Littorina rudis* and *Littorina arcana* (Prosobranchia: Littorinidae). *Biological Journal of the Linnean Society. 14*: 417-428.

Weir, B.S. 1990. *Genetic Data Analysis*. Sinauer Associates, Incorporated. Sunderland, Mass. 377 pp.

Weir, B.S. and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution. 38(6)*: 1358-1370.

Whittam, T.S., Ochman, H. and Selander. 1983. Geographic components of linkage disequilibrium in natural populations of *Escherichia coli*. *Molecular Biology and Evolution. 1*: 67-83.

Wickham, D.E. 1978. A new species of *Carcinonemertes* (Nemertea: Carcinonemertidae) with notes on the genus from the Pacfic coast. *Proceedings of the Biological Society of Washington. 91*: 197-202.

Wickham, D.E. 1979. Predation by *Carcinonemertes errans* on eggs of the Dungeness crab, *Cancer magister*. *Marine Biology. 55*: 45-53.

Wickham, D.E. 1986. Epizootic infestations by nemertean brood parasites on commercially important crustaceans. *Canadian Journal of Fisheries and Aquatic Sciences. 43*: 2295-2302.

Wickham, D.E. and Kuris, A.M. 1985. The comparative ecology of nemertean egg predators. *American Zoologist. 25*: 127-134.

Wickham, D.E., Roe, P. and Kuris, A.M. 1984. Transfer of nemertean egg predators during host molting and copulation. *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass. 167*: 331-338.

Wijnhoff, G. 1914. The proboscidean system in nemertines. *The Quarterly Journal of Microscopical Science. 60*: 273-312.

Wilfert, M. and Gibson, R. 1974. A new genus of hermaphroditic freshwater heteronemertean (Nemertinea). *Zeitschrift fur Morphologie der Tiere. 79*: 87-112.

Williams, K.A., Skibinski, D.O.F. and Gibson, R. 1983. Isoenzyme differences between three closely related species of *Lineus* (Heteronemertea). *Journal of Experimental Marine Biology and Ecology. 66*: 207-211.

Willmer, E.N. 1970. *Cytology and Evolution.* 2nd Edition. Academic Press, New York and London. 649pp.

Willmer, P. 1990. *Invertebrate Relationships. Patterns in Animal Evolution.* Cambridge University Press. Cambridge. 400pp.

Wills, C. 1981. *Genetic Variability.* Clarendon Press, Oxford.

Wilson, A.C., Carlson, S.S. and White, T.J. 1977. Biochemical evolution. *Annual Review of Biochemistry. 46*: 573-639.

Winston, J.E. 1982. Drift plastic - an expanding niche for a marine invertebrate? *Marine Pollution Bulletin. 13*: 348-351.

Wright, C.A. (Ed.) 1978. *Biochemical and Immunilogical Taxonomy of Animals*. Academic Press, London.

Wright, S. 1940. Breeding structure of populations in relation to speciation. *American Naturalist. 74*: 232-248.

Wright, S. 1943. Isolation by distance. *Genetics. 28*: 114-138.

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics. 15*: 323-354.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution. 19*: 395-420.

Wright, S. 1969. *Evolution and the Genetics of Populations. Vol. 2: The Theory of Gene Frequencies*. Chicago. Illonois: University of Chicago Press.

Wright, S. 1978. *Evolution and the Genetics of Populations. Volume 4. Variability in and among Natural Populations*. University of Chicago Press. Chicago, Ill.

Yang, S.Y., Soule, M and Gorman, G.C. 1974. *Anolis* lizards of the eastern Caribbean: a case study in evolution. I. Genetic relationships, phylogeny and colonisation of the roquet group. *Sytematic Zoology. 23*: 387-399.

Zouros, E. and Foltz, D.W. 1987. The use of allozyme variation for the study of heterosis. In: Rattazi, M.C., Scandalios, J.G. and Whitt, G.S. (Eds.) *Isozymes: Current topics in biological and medical research. Vol. 13*: 1-59.

Zuckerkandl, E. and Pauling, L. 1965. Evolutionary divergence and convergence in proteins. In: Bryson, V. and Vogel, H.J. (Eds.). *Evolving Genes and Proteins*. Academic Press, New York. 97-166.

Aspects of the genetics and taxonomy of marine nemerteans.

A.D. Rogers

Abstract

Starch gel electrophoresis was used to investigate the taxonomy and population genetics of several species of common littoral or shallow sublittoral nemerteans found around the North Atlantic coasts of the U.S.A., Canada, Sweden and Europe.

Levels of heterozygosity were estimated for eight heteronemertean and a single hoplonemertean species over a maximum of 13 enzyme loci. Mean observed heterozygosity was found to range between 0.044 for *Lineus ruber* (a low value for marine invertebrates) to 0.278 for *Riseriellus occultus* (a high level of genetic variability). For most species investigated, mean observed fell in a range between 0.08 - 0.12, values which are within the normal range for invertebrate phyla.

Genotype frequency data was used to investigate the genetic structure of populations of four common species of nemertean found around the North Atlantic. These were the heteronemerteans *Lineus ruber*, *Lineus viridis*, *Lineus sanguineus* and the hoplonemertean *Amphiporus lactifloreus*. These species all undergo a form of development which does not include a pelagic larval phase and *Lineus sanguineus* is reputed to only reproduce asexually by fragmentation. Contrary to expectations from other workers, genetic data suggested that in at least two species, *Lineus ruber* and *Lineus viridis* moderate levels of gene-flow were maintained between conspecific populations over a distance of 6,000 km or more. Passive dispersal is suggested as a possible explanation for this.

Lineus sanguineus did not show genetic evidence of asexual reproduction (e.g. a large deviation from genotype frequencies expected under Hardy-Weinberg equilibrium conditions i.e. large F_{IS} values).

A cryptic species and new genus of heteronemertean, *Riseriellus occultus*, discovered by use of electrophoresis, is described. This species resembles two known species of heteronemertean, *Lineus longissimus* and *Lineus viridis*. Genetic evidence for another cryptic species found occurring sympatrically with populations of *Lineus*

ruber and *Lineus viridis* is presented. A new species of palaeaonemertean, *Callinera monensis*, discovered in a harbour on the Isle of Man, is described.

A genetic investigation in to how related members of the genera *Lineus* and *Micrura* were revealed very few common alleles between congeneric species. It is proposed that this is due to fundamental flaws in the taxonomy of the Heteronemertea and that the order is in need of urgent revision.