BRACHIOPOD SERO-TAXONOMY AND THE APPLICATION OF IMMUNOLOGY TO GEOLOGY

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To my parents, Pat and Nat and my husband, Charlie.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 5

Chapter 6

Chapter 8

LIST OF TABLES

SUMMARY

Antisera were generated against representatives of a number of brachiopod families and these were tested by the enzyme-linked immunosorbent assay (ELISA) and the fluorescence ELISA to determine the relationships between the families. Cluster analyses were done and immunological distances calculated using antigens for which homologous antisera were available.

Monoclonal antibodies and hybridoma supernatants were generated against brachiopod extracts and these were tested against a wide range of Recent and fossil antigens.

Antisera were prepared against Kimmeridge clay and carbazole.

The results of this study indicated that the traditional distinction between short-looped and long-looped brachiopods, based on the brachial loop, was not valid and suggested that the distinction is between those long-looped forms in which a median septum is involved in ontogeny and those in which it is not. This work supported the suggestion by Cooper (1973a) that a separate superfamily status should be assigned to the cancellothyrides. The results presented here suggest that lineages split much later than was previously thought.

Phylogenetic information is present in the intra-crystalline macromolecules of brachiopods and can be used in sero-taxonomic studies. This study has shown that a revised taxonomy of brachiopods is required at the family and superfamily status. The integration of molecular taxonomy and morphological taxonomy would benefit both approaches to the study of phylogenetic relationships. This work has also shown that it is possible to apply immunological techniques to the detection of substances of geological interest in rock samples.

Chapter 1. GEOLOGY BACKGROUND

1.1 Preface.

In the initial stages of this project no equipment or facilities for immunological assay or tissue culture existed in the Department of Geology and Applied Geology and it was therefore necessary to rely upon outside sources of equipment at that time. Working visits to our collaborating laboratory in the University of Leiden, The Netherlands, during which different techniques were explored, helped with planning the present work.

A period of consultation with colleagues Drs Matthew Collins, Gerard Muyzer, and Timo Zomerdijk in Leiden led to decisions being made about which pieces of equipment would best serve the purposes of this study. The author, having had five years experience of monoclonal antibody technology, was able to choose tissue culture equipment.

By the end of the three years all the necessary facilities, including equipment for immunological assays, a tissue culture suite for the production of monoclonal antibodies and a gel electrophoresis system and blotting equipment, had been established in this department, the first time such a venture had been undertaken in a Geology department.

During the course of the project it was considered necessary to repeat experiments using as many taxa as possible and a more sensitive detection system for the immunological assay, coupled with a better method of determining relationships between taxa. This is seen in the development of the work reported in Chapters 4 to 6. In Chapter 4, the first part of the study, few taxa were available and the enzyme-linked immunosorbent assay technique was used along with cluster analysis, whereas in Chapter 5 more taxa were available, allowing a more sophisticated measurement of phylogenetic relationships using immunological distances. The work reported in Chapter 6 included more taxa and measurements of immunological distance were determined by a more sensitive immunological assay, the fluorescence enzyme-linked immunosorbent assay. The Dynatech Microfluor reader, for detection of the end product of the fluorescence assay, was tested by our colleagues, considered to be a more sensitive detection system, and was therefore purchased for this study.

1.2 General description and Lifecycle of Brachiopods.

The Phylum Brachiopoda (Duméril, 1806) is composed of marine invertebrate sessile filter feeders. All living brachiopods form part of the sessile benthonic fauna and almost all are epifaunal with brachiopods being permanently attached to the sea floor or to objects on it. During a short larval stage they are free to swim or drift with the currents into a new area and most of their functional organisation is related to this fixed way of life, in that they do not have organs of locomotion or highly developed sense organs. Their defence against predators or other dangers is entirely passive, they simply close their shell when danger threatens. They cannot actively search for food and must utilise whatever source of food approaches them suspended in sea water. Reproduction generally involves shedding of gametes into the surrounding water and relying on the subsequent chances of fertilisation and development outside the shell. Various species of brachiopod can be found living between the shoreline and the deep floor of the oceans, from the polar regions to the tropics and on many different types of sea bed.

Brachiopods are characterised by being solitary, bivalved, bilaterally symmetrical coelomates. The Phylum Brachiopoda is divided into two classes, the Articulata and the Inarticulata, differing morphologically and in the chemical composition of their shells. Articulates possess a hinge for opening the valves of the shell whereas inarticulates rely solely on muscles to open the shell. Articulate shells contain about 99% inorganic material, mostly calcium carbonate, and less than 1% organic material, mostly protein (Jope, 1971). Inarticulate shells contain calcium phosphate as the mineral phase instead of calcium carbonate and much more organic material, 25-55%, as a chitin-protein structure (Jope, 1971). The phylum has unbroken ancestry as two distinct groups back to the early Cambrian and the survival of the genus *Lingula*, unchanged since the Ordovician, provides an early example of phosphatic calcification. Together with the Phylum Phoronida and the Phylum Bryozoa brachiopods are sometimes placed in the lophophorate "superphylum" (Hyman,1959) since their adult body plans share a common theme, being trimerous with a feeding lophophore, although the brachiopod lophophore is more elaborate than that of the other two lophophorate phyla.

The large number of brachiopod genera and species (2,000 genera and 30,000 species) indicates the great diversity that has developed within a rather stable structural organisation. The brachiopod fossil record shows the remarkable diversity of form, and apparently also of habit, that can develop within the severe limitations imposed by a basically unchanging anatomical and physiological organisation. In this fact lies much of the evolutionary interest of the phylum.

1.3 Brachiopod anatomy.

1.3.1 The organisation of the brachiopod shell.

Like other "shellfish" the shell of a brachiopod is its most conspicuous part. Like bivalve molluscs, such as cockles and mussels, the shell consists of two separate parts, the dorsal and ventral valves, which enclose almost all the rest of the organism. The main function of the shell is probably to enclose and protect the organism. The shell is almost always perfectly bilaterally symmetrical but the plane of symmetry runs through the two valves and not, as in most bivalve molluscs, between them. This serves as a key to the conventional orientation of a brachiopod which is an essential aid to description. In articulates the calcareous shell usually has two or three distinct layers and well preserved fossil shells suggest that this arrangement has existed since very early in the history of the class. In calcareous and chitinophosphatic shells the outer surface is covered by a very thin sheet or network of organic material, the periostracum, which is not preserved in fossils (**Figure 1.1**). The very thin outer primary layer is usually composed of extremely fine granular calcite secreted by the mantle cells at the outside edge of the mantle lobe, thus the primary layer is confined to the outer surface of each valve. The inner secondary layer or fibrous layer is made up of slender calcite fibres stacked in a characteristic pattern and inclined at a low angle to the surface of the shell. These fibres are sheathed in protein and this layer is secreted by the whole surface of the mantle epithelium within the extreme marginal zone. Each cell secretes a single calcite fibre with its associated strip of protein and this secretion usually carries on throughout the life of the brachiopod which can be between two and fifty years. During this time the secondary layer increases in thickness away from the valve edge by secretion from the outer epithelium (Williams, 1965).

In a calcareous shell there is very little organic material and nearly all the inorganic material is calcium carbonate, almost always in the form of calcite. Calcite, and especially low magnesium calcite as in the case of brachiopods, is more resistant to diagenetic change than metastable aragonite which characterises many molluscs. This is partly responsible for the fine state of preservation of many fossil brachiopods. Brachiopods and corals are used in isotope studies of Palæozoic rocks because they are least changed over time.

The shell of a brachiopod gives a false idea of the real size of the organism. The actual body of the animal occupies only a small part of the shell cavity at the posterior side.

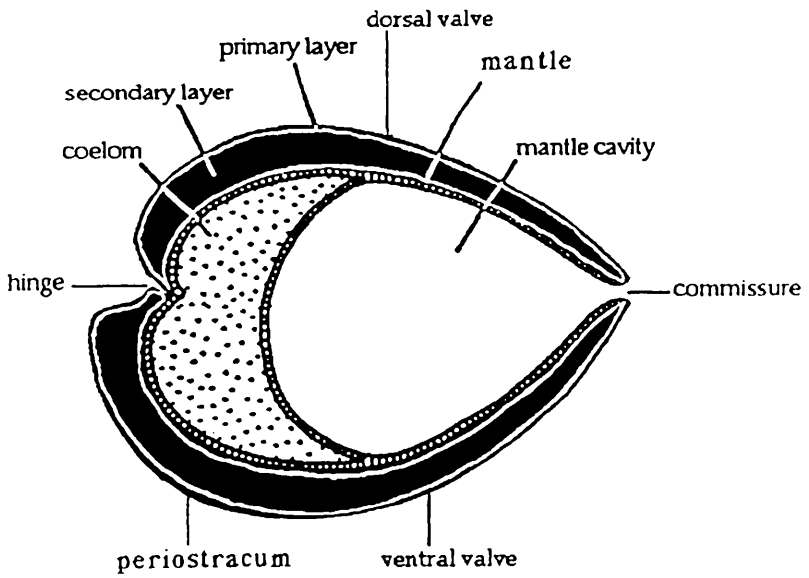


Figure 1.1 Relationship of brachiopod shell and mantle (adapted from Rudwick, 1970).

From the body two thin sheets of living tissue, the mantle lobes, extend forward and line the inner surface of the two valves. Between them is a large mantle cavity filled with sea water which communicates directly with the exterior wherever the valves gape apart.

Some articulate brachiopods possess tiny near-perforations of their shells called punctae. Owen and Williams (1969) suggested that these punctae produce a secretion which deters parasitic borers such as polychaetes.

1.3.2 Internal anatomy.

The mantle cavity contains a large and complicated organ termed the lophophore, see **Figure 1.2**. Like the shell and indeed like almost every organ of the brachiopod, the lophophore shows perfect bilateral symmetry. It consists of a pair of feathery brachia which project from the body at the back of the mantle cavity and are variously twisted within the cavity. It was thought at one time that these feathery arms could be uncoiled and protruded out of the shell and possibly even used for locomotion like the foot of a bivalve mollusc. This was the origin of the term Brachiopoda, i.e. "arm-footed".

The ciliated, filament-bearing lophophore is primarily a feeding organ, filtering small particles of food out of sea water, but it also plays an important role in respiration and other metabolic activities. In some articulate brachiopods the lophophore is partly supported on an internal skeleton, the brachidium or brachial loop, which is attached to the dorsal valve. The form of the brachidium gives some indirect evidence of the form of the lophophore in extinct brachiopods and has also been important in the high level classification of the Articulata, although recent work has thrown doubt on this (Collins *et al.*, 1988; Curry *et al.*, submitted; Collins *et al.*, in press).

The body of the brachiopod, from which the lophophore projects, contains not only the muscles that move the valves but also a simple digestive system, excretory and reproductive organs and the central parts of simple circulatory and nervous systems. All of these organs are suspended in a fluid-filled cavity, the coelom, which is separated from the mantle cavity by a thin body wall.

The coelom is divided into two principal areas, the larger one is the posteriorly located metacoel which forms the body cavity and which encloses most of the organs; the smaller one, the mesocoel, is the coelomic space inside the lophophore. (**Figure 1.2**). The coelom, and some of the organs within it, also extend into the mantle lobes and may leave more or less direct traces on the shells of fossil species. There are no highly developed

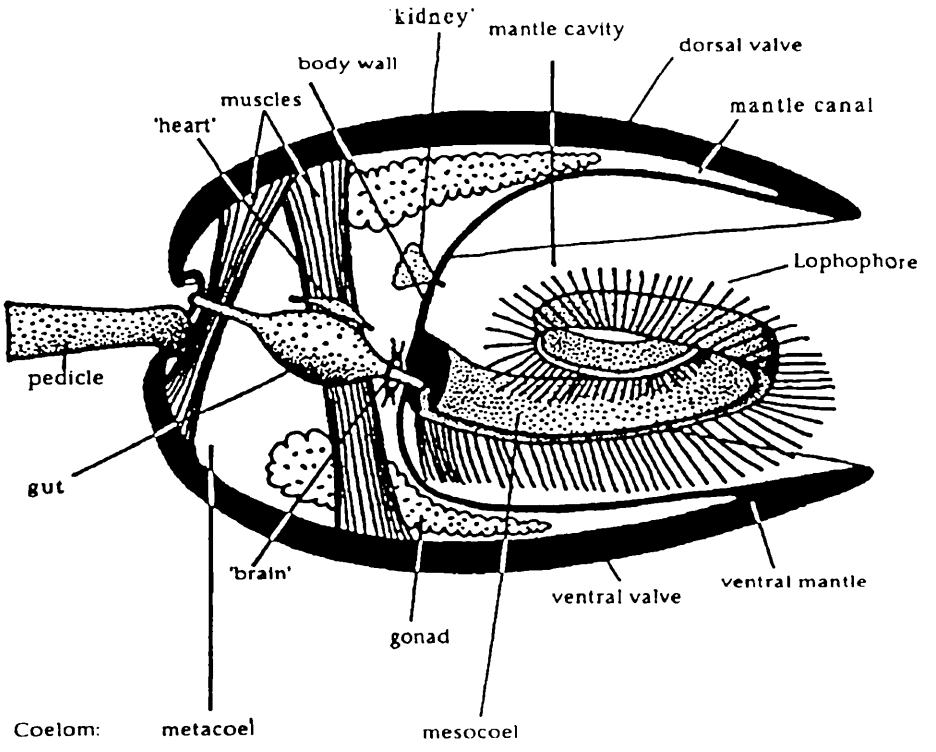


Figure 1.2 Generalised anatomy of a brachiopod (adapted from Rudwick, 1970).

sense organs and the sensitivity of the animal seems to be virtually confined to the edges of the mantle lobes, where the living tissues have their most direct contact with the external environment.

Most brachiopods are attached to the sea floor by a stalk, the pedicle, which projects between the valves on the posterior side of the shell. Two quite different organs are included under this name since the pedicles of articulates and inarticulates develop from different sources during ontogeny and differ significantly in structure and function. In both groups the point of emergence of the pedicle is often incorporated in the ventral valve as a pedicle foramen. The pedicle foramen is preserved in fossil brachiopods and is important in the high level classification of the phylum.

1.3.3 Lophophore ontogeny.

The amount of nutrition and oxygen the lophophore can absorb is directly related to the surface area of the organ. Brachiopods employ various geometrical devices to maximise that area. A simple circle of filaments will nourish a tiny shell, but with increase in shell size the ribbon must be twisted, coiled or indented or some combination of these to contain an ever lengthening lophophore within the confines of the shell. Each order of brachiopods seems to have solved the problem of surface area in a slightly different way, all beginning with a simple circle of filaments called a trocholophe. See **Figure 1.3**.

The lophophore increases in complexity during succeeding growth stages, lengthening by the addition of filaments anteriorly. As the valves grow, the lophophore also increases in size and indents towards the mouth as it lengthens. This stage is called the schizolophe (**Figure 1.3**) and, like the trocholophe, is almost universal in the small and early growth stages of living brachiopods. However in a few species, which become adult at a very small size, it is also the final form of the lophophore. Such species are found among the lingulides (*Pelagodiscus*), strophomenides (*Thecidellina*) and the terebratulides (*Argyrotheca, Pumilus*).

In the rhynchonellides and some terebratulides (terebratulaceans), crura grow forward and support the posterior side of the schizolophe. In other terebratulides (terebratellaceans) there is also a median septum which grows from the floor of the dorsal valve and supports the median indentation of the schizolophe. At this schizolophous stage, different genera of brachiopods have different growth patterns. In a schizolophe the brachial axes are fused throughout to the body wall and dorsal mantle. The first and perhaps most important line of development escapes from this limitation and allows the

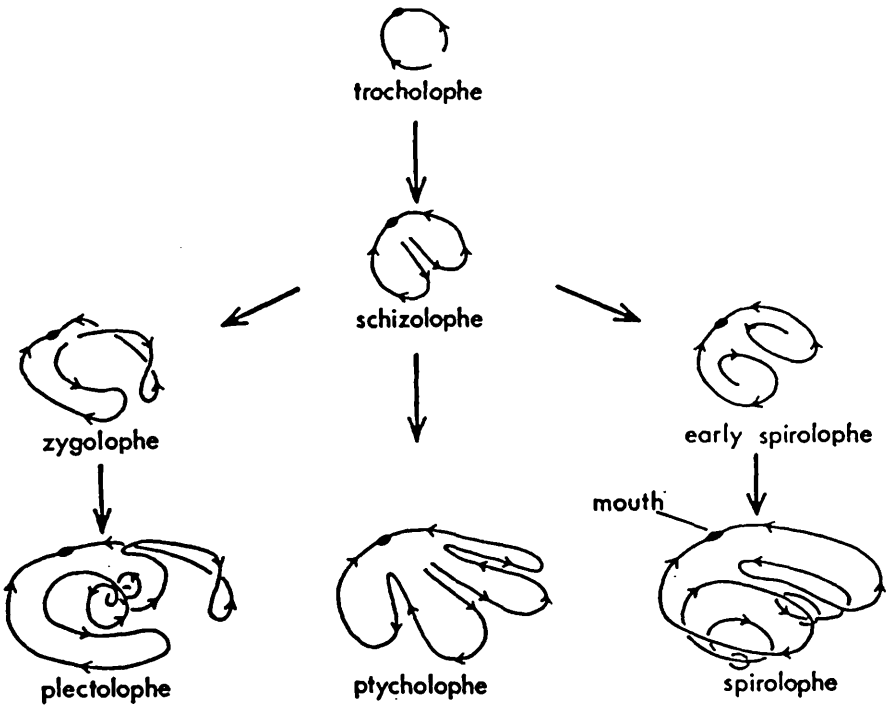


Figure 1.3 Ontogenetic pathways in the growth of the lophophore in living brachiopods (Rudwick, 1970).

brachia to be coiled freely in three dimensions within the mantle cavity. When a schizolophe develops into a spirolophe, (**Figure 1.3**), the tips of the brachia diverge from one another and from the mantle surface, until eventually the brachia grow into a pair of spirals. The tips of the filaments touch either the mantle or another part of the spiral, so that the whole spirolophe divides the mantle cavity into separate chambers. Spirolophes are characteristic of all the living inarticulates and rhynchonellides. No trace is left in fossil material since they are supported principally by a hydrostatic skeleton or other non-preservable supporting structures. However the wide distribution of spirolophes among living brachiopods suggests that this has been a very common form of lophophore throughout the history of the phylum.

According to Rudwick (1970) this may have been the first way in which the lophophore grew beyond the schizolophous stage, thus allowing brachiopods to become moderately large in size. Clear impressions of spirolophes have been found on the thickened valves of several Palæozoic strophomenides, which would otherwise show no sign of the form or even existence of the lophophore.

The second of the possible lines of development from the schizolophe also allows the brachia to be coiled in three dimensions. But, in addition, the fusion of certain parts of the supporting structures allows more effective use of the available space in the mantle cavity. The first stage involves a lateral twisting of the lobes of the schizolophe, resulting in the reversal of the original arrangement of the apertures, as in the growth of a spirolophe. These lateral lobes then grow away from the dorsal mantle surface and project freely into the mantle cavity, but the brachial axes remain united across the floor of each lobe, so that the current system continues to be effective. The uniting of the axes is due to the fusion of the otherwise doubled 'great brachial canal' into a single supporting tube. This is the first novel feature which makes this line of development possible. This stage of growth is termed the zygolophe. Among living brachiopods it is confined to the terebratulides and is never more than a transient stage. It is always supported by some sort of brachidium. In the terebratulaceans this consists of lamellae extending from the crura and later uniting on the dorsal side of the lophophore to form a short loop which supports the base of the zygolophe where it is attached to the body wall. In most members of the other extant group, the terebratellaceans, the zygolophe is supported partly by lamellae extending from the crura and partly by some projecting from the crest of the median septum. In one small group (kraussinides) the support is only of the latter kind.

The first development of zygolophes can probably be located at the origin of the terebratulides themselves, early in the Devonian period. The loops of these early

terebratulides and the spiral brachidia of contemporary spiriferides have little in common in their adult forms but their early growth stages are quite similar. This suggests that the first zygolophes may have evolved from the schizolophes of some small neotonous spiriferides.

In living terebratulides the zygolophe is soon transformed into a plectolophe by the further growth of the brachial axes. The tips of the brachial axes, which have remained close together at the back of the mantle cavity, now grow away from the body wall again and eventually form a large plano-spiral median coil between the lateral lobes. In the median coil the axes are united across the median plane by a diaphragm of connective tissue and the filaments arch over and touch each other so that the coil forms a tunnel full of inhalant water. The development of the median coil allows the lophophore to fully occupy the mantle cavity but it is dependant on the existence of the diaphragm, without which the current system could not be effective.

The plectolophes of living terebratulides are supported by a variety of brachidia, which are developments of those of the zygolophous stage. The base of the plectolophe is supported by a short loop in terebratulaceans. Alternatively, by a complex metamorphosis, the lamellae connected to the crura and to the septum may link up with each other and then both become more or less detached from the septum, forming the long loop of the terebratellaceans. In long-looped forms the lamellae run parallel to the brachial axes in the lateral lobes but are still enclosed within extensions of the body wall accompanied by extensions of the coelom (the brachial pouches). Long loops only support the base of the median coil, where it is attached to the body wall, and never extend into the median coil itself.

Loops analogous to those which support plectolophes in living species first appeared among the earliest terebratulides in the Devonian period. This may mark the first acquisition of the diaphragm that made a median coil and therefore a plectolophe an effective possibility. The plectolophe is by far the most abundant and successful form of lophophore among living brachiopods.

The third and last mode of development of the lophophore seems, by contrast with the others, inherently less effective. It simply entails the expansion of a bilobed schizolophe into a multi-lobed ptycholophe. More indentations of the brachial axes are added laterally, and their filaments form additional exhalant tunnels; otherwise the current system remains much the same. Only two living genera (*Lacazella* and *Megathyris*) have ptycholophes; in both of these genera the lophophore never develops beyond the four-lobed stage and the

adult shells are only slightly larger than related schizolophous species. One of them, *Lacazella*, has fossil relatives (thecideaceans) which developed more complex ptycholophes with up to twenty lobes and correspondingly grew to a larger adult size. In this group the brachial grooves allow the lophophore to be reconstructed with virtual certainty. These complex ptycholophes seem to have evolved during three periods in the history of the group, first in the late Triassic, in a different form in the early Jurassic, and again much later in the Cretaceous.

1.4 Importance of the loop.

As described above, (in **1.3.3**), the lophophore can take a variety of forms and some of these are reflected in preservable skeletons. If there were no living terebratulides with both the calcareous loop and the fleshy lophophore to study, reconstruction of the plectolophe in fossils would be almost impossible. The loop does not follow the course of the plectolophe, but in the extinct fossil groups where a calcareous brachidium is preserved, the assumption is made that it indicates the shape of the missing fleshy lophophore. Without living rhynchonellides there would be no clue as to the spiral shape of the lophophore because only short crura are preserved in fossils and the lophophore is entirely fleshy with no complete calcareous support.

Significant evolutionary changes in the lophophore, usually indicating the importance of that structure for survival, have been noteworthy in articulate brachiopods. In fact, classification of the brachiopods on the basis of the configuration of the lophophore and its structural supports has been attempted frequently in the past for taxonomic differentiation of the phylum.(Elliott, 1953; Williams, 1956; Williams & Rowell *in* Williams *et al.*, 1965). Taxonomic treatment based on pattern of loop development was suggested by Muir-Wood (1955) and confirmed by the Treatise (1965). The Order Terebratulida is currently divided into three suborders on the basis of ontogeny and form of loop development.

1.5 Importance of Fossil Brachiopods.

The phylum has several features which make it especially suitable for a study of the broader aspects of the evolutionary process. It has an exceptionally long recorded history; no group with a satisfactory record of preservation has a longer history. The continuity of the record is also exceptionally good. Brachiopods can be found in variety and often in large numbers, at least somewhere in the world, in rocks of almost every geological age from Cambrian i.e. 590 Million years ago to Cenozoic i.e. the present day. Although the most widely known brachiopod genus *Lingula* is a textbook example of evolutionary stability, the phylum as a whole was far from static throughout this time. During this period of around 600 Million years some groups rose slowly to dominance while others declined, there were bursts of rapid diversification as well as massive extinctions and this continual change in the detailed and overall character of the phylum makes the study of brachiopods important for the dating and correlation of strata. See **Figure 1.4**. Thus fossil brachiopods can be used to date and correlate rocks and their distribution and associations are vital clues to the reconstruction of ancient oceans. Brachiopods have been successfully used for many years to provide local, regional and worldwide biostratigraphic control.

Any meaningful interpretation of evolutionary patterns involves some understanding of the biology of the organisms as they are in the living state. Only when they are considered as living organisms will the evolutionary significance of their structural transformations become clear. Here too the brachiopods offer unusually attractive possibilities. Their skeletal structures are often very well preserved and bear the imprint or traces of many of the other important organs of the body. The relation between the hard and soft parts of the anatomy-between what is commonly preserved in the fossil state and what is not-is much more intimate than in most other groups with a skeleton of ectodermal origin. Much of the anatomy of extinct brachiopods can be reconstructed with a fair degree of confidence. The main key for this is a knowledge of the surviving species; it is fortunate that although the phylum is much reduced from its former abundance it is not extinct and sufficient taxonomically diverse species have survived to provide a fairly satisfactory key to the anatomy of the extinct species. The living species also provide a partial key to the adaptations of extinct species which enhances the sometimes slender evidence of adaptation recorded in the structure of the fossil organism itself. Thus the evolution of brachiopods can be interpreted in terms of their changing modes of life. An account of the evolutionary history of brachiopods is given in Chapters 4, 5, and 6.

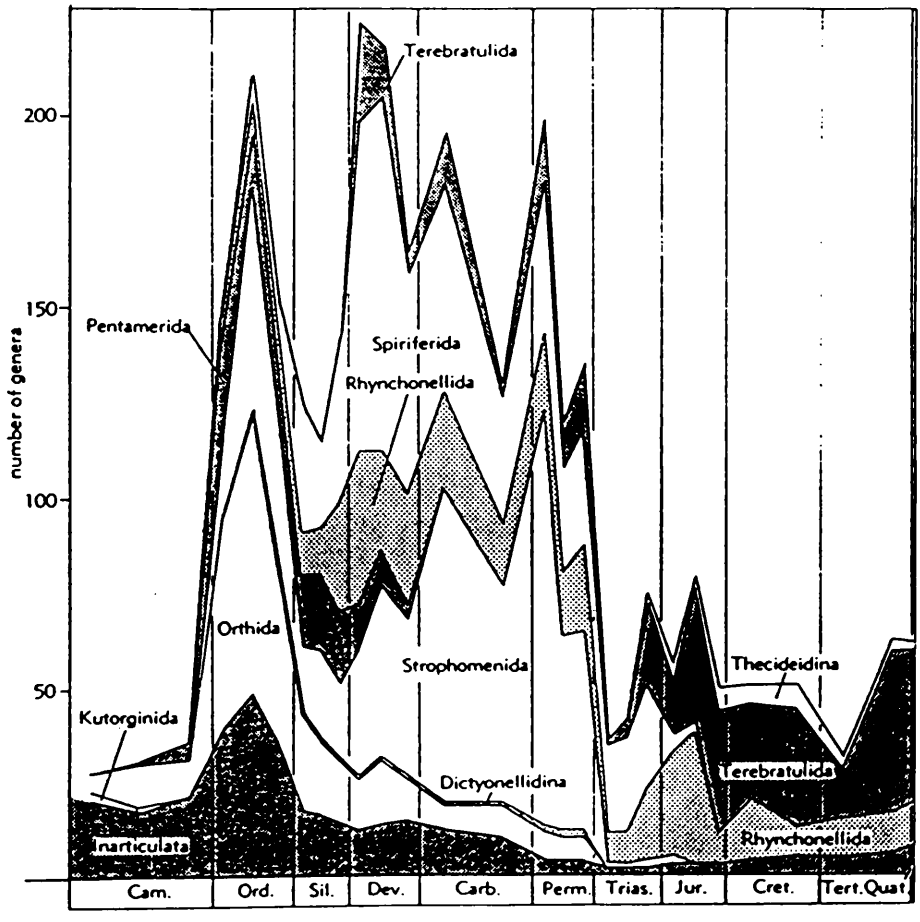


Figure 1.4 Stratigraphic distribution of Brachiopoda according to number of genera (Williams, 1965).

1.6 Phylogeny.

1.6.1 Monophyly versus polyphyly.

The traditional view of brachiopods is that they constitute a monophyletic clade (Williams & Rowell, *in* Williams *et al.*, 1965, Williams & Hurst, 1977) whose closest relatives are the other lophophorates, i.e. the phoronid worms and bryozoans (Hyman, 1959). It is commonly accepted that these lophophorates radiated from a trimerous, tubicolous coelomate ancestor (Clark, 1979), which is usually regarded as being a phoronid-like organism. The implication is that brachiopods share a common genealogical history such that their most recent common ancestor was itself a brachiopod. See **Figure 1.5**.

An alternative view of the early phylogeny of brachiopods exists. Valentine (1973) accepted that the brachiopods arose from infaunal phoronid-like ancestors, but suggested that the various early brachiopod lineages developed separately and independently from different groups of phoronid-like forms. With this interpretation brachiopods are regarded as a grade of organisation and any formal taxon that unites them, but excludes their ancestors, must be considered as polyphyletic. (**Figure 1.6**). The corollary, as Valentine (1973) recognised, is that several monophyletic taxa would be needed to classify the organisms that are presently termed brachiopods. The views of Wright (1979) on the Lower Palæozoic brachiopod radiation are similar in some respects to those of Valentine. Wright (1979) concluded that brachiopods were not a monophyletic clade and that they may have originated from as many as seven different brachiophorate stocks. Wright (1979) described them as infaunal worm-like creatures with lophophores projecting freely from their tubes.

These two views of the origin of the brachiopods, whether they arose monophyletically or polyphyletically, are mutually exclusive. Rowell (1986) argued that the differences are not as major as they might seem at first glance. Paradoxically there is no significant dispute over the empirical palæontological evidence; the disagreement is confined to its interpretation.

Those who support a polyphyletic origin draw attention to the differences between the various inarticulate stocks and the Articulata and infer that they are too large to have arisen by divergence from a common ancestral brachiopod lineage.

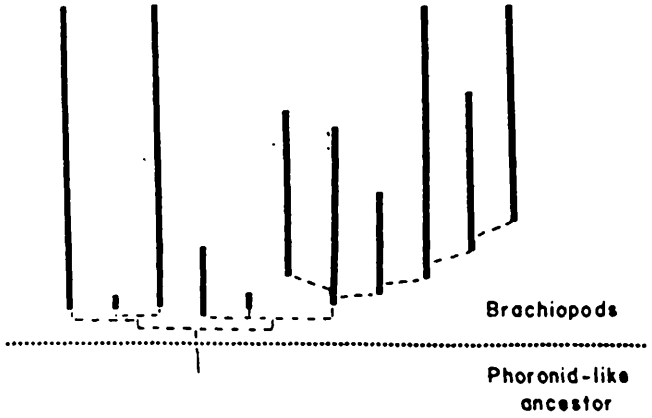


Figure 1.5 Diagrammatical representation of a monophyletic origin of the brachiopods from a phoronid-like ancestor. Heavy bars depict stratigraphic ranges of the principal orders. The six bars on the right of the diagram together are the Articulata (Rowell, 1981).

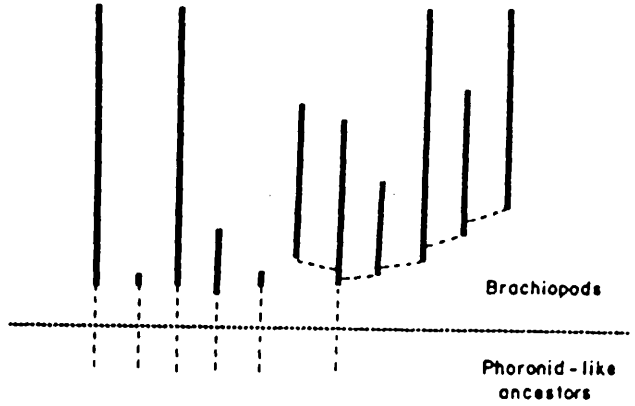


Figure 1.6 Diagrammatical representation of a polyphyletic origin of the brachiopods from several phoronid-like ancestors. Stratigraphic ranges as in **Figure 1.5** (Rowell, 1981).

In trying to decide between a monophyletic or polyphyletic origin it is not enough to point out differences or stress similarities. The differences may have arisen as a consequence of later evolutionary divergence. Williams and Rowell (*in* Williams *et al.*, 1965) argued that many of the features that differentiate living articulates from inarticulates did not appear with the origin of the orthides. They suggested, for example, that mantle reversal did not occur until the evolution of the rhynchonellides, although it is characteristic of all living articulates. However, similarities alone are also an inadequate basis for postulating a monophyletic origin. The important question is how did the similarities arise? Answering this question is part of the basic approach of cladistic analysis.

1.6.2 Cladism and the origin of Brachiopods.

One of the objectives of cladistic analysis is an understanding of genealogical relationships. Cladistic techniques were first developed by neontologists concerned with phylogenetic relationships among forms with a poor fossil record (Hennig, 1966). In general, cladistic techniques and philosophy have not been well received by palæontologists. Conflict still exists between stratophenetic philosophy (Gingerich, 1979), the basis of methods employed in most palæontological investigations, and cladistic theory. The differences of opinion regarding the merits of these two approaches are greatest when trying to reconstruct phylogenetic trees as opposed to cladograms (Bretsky, 1979; Eldredge, 1979; Wiley, 1979).

The stratophenetic approach is not applicable in trying to address the problem of the origin of the brachiopods. With the present knowledge of the stratigraphic record of brachiopods there are wide morphological gaps between the various orders and one cannot be traced evolving into another. Future collecting may fill these gaps but it is probable that the first appearance of the various lineages marks the time at which they developed the ability to secrete mineralised skeletons, not the time at which the lineage arose. They may have had a significant prior evolutionary history as small forms whose mantles secreted only organic material.

Cladistic techniques are helpful in trying to resolve the pattern of evolution. One of the main contributions of the cladistic school has been the clear recognition that when the effects of resemblance due to convergence are removed two types of evolutionary similarity are possible (Eldredge, 1979). At any given level of analysis, the resemblance between two taxa may be due to shared evolutionary novelties inherited from an immediate common ancestor and thus not found in any other taxon (Eldredge, 1979). Such similarities are termed synapomorphs and they are in contrast to the other forms of

similarity, symplesiomorphs, which are relatively primitive similarities inherited from some more remote common ancestor that may also be found in other descendant taxa. Only synapomorphs, shared evolutionary novelties, provide information on the close phylogenetic relationship between two taxa. Their presence effectively labels members of the new lineage.

To look at the origin of brachiopods it is necessary to analyse the similarities shared by brachiopods and to evaluate which, if any, of them are evolutionary novelties relative to other lophophorates. If brachiopods are monophyletic they should share one or more evolutionary novelty(ies) that unite the group. If they arose polyphyletically then all similarities should be either false similarities and the results of convergence, or symplesiomorphic, inherited from some more distant ancestor that was not itself a brachiopod.

One of the strengths of cladistic methodology is that both living and fossil forms may be used to recognise shared evolutionary novelties. Thus the distribution of synapomorphs in living brachiopods may provide information on the earliest evolutionary history of the group.

The brachiopods and phoronids together with the bryozoa share many synapomorphs that unite them as lophophorates. The principal evolutionary novelties at this level include a mesosomal lophophore, which bears hollow ciliated filaments, and is partially or completely separated from a nonsegmented metacoel.

If the synapomorphs of **Figure 1.8** are correct then the Paterinida, Kutorginida and Orthida (together with the remainder of the Articulata) are the sister group of the Lingulida and Obolellida and Acrotretida. In turn the Lingulida and Obolellida are the sister group of the Acrotretida. Each of these three major branches has a lineage that is represented today by living brachiopods. Therefore any evolutionary novelty present in all recent brachiopods must have been an evolutionary novelty common to all brachiopods.

If even one of the supposed synapomorphs illustrated in **Figures 1.7** and **1.8** is correctly identified as a synapomorph it would follow that brachiopods are monophyletic. Two questions need to be asked of these postulated 'derived evolutionary novelties.' Are they true similarities and not merely the result of convergence or parallelism ? Secondly, if they are homologous features are they indeed synapomorphs and not merely symplesiomorphs ?

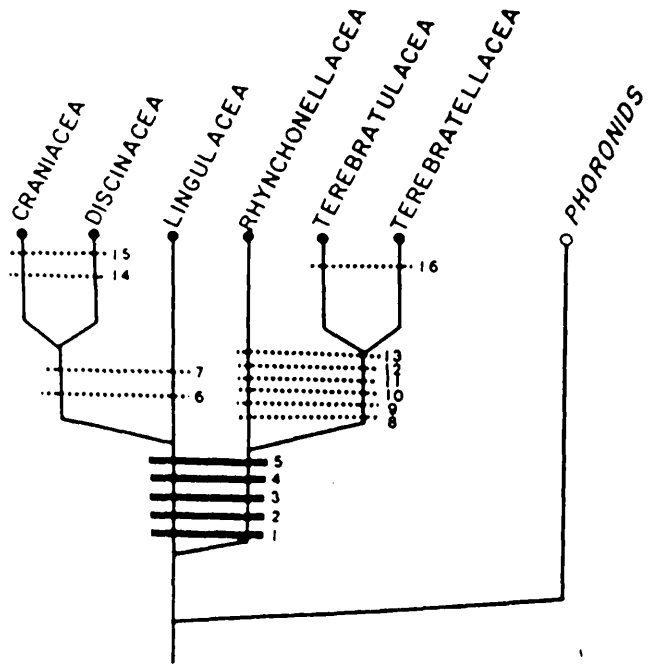


Figure 1.7 Cladogram showing relationships between major taxa of extant brachiopods. Synapomorphies, shown by bars connecting taxa, include 16 Presence of loop (Rowell, 1981).

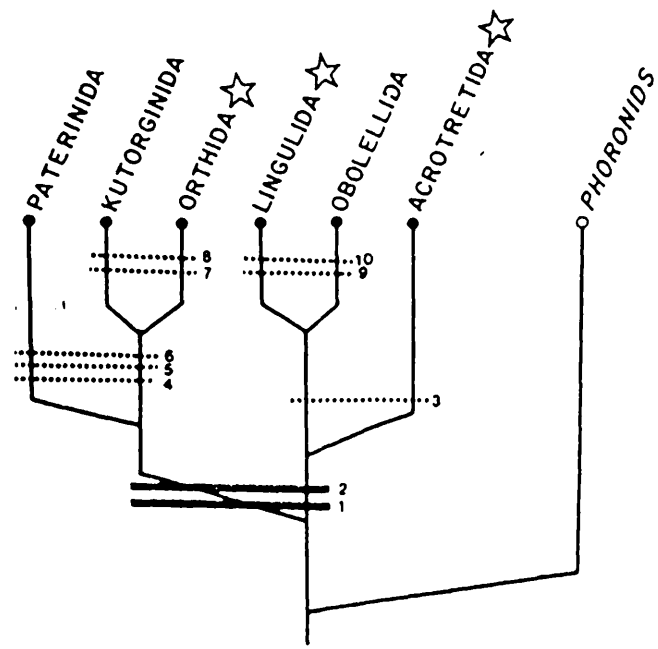


Figure 1.8 Cladogram showing relationships between principal taxa of Cambrian brachiopods. Synapomorphies are shown by bars connecting taxa (Rowell, 1981).

There are clear differences between phoronid and brachiopod lophophores. The structural features of brachiopod lophophores, however, are almost identical and there can be little question that the similarities are true homologies. In phoronids the adult lophophore is typically spirally coiled and bears a single palisade of filaments on both sides of the axis (Hyman, 1959). In contrast, all brachiopods fundamentally have only a single palisade of filaments about the lophophore axis. All brachiopods have two coelomic spaces in the lophophore, phoronids have only one. All brachiopods have a brachial lip bounding the food groove, in phoronids the opposing palisade of filaments is in this position. Virtually all brachiopods have the adult filaments of the palisade arranged in a double row alternating in position, in phoronids they are invariably in a single row. A fifth similarity, common to all brachiopods and unknown in phoronids, is the possession of mantle canals. These are fingerlike extensions of the body cavity into the mantles found in all living brachiopods and commonly reflected in the shell of fossil forms.

Two features are shown as potential synapomorphs uniting all brachiopod lineages in **Figure 1.8**. One of these may have arisen by convergence and would not be a true similarity. Although it is an empirical observation that brachiopod valves are always secreted by mantles that are dorsal and ventral, and never left and right, it is possible that this orientation could have arisen independently in different stocks being controlled by the orientation of the lophophore. The detailed morphological resemblance of the second potential synapomorph, the development of slender, bristle-like sensory setae along the margin of both mantles, suggests that this is true similarity, not the consequence of parallelism or convergence. These setae rarely occur in the fossil state because of their delicacy, but are known in living representatives of each of the three major branches of brachiopods and also occur in Cambrian Paterinida from the Burgess Shale (Walcott, 1912).

Given that there are six, possibly seven, similarities shared by all brachiopods, the next question is are they indeed 'derived evolutionary novelties'? The cladograms of **Figures 1.7** and **1.8**, although they show the nested distribution of various similarities among brachiopods, do not show the direction of the ancestral-descendant relationships between brachiopods and phoronids. This information is crucial to deciding whether the shared similarities of brachiopods are 'derived evolutionary novelties' or symplesiomorphic.

The conventional view is that phoronid-like organisms gave rise to brachiopods. The general aspects of the adaptive model of change from an infaunal to an epifaunal existence (Valentine, 1975) make sense only with this ancestral-descendant relationship. Although no palæontological range data or meaningful outgroup comparisons (Eldredge & Cracraft,

1980) are available to verify this hypothesis, it is supported by limited ontogenetic information among the features that have been discussed. In most living brachiopods the early stages of lophophore development resemble those of phoronids in having only a single row of filaments. The double row of filaments characteristic of most adult brachiopods appears later in development supporting the view that the phoronid condition is primitive.

If phoronid-like animals gave rise to the brachiopods then the six or seven similarities common to all brachiopods are 'derived evolutionary novelties' and the brachiopods are monophyletic. To refute this statement it would be necessary to show that one or more brachiopod order(s) is phylogenetically more closely related to some other group of organisms than it is to the remaining brachiopods.

Before attempting to demonstrate that this was the case it would be necessary to show that all seven features that have been regarded as synapomorphs were merely the results of convergence or parallelism. The near identity of these features in different brachiopod lineages suggests that this is unlikely.

The model of the adaptive radiation of brachiopods suggested by Valentine (1975) tolerates the assertion that the group is monophyletic. It is necessary to modify the model only to the extent that the synapomorphic features of the group were acquired before the radiation of the organisms that are presently recognised as brachiopods. There may be an adaptive explanation for the development of these features. A brachiopod lophophore appears to be mechanically better organised for pumping, filtration and separation of inhalant and exhalant currents in a partially enclosed space than is the phoronid type, which functions in an open environment.

If brachiopod lineages differentiated before the acquisition of a mineralised shell, then this is an example of convergence within the group. Terms like monophyly and polyphyly, however, are determined by group relationships, not by level of development of one or more characters (Patterson, 1978). Consequently, the theory of Wright (1979) and that of Rowell (1986), may be reconciled only by regarding shell-less forms that possessed any of the features that are synapomorphic for brachiopods as brachiopods, not brachiophorates or phoronid-like worms.

1.6.3 Ecophenotypic versus Genetic variation.

The variation between individual phenotypes reflects differences in the genotype, age and sex as well as non-genetic differences due to environmental factors. The same genotype may produce different phenotypes under different environmental conditions, while different genotypes may result in similar phenotypes when their reaction ranges overlap. There is no simple solution to the problem of genotypic and ecophenotypic variation in fossils and it is never possible to prove one or the other, but only to deal with probabilities.

Genotypic variation may be polytypic or polymorphic; in polytypic variation populations can be distinguished from one another and may be separated geographically, ecologically or stratigraphically and may ultimately give rise to races or subspecies with clines present when there is continuous variation between neighbouring races. In polymorphism two or more distinct morphs are present in a population. Balanced polymorphism indicates a balance of selective forces maintaining the different morphs in the population.

Ecophenotypic variation is regarded as non-genetic plasticity allowing an individual to adapt to its environment during its development and where environmental factors influence the final morphology of the phenotype. The potential phenotypic expression is dependent upon the genotype so the latter determines the possible morphological range.

There are two important ways in which it may be possible to distinguish between genotypic and ecophenotypic variation. The first is by direct comparison with living relatives, the more closely related the better, but this is less useful as time increases. The second method is to determine whether there is any correlation between morphological change and environmental gradient; for this to be convincingly portrayed the morphological change should be repetitive. It is useful if the variation being surveyed can be quantified and examined statistically. Ecophenotypic changes are often correlated with possible environmental parameters, but even with living organisms the cause of the variation is rarely established because the necessary genetic research has not been done.

1.6.4 Extinction.

Explanations of extinctions include both large scale competition and extinction followed by a new adaptive radiation. There is increasing evidence that major physical changes have caused more large-scale evolutionary changes than competition has. During adaptive radiation the probability of speciation may be enhanced by the acquisition of a new

adaptive innovation (Benton, 1983). Benton concludes that changes are due to stochastic factors, i.e. a random mixture of biological and physical factors. If phyletic gradualism is taken as the mode of speciation then Darwinian reasoning may be applied, i.e. species are adjusting to the environment and changing on a regular basis under the control of natural selection. If the duration frequencies of genera are plotted on a logarithmic scale they plot close to a straight line- this being the basis of Van Valen's Law of Constant Extinction- indicating that old and young species are equally vulnerable (Van Valen, 1973). This fits perfectly with the Synthetic Theory, species being adapted to their ambient environment, not to some unknowable future possibility. Add to this the possible causes of extinction in this mode-competition and environmental changes-with some species being capable of both winning a competition and surmounting environmental changes- then the net result would be a regular flow of extinctions.

If punctuated equilibrium is employed then species change quickly at or around the time of their production, but not thereafter, thus some other means of sorting out species must exist. In such a mode the main method of extinction would be a relatively major abiotic event (sea level change, climatic change,etc.) as a ground-clearing operation thus allowing space for other or new species to enter. Viewed in this way extinction is not constructive but selective and can be considered as a necessary motive force in evolution. Moreover extinctions would be clustered rather than regular, relatively large regional abiotic events being the exception rather than the rule.

Evidence for both modes of extinction exists within the fossil record. Background extinction, the normal or spontaneous rate of replacement of one species by another, is estimated as amounting to an average of eight or fewer familial extinctions per million years. The more episodic form of extinction determines most major geological boundaries (stage and system boundaries).

The pattern of extinction shown by the brachiopods in the earlier part of their history can be interpreted as ecological replacement by groups with clear adaptive advantages. Among the strophomenides, later forms which had tubular spines were better adapted to a quasi-infaunal life in soft substrates and therefore replaced the spine-less superfamilies.

The Permo-Triassic extinction had a drastic effect on several other marine phyla as well as the brachiopods but among the brachiopods it was significant in the almost total elimination of the strophomenides. The strophomenides were highly adapted in terms of locomotory ability, feeding habits and articulation and represented a level of adaptation unparalleled at any other time in brachiopod evolution.

The idea that the seas withdrew from the continents to an exceptional extent and that their surface waters also became brackish at this time explains the relatively catastrophic extinction of the brachiopods. Relics of the stenohaline faunas should have survived only in inland seas or other areas of normal salinity.

1.6.5 Molecular approach to speciation.

There is increasing evidence that, unlike morphological change, the rate of DNA base substitutions, and hence amino acid substitutions in proteins, is a fairly constant function of time, so that the number of differences in the comparable DNA or proteins of two species is a measure of their time of divergence from a common ancestor (Wilson *et al.*, 1977). Frogs have changed morphologically relatively little in the past 100 million years (Ma) in comparison to all the placental mammals, in particular the primates. Yet the frog proteins have undergone as much change during this interval as those of mammals (Wilson *et al.*, 1977).

Phylogenetic trees constructed from the biochemical similarities of homologous DNA and proteins have helped to clarify the evolutionary relationships of living species (Ayala, 1976), but our understanding of fossil species has continued to depend almost exclusively on their anatomical characteristics. In some cases, the conclusions drawn from biochemical and anatomical data are reconcilable, as in the question of the phyletic status of the Miocene hominoid, *Ramapithecus*, considered by many anthropologists to be a hominid (Simons, 1977). Analysis of DNA and 40 different proteins shows 99% identity between humans and chimpanzees (King & Wilson, 1975). Sarich and Cronin (1976) find equal closeness between human, chimpanzee and gorilla proteins, based on the immunological cross reactions of the albumins and transferrins, and conclude that the three species diverged from a common ancestor about 5 Ma ago. If this is correct, then *Ramapithecus*, which lived some 8-20 Ma ago, could not have been 'human' (Zihlman & Lowenstein, 1979). The issue continues to be disputed whether DNA and proteins, or fossil jaws and teeth, are the best criteria for phyletic status. This controversy, like many others in evolution, might be clarified if biochemical as well as morphological comparisons between fossil and living species could be made.

Among the higher ranks of the green algal Cladophorales complex (Cladophorales and Siphonocladales inclusive) current understanding of evolutionary relationships is based on ultrastructural studies of cytokinesis, flagellar apparatus and chloroplasts, as well as accessory pigment composition, cell wall configuration and life history. Although

ultrastructural data have been useful at the ordinal ranks and above, they have as yet been unable to resolve differences among taxa at lower ranks. Virtually all lower ranking classifications of algae are phenetic, that is they are based on overall similarity of morphological form with little regard given to convergence and parallelism. This is an important taxonomic consideration in these algae in which anatomical simplicity is marked. Usefulness of traditional classifications is not disputed, in so far as they provide the basis for identification, but they are limited in that they do not provide information on evolutionary relatedness which is intrinsic to biosystematics in general.(Olsen-Stojkovich *et al.*, 1986).

Protein phylogenies have been very useful in assessing relationships among numerous groups of animals and, to a lesser extent, plants. A comprehensive review of the theory and applications can be found in Wilson *et al.* (1977) and Thorpe (1982). Molecular data provide a complementary line of evidence that allows more objective choices to be made among competing hypotheses of evolutionary relationships drawn from morphological studies alone, as well as to present hypotheses outside the bounds of current thinking. For the most part molecular phylogenies have agreed with those derived from comparative morphology. Marked exceptions generally occur in those cases where morphometric characters are few and uninformative, or where phenetic relationships have been superimposed on phylogenetic ones. Where morphology is almost entirely lacking (e.g. amoebae), molecular phylogenies have been invaluable (Friz, 1984). Molecular phylogenies are able to provide insights into relative divergence times and, in those cases where single protein or single copy nuclear DNA measurements can be compared with the fossil record, or a known vicariant event, a molecular clock can be set with some precision. Although tests of dispersal and vicariance models in historical biogeography (Pielou, 1979 and references therein) for benthic algae are still in the future, success will depend, in part, on an understanding of their phylogeny.

In a study of African jackals morphological and molecular techniques were used in combination to determine how three virtually identical species of jackal were able to share the same habitat. The mitochondrial DNA in blood samples taken from the jackals was analysed to give a measure of the time since the species diverged. Unlike nuclear DNA, which is a mixture of the mother's and father's DNA, mitochondrial DNA is passed intact from a mother to her offspring. In turn, her daughters pass it on intact to their offspring, and so on. Thus changes in mitochondrial DNA document the mutations that accumulate in the females of an evolving lineage. Work by Wallace *et al.* (1990) suggests that the percentage of changes in the mitochondrial DNA between two forms can be used as a rough clock to determine divergence times. Cann *et al.* (1990) have calculated that, on

average, mitochondrial DNA will accumulate divergences of only between two and four per cent in one million years.

The fossil record showed that the jackals occupied the same geographical area at least two million years ago indicating that the jackals had been sympatric for a considerable length of time. It seemed that food was not in short supply and therefore was not a limiting factor in this case. The most common mechanism for avoiding competition, divergence in size, did not seem to be an option for these jackals since there were seven larger and twenty smaller carnivores in the same area.This study reinforced the idea that morphology reflects the genotype only to the extent that ecological factors permit. In the case of these three species of jackal, it was not just the habitat but the entire guild of sympatric carnivores that constrained the morphological evolution. In this study it is clear that neither the molecular data nor the morphological and ecological data would be as revealing if considered in isolation.

An ecologist, knowing nothing of the divergence data or quantitative measures of morphology, might postulate that the small-scale differences in the use of habitat or "activity patterns" were somehow sufficient to avert competition. Only an extremely long-term ecological study would highlight the entire carnivore guild as a potent evolutionary force shaping the jackals' adaptations. A morphologist, unaware of the molecular data, might postulate that the species had not had a sufficient period of time in which to diverge. A geneticist faced with only this information might conclude that the species were not very different-which they are not-and might fail to see that a problem existed at all. This study shows the value of combining information from morphological and molecular studies.

In the present work molecular information derived from brachiopods using immunological techniques is used to elucidate phylogenetic similarities among terebratulide families and this is compared with the existing morphological classification.

Immunological techniques are also used in the detection of compounds of geological interest which may be important in gas or oil exploration.

2.1 Background to present study.

Genetic relationships between living species can be deduced from biochemical as well as morphological similarities, but until recently our understanding of fossil species has relied completely on their morphology. Evidence is now accumulating that indicates that biochemical information can be preserved in the fossil record to a much greater extent than was previously thought possible and this information is amenable to study by both biochemical and immunological techniques.

2.2 Previous biochemical and immunological experiments.

Abelson (1954) isolated preserved amino acids from 360 million year old fossil shells and other workers have identified amino acids in fossil shells of different geological ages (Hare & Mitterer, 1967 ; Wyckoff, 1972).

Abelson (1955) was first to recognise the potential value of utilising fossil material preserved in shells to study evolution directly from the fossil record. Comparisons of amino acid compositions of fossil proteins of different geological ages yielded information about the rates and types of mutations a single gene had undergone.

In the first example of the application of immunological techniques to palæontology, de Jong *et al.* (1974) found preserved antigenic determinants in fossil shell matrix components and used these to investigate phylogenetic relationships.

Shell glycoproteins of distinct molecular weight were described in an 80 million year old fossil mollusc (Weiner *et al.*, 1976). These glycoproteins comprised a repeating amino acid sequence shared with a living representative species of the same superfamily.

Immunological cross-reactivity between a late Cretaceous belemnite and a recent cephalopod, *Nautilus*, occurred when these were tested using an antiserum generated against *Nautilus*, (Westbroek *et al.*, 1979), indicating that the antigens shared identity. Organic matrix components of a fossil ammonoid shell from the Upper Cretaceous were separated into sub-fractions, using ion exchange chromatography, and these were generally comparable to those found in extant *Nautilus* (Weiner *et al.*, 1979). Weiner *et al.*(1979) concluded that at least portions of these matrix components were sufficiently well-preserved to interact in a characteristic manner with the ion-exchange resin, despite

the fact that the amino acid compositions of these sub-fractions did not resemble *Nautilus* organic matrix sub-fractions, indicating that there had been considerable diagenetic alteration of the material.

The extent of amino acid racemisation depends on temperature and time, the L form changing to the D form as time progresses. Under appropriate conditions the amino acid enantiomeric (D/L) ratios of Quaternary calcified tissue can be used for the estimation of age and palæotemperature. In still older materials D/L ratios approach equilibrium values and do not provide meaningful geological information. (Hare *et al.*, 1980; Bada, 1982; Wehmiller, 1982).

Wholly organic fossils are rarely preserved in anything approaching their original state. However, carbonate skeletons, or fragments of those skeletons, with their incorporated organic matrix, may survive for periods of time long enough to be of geological interest, in some instances with remarkable preservation of ultrastructural detail and with essentially original elemental and isotopic compositions (Popp *et al.*, 1985). Even in cases of calcite recrystallisation or calcite replacement of aragonite some organic relics are often preserved. Collagen, albumin and serum factors were detected in fossil mammoth bones which had been exposed to ambient temperatures and to the effects of water, chemical and bacterial action for thousands of years (Lowenstein, 1981). Lowenstein (1981) suggested that the calcium apatite matrix in which the proteins were embedded provided considerable protection against the destructive effects of temperature and chemical agents. Proteins do not have to survive intact to produce a reaction since most immunological determinants consist of a few adjacent amino acids, therefore fragments could still be immunologically reactive (Lowenstein, 1981).

Because antibodies recognise a small part of a macromolecule they may be capable of recognising a target antigen even after the macromolecule is partially destroyed (Rybicki & Von Wechmar, 1982). Muyzer *et al.* (1984) conclude that immunology is ideally suited to the study of the preservation and degradation of macromolecular structures over geological time.

Studies on the isotopic and elemental compositions of brachiopod shells,which are low magnesium calcite, indicated that compositions that are closely related to the original remain in texturally well-preserved mid- and probably also early Palæozoic specimens (Popp *et al.*, 1985). Amino acid assemblages have been recovered from Jurassic brachiopods in proportions which correlate closely with those determined in taxonomically-related Recent species (Kolesnikov & Prosorovskaya, 1986).

Up to 50% of the tissue mass of living adult brachiopods is situated within the shell (Curry & Ansell, 1986) and although this is not available in fossil organisms it is useful in work using Recent species. Material trapped in the shell fabric of living brachiopods contains phylogenetic information detectable by immunological techniques (Collins *et al.*, 1988).

The fact that so much of the tissue mass of living brachiopods is found in the shell and that the shell is a protected microenvironment where fossil material can be preserved means that it should be possible to apply immunological techniques to palæontology in a study of living and fossil brachiopods. The extensive fossil record of many living brachiopods favours their selection for such a study.

2.3 A brief history of immunology.

If early attempts at smallpox inoculation and Jennerian vaccination are excluded, the science of immunology can be thought of as being only 110 years old, being born in 1880 with the discovery by Pasteur of specific acquired immunity to chicken cholera. This was followed by similar observations for anthrax, rabies and numerous other diseases.

The rôle played by phagocytic cells in clearing away and destroying bacteria was recognised by Metchnikoff in 1884. An antibacterial substance or factor in the blood of animals immunised against tetanus and diphtheria organisms was demonstrated by von Behring and Kitasato in 1890. The neutralising ability of such blood serum for the bacterial toxins was the first demonstration of the effect of what is now recognised as antibody. In 1899 Pfeiffer and Bordet demonstrated the activity of a serum factor called complement that participates with antibody in the destruction of bacteria and has now been shown to have a wide variety of important biological activities. Paul Ehrlich suggested the first theory of antibody formation, the side chain theory, which proposed the existence of receptors on the surface of cells that could be released into the blood to neutralise bacterial toxins in 1897.

Later the helpful effect of antibody in encouraging phagocytosis became apparent, thus reconciling two opposing schools of thought on immune mechanisms: one believing the process to be the sole result of blood factors, i.e. by humoral immunity, and the other upholding an entirely cellular viewpoint.

Tissues and cells capable of exhibiting what is now recognised as an adaptive immune response have an evolutionary history of 400 million years and the forms taken by the response during this period have maintained a remarkable constancy both at the molecular and the functional level. The basic pattern of the protein molecules involved in this adaptive immune response has been retained with the diversification that has occurred through evolutionary selective pressures being superimposed on this basic pattern. When a foreign substance, i.e. an antigen, such as a bacterium or virus, enters a host the immune system is activated and bone marrow-derived cells, called B cells, differentiate into antibody-producing plasma cells. In an experimental system an animal, injected with the material of interest, will produce antibody which can be detected by the enzyme-linked immunosorbent assay (ELISA) (Engvall & Perlman, 1971).

The specificity of the antibody for the antigen which induced its formation led to the use of antibody as an analytical tool. Thus the antigenic characteristics of bacterial and non-bacterial substances were determined and systems of classification of microorganisms were developed on this basis. In 1901 Landsteiner used antigen-antibody interactions to define the ABO blood grouping system on the basis of antigenic differences in red cell membranes and was also responsible for carrying out the ground work on the chemical basis of antigenic specificity.

If an antibody prepared specifically against a particular determinant on the antigen under scrutiny was used the likelihood of cross-reactions would be minimised. Such an antibody is called a monoclonal antibody, i.e. it recognises only one determinant or epitope on the antigen in question. Monoclonal antibodies were first developed by Köhler and Milstein (1975) by the fusion of spleen cells with mouse tumour cells.

Using monoclonal antibody technology it is possible to develop a cell line which will produce a constant supply of homogeneous antibody which, because it is highly specific and standard in its characteristics, could be a very powerful tool in a taxonomic study.

2.4 Application of immunology to phylogenetic studies.

2.4.1 Previous work.

Immunological cross-reactions among sera of many animals were used to estimate their relative genetic distances (Nuttall, 1904). Immunodiffusion was used by Goodman (1963) to study evolutionary relationships among primates. Immunological distances between taxa can be measured by producing an antibody against one taxon and then determining the degree of cross-reactivity of that antibody with other taxa (Sarich & Wilson,1966). The microcomplement fixation method was used to construct immunological family trees for specific proteins such as albumin and transferrin (Sarich & Wilson, 1967) and a correlation was found between immunological distance and the divergence time of common ancestors. Measurements of immunological distance are recognised as useful taxonomic indicators (Thorpe, 1982) and a direct linear relationship occurs between antigenicity and amino acid substitution (Maxson & Maxson, 1986).

A panel of monoclonal antibodies with a range of specificities proved to be useful in work on bivalve families (Muyzer *et al.*, 1984). In an artificial diagenesis experiment Muyzer *et al.* (1984) found that diagenetic degradation of biopolymers could be followed over geological time using a suite of appropriate monoclonal antibodies.

2.4.2 Present study.

Antisera.

Antisera were generated against representatives of the Order Terebratulida to try to elucidate phylogenetic relationships between families of terebratulide brachiopods using immunological distance measurements.

Antisera were also generated against small molecular weight compounds of geological interest with a view to using these to detect the compounds in rock samples.

The antibodies in an antiserum are polyclonal since they are produced by between 5,000 and 10,000 B cells which are stimulated by the presence of the antigen. All of the B cells produce slightly different immunoglobulins with varying affinities and specificities. Antigens usually have a number of different haptenic determinants or epitopes with which different antibodies react. An antibody may recognise different determinants, if these are very similar, and in an antiserum there is a range of molecularly different yet specific

antibodies against each determinant. Thus antisera may exhibit a certain degree of cross-reactivity, i.e. they may react with a number of different antigens if they are sufficiently alike.

One advantage of using antisera is that they are relatively quick and simple to prepare but a disadvantage, as mentioned above, is that they may cross-react to a certain extent with similar antigens. This can be overcome by absorbing the serum with selected antigens.

Monoclonal antibodies.

Monoclonal antibodies have been successfully used in medical and biological studies but as yet have not been systematically applied to palæontology. Monoclonal antibodies have been used in the present work to determine phylogenetic relationships.

Although monoclonal antibodies can be very useful they have two main drawbacks. Firstly, they are time-consuming and labour-intensive to prepare and secondly they are sometimes too specific for the antigen which was used to stimulate them. This level of specificity sometimes proves to be too great to be of use and a number of monoclonals must be used in combination before a meaningful result is obtained.

Compounds of Geological interest.

Antisera were prepared against a sample of Kimmeridge Clay and against carbazole, a small molecular weight compound. Substances of molecular weight less than 1000 are not generally antigenic. Antibodies can be raised to small molecules by immunisation with conjugates made up of low molecular weight substances (haptens) covalently linked to proteins.
Small molecules frequently provide good epitopes for binding to B cells but are unable to induce a response because they are too small to contain the determinants necessary for simultaneous binding with class II proteins and T-cell receptors. Such molecules are known as haptens. To elicit a good antibody response, haptens must be coupled to other molecules that can be processed to provide suitable sites for T-cell receptor binding. Such molecules are called carriers and provide the class II-T-cell receptor binding sites. One of the requirements for the production of a strong antibody response is the cell to cell contact between B cells and helper T-cells and between helper T-cells and antigen-presenting cells. This contact is mediated by a fragment of the antigen that has binding sites for both class II protein binding and T-cell receptor binding. Any antigen that does not have a region that can bind to both these proteins will not elicit a good response. However, these

sites can be added to the protein by coupling them to molecules that do have the proper site. This can be done by coupling protein antigens to other proteins that have good class II-T-cell receptor sites. Keyhole limpet hæmocyanin is a good protein for coupling since it is highly immunogenic.

Chapter 3 MATERIALS AND METHODS

Materials.

Thanks to Drs M. Collins and G. Muyzer for some rabbit antisera and antigens.
Thanks to Mr C. Downes and Dr N. Evans of British Gas for samples of Kimmeridge, Oxford and Lower Lias clays and carbazole.
New Zealand White rabbits were obtained from S. Merry, The Poultry Farm, Whiterig, Near Airdrie ML6 7SE and W. H. Mellor, HG Rabbitry, Oldham, Lancs OL1 2SP.
BALB/c mice were provided by the Animal House, Department of Physiology and Biochemistry, University of Glasgow.

Item	Supplier
Tissue culture:-	
RPMI medium 1640 1x	Northumbria Biologicals Limited
L-glutamine	Northumbria Biologicals Limited
Fœtal bovine serum	Imperial Laboratories
Penicillin	Flow
Streptomycin	Flow
Trypan blue	Gurr, BDH
Tissue culture flasks, 25 cm^2	Falcon, Becton Dickinson Ltd.
Tissue culture flasks, 75 cm^2	Flow
Petri dishes, 60 mm.x15 mm.	Falcon, Becton Dickinson Ltd.
Tissue culture plates, 24 well & 96 well.	Falcon, Becton Dickinson Ltd.
Pipettes, 1 ml, 5 ml and 10 ml.	Sterilin
Minisorp tubes	Gibco
Conical tubes, 13 ml capacity	Falcon, Becton-Dickinson Ltd.
Centrifuge tubes, 50 ml capacity	Falcon, Becton-Dickinson Ltd.
Monoclonal antibody work:-	
HAT supplement	Sigma
HT supplement	Sigma
Dimethylsulphoxide (DMSO)	BDH
Polyethylene glycol 4000	Merck, BDH
Bio-freeze vials	Northumbria Biologicals Ltd.

SMI aliquoter	Alpha Laboratories.
X63-Ag8-653 mouse myeloma cells	Biochemistry, Univ. Glasgow

Antigen Preparation:-

YM 10 filters	Amicon Ltd.
EDTA (ethylene diamine tetra-acetic acid)	Boehringer Mannheim, BCL
Sodium hypochlorite	Sigma

Enzyme-linked immunosorbent assay and FELISA:-

Diethanolamine	Sigma
Gelatin	Sigma
Magnesium chloride	Sigma
Goat anti-mouse IgG (whole molecule) conjugated to alkaline phosphatase	Sigma
Goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase	Sigma
Disodium p-nitrophenyl phosphate	Sigma
MUP 4-methylumbelliferyl phosphate dilithium salt	Boehringer Mannheim, BCL
Tween 20 (Polyoxyethylene sorbitan monolaurate)	Sigma
Sodium azide	Sigma
Falcon Microtest III flexible assay plate	Falcon, Becton Dickinson Ltd.
Microfluor B plates (black) flat bottom	Dynatech

Immunisation:-

Complete Freund adjuvant Bacto	Difco Laboratories
Incomplete Freund adjuvant Bacto	Difco Laboratories
Syringes	Becton Dickinson Ltd.
Needles	Becton Dickinson Ltd.
Rocket syringes	Vicarey Davidson

Serum Preparation:-

Glass universals	Sterilin
Eppendorf tubes	Elkay
Ammonium sulphate	Sigma

Chemicals:-

Tris Sigma 7-9	Sigma
Sodium chloride	Fisons
Di-sodium hydrogen orthophosphate anhydrous	Griffin & George
Sodium di-hydrogen orthophosphate.$2H_2O$	Sigma

Formulae:-

Phosphate buffered saline PBS pH 7.3:-
8.5 g Na Cl; 1.07 g Na_2 HPO_4 anhydrous; 0.39 g Na H_2 $PO_4.2H_2O$: in 1 l dist. H_2O.

Tris buffered saline TBS (10 mM):-
1.21 g Tris; 9 g Na Cl: made to 900 ml with dist. H_2O; pH adjusted to 7.5 and made to 1l.

Substrate buffer for ELISA:-
97 ml diethanolamine; 0.2 g Na N_3: made to 800 ml with dist. H_2O; pH adjusted to 9.8 and made to 1 l.

Substrate buffer for Fluorescence ELISA:-
1.051 ml diethanolamine (10 mM); 0.2033 g Mg Cl_2: made to 900 ml with dist. H_2O; pH adjusted to 9.8 and made to 1 l.

Addresses of Suppliers

Alpha Laboratories, 40, Parham Drive, Eastleigh, Hants SO5 4NU.
Amicon Ltd., Upper Mill, Stonehouse, Glos GL10 2BJ
BCL, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex BN7 1LG
BDH Ltd., Burnfield Avenue, Thornliebank, Glasgow G46 7TP
Becton Dickinson U. K. Ltd., Between Towns Road, Cowley, Oxford OX4 3LY
A. & J. Beveridge, 5, Bonnington Road Lane, Edinburgh EH6 5BP
Difco Laboratories Ltd., P.O. Box 14 B, Central Avenue, East Molesey, Surrey KT8 0SE
Dynatech Laboratories Ltd., Daux Road, Billingshurst, Sussex RH14 9SJ
Elkay Laboratory Products (U.K.) Ltd., Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hampshire RG21 2YH
Fisons plc, Loughborough LE11 0RG
Flow Laboratories Ltd., Woodcock Hill Industrial Estate, Harefield Road, Rickmansworth, Herts WD3 1PQ
Gibco Ltd., P.O. Box 35, Washington Road, Abbotsinch Industrial Estate, Paisley PA3 4EP
Griffin & George, Bishop Meadow Road, Loughborough, Leicester LE11 0RG
Imperial Laboratories (Europe) Ltd., West Portway, Andover, Hampshire SP10 3LF
Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramington, Northumberland NE23 9HL
Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset
Sterilin House, Clockhouse Lane, Feltham, Middlesex TW 14 8QS
Vicarey Davidson, 162, Bath Street, Glasgow G2 4TD

Methods.

3.1 Preparation of Complete Medium (CM) for Cell Culture.
3.2 General Care of Mouse Myeloma Cell lines.
3.3 Estimation of Viability of Cells.
3.4 Freezing down Cell Lines.
3.5 Bringing up Cell lines from Liquid Nitrogen storage.
3.6 Preparation of shell fibres.
3.7 Preparation of antigen for ELISA, FELISA and immunisation.
3.7.1 Secondary fibres.
3.7.2 Crude antigen.
3.7.3 Body tissues.
3.7.4 Heat-treated secondary fibres.
3.7.5 Clay samples.
3.8 Immunisation protocols.
3.8.1 Intraperitoneal route.
3.8.2 Subcutaneous route.
3.9 Isolation of serum from blood.
3.10 Absorption of sera.
3.11 Isolation of IgG from serum by ammonium sulphate precipitation.
3.12 Enzyme-linked immunosorbent assay (ELISA).
3.13 Fluorescence enzyme-linked immunosorbent assay (FELISA).
3.14 Cell fusion procedure.
3.15 Preparation of HAT and HT media.
3.16 General Care of Hybridomas.
3.17 Screening of Hybridoma supernatants.
3.18 Subcloning by Limiting Dilution.
3.19 Preparation of Mouse Spleen cells as Feeders for Subcloning.
3.20 Coupling of small molecular weight compounds through diazo bonds.

3.1 Preparation of Complete Medium (CM) for Cell Culture.

Complete medium (100 ml) was prepared by mixing 80 ml of RPMI 1640 (1x), 1ml of L-glutamine (200 mM) and 20 ml of Fœtal bovine serum in a sterile 75 cm^2 tissue culture flask. Penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added. The whole procedure was carried out in a vertical laminar flow hood (Gelaire) using sterile technique.

3.2 General Care of Mouse Myeloma Cell lines.

Cells of the mouse myeloma cell line X63.Ag8.653 were grown in continuous culture in Complete medium containing 20% Fœtal bovine serum. The cells were kept in small (25 cm^2) tissue culture flasks in a Flow IR 1500 CO_2 incubator delivering 5% CO_2 / 95% air at 37°C.

When the medium became yellow, i.e. was expended, the cells were resuspended, 3/4 of the volume discarded and replaced with the same volume of Complete medium, and the cells cultured as before.

3.3 Estimation of Viability of Cells.

The tissue culture flask was transferred from the CO_2 incubator to an alcohol-cleaned laminar flow hood where the cells were resuspended by inversion of the flask. A sample of the suspension (200 µl) was transferred into a minisorp tube using a sterile pipette. A small amount (50 µl) of the suspension was placed in a second minisorp tube and to this was added 50 µl of 0.2% trypan blue in phosphate buffered saline (PBS) (pH 7.3).

The samples were mixed and applied to an Improved Neubauer hæmocytometer. The living and dead cells in the complete 5 x 5 square were counted and the number of living cells determined.

3.4 Freezing down Cell lines.

The viability of the cell suspension to be frozen down was estimated. See **3.3.**

A suitable volume of the cell suspension was centrifuged at 350 ***g*** for 5 min at 25°C. in an Heraeus Omnifuge 2.0 RS.

The supernatant was discarded and the cells resuspended in a chilled mixture of Fœtal bovine serum (85% v/v), Dimethyl sulphoxide (DMSO) (15% v/v) at a concentration of $5x10^6$ cells per ml in a Bio-freeze vial. Preparation of more than one vial necessitated that the vials were held on ice at this stage.

The vial was placed in a Nunc ampoule box and incubated at -70°C for 24 h, then transferred to a cane and stored in a liquid nitrogen vat.(-173°C.).

3.5 Bringing up Cell lines from Liquid Nitrogen storage.

Complete medium (20 ml) was placed in each of two Falcon centrifuge tubes (50 ml capacity).

The contents of the vial were thawed quickly and the vial was doused with 70% alcohol.

The contents of the vial were divided between the two Falcon tubes:- approximately 0.5ml per tube, and the tubes centrifuged at 350 *g* for 5 min at 25°C.

The supernatant was discarded and each set of cells resuspended in 5 ml of complete medium, added to a small tissue culture flask containing 5 ml of complete medium, and incubated in a Flow IR 1500 CO_2 incubator delivering 5% CO_2 / 95% air at 37°C.

3.6 Preparation of Shell fibres.

Shells were soaked in sodium hypochlorite (10% w/v active chlorine) for 48 h to remove epifauna, endoliths, body tissues and the organic matrix of the shell.

The shells were agitated and the fibres isolated from the other elements on the basis of differential suspension rates. The purified fibres were sonicated in a weak hypochlorite solution, thoroughly rinsed in distilled water, drained and freeze-dried.

3.7 Preparation of antigens for ELISA, FELISA and immunisation.

3.7.1 Secondary fibres.

Organic material was extracted from the fibres prepared as in **3.6** by decalcification in excess 10% w/v EDTA (ethylene diamine tetra-acetic acid) at pH 8 and centrifugation at 10,000 *g* for 20 min at 4°C. The EDTA-calcium complex was removed from the

supernatant by ultrafiltration across YM 10 filters (10,000 kDa cutoff) and the remainder used for immunisation.

3.7.2 Crude antigen.

Crude antigen preparations for coating ELISA and FELISA plates were made by decalcification of 46 g of powdered shell material in 1 litre of 20% w/v EDTA (pH 8).

3.7.3 Body tissues.

The body tissues of 10 fresh specimens of *Terebratulina retusa* were extracted, the guts discarded and the tissues homogenised with 10 ml PBS (pH 7.3).

The mixture was centrifuged at 1,500 *g* for 10 min at 25°C and the supernatant used for immunisation.

3.7.4 Heat-treated secondary fibres.

Secondary fibres prepared as in **3.7.1** were incubated at 140°C for 96 h to mimic conditions which the shells may have undergone during fossilisation and these were used for immunisation.

3.7.5 Clay samples.

A borehole sample from the Kimmeridge Clay of the Dorset/North Channel Basin area was solvent extracted and the polar fraction isolated by liquid chromatography. The solvent was evaporated off and air in the vial displaced with nitrogen. This sample and similar samples of Oxford and Lower Lias Clays were provided by British Gas.
Samples were air-dried and reconstituted in sterile saline (0.9% NaCl) or in methanol for immunization and FELISA.

3.8 Immunisation protocol.

BALB/c mice, 7-10 weeks old, were immunised by the intraperitoneal route and New Zealand White rabbits were immunised by the subcutaneous route. These animals were bled before being immunised to provide pre-immune sera for use as negative controls in the ELISA and FELISA.

3.8.1 Immunisation of Mice by the Intraperitoneal route.

The antigen to be injected was emulsified with an equal volume of Freund's Complete adjuvant . In each case 50 μg of protein antigen was administered in a final volume of 0.3 ml to one mouse.

The emulsion was made by repeatedly passing the mixture between two Rocket 2ml glass syringes, attached by a double needle assembly, until the mixture was thick and creamy and a drop of it placed on the surface of PBS (pH 7.3) did not disperse. The immunising dose was drawn up into a 1ml syringe fitted with a 23G needle and was administered by inserting the tip of the needle straight through the fur and skin into the peritoneum.

One month later the procedure was repeated using Freund's Incomplete adjuvant.

One month later the procedure was repeated using 100 μg protein antigen in PBS (pH 7.3).
Fifteen days after each immunisation the mouse was bled from the tail and the titre of the serum was tested by FELISA. See **3.13.**

If the serum titre, i.e. the amount of circulating antibody present in the serum, was sufficiently high, a fusion, using the spleen of the immunised mouse, was performed four days after the third immunisation.

3.8.2 Immunisation of Rabbits by the Subcutaneous route.

An emulsion of the antigen in Freund's complete adjuvant was prepared as described in **3.8.1** so that 1 mg protein antigen was administered in 1 ml to one rabbit. The emulsion was injected subcutaneously into the scruff of the neck in 5 sites.

One month later the procedure was repeated using 0.5 mg protein antigen in Freund's incomplete adjuvant.

The procedure was repeated as above at 2 week intervals on a further two occasions.

Blood was collected from the ear vein 2 weeks after the fourth immunisation and tested for the presence of antibody by the FELISA.

3.9 Isolation of Serum from Blood.

Blood was collected in a glass universal bottle and incubated at 25°C for 1 h. The clot was teased from the sides of the universal and the sample incubated at 4°C for 23 h. The clot-free liquid was poured into a Falcon centrifuge tube (50 ml capacity) and centrifuged at 1,500 *g* for 20 min at 4°C. The serum was decanted off, aliquoted and stored at -20°C.

3.10 Absorption of Sera.

Aliquots of dilute sera were incubated with secondary fibres of chosen antigens for 1 h. The suspensions were centrifuged and the supernatants subjected to the same treatment with fresh amounts of shell fibres (Muyzer, 1984). This was done to remove the antibody activity against the chosen antigen in order to increase the specificity of the antiserum to a particular antigen.

3.11 Isolation of IgG from serum using ammonium sulphate precipitation.

A saturated solution of ammonium sulphate was prepared by dissolving 75 g of ammonium sulphate in 100 ml distilled water at 25°C. The solution was stored at 4°C and ammonium sulphate crystals reformed.

Saturated ammonium sulphate was added dropwise to serum in an eppendorf tube to give a final ratio of 1:1.

The mixture was incubated at 25°C for 30 min and centrifuged at 3,888 *g* for 10 min at 10°C.

The supernatant was discarded and the pellet resuspended in TBS (10 mM) pH 7.5. Sodium azide was added to give a final concentration of 0.01% v/v and the preparation was aliquoted and stored at -20°C.

3.12 Enzyme-linked immunosorbent assay (ELISA).

The Falcon Microtest III plate was coated with antigen at a concentration of 460 µg shell powder in 100 µl 20% EDTA and incubated at 4°C overnight.

The plate was washed twice with TBS (pH 7.5).

Non-specific binding sites on the wells were blocked with 100 µl per well of 2% gelatin in TBS and incubated at 37°C for 30 min. Wells with fossil material were blocked with 100 µl per well of 3% donkey serum in TBS and incubated at 37°C for 30 min.

The plates were washed twice with TBS containing 0.02% Tween.

Mouse or rabbit antiserum (100 µl), diluted in 0.2% gelatin in TBS containing 0.02% Tween 20, or undiluted monoclonal antibody was added to each well and incubated at 37°C for 1.5 h.

The plates were washed once with TBS, twice with TBS containing 0.02% Tween 20 and once with TBS.

Goat anti-mouse or anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase, diluted 1: 1000 in 0.2 % gelatin in TBS containing 0.02% Tween 20, (100 µl) was added to each well and incubated at 37°C for 1.5 h.

The plates were washed 5 times with TBS containing 0.02% Tween 20.

Disodium p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets) (5 mg) in 10 ml substrate buffer was added at 100 µl per well to each well and incubated at 37°C for 20 min. The reaction was stopped by the addition of 100 µl 1N Na OH to each well. The resulting absorbance was read on a Titertek Multiskan Plus reader (Flow). The value of a blank well containing only phosphatase substrate was automatically subtracted from all the absorbance values.

Each ELISA was performed in duplicate. A positive control was provided by the inclusion of a hyper-immune serum at a suitable concentration, while a negative control for antigen was either 20% w/v EDTA or TBS (10 mM) pH 7.5, and for antibody was pre-immune serum.

3.13 Fluorescence Enzyme-linked immunosorbent assay (FELISA).

The Dynatech Microfluor B plate was coated with antigen at a concentration of 460 µg shell powder in 100 µl 20% EDTA or 5µg carbazole in 100 µl TBS (10 mM) pH 7.5 or a range of concentrations of Kimmeridge, Oxford and Lower Lias clays and incubated at 4°C overnight.

The plate was washed twice with TBS (pH 7.5).

Non-specific binding sites on the plates were blocked with 100 µl per well of 2% gelatin in TBS and incubated at 37°C on a shaker-incubator (Dynatech) for 30 min. Wells with fossil material were blocked with 100 µl per well of 3% donkey serum in TBS and incubated at 37°C on a shaker-incubator for 30 min.

The plates were washed twice with TBS containing 0.02% Tween on an Ultrawash II (Dynatech).

Mouse or rabbit antiserum (100 µl), diluted in 0.2% gelatin in TBS containing 0.02% Tween 20, or undiluted monoclonal antibody was added to each well and incubated at 37°C on the shaker-incubator for 1.5 h.

The plates were washed once with TBS, twice with TBS containing 0.02% Tween 20 and once with TBS.

Goat anti-mouse or anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase, diluted 1:1000 in 0.2 % gelatin in TBS containing 0.02% Tween 20, (100 µl) was added to each well and incubated at 37°C on the shaker-incubator for 1.5 h.

The plates were washed 5 times with TBS containing 0.02% Tween 20 and once with substrate buffer.

MUP (4-methyl umbelliferyl phosphate dilithium salt) (2 mM) in substrate buffer was added at 100 µl per well to each well and the resulting fluorescence read on a Microfluor reader (Dynatech) at 0 min, 5 min, 10 min, 15 min and 20 min, the plates being kept in the dark between readings. The value of a blank well containing only MUP was automatically subtracted from all the fluorescence values. The optimal incubation time was found to be 20 min.

Each FELISA was performed in duplicate. A positive control was provided by the inclusion of a hyper-immune serum at a suitable concentration, while a negative control for antigen was either 20% w/v EDTA or TBS (10 mM) pH 7.5, and for antibody was pre-immune serum.

3.14 Cell Fusion procedure.

The last immunising dose was administered and, four days later, the mouse was killed. Blood was collected by cardiac puncture and the spleen was transferred to a petri dish containing 5 ml of complete medium.

The spleen was decapsulated and the cells teased apart using two 18G needles. The cells were aspirated into a 10 ml syringe fitted with a 21G needle, expelled and then aspirated into the 10 ml syringe fitted with a 25G needle and expelled into a Falcon centrifuge tube (13 ml capacity).

The cell suspension was incubated at 25°C for 5 min and centrifuged at 350 ***g*** for 5 min at 25°C.
The supernatant was discarded and the pellet resuspended in 5 ml of complete medium. It was estimated that one spleen yielded $100x10^6$ cells.

The viability of the mouse myeloma cells was estimated and that volume containing $30x10^6$ viable cells was centrifuged at 350 ***g*** for 15 min at 10°C. The fusion was postponed if the cells were less than 70% viable.

The myeloma cells were mixed with the spleen cells in a Falcon centrifuge tube (50ml capacity) and centrifuged at 350 ***g*** for 5 min at 25°C.

The supernatant was discarded, 2 ml of polyethylene glycol (PEG) 4000 (42% w/v) in RPMI, warmed to 37°C, added and the cells resuspended for 30 s then incubated at 25°C for 30 s.

Complete medium (5 ml) was added dropwise over 90 s with constant flicking of the tube. Another 5 ml of complete medium were added immediately and the contents mixed by inversion then incubated at 25°C for 3 min.

The mixture was centrifuged at 350 ***g*** for 5 min at 25°C, the supernatant decanted and the pellet gently resuspended in 10 ml of complete medium. Care was taken not to break up all the cell clumps.

Samples of the cell suspension (100 µl) were dispensed, using a SMI aliquoter, into each well of 4 Falcon 24-well plates each of which contained 1 ml of HAT medium. The

fusion plates were incubated at 37°C in a Flow IR 1500 automatic CO_2 incubator delivering 5% CO_2 / 95% air.

3.15 Preparation of HAT and HT medium.

HAT (50x) was reconstituted in sterile distilled water and 2ml added to 100 ml of complete medium to give a final concentration of 10^{-4} M hypoxanthine, $4x10^{-7}$ M aminopterin and $1.6x10^{-5}$ M thymidine. HAT medium is selective for cells which have fused and are capable of utilising an alternative metabolic pathway. Thus unfused tumour or spleen cells will not continue to grow when fed under these conditions.

HT (50x) was reconstituted in sterile distilled water and 2 ml added to 100 ml of complete medium to give a final concentration of 10^{-4} M hypoxanthine and $1.6x10^{-5}$ M thymidine.

3.16 General Care of Hybridomas.

HAT medium (0.5 ml) was added to each well 7 days after fusion.
The medium was removed from each well 14 days after fusion and HT medium (1 ml) was added to each well.

HT medium (0.5 ml) was added to each well 21 days after fusion.
The medium was removed from each well 28 days after fusion and complete medium (1ml) was added to each well.

3.17 Screening of Hybridoma supernatants.

Fusion plates were examined daily for cell growth and when a cell clump measuring at least 2 mm in diameter was noticed supernatant (100 µl) was removed for testing by the fluorescence enzyme-linked immunosorbent assay (FELISA). See **3.13**. Supernatant was not removed from a well which had been fed within the last 3 days, this was to allow the concentration of antibody to increase after being diluted by the addition of fresh medium.

3.18 Subcloning by limiting dilution.

Those cells in a well containing antibody which was positive by FELISA were counted and the number of cells adjusted to give ten cells per 100µl, one cell per 100µl and 0.3 cells per 100µl of medium. Samples of these cell suspensions (100µl) were dispensed

into each well of ten 96 well tissue culture plates containing 10^5 mouse spleen cells (Feeders) in 100µl of medium. The medium used for dilution of hybridoma cells and spleen cells was HAT medium until Day 14 post fusion, HT medium between Day 14 and Day 28 post fusion and complete medium thereafter.

The wells of the subcloning plates were examined on Day 10 and supernatant from those wells containing clumps of at least 2 mm in diameter.was tested by FELISA. Only wells with a single clump of cells were tested.

The cells of a positive subcloning well were expanded into one well of a 24 well plate containing either 0.5 ml or 1 ml of the appropriate medium. The cells were allowed to multiply for a few days and the supernatant was tested by FELISA. Any positive cells were subcloned by limiting dilution as before.

The subcloning procedure was carried out three times before the cells were considered to be derived from a single ancestor and therefore to be producing monoclonal antibody.

After the third subcloning and expansion the cells were expanded into a second well of the 24 well plate and when the number of cells had increased sufficiently the cells from both wells were transferred into a 25 cm^2 tissue culture flask and fed as necessary.

The cells were cultured continuously and the supernatant tested frequently to ensure that the cells were still producing antibody. When sufficient cells were present some of the cells were frozen down as described in **3.4**.

3.19 Preparation of Mouse Spleen cells as Feeders for Subcloning.

A non-immunised, i.e.'normal', mouse was killed and the spleen decapsulated in a petri dish containing 5 ml of complete medium.

The cells were teased apart using two 18G needles. The cells were aspirated into a 10ml syringe fitted with a 21G needle, expelled and then aspirated into the 10 ml syringe fitted with a 25G needle and expelled into a Falcon centrifuge tube (13 ml capacity).

The cell suspension was incubated at 25°C for 2 min and the supernatant drawn up into the syringe and transferred to a 75 cm^2 tissue culture flask containing approximately 96 ml of medium.

One mouse spleen was estimated to yield $100x10^6$ cells, therefore one spleen was used to supply feeders for ten 96 well plates at 10^5 cells per 100 µl per well. If the subcloning was to be done before the fusion was 14 days old HAT medium was used, if the fusion was between day 14 and day 28 HT medium was used and after day 28 Complete medium was used.

3.20 **Coupling of small Molecular weight compounds through Diazo bonds**.modified from Handbook of Experimental Immunology Vol.1 4th. Edition Weir. 1986.

Carbazole (30 mg) was added to 100 ml of 0.1M hydrochloric acid then 1% sodium nitrite was added slowly dropwise with constant stirring on ice in a fume hood. The presence of free nitrous acid was detected using starch-iodide paper. The end-point of the addition of sodium nitrite was taken as the first positive starch-iodide test 15 min after the last addition of sodium nitrite.

Keyhole Limpet Hæmocyanin (KLH) (50 mg) was dissolved in 100 ml sodium carbonate buffer (pH 9.0) (2 mM) and the pH adjusted to 9.5 with 0.1M sodium hydroxide. The coupling reaction was carried out on ice between pH 9 and 9.5 with continuous stirring. The carbazole was added slowly to the KLH by running it down the side of the beaker. The pH was monitored during this addition and if it fell below 7 it was adjusted to 9.5 by running 0.1 mol/l sodium hydroxide down the side of the beaker. This was continued until all the carbazole was added. A final adjustment of pH to 9.0 was made and mixing continued for 2 h, checking the pH was still 9.0. The mixture was incubated at 4°C overnight and dialysed against 5 changes of 5 l of 0.15 mol/l sodium chloride. The azoprotein was made up to 600 ml with saline, giving a final protein concentration of 0.08 mg/ml, sterile filtered, aliquoted and stored at -20°C.

Chapter 4 SERO-TAXONOMY OF SKELETAL MACROMOLECULES IN LIVING TEREBRATULIDE BRACHIOPODS

4.1 Introduction.

The use of immunological distances as taxonomic indicators is a well established branch of molecular systematics. The technique involves the production of antibodies against one taxon and the assessment of taxonomic 'relatedness' by measuring the extent to which these antibodies cross-react with homologous compounds from other taxa.

Serological techniques have been applied to fossil material (Lowenstein, 1986) and species specific cross-reactivity has been demonstrated for a variety of mammalian fossils including Pleistocene bison (Lowenstein, 1986) and Pleistocene bivalve shells (Muyzer *et al.*, 1988). Treponemes, the organisms responsible for syphilis, have been recognised in bone lesions from Pleistocene bear using immunological techniques (Rothschild & Turnbull, 1987).

Analysis has shown that most skeletal carbonates lose organic material relatively rapidly, primarily by hydrolysis in the presence of water (Wyckoff, 1972). It has been suggested that greater emphasis be placed on investigation of the preservation of intra-crystalline rather than inter-crystalline material (Towe, 1980).

The work described in this chapter, which has been published in another form in *Historical Biology* by Collins *et al.* (1988), followed this suggestion by attempting to isolate intra-crystalline macromolecules and prepare antibodies to antigenic determinants protected within a biomineral. Most articulate brachiopods have a simple ultrastructure composed predominantly of large fibres of micro-crystalline low magnesium calcite (**Figure 4.1**) (Williams, 1968), making them particularly suitable for investigation of protected macromolecules. This study was carried out to determine if antigenic material isolated from secondary layer fibres contained phylogenetic information detectable by immunological techniques.

Terebratulide brachiopods were chosen because of the availability of extant genera for cross-reactivity experiments. The shell of a terebratulide brachiopod is characterised by a delicate internal calcareous structure, the 'loop', which in life supports the food-gathering lophophore. Variations in loop geometry and ontogeny are considered to be of primary taxonomic importance (Williams & Hurst, 1977) (**Figure 4.2**). The order was classified in the Brachiopod Treatise (Williams *et al.*, 1965) into two superfamilies:- the short-

Table 4.1 Localities of taxa used in the investigation.

Genus	Locality
Lingula unguis (Linnæus)	Hong Kong
Neocrania anomala (Muller)	Firth of Lorn, Argyll, Scotland
Hemithyris psittacea (Gmelin)	Friday Harbor, Washington, USA
Liothyrella uva notorcadensis (Broderip)	Ross Island, Antartica
Gryphus vitreus (Born)	Corsica, Mediterrenean
Terebratulina retusa (Linnæus)	West Coast of Scotland, Tromso, Roscoff & Corsica
Terebratulina septentrionalis (Couthoy)	Bay of Fundy and Simpson Island, N. Brunswick, Nova Scotia, Canada
Terebratulina unguicula (Carpenter)	Friday Harbor, Washington, USA
Agyrotheca barretiana (Davidson)	Jamaica
Mergerlia truncata (Gmelin)	Corsica, Mediterranean
Kraussina rubra (Pallas)	Southern tip of S. Africa
Macandrevia cranium (Muller)	Hebrides Shelf, Scotland
Dallina septigera (Lovén)	Hebrides Shelf, Scotland
Coptothyris grayi (Davidson)	Japan
Terebratalia transversa (Sowerby)	Friday Harbour, Washington, USA
Laqueus californianus (Koch)	Friday Harbour, Washington, USA
Magellania flavescens (Lamarck)	Port Jackson, Australia
Waltonia (Terebratella) inconspicula (Sowerby)	Christchurch, New Zealand
Terebratella sanguinea (Leach)	Marlborough Sound, New Zealand
Neothyris lenticularis (Deshayes)	Foveaux Strait, New Zealand

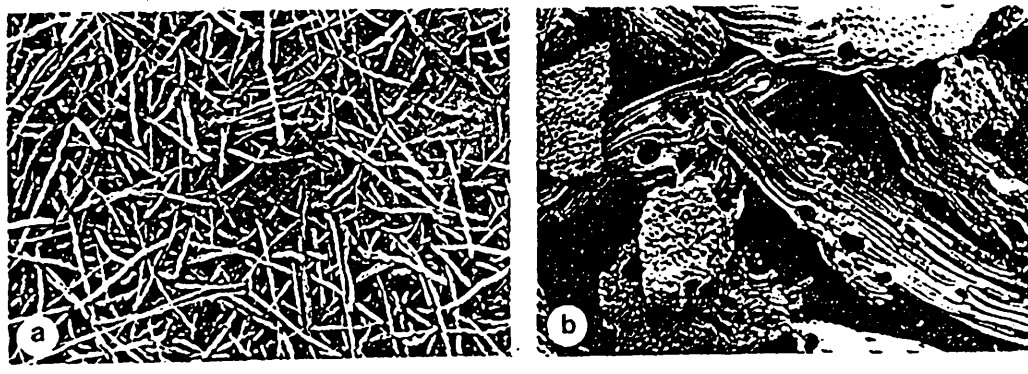


Figure 4.1 Fibre preparation of *Terebratulina septentrionalis*.
(a) organic material for immunisation and cross-reactivity experiments was isolated from the secondary layer fibres only (x100).
(b) residual fragments were composed predominantly of primary layer.

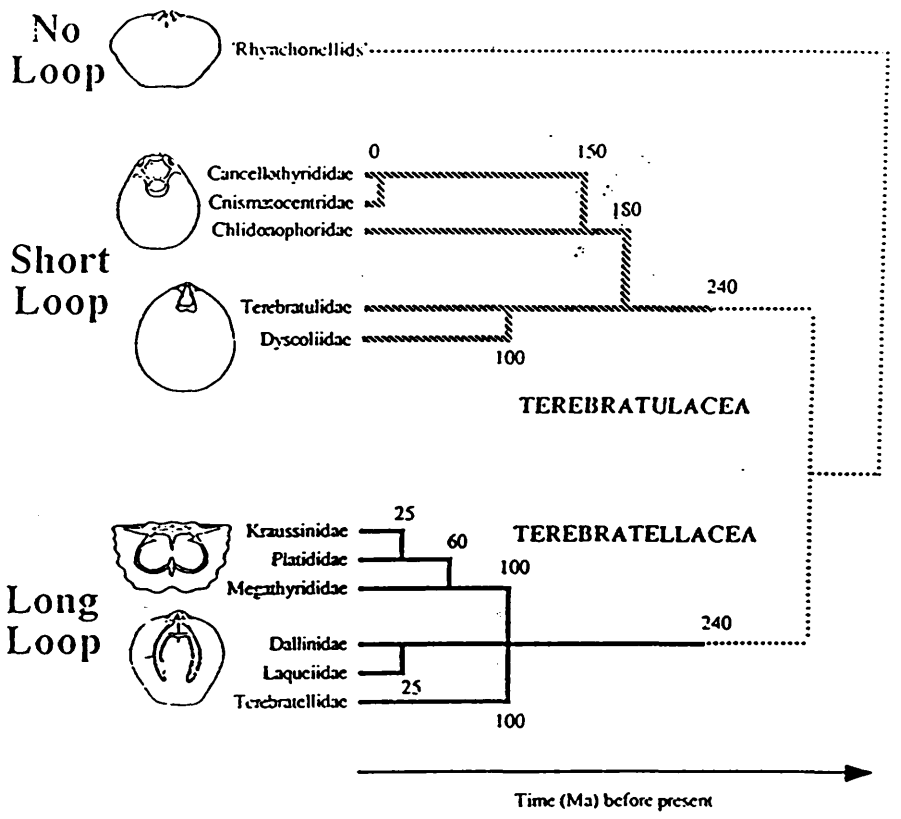


Figure 4.2 Traditional interpretation of terebratulide evolution (Williams *et al.*, 1965), showing the division into short-looped Terebratulacea and long-looped Terebratellacea. The figures give the approximate time of origin of each group in millions of years before present and illustrate their inferred relationships. Rhynchonellides, a separate order with no calcareous loop, are included schematically as an outgroup.

looped Terebratulacea and the long-looped Terebratellacea (**Figures 4.2** and **4.3**). Subsequent revision of the articulates has been incomplete, but has included reviews of loop and cardinal development in Mesozoic terebratulides (Baker, 1972; Dagys, 1974) and Recent terebratellides, (Richardson, 1975; La Barbera, 1986; Gunji, 1987), and a review of the loop of Mesozoic and Recent terebratulides (Cooper, 1982).

4.2 Materials and Methods.

In the case of *Gryphus vitreus*, secondary layer fibres could not be prepared since this species only produces a tertiary layer, therefore shell powder was used in place of shell fibres which were used for *Terebratulina retusa* and *Dallina septigera.*

A rabbit antiserum was prepared against each of *Terebratulina retusa, Dallina septigera* and *Gryphus vitreus* and a mouse antiserum (M1) was prepared against *Terebratulina retusa.*

The antiserum against *T. retusa* was absorbed with the antigens of six *T. retusa* populations, two populations of the West Atlantic species *T. septentrionalis* and a Pacific species *T. unguicula.*

Crude antigen preparations of 18 of the genera listed in **Table 4.1** were used to determine the level of cross-reactivity of the antisera, tested by ELISA and FELISA.

Immunological distances could not be calculated for this data set due to the lack of reciprocal antisera for most genera, therefore the data were not amenable to tree-building programmes such as FITCH (Felsenstein, 1984). A distance matrix was derived from levels of cross-reactivity using the euclidian metric and analysed using hierarchical clustering (Becker & Chambers, 1984). The level of cross-reactivity of individual genera to an antiserum is a coarser measure of 'relatedness' than are immunological distances, and only higher level clusterings were considered.

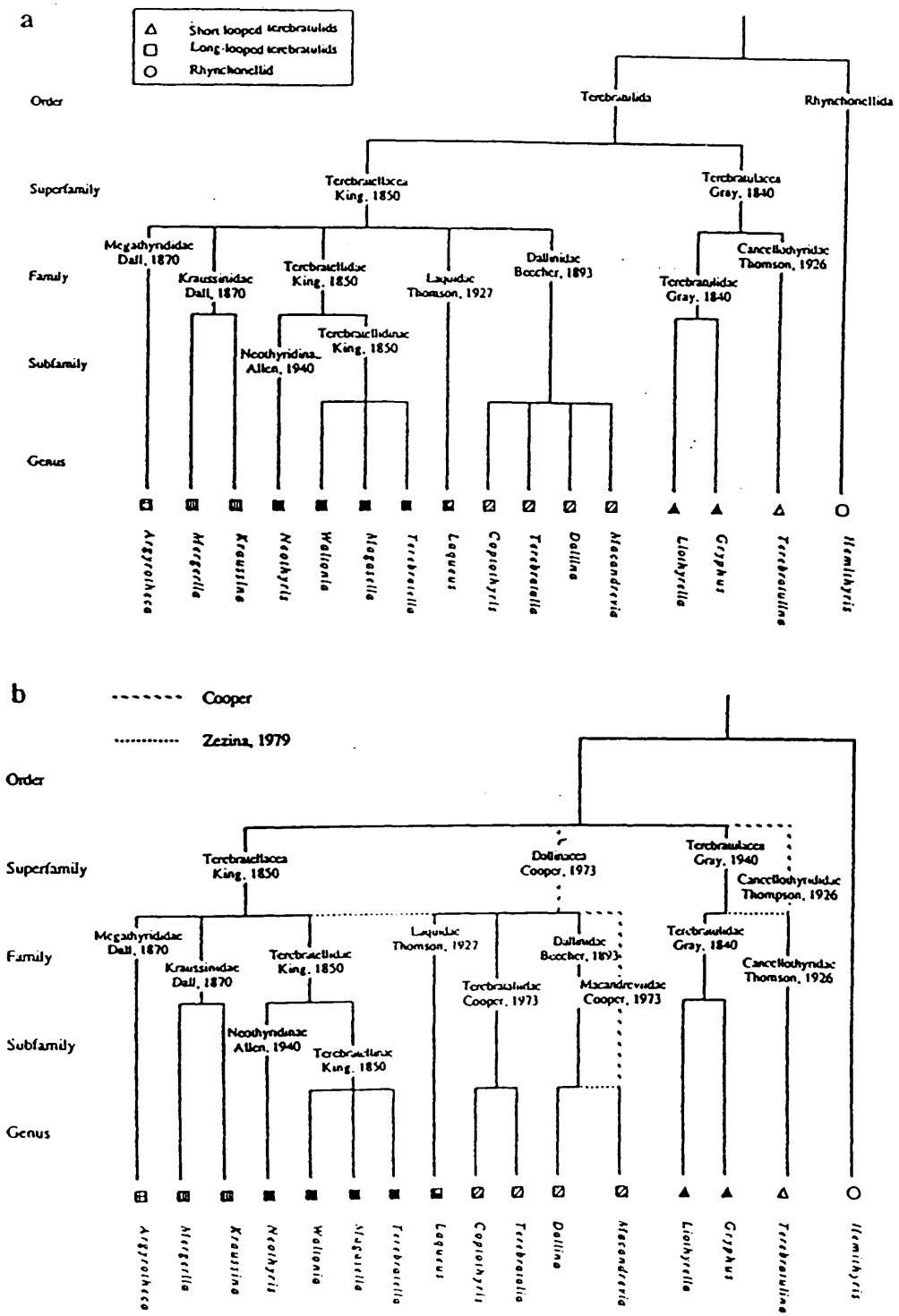


Figure 4.3 (a) Taxonomic relationships between the genera used in this study, based on the Treatise (Williams *et al.*, 1965). *Hemithyris* is a member of the Rhynchonellida, a separate order with no calcareous loop, which is included for outgroup comparison.

(b) One possible post-Treatise interpretation of the terebratulides, based on Zezina (1976) and Cooper (1973a, 1973c and 1981).

4.3 Results.

4.3.1 Antisera prepared against *Terebratulina retusa*.

Both antisera prepared against the short-looped cancellothyride *T. retusa* clustered members of the genus *Terebratulina* (**Figure 4.4**). Data are given in **Table 4.2.** However the cluster analysis did not readily distinguish other species of *Terebratulina* from populations of *T. retusa*. Cluster analysis demonstrated that the absorbed antisera were capable of distinguishing *T. retusa, T. septentrionalis* and *T. unguicula* (**Figure 4.5**). Data are given in **Table 4.3.**

Rabbit antiserum 4962, prepared against *T. retusa*, showed highest levels of cross-reactivity with three terebratellacean families (Terebratellidae, Laqueidae and Dallinidae excluding *Macandrevia*- the TLD group). The least cross-reactive of this TLD group, *Laqueus*, had a similar level of cross-reactivity to the short-looped terebratulacean *Liothyrella*. However the mouse antiserum prepared against *T. retusa* showed higher levels of cross-reactivity to *Liothyrella* than to any terebratellacean (**Table 4.2**).

Rabbit antiserum 4962 cross-reacted much less with Kraussinidae than with the TLD group of terebratellaceans or the Terebratulida. *Macandrevia* and the megathyrid *Argyrotheca* were weakly reactive with this antiserum, although this was the highest level of activity of the *Argyrotheca* antigen.

The differences between relationships derived using rabbit and mouse anti-*T. retusa* sera were striking. There was not enough mouse antiserum to afford duplicate results and the titre was low, being 1: 500, so the results cannot be considered as conclusive. The overall pattern and grouping of cross-reactivity levels within families was broadly similar but the relative positions of the Terebratulida and the 'complex-looped' group of terebratellaceans were reversed (**Table 4.2**).

4.3.2 Antiserum prepared against *Dallina septigera*.

With the rabbit antiserum against *Dallina*, 5007, *Macandrevia* is an exception contrasting as it does with the high levels of cross-reactivity of the TLD group (**Table 4.2**). The subtle divisions within the TLD group proposed by Richardson (1975) were not resolved by this study. *Terebratulina* is the only other genus to show marked reactivity with 5007. Terebratulidae, Kraussinidae, Megathyridae and *Macandrevia* were all poorly reactive with this antiserum (**Table 4.2**).

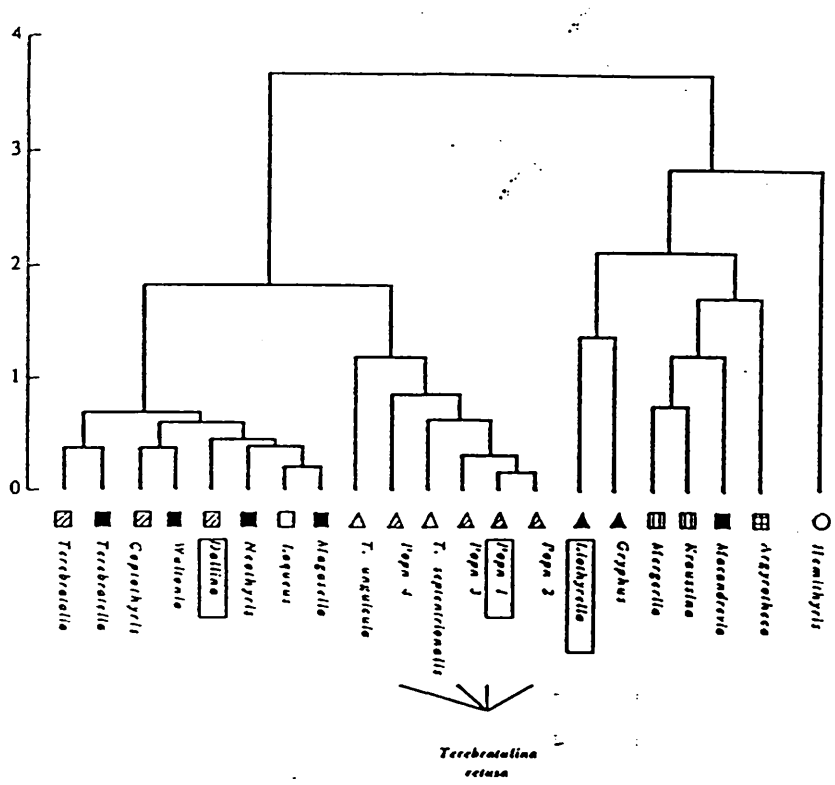


Figure 4.4 Cluster analysis based on the combined results of cross-reactivity experiments. The brachiopods to which the four antisera were generated are outlined, other symbols used are the same as in Figure 4.3.

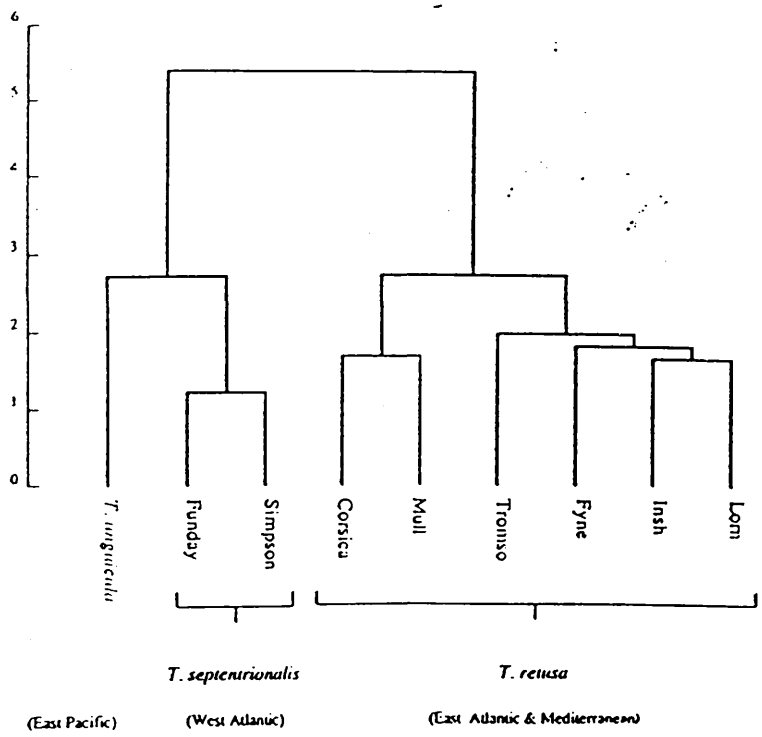


Figure 4.5 Cluster analysis based on the cross-reactivity of absorbed *T. retusa* serum with a variety of *Terebratulina* populations and species.

Table 4.2 Cross-reactivity of genera to the four antisera prepared against skeletal macromolecules.

Class	*Order*	*Superfamily*	*Family*	*Genus*	*Antiserum*						
					K4962		*K5007*		*K5010*		*M1*
					Mean	SD	Mean	SD	Mean	SD	
Inarticulata	Craniida	Craniacea	Craniidae	*Neocrania**	3.5	0.8	4.7	0.6	1.1	1.2	1.8
Inarticulata	Lingulida	Lingulacea	Linguliidae	*Lingula**	0.8	0.2	5.7	0.7	-	-	1.8
Articulata	Rhynchonellida	Rhynchonellacea	Hemithyrididae	*Hemithyris*	2.4	1.2	3.0	1.5	1.4	1.3	21.2
Articulata	Terebratulida	Terebratulacea	Terebratulidae	*Liothyrella*	23.6	6.8	7.8	2.5	†††	5.1	32.4
Articulata	Terebratulida	Terebratulacea	Terebratulidae	*Gryphus**	11.1	1.5	2.8	0.9	45.3	9.2	-
Articulata	Terebratulida	Cancellothyridacea	Cancellothyrididae	*Terebratulina*	†††	3.6	38.5	3.7	4.1	0.6	†††
Articulata	Terebratulida	Terebratellacea	Megathyrididae	*Argyrotheca*	9.5	1.2	6.0	-	4.1	1.8	-
Articulata	Terebratulida	Terebratellacea	Kraussinidae	*Mergerlia**	10.1	1.7	5.6	1.0	25.7	5.5	5.1
Articulata	Terebratulida	Terebratellacea	Kraussinidae	*Kraussina*	11.2	2.2	7.8	2.9	14.0	9.1	2.1
Articulata	Terebratulida	Terebratellacea	Terebratellidae	*Neothyris*	36.5	4.5	81.0	7.3	1.6	1.7	11.3
Articulata	Terebratulida	Terebratellacea	Terebratellidae	*Waltonia*	42.6	0.9	95.7	7.4	4.0	1.5	-
Articulata	Terebratulida	Terebratellacea	Terebratellidae	*Magasella*	31.8	0.5	86.0	-	3.0	0.6	-
Articulata	Terebratulida	Terebratellacea	Terebratellidae	*Terebratella*	28.5	5.9	99.8	2.8	1.1	1.4	6.4
Articulata	Terebratulida	Dallinacea	Laquidae	*Laqueus*	23.6	8.0	92.0	14.2	1.8	1.8	6.8
Articulata	Terebratulida	Dallinacea	Laquidae	*Coptothyris*	56.0	1.7	86.8	-	3.5	0.8	-
Articulata	Terebratulida	Dallinacea	Terebratalidae	*Terebratella*	33.5	3.1	76.4	3.1	1.1	1.2	3.8
Articulata	Terebratulida	Dallinacea	Dallinidae	*Dallina*	31.0	5.6	†††	9.8	1.5	1.9	20.4
Articulata	Terebratulida	Dallinacea	Macandreviidae	*Macandrevia*	5.0	1.9	4.1	2.0	25.3	4.3	0.7

Cross-reactivity levels as a percentage of that of the homologous antigen (†††); values not determined(-).
*Antigens prepared from powdered shells rather than fibres.

Table 4.3 Reactions of absorbed anti-*T. retusa* serum with *Terebratulina* populations.

Anti-*Terebratulina* serum (K4962) pre-absorbed against:-

Antigens	*Lorn*	*Mull*	*Insh*	*Fyne*	*Corsica*	*Tromso*	*T. sept.*	*T. ung.*
Firth of Lorn[2]	**100.0**	98.2	85.9	94.6	119.1	88.1	311.7	433.2
Sound of Mull[2]	106.7	**100.0**	87.3	77.2	121.3	147.7	321.3	495.3
Insh Island[2]	138.1	111.9	**100.0**	100.4	188.2	158.4	283.4	475.4
Loch Fyne[2]	140.0	127.5	85.3	**100.0**	111.8	124.1	233.8	478.4
Corsica	82.4	87.8	66.5	39.2	**100.0**	90.1	201.9	317.8
Tromso	123.5	84.9	73.0	82.5	143.0	**100.0**	207.2	370.2
T. septentrionalis	64.5	63.4	49.5	48.7	74.6	48.2	**100.0**	340.2
T. unguicula	106.0	79.7	67.8	64.1	91.4	74.2	169.3	**100.0**

[1]Cross-reactivity relative to homologous pre-absorbed sample (as 100).
[2]Populations from the west coast of Scotland are all within a 40 mile radius of Oban, Argyll.

4.3.3 Antiserum prepared against *Liothyrella uva notocadensis*.

Rabbit antiserum against *Liothyrella*, 5010, reacted most strongly with the short-looped terebratulacean *Gryphus*, although this reaction was low in comparison to the reactions of the family homologues with the anti-*Dallina* serum. Kraussinidae and *Macandrevia* were moderately reactive but *Terebratulina* species were weakly reactive and there was no significant activity with the TLD group of terebratellaceans.

4.4 Conclusions.

The cluster analysis of available serological data recognised five main groupings (**Figure 4.4**) which all behaved in a similar fashion against each of the antisera (**Table 4.2**). The groupings are somewhat arbitrary, being more accurate where greater numbers of genera were available. This is illustrated by the positioning of the rhynchonellide *Hemithyris*, which is shown clustering on one side of the dichotomy along with some terebratulides and the non-TLD terebratellaceans. This is an artefact of the clustering procedure, due to the fact that no antiserum was prepared against *Hemithyris* and because all other sera have low cross-reactivity with rhynchonellides.

Argyrotheca was poorly cross-reactive with all sera but the reaction with the mouse anti-*T. retusa* serum put it into an outgroup with *Hemithyris*.

Cluster analysis from serological data can be compared with existing biostratigraphical and morphological evidence. The serological data set used here is too limited to fully resolve family assignments but suggests the following revisions of the higher groupings:-

1. *Terebratulina* (and by extension the Cancellothyridacea) is only distantly related to present day terebratulaceans.
2. *Argyrotheca* (and by extension the Megathyridae) is not closely related to either the modern day terebratulaceans or terebratellaceans.
3. the kraussinides arose from terebratulacean rather than terebratellacean stock.
4. *Macandrevia* arose from terebratulacean rather than terebratellacean stock.
5. the TLD group of terebratellaceans is monophyletic.

Chronologically, as Elliott (1957) stated, the appearance of the terebratellacean families, although complicated by lack of information on internal structures, does not immediately suggest the evolutionary connections implied by traditional taxonomy (**Figure 4.2**).

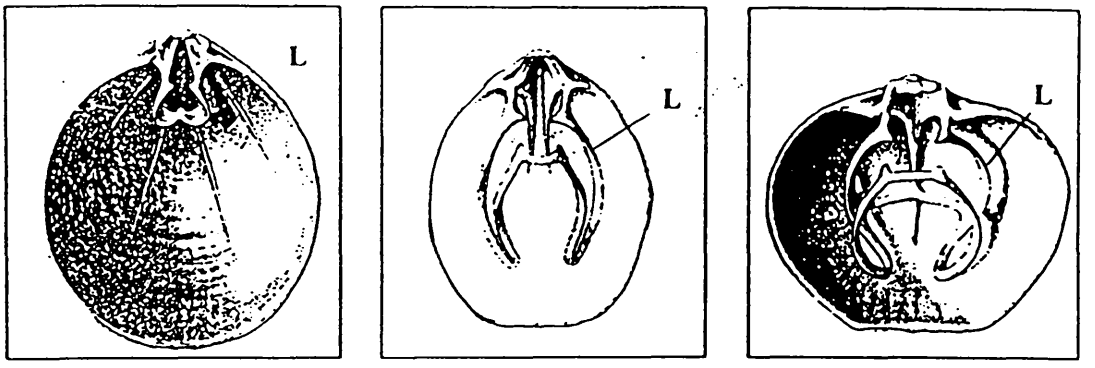
A number of taxonomic studies published since the Treatise afford comparison with the revisions suggested by serology. Three of the four antisera used here suggest that *Terebratulina* is closer to terebratellacean stock than to cancellothyride stock and the re-analysis of the cancellothyrides (Cooper, 1973a) supports a more distant relationship between them and the terebratulides than is inferred in the Treatise. A separate superfamily status for the cancellothyrides (**Figure 4.3b**) (Cooper, 1973a) is in line with the results of the serological study.

The loops of the kraussinides and megathyrides are simpler than those of the TLD brachiopods but there has been little detailed analysis of morphology or loop ontogeny of these families. Because the loops of the three non-TLD terebratellacean families are so simple it is difficult to assign them in a classification which relies so heavily on loop structure.

A dichotomy between the loop form of the TLD group and other terebratellacean families was recognised by Elliott (1976); this dichotomy has not yet been expressed taxonomically. Thus the assignment, from serological results, of the kraussinides to a terebratulacean stock and *Argyrotheca* to outgroup status is not contradicted by the available morphological evidence. Serology is in general agreement with traditional taxonomy in suggesting a monophyletic origin for the TLD group of terebratellaceans (**Figures 4.2** and **4.3a**) (Elliott, 1976) but see **Figure 4.3b**.

Indications of a terebratulacean origin for *Macandrevia*, independent of the TLD group, is perhaps the most unusual result of the serological study. Stratigraphically *Macandrevia* is a relatively young genus first recorded from the Miocene of Japan (Williams *et al.*, 1965). There is a great similarity between the adult loop of this brachiopod and those of the TLD group (**Figure 4.6**). However, comparison of the cardinalia (Cooper, 1973a; Richardson, 1975) and loop ontogeny (Richardson, 1975) has led both authors to conclude that, relative to other members of the TLD group, this genus is aberrant (**Figure 4.3b**). In fact Cooper (1973c) created a new family, the Macandreviidae, based on the aberrant cardinalia of *Macandrevia*.

While available morphological evidence does not fully support the results of this limited sero-taxonomy neither does it contradict it. Differences between the Treatise and serological results can be thought of as amplifications of divisions expressed or suggested since the publication of the Treatise in 1965.



Gryphus *Macandrevia* *Terebratella*

Figure 4.6 Comparison of the loops (L) of *Gryphus*, *Macandrevia* and *Terebratella* (from Davidson, 1886-8). Differences between the short loop of *Gryphus* and the long loops of *Macandrevia* and *Terebratella* are clear, however, serologically, *Macandrevia* is more closely related to *Gryphus*.

The serological data contradicts a primary division of terebratulides into long-looped and short-looped forms (Stehli, 1956; Williams, 1956; Williams & Hurst, 1977). It also predicts non-intuitive relationships (such as the origin of the kraussinides, *Argyrotheca* and *Macandrevia*) which are not contradicted by current taxonomic evidence.

If the immunological analysis is correct then a re-appraisal of the systematics of the Order Terebratulida at the family and superfamily level is necessary. Current classification of the order at this level is dominated by the loop, the taxonomic significance of which is not fully understood. The need to maintain lophophore function throughout ontogeny will constrain loop development (La Barbera, 1986) and it is possible that such constraints are so severe as to mask homeomorphy, i.e. close evolutionary convergence. A *Macandrevia* loop that was demonstrably homeomorphic would not only give insight into developmental constraints such as the rôle of a median septum but would also help to identify subtle morphological features which are more precise indicators of ancestral relationships. The posteriorly bifurcating median septum of *Macandrevia* may be such an example as it recalls the septa of Mesozoic terebratellaceans (Richardson, 1975).

Williams (1956) stated that the lophophore of *Macandrevia* has two brachial canals per side arm unlike *Terebratulina* and *Terebratella* (*Waltonia*) which have only one. The taxonomic significance of the loop may subsequently be downgraded, as a number of authors have argued (Williams, 1956), as other features such as the cardinalia (Dagys, 1974) prove to be more reliable.

The results of this investigation show, both in the consistency of the reactions within groups (e.g. the TLD group) and in the variation parallelled by morphology (e.g. the cases of *Macandrevia*, *Kraussina*, *Megerlia* and *Argyrotheca*), that phylogenetic information can be obtained from material trapped within the shell fabric of terebratulide brachiopods.

Chapter 5 AN IMMUNOLOGICAL INVESTIGATION OF THE RELATIONSHIPS WITHIN THE ORDER TEREBRATULIDA

5.1 Introduction.

Inadequacies within the systematics of the Order Terebratulida have been recognised (Richardson, 1975; Elliott, 1976; Williams & Hurst, 1977) but the essential sub-division between the short-looped Terebratulacea and the long-looped Terebratellacea has remained unchallenged except by the immunological investigation described in Chapter 4 and in Collins *et al.* (1988).

The work described in this chapter is an extension of the study in Chapter 4. In this case more antisera were prepared to allow immunological distances to be calculated. Nine species of Recent articulate brachiopods were tested by ELISA, using intra-crystalline macromolecules extracted from each species, to assess the relationships within the Order Terebratulida. The genera used in the investigation were determined primarily by the availability of sufficient numbers of shells which could be used to provide the necessary quantities of intra-crystalline organic material for immunisation. Nevertheless it was possible to include taxa representative of all major groups of living articulate brachiopods. The number of genera available from each group closely reflected their relative abundance in Recent brachiopod faunas. In this study five genera were available from the long-looped terebratulides, which dominate present day faunas, four of these were assigned to two families of the Superfamily Terebratellacea, while the remaining genus was classified within the Superfamily Dallinacea. Three short-looped terebratulides were available, representing two discrete families which, depending on interpretation, represented either one or two discrete superfamilies. The rhynchonellide genus *Notosaria* was included as an outgroup. Thus about 10% of all living brachiopod genera were included in this first comprehensive investigation of the biochemical systematics of brachiopods.

Immunological distance measurements have been used in taxonomic investigations, either using whole organisms (Olsen-Stojkovich *et al.*, 1986) or purified macromolecules (Sarich & Wilson, 1967; Sarich & Cronin, 1976; Lowenstein *et al.*, 1981). The technique assumes that the rate of evolution averaged over a large number of antigenic sites is uniform enough to give an accurate portrayal of the evolutionary branching pattern of the groups examined. The method permits highly quantitative comparisons of taxa that have diverged up to several hundred million years ago, using very small quantities of macromolecules. These are particularly useful attributes for a study of brachiopod

evolution since the availability of many modern taxa is limited, due to their restricted worldwide distribution and relative rarity.

5.2 Materials and Methods.

Shell fibres were prepared as in **3.6** except for *Megerlia truncata*, which was dialysed in EDTA using the technique of Weiner and Lowenstam (1980) because very little of it was available. For *Neothyris lenticularis* a single protein band of 45 kD, excised from a sodium dodecyl sulphate-polyacrylamide gel, was used for immunisation.

Rabbit antisera were prepared for each of the antigens listed in **Table 5.1**.

In this experiment the IgG fraction of each antiserum, prepared as in **3.11**, was used to try to improve specificity of the antisera.

The ELISA was used to determine the reaction between each antigen and antiserum.

5.3 Calculation of Immunological distances.

Homologous antisera were available for all antigens used so that it was possible to determine immunological distances from the results of ELISA carried out for all antigen-antibody combinations. Immunological distance (ID) was calculated as:-

$ID=100 \times \log_{10} (100/\Delta)$ where Δ is the mean reciprocal % cross-reactivity (taking the reaction of antiserum with homologous antigen as 100%). These distances were obtained from the linear regions of semi-logarithmic binding curves plotted using a series of antibody concentrations for each combination of antigen and antibody. The average of reciprocal distances for each combination was taken and means of duplicates were used for clustering.

Tree diagrams were constructed using either the method of UPGMA (Sneath & Sokal, 1973) or Fitch and Margoliash (1967) as represented by FITCH in the program package PHYLIP written by Joseph Felsenstein (University of Washington, Seattle).

Table 5.1 Localities of species used in the immunological distance study.

Taxon	Locality	Prep.	Abbrev.	Serum
Terebratellidae				
Waltonia (Terebratella) inconspicua (Sowerby)	Christchurch, N. Zealand	Fibre	Wi	K5040
Neothyris lenticularis (Deshayes)	Foveaux Strait, N. Zealand	Protein	Nl	427
Dallinidae				
Dallina septigera (Lovén)	Hebridian Rise, Scotland	Fibre	Ds	K5007
Kraussinidae				
Kraussina rubra (Pallas)	Southern Tip, S. Africa	Fibre	Kr	801
Megerlia truncata (Gemlin)	Corsica, Mediterranean	Fibre	Mt	K5053
Cancellothyridae				
Terebratulina retusa (L.)	Firth of Lorn, Scotland	Powder	Tr	K4962
Terebratulidae				
Liothyrella neozelandica (Thomson)	Foveaux Strait, N. Zealand	Fibre	Ln	802
Gryphus vitreus (Born)	Corsica, Mediterranean	Powder	Gv	803
Rhynchonellida				
Notosaria nigricans (Sowerby)	Christchurch, New Zealand	Fibre	Nn	K5038

5.4 Results.

Immunological distances among genera of the Order Terebratulida are presented in **Table 5.2** and the UPGMA and Fitch-Margoliash dendrograms based on these are given in **Figures 5.1** and **5.2.**

The outgroup rhynchonellide *Notosaria nigricans* was the least reactive with all 8 terebratulide antisera and was well separated from the terebratulides in both dendrograms (**Figures 5.1** and **5.2**). All confamilial genera clustered together in both forms of analysis, e.g. *Megerlia* with *Kraussina*, *Gryphus* with *Liothyrella* and *Neothyris* with *Waltonia* . These results are consistent with established morphology-based brachiopod systematics and reinforce the contention that intra-skeletal macromolecules are an important source of phylogenetic information (Collins *et al.*, 1988).

Immunological distances distinguished three main clusters within the terebratulides (**Figures 5.1** and **5.2**). The novel aspect of these three main clusters is that they do not coincide with the long- and short-looped stocks which currently represent the primary subdivisions of the terebratulides. Instead two represent respectively the Cancellothyrididacea, which was raised to superfamily status by Cooper in 1973, and a subgroup of the Terebratellacea, the 'TLD' group of Collins *et al.*, 1988. The third is more heterogeneous including both a long-looped family, the Kraussinidae, and the short-looped family Terebratulacea. The third cluster is even more diverse than the cluster analyses suggest as it also contains an aberrant long-looped genus *Macandrevia* (Collins *et al.*, 1988). The fact that the three way clustering pattern is verified by two different methods of data analysis (**Figures 5.1** and **5.2**) and is based on fully reciprocal immunological distances confirms that this is an accurate reflection of the phylogenetic relationships among living terebratulides.

There is a good correlation between immunological distances of the major nodes of branching and the estimated divergence times (**Table 5.3**). Divergence times are based on the first appearance of members of genera or families and these are not always well-defined, being subject to significant change as continuing investigation of fossil brachiopods extends or reduces the known range of taxa, or redefines their higher level taxonomic assignment. Since the first appearance of major groups is used to calibrate immunological distances, it follows that the two are in good agreement. The greatest interest is in those data points that do not correlate; two such points are the unexpectedly small distances between the members of the TLD group and the

Table 5.2 Immunological distances among the articulate brachiopods. Distances represent means of reciprocal distances obtained from ELISA binding curves. See **Table 5.1** for species abbreviations.

	Wi	Ds	Nl	Tr	Gv	Li	Mt	Kr	Nn
Waltonia inconspicua	0	15	6	100	169	177	204	117	301
Dallina septigera		0	18	77	118	129	154	170	168
Neothyris lenticularis			0	88	156	149	130	132	239
Terebratulina retusa				0	134	70	146	206	253
Gryphus vitreus					0	14	45	15	193
Liothyrella neozelandica						0	34	54	272
Megerlia truncata							0	16	284
Kraussina rubra								0	306
Notosaria nigricans									0

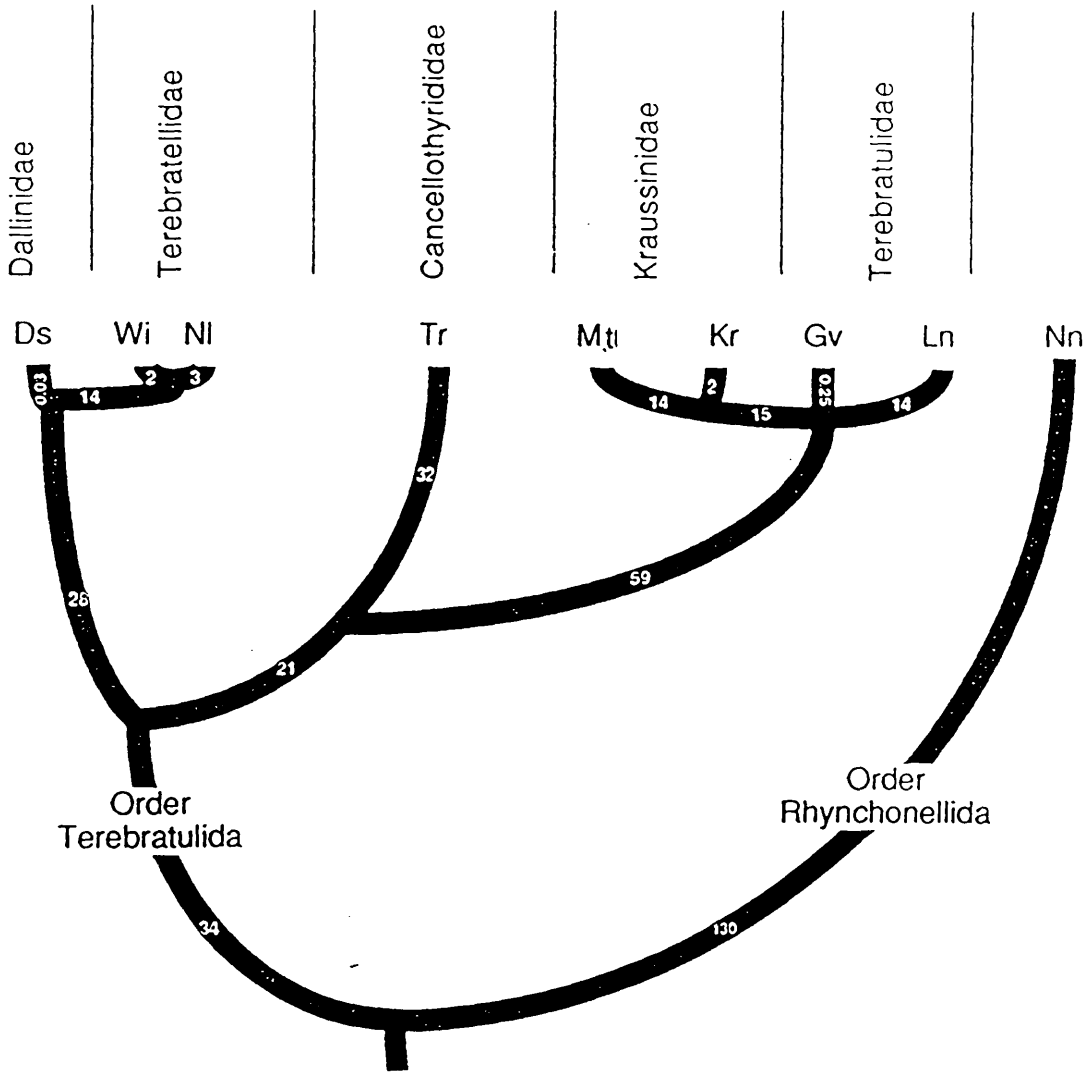


Figure 5.1 UPGMA dendrogram of the immunological distances of nine genera of articulate brachiopods.

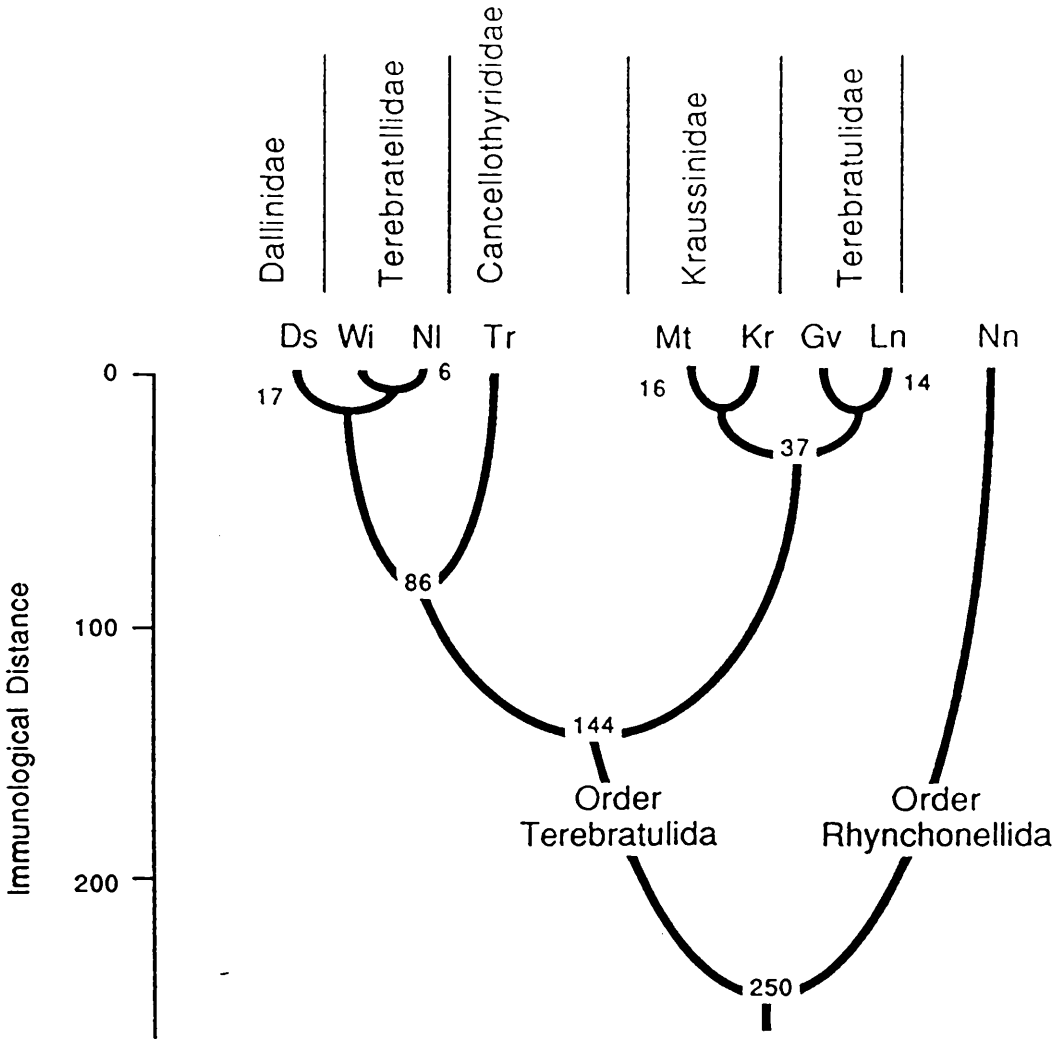


Figure 5.2 Fitch-Margoliash dendrogram of the data from **Table 5.2.**

equidistance of the three major lineages (**Figures 5.1** and **5.2**). The equidistance leads to contradictory interpretations of the evolutionary relationships between the lineages when different methods of clustering are used. The precise relationship between the three lineages cannot be derived from the existing data but, until additional information is available, the best interpretation is a trichotomous split dating from a period encompassing the end Triassic and early Jurassic (195 Myr).

5.4.1 Taxonomic implications.

Evolution of the Order Terebratulida.

The Order Terebratulida, first recorded from the Lower Devonian (410 Myr), is characterised by the possession of a distinctive internal skeleton, the 'loop'. Traditional interpretation of evolution within this order is of considerable 'experimentation' with the internal skeleton in the early Devonian. The period of diversification was relatively short (Stehli, 1965; Rudwick, 1970; Williams & Hurst, 1977) with two distinct lineages diverging in the late Devonian, giving rise to the present day Terebratulacea (short loop) and the Terebratellacea (long loop).

The serotaxonomic data indicate that this interpretation is in need of revision. Immunological distances suggest a period of diversification in the late Triassic early Jurassic (200 Myr), almost 200 Myr after the major split is traditionally interpreted to have occurred. The early Mesozoic diversification resulted in at least three discrete lineages which have survived until the present day (TLD terebratellaceans, cancellothyrides and 'terebratulaceans', the last including Kraussinidae). The youngest of these three lineages, the Cancellothyrididacea, first appeared in the mid-upper Jurassic.

From this study the revised interpretation is that all three major extant terebratulide lineages evolved from a single Mesozoic ancestor. The end Permian extinction event which coincided with the formation of Pangea dramatically reduced brachiopod stocks. A second extinction event at the end Triassic acted as a further filter (**Figure 5.3**) before diversification in the Jurassic, as Pangea began to break up. Following these two extinction events the terebratulides have become the most diverse and successful of surviving brachiopod orders.

The three major lineages have undergone different patterns of evolution. The most homogeneous have been the cancellothyrides, which have changed very little since the late Jurassic. Immunological distance measurements indicate that the modern day

Kraussinidae split from the main terebratulacean lineage at the end of the Cenozoic (ID 37; 67 Myr). Members of the family Kraussinidae have a worldwide distribution, but a geological record which stretches back only into the Miocene. Additional serotaxonomic and palæontological investigation is necessary to determine whether early Cenozoic ancestors of the Kraussinidae exist.

A major period of diversification for both the TLD and terebratulide lineages occurred in the mid-Cenozoic. From the serotaxonomic data it would appear that a number of events occurred at the same time in the early to middle Oligocene (ID 14-17; 25-31 Myr). The unusual geographical location of the TLD lineages, restricted to either the northern (Dallinidae and Laqueidae) or southern (Terebratellidae) hemispheres, suggest diversification from an equatorial region. At the end Eocene the formation of the psychrosphere, the cold layer of bottom water in the oceans produced by the polar submergence of dense refrigerated waters, caused high latitude bottom waters to be moved equatorially. The changes in ocean temperature and circulation which coincided with the initiation of the circumantarctic gyre and the associated drop in sea level in the mid Oligocene (32.5 Myr) could account for the separate evolution of northern and southern lineages.

Terbratulide taxonomy.

While immunological distances correspond well with major periods of change in the marine régime, enabling a plausible phylogenetic history of the terebratulides to be sketched, it is difficult to relate the immunological distances to morphological taxonomy.

The simplified cardinalia and distinctive loop of the cancellothyrides make this the most homogeneous of the extant brachiopod lineages. Heavy spiculation is characteristic of the Cancellothyrididae and the Terebratulidae (including Kraussinidae) but is probably plesiomorphic rather than synapomorphic, being lost or rare in most TLD terebratellaceans. This characteristic is not of use in fossil brachiopods, since spicules are preserved only in exceptional circumstances.

The TLD terebratellides are characterised by supporting hinge plates on the median septum, which distinguish these terebratellides from the aberrant genus *Macandrevia* , but this latter organisation is also seen in some Palæozoic terebratulides and in other articulate brachiopod orders.

The loop is cited as the major discriminatory feature in the terebratulides although ontogenetically there is a strong relationship between long- and short-looped forms. The earliest stages in the development of the descending branches of the calcareous loop up to their fusion with the median septum are the same for all three extant lineages. Elliott (1953; 1957) noted that the first formed calcareous support for all modern long-looped brachiopods (with the exception of *Argyrotheca*) always includes a dorsal median septum. Of the Palæozoic superfamilies of terebratulides believed to be ancestral to modern forms all contain genera which possess median septa, but there is no clear evidence of the involvement of the septum in the ontogeny of the long loop in either the Stringocephalacea or Zeilleriacea. If in these two superfamilies a long loop was derived simply by anterolateral growth of a short dielasmatacean-like loop, then the rôle of the median septum in all modern long-looped lineages is significant.

As long-looped forms have evolved from short-looped forms repeatedly in the history of this order, by a slight dorsal shift of the trocholophe and accelerated growth of the descending branches, a dielasmatacean ancestor of modern terebratulides is preferred. It is by no means clear that the distinction between the zeilleriaceans and dielasmataceans, based as it is primarily on loop length, is valid. The Dielasmatacea were undergoing considerable variation in the relationship between the median septum, the cardinalia and the loop in the mid-Triassic. The fixing of one novel character, a link between an acceleration in the time and rate of growth of the median septum coincident with that of the descending branches was probably all that was necessary to pave the way for the Mesozoic and Cenozoic diversifications (**Figure 5.3**).

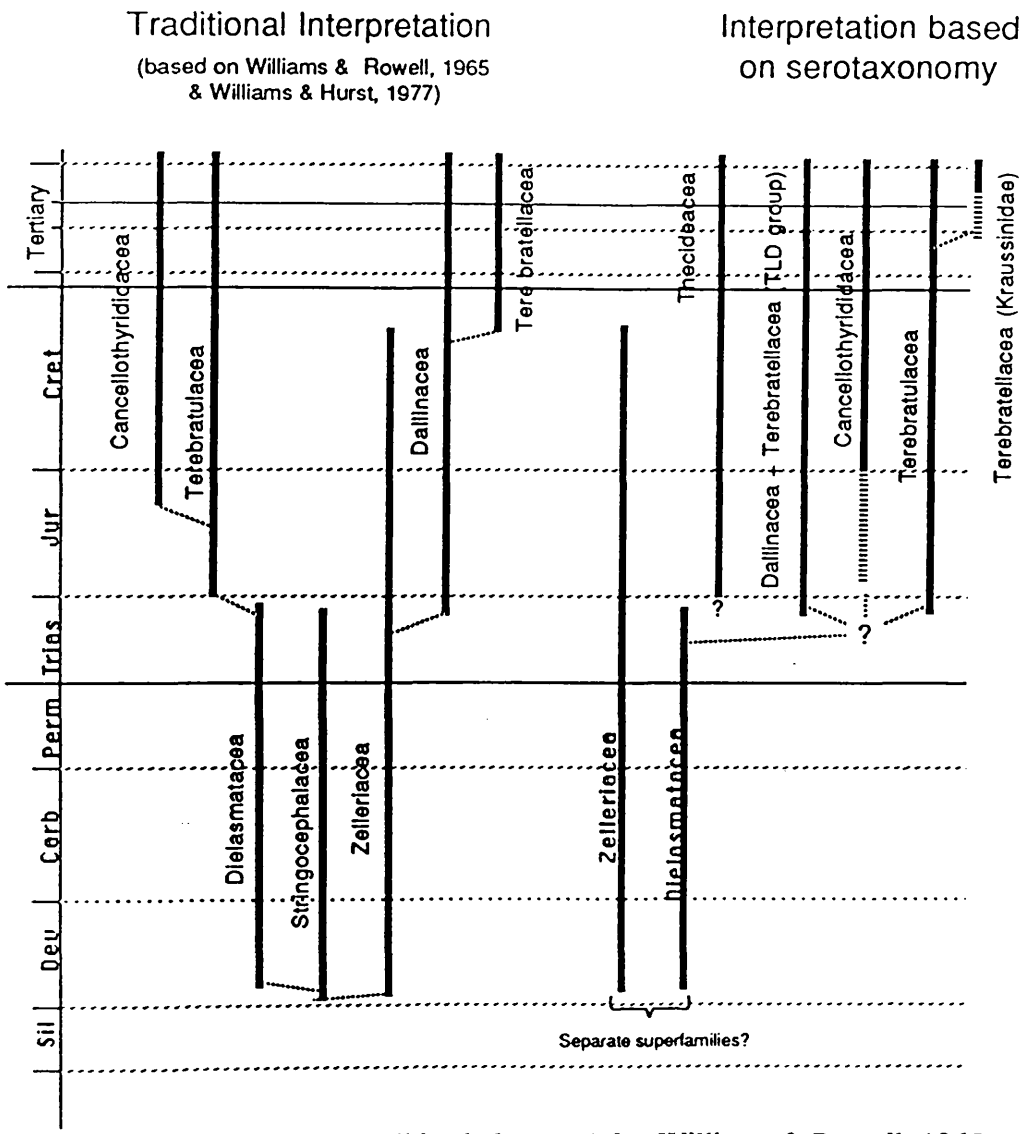


Figure 5.3. Terebratulide phylogeny (after Williams & Rowell, 1965 and Williams & Hurst, 1977) reinterpreted in the light of the sero-taxonomic data.

Table 5.3 Estimated divergence time of major branches of brachiopods

Divergence	mean ID	Time (Ma)	1st occurrence of ....
No divergence	0	0	
Wl from Nl	6	15	genus *Neothyris*
	6	?	genus *Waltonia*
Ds from Wl, Nl	17	35	subfamily Neothyridinae
	17	18	genus *Dallina*
Mt from Kr	16	0	genus *Kraussina*
Mt, Kr from Ll, Gv	37	15	family Kraussinidae
	37	35	genus *Gryphus*
Tr from Ds, Wl, Nl	100	150	the genus *Terebratulina*
	100	200	family Dallinidae
Mt, Kr,Ll, Gv from Tr	139	200	genus *Terebratulina*
Mt, Kr,Ll, Gv from Ds, Wl, Nl	163	200	subfamily Terebratulinae/family Dallinidae
	163	120	subfamily Dallininae
Nn from others	295	460	Order Rhynchonellida

5.5 Conclusions.

Immunological data indicated that the sub-division of the Order Terebratulida into three suborders, based on the characteristics of the internal skeleton, particularly the brachial loop, is not valid. Four separate groups were recognised within the Terebratulida but within these four subgroups traditional systematics were confirmed. The immunological data suggest that lineages split much later than was previously predicted and within each lineage there was a much greater degree of subsequent differentiation than was previously recognised. Distinction within the Order Terebratulida is not between long- and short-looped forms but between those long-looped forms in which a median septum is involved in the ontogeny of the loop and those in which it is not.

The primary distinction within the Order Terbratulida is, from the results of this work, the accelerated development of the median septum and descending branches; this character, or the ability to express it, is present in all extant terebratulides. Within each of the three lineages identified it is very difficult to identify high level anapomorphy. The articulate brachiopod shell is structurally simple and evolutionarily conservative, the term 'homeomorphy' having been coined for use in brachiopod studies.

Only Recent taxa have been used in the immunological distance experiments so immunological data alone cannot determine the taxonomic relationships of terebratulide brachiopods. The data only relate to the phylogenetic relationships of the families to which the genera used in the study are assigned and these assignments may be incomplete or wrong (e.g. *Macandrevia*, Collins *et al.*, 1988). Nevertheless the immunological distances provide a new perspective on terebratulide evolution and subsequent interpretations would benefit from integration of this information. From the results of this work it would appear necessary to erect a new high level taxonomy of the Order Terebratulida.

Chapter 6 IMMUNOLOGICAL RESPONSES FROM BRACHIOPOD SKELETAL MACROMOLECULES AND THEIR PHYLOGENETIC IMPLICATIONS

6.1 Introduction.

In the light of the conclusions of the previous chapters, it was considered necessary to further verify those results. This was done by including more taxa and using a more sensitive assay system, the fluorescence ELISA. Two Japanese genera were available, *Laqueus rubellus* and *Pictothyris picta*, both members of the Family Laqueidae, and these and their corresponding antisera were included in the immunological distance experiments. Additional taxa were included in this study to determine if the patterns found in the immunological distance experiments were consistent in other representatives of the groups investigated.

6.2 Materials and Methods.

A total of eleven brachiopod genera were available from worldwide locations (see **Table 6.1**) representing 10% of all living brachiopod genera. Most of these were terebratulide brachiopods which dominate present day brachiopod faunas, and the availability of antibodies against skeletal macromolecules of ten genera from this order allows a comprehensive investigation of the extent of molecular similarity within this major grouping. The rhynchonellide brachiopod *Notosaria nigricans* was included to represent a separate brachiopod order without a brachial loop. The bivalve mollusc *Mercenaria mercenaria* was used as an outgroup.

Small fragments of a wide range of additional taxa were obtained from private and museum collections. Immunological distances could not be calculated for these taxa as they were in such short supply that it was not possible to prepare antisera against them. Nevertheless it was possible to assess the similarity between molecular extracts of these taxa and the existing panel of brachiopod antibodies and to carry out a simple clustering exercise to investigate their relationships.

The relationships between the various antigens and antisera were quantified using the fluorescence enzyme-linked immunosorbent assay. The testing was done in duplicate and the readings and mean of results minus the blank readings are given in **Appendix I**. Blank wells were treated as normal but antigen was replaced with 20%EDTA and antiserum was replaced with diluent. Graphs of the reaction between each antigen and

GENUS	LOCALITY
Dallina septigera (Loven)	Scotland
Gryphus vitreus (Born)	Mediterranean
Kraussina rubra (Pallas)	S. Africa
Laqueus rubellus (Sowerby)	Japan
Liothyrella neozelandica (Thomson)	New Zealand
Megerlia truncata (Gmelin)	Mediterranean
Neothyris lenticularis (Deshayes)	New Zealand
Notosaria nigricans (Sowerby)	New Zealand
Pictothyris picta (Dillwyn)	Japan
Terebratulina retusa (Linnaeus)	Scotland
Waltonia inconspicua (Sowerby)	New Zealand

Table 6.1 Localities of brachiopod genera used in this study.

five serial dilutions of each antiserum were drawn (**Appendix II**). Measurements obtained from the linear regions of semi-logarithmic binding curves are given in **Appendix III**. Immunological distances were determined using the formula

$ID=100x\log_{10}(100/\Delta)$ where Δ is the mean reciprocal % cross-reactivity (taking the reaction of antiserum with homologous antigen as 100%) and are shown in **Table 6.2**. The data were processed by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) (Sneath & Sokal, 1973) (**Figure 6.1**).

6.3 Results.

6.3.1 Comparison of immunological results with existing terebratulide classification and the fossil record.

At the higher taxonomic levels the pattern of antibody reactivity is in agreement with the current classification. The bivalve *Mercenaria* consistently shows the least immunologically-detectable molecular similarity with, and hence the greatest immunological distance to, the brachiopod genera (**Figure 6.1**). It seems probable that the skeletal organic components, which are common to such distantly related groups, have some fundamental rôle in biomineralisation in calcareous-shelled organisms. The major Precambrian radiation of metazoan phyla cannot be dated by direct palæontological evidence, but this event, which must have provided the last common ancestors of brachiopods and bivalves, has been dated between 700 and 900 Myr ago.

Within brachiopod stocks the rhynchonellide *Notosaria*, lacking a loop, is strongly resolved from all the remaining loop-bearing terebratulide taxa (**Figures 6.1** and **6.2**). Rhynchonellides are a very distinctive and conservative group of brachiopods, the morphology of which has changed very little throughout a geological history extending back approximately 500 Myr to the early Ordovician (Williams & Rowell *in* Williams *et al.*, 1965). The rhynchonellides are thought to be the oldest and least specialised of a third major phase of anatomical development which characterised the Palæozoic history of brachiopods (Williams & Rowell *in* Williams *et al.*, 1965). The terebratulides and spiriferides are the other major components of this third evolutionary phase and various lines of evidence suggest that these three orders were originally closely related, with the rhynchonellides possibly giving rise to the spiriferides in the mid Ordovician (about 460 Myr ago) and the spiriferides in turn acting as the parent group of the terebratulides in the late Silurian (about 410 Myr ago) (Williams & Rowell *in* Williams *et al.*, 1965).

	Gr	Lio	Meg	Kr	Dal	Walt	Neo	Pict	Laq	Tere	Not	Merc
Gryphus	x											
Liothyrella	8	x										
Megerlia	24	71	x									
Kraussina	40	114	71	x								
Dallina	187	178	131	178	x							
Waltonia	188	239	189	188	33	x						
Neothyris	179	116	176	165	48	39	x					
Pictothyris	74	149	128	119	69	64	61	x				
Laqueus	25	180	225	158	58	60	90	65	x			
Terebratulina	186	147	194	243	134	173	165	155	183	x		
Notosaria	188	324	342	386	280	392	109	282	322	287	x	
Mercenaria	249	327	352	362	447	470	151	362	313	408	324	x

Table 6.2 Immunological distances among brachiopods and the bivalve *Mercenaria*.

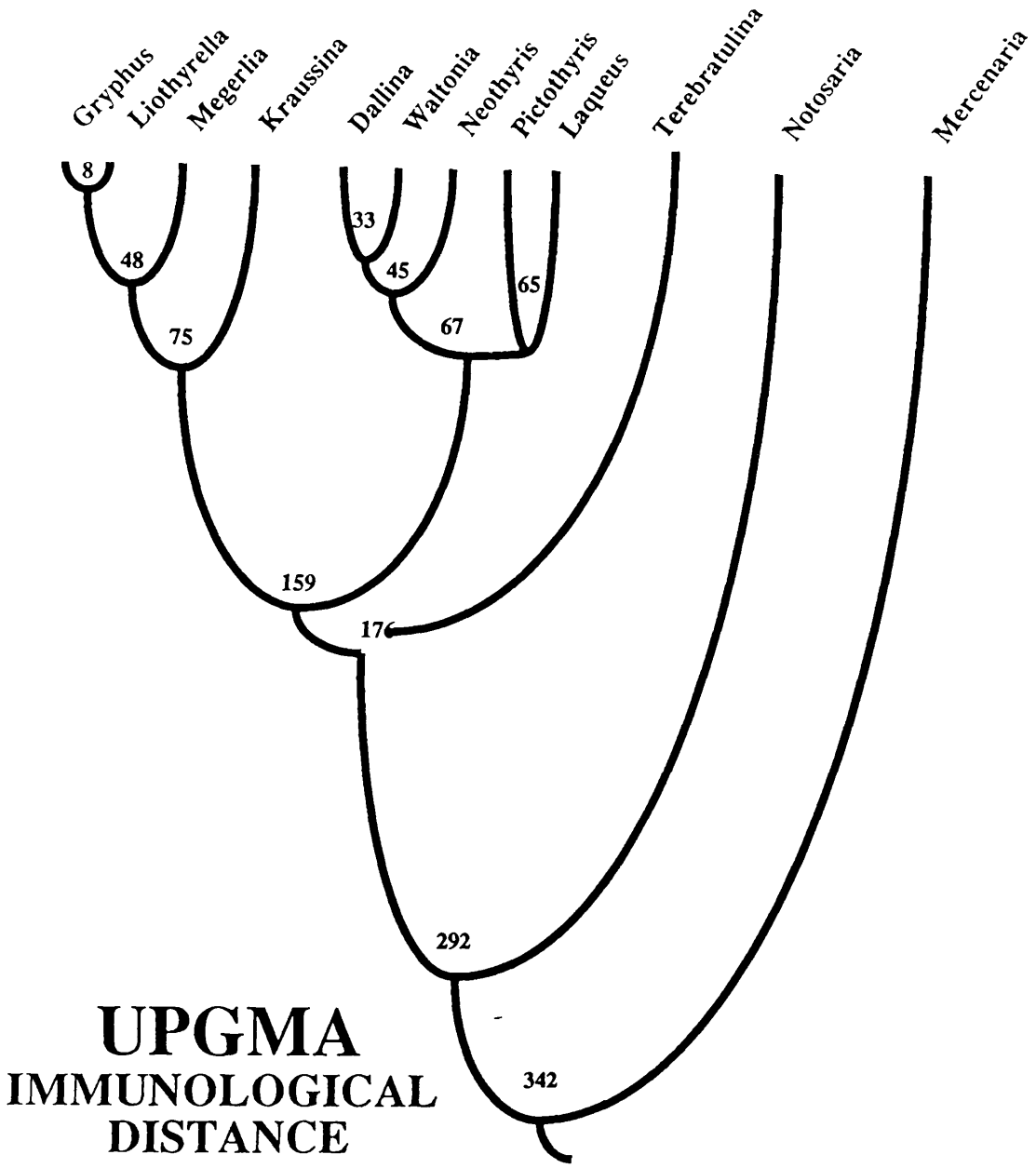


Figure 6.1 UPGMA dendrogram based on immunological distance data in **Table 6.2**.for the brachiopod genera listed in **Table 6.1**.

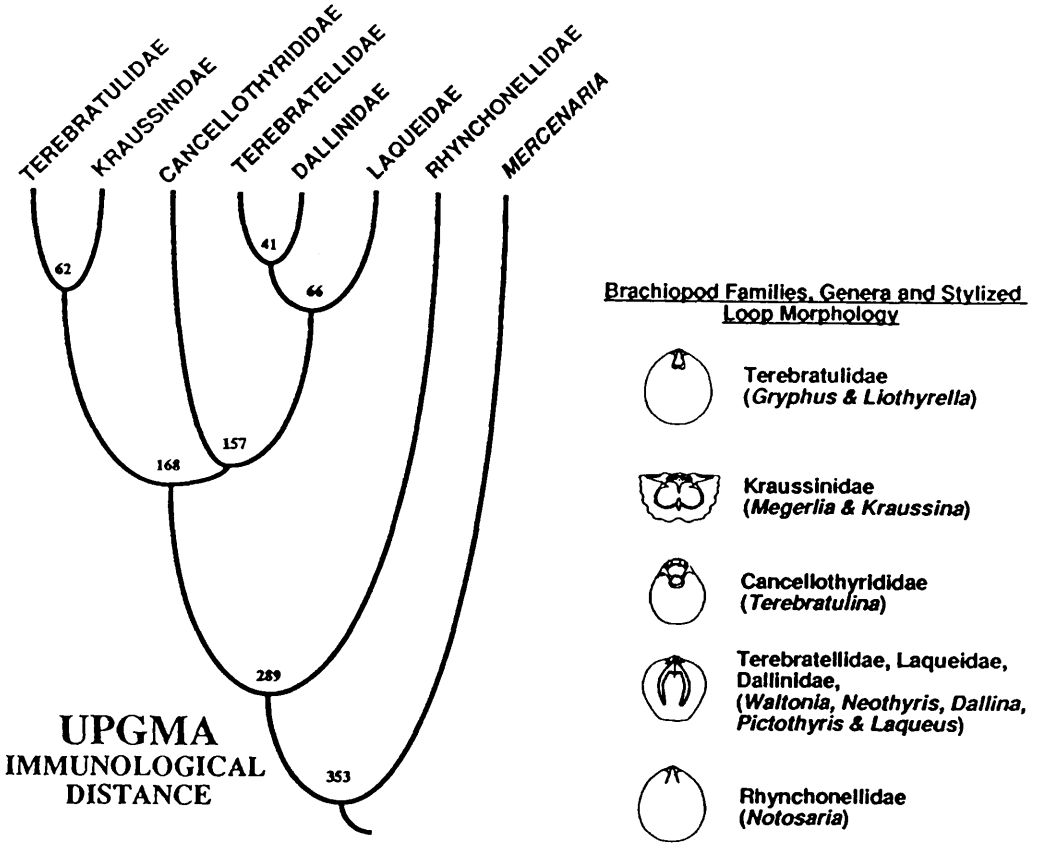


Figure 6.2 UPGMA dendrogram of immunological distance data from Table 6.2 grouped by family. Family assignment illustrated in key, along with stylised representation of loop morphology within each grouping.

The immunological data indicates a three-fold subdivision of the terebratulides irrespective of whether the data are plotted for genera (**Figure 6.1**) or for families by using mean data for all available constituent genera in each family (**Figure 6.2**). In contrast the last major summary of brachiopod classification (in the Treatise on Invertebrate Palæontology) adopted a two-fold division of the order based on whether the length of the loop was long (Superfamily Terebratellacea) or short (Superfamily Terebratulacea) in relation to the length of the shell (Williams *et al.*, 1965). As illustrated schematically in **Figure 6.2**, the molecular data yield clusters in which the simple subdivision into short- and long-looped taxa has broken down to the extent that the short-looped Terebratulidae cluster with the long-looped Kraussinidae, and the short-looped Cancellothyridae form a third major grouping virtually equidistant from the Terebratulidae-Kraussinidae cluster and the long-looped Terebratellidae, Laqueidae and Dallinidae (**Figure 6.2**). The oldest geological representatives of the three groups are of very similar age (Upper Triassic-Lower Jurassic-approximately 210 Myr ago) suggesting that there may be a rough correlation between immunological distance and the timing of divergence of common ancestors as has been reported in other sero-taxonomic studies (Sarich & Wilson, 1967).

Some elements of the immunological clustering can readily be reconciled with developments in morphology-based taxonomy since the publication of the Treatise. Cooper (1973a) suggested that the morphology of the short-looped Family Cancellothyrididae was sufficiently distinct to warrant its elevation to Superfamily status. The immunological findings are consistent with this, in that the cancellothyride representative plots well away from the other short-looped brachiopod stocks with which it was previously classified (**Figure 6.2**). The combination of independent molecular data, along with distinctive morphological characters and a separate geological history stretching back to the late Jurassic, provides multidisciplinary justification for the establishment of the Superfamily Cancellothyridacea (Cooper, 1973a). A new superfamily has been proposed for the Dallinidae (Cooper, 1981), but this group plots closely with other long-looped taxa in the immunological clustering, hence the molecular data provide no support for the proposed Superfamily Dallinacea and in fact constitute evidence against such a grouping, at least among living stocks. The dallinide pattern of loop development is distinctive and is known from some of the earliest terebratelloids in the fossil record, but such a growth pattern may have appeared several times and its appearance in living brachiopods could also be due to convergence.

By far the most complicated immunological result to explain is the close clustering of the long-looped Kraussinidae with the short-looped Terebratulidae. The Kraussinidae and

related Megathyrididae are thought to have arisen by neoteny from terebratelloid ancestors, but such living representatives as *Megerlia* and *Kraussina* are immunologically much more similar to short-looped Terebratulidae (*Gryphus* and *Liothyrella*) than to present day terebratelloids such as *Dallina*, *Waltonia*, *Neothyris*, *Pictothyris* and *Laqueus* (**Figure 6.1**). The molecular data presented here suggest that the relationships between these groups warrant further detailed investigation. The two groups of long-looped brachiopods distinguished by the immunological data are known to have different patterns of loop development and this may reflect a much more fundamental separation than has previously been recognised. In particular the data may indicate that the kraussinides and related stocks were derived from short-looped brachiopods rather than the long-looped terebratelloids as has generally been accepted. Elliott (1950) found both long- and short-looped adult forms within a single terebratelloid species from one Jurassic locality, and such discoveries may be indicative of an underlying plasticity in loop morphology in some stocks.

6.3.2 Results with additional brachiopod genera.

The results of a single-linkage clustering of data obtained from small amounts of additional taxa (equivalent to the nearest-neighbour method of Sneath & Sokal, 1973) reinforce the major conclusions of the immunological distance experiments. See **Figure 6.3**. Two additional cancellothyride genera were available, (*Cancellothyris* and *Chlidonophora*). They plotted, along with *Terebratulina*, in a tight cluster well separated from other short- and long-looped brachiopods. Additional dallinid, laqueid and terebratellid genera clustered together as a coherent group, while the mixed long- and short-looped terebratulide-kraussinide-megathyride grouping remained intact with the incorporation of a larger number of genera. The only inconsistencies were the positioning of *Macandrevia* and *Ecnomiosa* well away from the terebratelloid genera with which they are currently classified. Compared to other terebratelloids, *Macandrevia* does have some unusual morphological features (Cooper, 1973b; Richardson, 1976) and the loop and cardinalia of *Ecnomiosa* have been described as "unique" and "so unusual as to set the genus apart from all others known" (Cooper, 1977). In the light of the immunological data, these distinctive features may indicate that the terebratelloid-like loops of *Macandrevia* and *Ecnomiosa* reflect evolutionary convergence rather than a common ancestry.

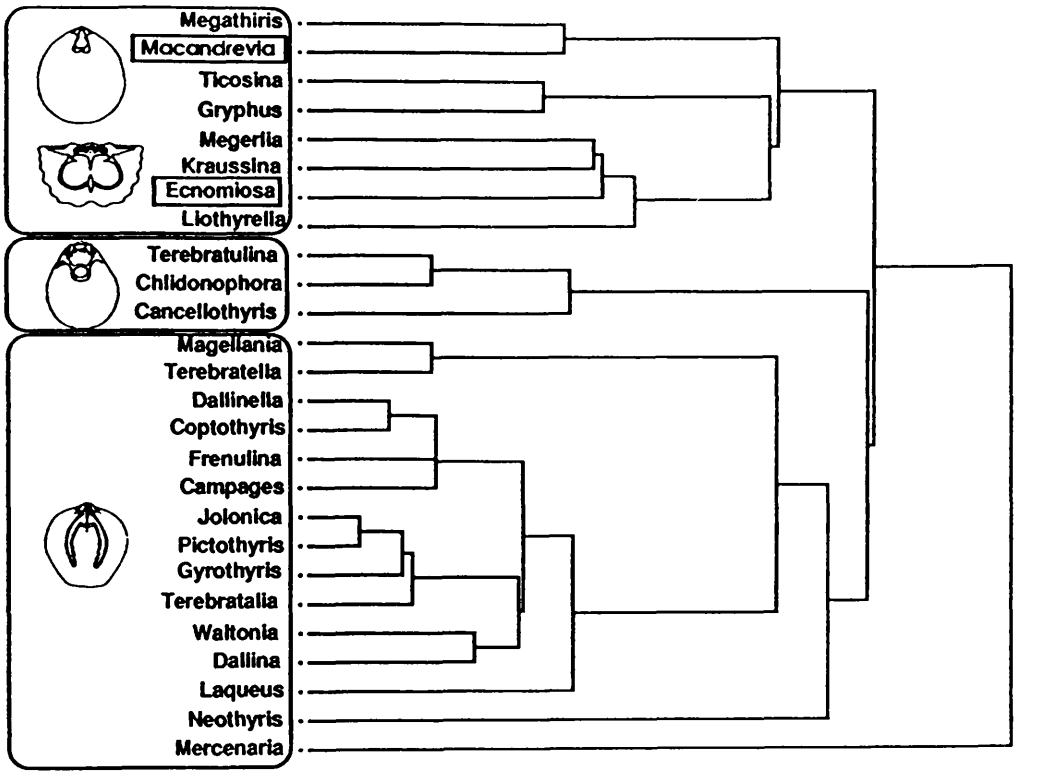


Figure 6.3 Single-linkage cluster analysis of 25 terebratuloid brachiopods with the three major groups outlined and represented by stylised loop sketches. The anomalous positions of *Macandrevia* and *Ecnomiosa* are indicated by boxes.

6.4 Conclusions.

Calculations of immunological distances between representatives of the Order Terebratulida have revealed a three-fold division which correlates with a proposed subdivision of the order into three superfamilies, but refutes attempts to establish a fourth superfamily.

The immunological results also indicate a fundamental subdivision of the long-looped brachiopods, with one group possibly being derived from short-looped terebratuloids rather than long-looped terebratelloids.

The results of this investigation indicate that skeletal macromolecules from brachiopods vary taxonomically to an extent which can be detected by immunological techniques. Although the molecular basis for such discrimination is unknown, the sero-taxonomy data have provided an invaluable molecular perspective in a group in which evolutionary inter-relationships have primarily been determined from morphological characteristics of the shell. There is a good correlation between the morphological and molecular approaches to brachiopod taxonomy and a combined morphological-molecular synthesis, using information from the fossil record on the stratigraphic and geographic distribution of the ancestors of living brachiopods, offers a good opportunity of accurately tracing evolutionary histories within the phylum.

Chapter 7 REACTIONS OF MONOCLONAL ANTIBODIES AND MOUSE SERA

7.1 Introduction.

The specificity of reaction afforded by monoclonal antibody as opposed to polyclonal antibody may be of use in phylogenetic studies. Monoclonal antibodies were used by Muyzer *et al.*(1984) to study bivalve families and diagenetic degradation of biopolymers. The use of rabbit polyclonal antisera has proved useful in taxonomy studies reported in the present work. For these reasons it was decided to prepare monoclonal antibodies and mouse antisera against brachiopod extracts. Unfortunately at this stage brachiopod material was in very short supply so the choice of antigens was limited.

7.2 Monoclonal antibody production.

7.2.1 Materials and Methods.

Nine fusions were carried out using the spleens of mice immunised against brachiopod extracts and 5 putative monoclonal antibodies were produced. The antigens used in this study are listed in **Table 7.1**.

The reactions of these antibodies with a panel of antigens which included all available Recent and fossil material are presented in **Table 7.2**.

7.2.2 Results.

The readings obtained using monoclonal antibody supernatants were low, this may have been because in some cases the hybridomas were not fully monoclonal and were producing a number of antibodies which were masking the reaction. Time did not allow culturing of the antibody-producing cells to the monoclonal stage in some of the experiments. The low readings may also be due to the age of the conjugate used in this study which may have begun to deteriorate. Time did not allow these experiments to be repeated with new conjugate.

The results are given as positive if the reading was above the level of reaction with the homologous antigen to which the monoclonal antibody was prepared.

ANTIGEN	SUPERNATANT	ANTISERUM
T.retusa	**1** H	**1**
T. transversa	n.d.	**2**
T. retusa body tissues	**3** M	**3**
T. retusa heat treated	**4a** M	**4**
T. retusa heat treated	**4b** M	n.d.
Notosaria nigricans	**8** H	**5**
Waltonia inconspicua	**9** H	**6**
Dallina septigera	n.d.	**7**

where H denotes Hybridoma supernatant, M denotes Monoclonal antibody supernatant, and n.d. signifies not done.

Table 7.1 Antigens against which monoclonal antibodies and mouse antisera were prepared.

Table 7.2 FELISA reactions of supernatants with Recent and fossil material.

	SUPERNATANT					
GENUS	**1H**	**3M**	**4aM**	**4bM**	**8H**	**9H**
Argyrotheca	-	-	-	-	-	-
Codakia orbicularia	-	-	-	-	+	-
Coptothyris grayii	-	-	-	-	-	-
Dallina septigera	-	-	-	-	-	-
Gryphus vitreus	-	-	-	-	+	-
Kraussina rubra	-	-	-	-	-	-
Lacazella	-	-	-	-	-	-
Laqueus rubellus	-	-	-	-	-	-
Lingula	-	-	-	-	-	+
Liothyrella neozelandica	-	-	-	-	+	-
Liothyrella uva notocadensis	-	+	+	-	-	-
Macandrevia cranium	-	-	-	-	-	-
Magasella flavescens	-	-	-	-	-	-
Megerlia truncata	-	-	-	-	-	-
Mercenaria mercenaria	-	-	-	-	+	-
Neocrania anomala	-	-	-	-	-	+
Neothyris lenticularis	-	-	-	-	-	-
Notosaria nigricans	-	-	-	-	-	-
Pictothyris picta	-	-	-	-	-	+
Terebratalia transversa	-	-	-	-	+	-
Terebratella dorsata	+	-	+	-	+	-
Terebratella sanguinea	+	-	+	-	+	+
Terebratulina retusa	+	+	+	+	-	-
Thecidellina	+	-	+	-	-	+
Waltonia inconspicua	+	-	-	-	-	+
Fossil barnacle	-	-	-	-	-	-
Fossil cockle	-	-	-	-	-	-
Carboniferous coral	-	-	-	-	-	-
Oligocene coral	-	-	-	-	-	-
Fossil gastropod *Turitella*	-	-	-	+	+	+

The results of the hybridoma and monoclonal antibody supernatant testing were as follows:-

1. Hybridoma supernatant **1H** produced against the soluble fraction of the cancellothyride *Terebratulina retusa* identified the TLD group of the terebratellaceans and *Thecidellina.*

2. Monoclonal antibody supernatant **3M** produced against the cancellothyride *T. retusa* body tissues identified only the terebratulid *Liothyrella uva notocadensis.*

3. Of two monoclonal antibodies prepared against heat-treated *T. retusa* secondary fibres, **4a**M and **4b**M, one identified *Liothyrella uva notocadensis*, the TLD terebratellaceans and *Thecidellina* and the other picked out only the fossil gastropod *Turitella.*

4. The hybridoma supernatant **8H** prepared against *Notosaria nigricans* identified the TLD group of terebratellaceans, terebratulaceans, the bivalve *Mercenaria* and fossil *Turitella.*

5. Hybridoma supernatant **9H** prepared against *Waltonia inconspicua* recognised the TLD group of terebratellaceans, *Lingula, Neocrania anomala, Thecidellina* and fossil *Turitella.*

7.2.3 Conclusions.

The short-looped cancellothyride antibodies recognised the long-looped terebratellaceans and the short-looped terebratulacean *Liothyrella uva notocadensis, Thecidellina* and fossil *Turitella.* The rhynchonellide *Notosaria nigricans* hybridoma supernatant recognised the TLD group of long-looped terebratellaceans and the short-looped terebratulaceans as well as fossil *Turitella*, but since this was a hybridoma supernatant these results are not conclusive.

The terebratellacean *Waltonia* hybridoma supernatant identified the TLD group of long-looped terebratellaceans, the inarticulate antigens (*Lingula* and *Neocrania*), *Thecidellina* and fossil *Turitella.*

From these results it can be seen that the hybridoma supernatants are not as specific as the monoclonal antibody supernatants in their identification of different genera. The potential of a range of monoclonal antibodies, while not realised here, is suggested by this preliminary study.

7.3 Mouse serology.

7.3.1 Materials and Methods.

Seven antisera were prepared against brachiopod extracts. The antigens against which these antisera were prepared are listed in **Table 7.1.**

The reactions of these antisera with a panel of antigens which included all available Recent and fossil material are presented in **Table 7.3.**

7.3.2 Results.

Since the readings were low the results are expressed as positive if the the reading was above the level of reaction obtained with the homologous antigen.

The results of the antisera testing were as follows:-

1. Antiserum **1** prepared against *Terebratulina retusa* soluble fraction identified the TLD group of terebratellaceans and *Lacazella.*

2. Antiserum **2** prepared against *Terebratalia transversa* secondary fibres identified the TLD group of terebratellaceans and *Thecidellina.*

3. Antiserum **3** prepared against *Terebratulina retusa* body tissues identified the TLD group of terebratellaceans, *Lacazella,* terebratulaceans, inarticulates, *Mercenaria mercenaria,* some kraussinides and *Notosaria nigricans.*

4. Antiserum **4** prepared against *Terebratulina retusa* heat-treated secondary fibres identified *C. g* , *Mercenaria mercenaria* and *Neocrania anomala.*

5. Antiserum **5** prepared against *Notosaria nigricans* did not react at a significant level with any of the antigens tested.

6. Antiserum **6** prepared against *Waltonia inconspicua* identified *Argyrotheca, Thecidellina* and *Notosaria nigricans.*

7. Antiserum **7** prepared against *Dallina septigera* identified *Argyrotheca, Terebratulina retusa, Lacazella, Liothyrella neozelandica, Pictothyris picta* and fossil coral.

Table 7.3 FELISA reactions of mouse antisera with Recent and fossil material.

	ANTISERUM						
GENUS	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Argyrotheca	-	-	-	-	-	+	+
Codakia orbicularia	-	-	-	-	-	-	-
Coptothyris grayii	+	-	-	+	-	-	-
Dallina septigera	+	-	+	-	-	-	+
Gryphus vitreus	+	-	+	-	-	-	-
Kraussina rubra	-	-	+	-	-	-	-
Lacazella	+	-	+	-	-	-	+
Laqueus rubellus	+	-	+	-	-	-	-
Lingula	-	-	+	-	-	-	-
Liothyrella neozelandica	-	-	+	-	-	-	+
Liothyrella uva notocadensis	-	-	-	-	-	-	-
Macandrevia cranium	-	-	-	-	-	-	-
Magasella flavescens	+	-	-	-	-	-	-
Megerlia truncata	+	-	+	-	-	-	-
Mercenaria mercenaria	-	-	+	+	-	-	-
Neocrania anomala	+	-	+	+	-	-	-
Neothyris lenticularis	-	-	+	-	-	-	-
Notosaria nigricans	+	-	+	-	+	+	-
Pictothyris picta	-	+	+	-	-	-	+
Terebratalia transversa	-	+	+	-	-	-	-
Terebratella dorsata	-	-	-	-	-	-	-
Terebratella sanguinea	-	-	-	-	-	-	-
Terebratulina retusa	+	-	+	-	-	-	+
Thecidellina	-	-	-	-	-	+	-
Waltonia inconspicua	+	-	+	-	-	+	-
Fossil barnacle	-	-	-	-	-	-	-
Fossil cockle	-	-	-	-	-	-	+
Carboniferous coral	-	-	-	-	-	-	-
Oligocene coral	-	-	-	-	-	-	-
Fossil gastropod *Turitella*	-	-	-	-	-	-	-

7.3.3 Conclusions.

One of the three anti-terebratellacean sera reacted strongly with long-looped terebratellaceans and with *Thecidellina*. Another of the anti-terebratellacean sera reacted with *Argyrotheca* and the rhynchonellide *Notosaria nigricans*. The third of the anti-terebratellacean sera identified *Argyrotheca* and a mixture of other genera at lower levels.

The antisera to long-looped terebratellaceans reacted most strongly with other long-looped genera and also picked out *Argyrotheca* and *Thecidellina*, suggesting that *Argyrotheca* is more closely related to the terebratellacean stock than to the kraussinides as was previously thought to be the case and also suggesting that *Thecidellina*, given a terebratulacean origin in the Treatise, may be more closely related to terebratellacean stock.

Two of the three anti-short-looped cancellothyride sera reacted with the TLD group of long-looped terebratellaceans and *Lacazella*, whereas the third reacted with an inconclusive mixture of genera. This would place *Lacazella* , given a terebratulacean origin in the Treatise, with the short-looped cancellothyrides.

Anti-rhynchonellide serum reacted with none of the antigens tested.

Antiserum **4**, prepared against heat-treated secondary fibres of *T. retusa*, reacted with a wide range of genera and this may have been because the heat-treatment denatured the antigen to some extent and made more non-specific antigenic sites available for antibody recognition.

These results, while they give some clue to the affinities of some genera and of difficult-to-assign groups such as the Thecidellinides, are preliminary and many more antisera would have to be prepared and tested before the results could be considerd to be conclusive.

Chapter 8 ANTIBODIES TO COMPOUNDS OF GEOLOGICAL INTEREST.

8.1 Introduction and background to present study.

The aim of the work described in this chapter was to determine if it was possible to prepare antibodies to compounds of geological interest, such as samples of Kimmeridge Clay and carbazole, which may be important in the detection of commercially viable gas or oil deposits.

Ho *et al.*(1974) studied a suite of 78 crude oils and on the basis of the relative amounts of the different sulphur compounds the oils were grouped into three categories:- immature, mature and altered. Immature oils were characterised by high relative amounts of thermally unstable non-thiophenic sulphur compounds, whereas the mature oils were marked by a high relative abundance of the more stable benzo- and dibenzothiophenes. It was found that the relative change in benzothiophene to dibenzothiophenes was useful as a maturity indicator for crude oils, i.e. the benzothiophene to dibenzothiophene ratio decreased with increasing depth of burial.

Radke and Welte (1971) developed a methylphenanthrene index, derived from the distribution of phenanthrene and methylphenanthrene isomers, which showed a good correlation with the mean vitrinite reflectance values within the oil window. The methylphenanthrene index could be used to analyse and interpret the maturity of the soluble organic matter from sedimentary rocks. In 1987 Schou and Myhr analysed crude oils and Upper Jurassic sediments to evaluate the possibility of using the distribution of sulphur compounds as maturity parameters and concluded that the variations in the relative distribution of dibenzothiophene and methyldibenzothiophene were useful as maturity indicators.

Part of the work reported here is concerned with the possibility of using a nitrogen-bearing compound as a maturity parameter. Carbazole is a nitrogen-bearing compound of 167 MW found in crude oils. The ability to detect such a compound may be useful as a molecular maturity parameter, bearing in mind that degraded oils contain a higher percentage of nitrogen.

Work by Li *et al.* (1989) has shown a favourable comparison between the enzyme-linked immunosorbent assay (ELISA) and gas chromatographic procedures in the determination of molinate residues. It may be that the ELISA will prove a useful alternative to gas chromatography, in terms of speed of detection, cost and convenience, in the testing of

samples taken at exploration sites since it may be possible to develop a field assay kit for on-site testing of samples.

Substances with a molecular weight of less than 1000 are not usually antigenic, but antibodies can be prepared against small molecules by immunisation with conjugates composed of low molecular weight substances (haptens) covalently linked to proteins. This procedure was carried out for carbazole using a modification of the method of Weir (1986) based on work on the diazotisation of proteins by Landsteiner (1927).

8.2 Materials and Methods.

Two rabbit antisera were prepared, one against the polar fraction of Kimmeridge Clay and the other against carbazole. Since carbazole is a very small molecular weight compound it was necessary to couple it to a protein carrier, in this case keyhole limpet hæmocyanin (KLH), before using it to immunise a rabbit. This was done as described in **3.20.**

The reactions of three test bleeds of the antiserum against Kimmeridge Clay and the antiserum against carbazole were determined by FELISA.

8.3 Results.

8.3.1 Antiserum to Kimmeridge Clay.

Figures 8.1-8.8 show the reactions between three test bleeds of the antiserum prepared against the polar fraction of Kimmeridge Clay sample A1 and Kimmeridge Clay samples A1, A2 and A3, Oxford Clay samples B1, B2 and B3 and Lower Lias Clay samples C1 and C3 tested by FELISA. The value of the reaction of pre-immune serum with these samples was subtracted and the mean of two experiments plotted.

From **Figures 8.1-8.8** it can be seen that the level of circulating antibody increases from the first test bleed to the third test bleed for most of the clay samples tested, indicating that the immune response was enhanced with repeated immunisations.

The antiserum reacted to some extent with all the clay samples tested. The fact that the antiserum prepared against Kimmeridge Clay reacted with Oxford and Lower Lias Clays is not unexpected since the Kimmeridge and Oxford Clay are adjacent in the Upper Jurassic of the stratigraphic column. The conditions of deposition during the

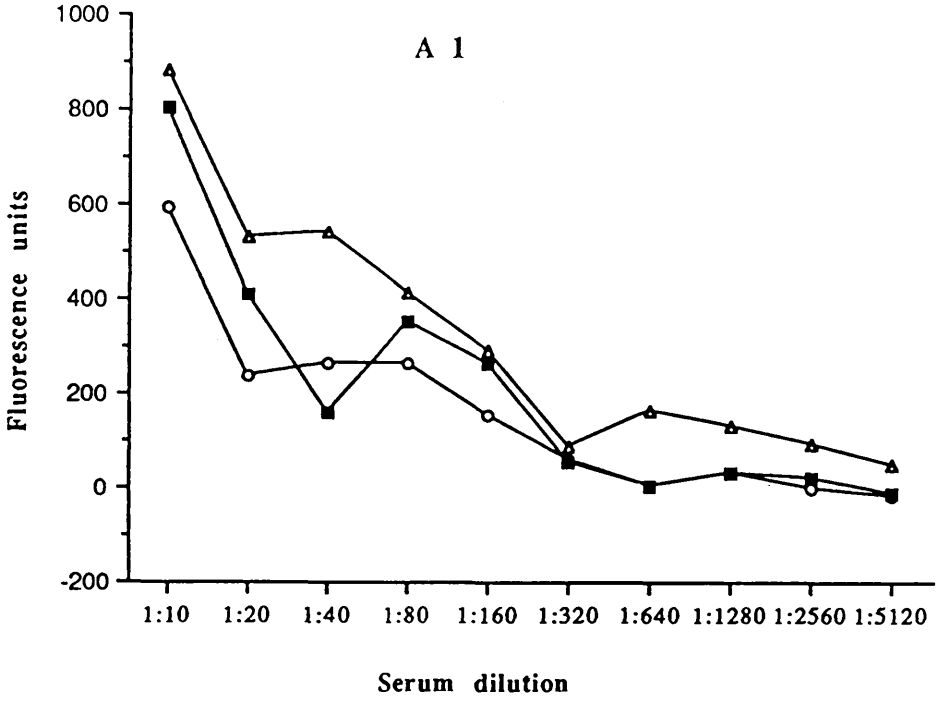


Figure 8.1 Reaction of anti-Kimmeridge clay serum with Kimmeridge clay fraction A1, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

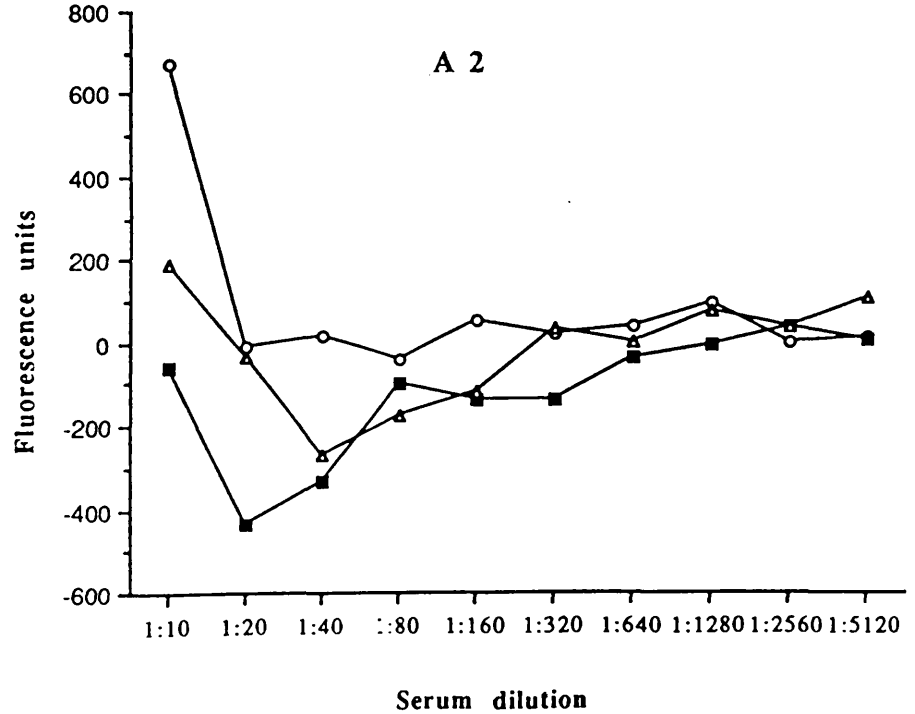


Figure 8.2 Reaction of anti-Kimmeridge clay serum with Kimmeridge clay fraction A2, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

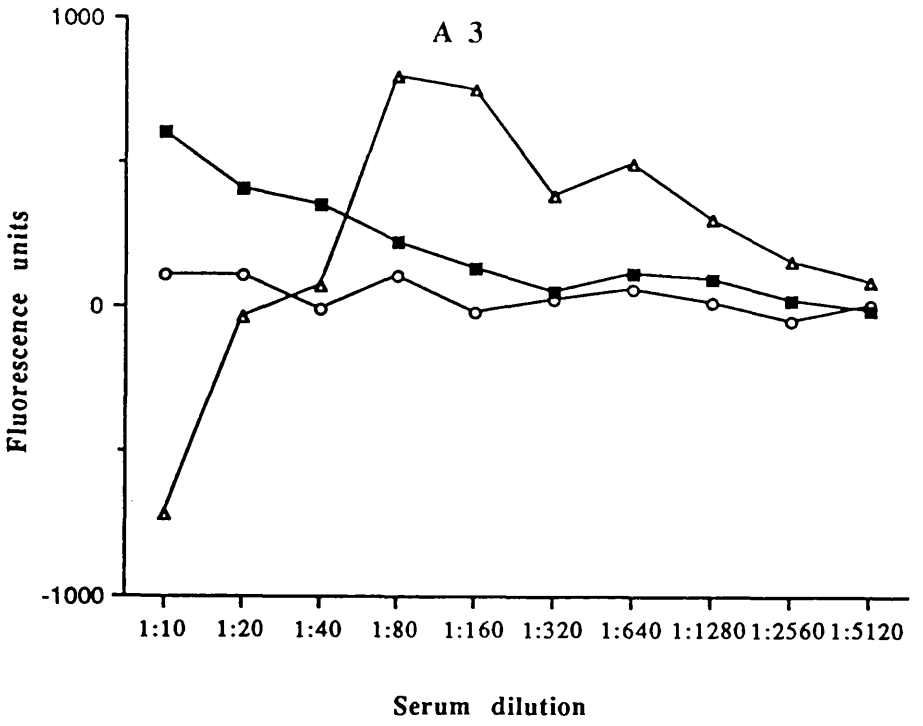


Figure 8.3 Reaction of anti-Kimmeridge clay serum with Kimmeridge clay fraction A3, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

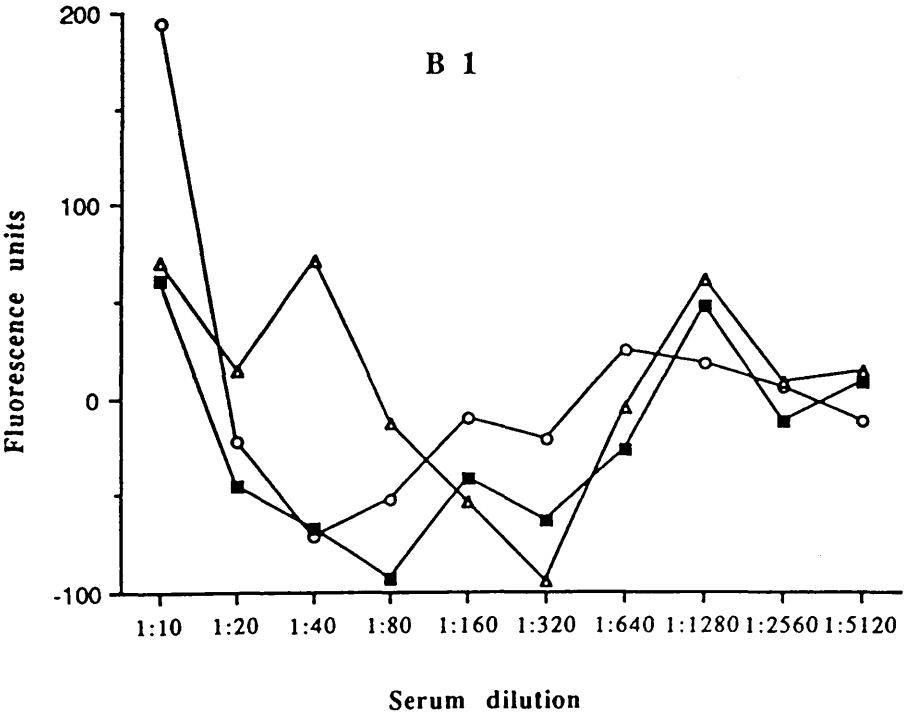


Figure 8.4 Reaction of anti-Kimmeridge clay serum with Oxford clay fraction B1, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

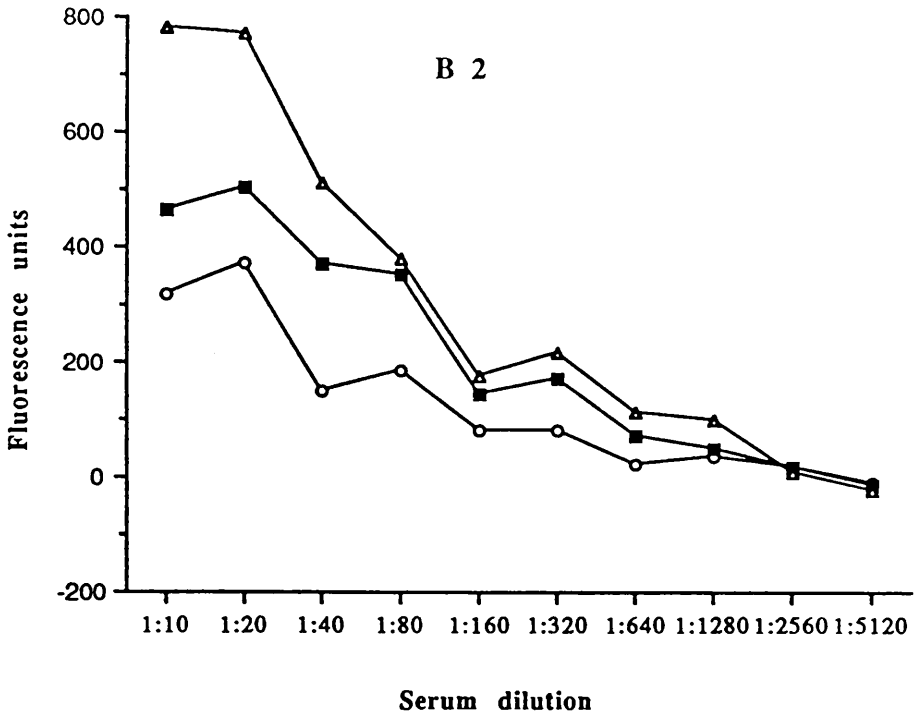


Figure 8.5 Reaction of anti-Kimmeridge clay serum with Oxford clay fraction B2, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

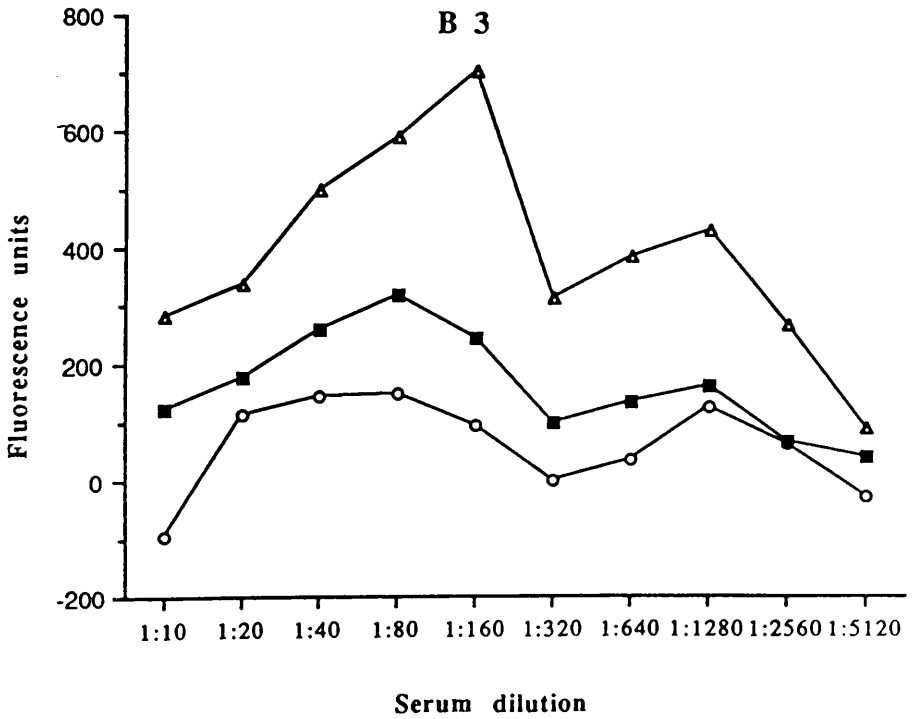


Figure 8.6 Reaction of anti-Kimmeridge clay serum with Oxford clay fraction B3, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

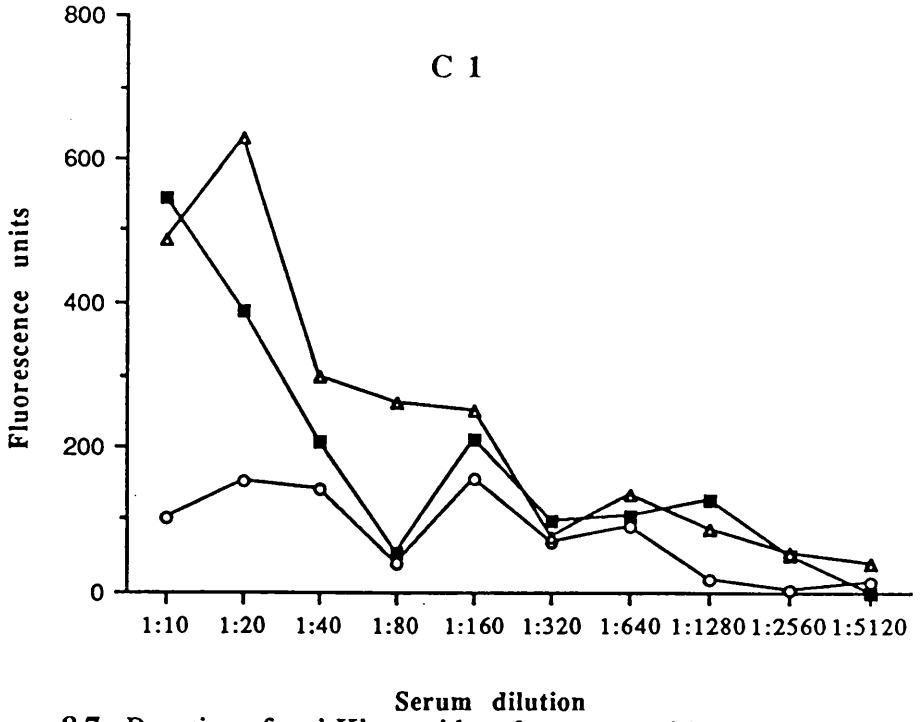


Figure 8.7 Reaction of anti-Kimmeridge clay serum with Lower Lias clay fraction C1, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

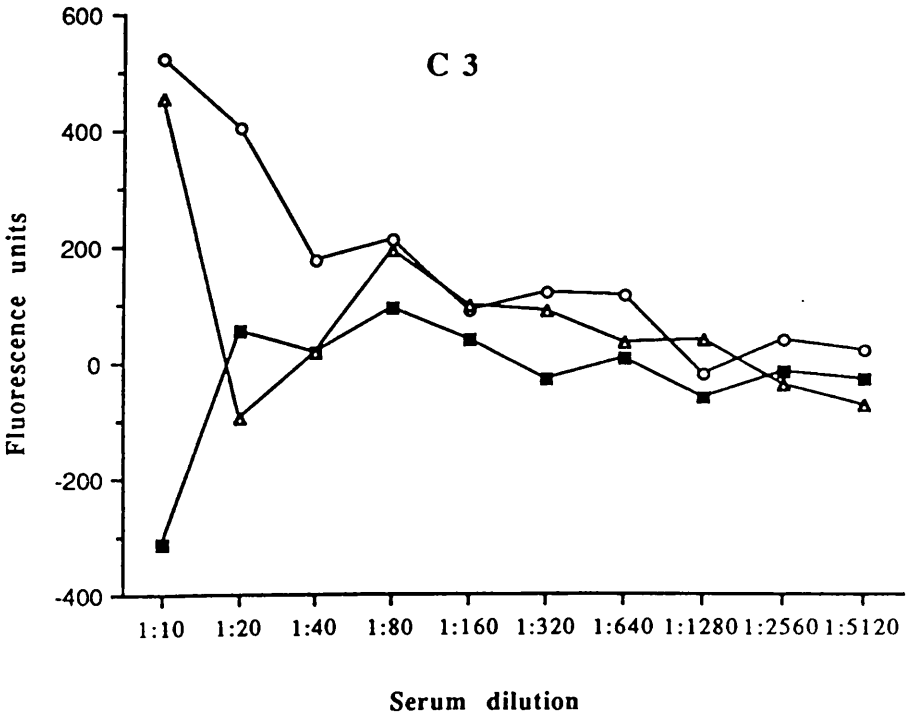


Figure 8.8 Reaction of anti-Kimmeridge clay serum with Lower Lias clay fraction C3, where o , ■ and △ represent Test bleeds 1, 2 and 3 respectively.

Kimmeridgian closely resembled those which prevailed during the deposition of the Oxford Clay, which in turn was deposited in conditions generally similar to those of the Lias of the Lower Jurassic (Bennison & Wright, 1969).

The strongest reaction of the antiserum was with A1, against which it was prepared. The next strongest reaction was against A3 and then B2. Thus the antiserum was more reactive with Kimmeridge and Oxford Clays than with Lower Lias Clay.

8.3.2 Antiserum to Carbazole.

The antiserum prepared against carbazole and the corresponding pre-immune serum were tested by FELISA against 0.9% NaCl, against which the conjugate was dialysed, carbazole at 50 μg/ml, carbazole/KLH at 80 μg/ml, KLH, the protein carrier, at 83 μg/ml and sodium carbonate buffer in which the coupling reaction took place.

The value of the reaction of pre-immune serum with each of these samples was subtracted from the anti-carbazole serum value which was then plotted in **Figure 8.9**. The values for NaCl and buffer are negligible. The reaction with carbazole/KLH and KLH reached a plateau at a concentration of antiserum of 1: 1600 suggesting that the fraction of the antibody which is reacting with these compounds is not diluted out and continues to react even at a serum dilution of 1: 12800.

The result for carbazole itself is a more characteristic antibody response where decreasing serum concentrations decrease the activity of the antibody. While the level of reaction of the anti-carbazole serum with carbazole is not high it must be remembered that only one immunizing dose was administered in this case. Repeated immunisations would increase the antibody concentration of the serum.

The intention was to continue this experiment by testing the antiserum against a panel of twelve rock samples, some containing carbazole, which were to be supplied by British Gas. These were to be tested unlabelled as to their carbazole content and the results were to be checked by British Gas. Unfortunately these samples were never sent and the experiment was discontinued. Thus it was not possible to determine if the anti-carbazole serum were capable of detecting carbazole in rock samples.

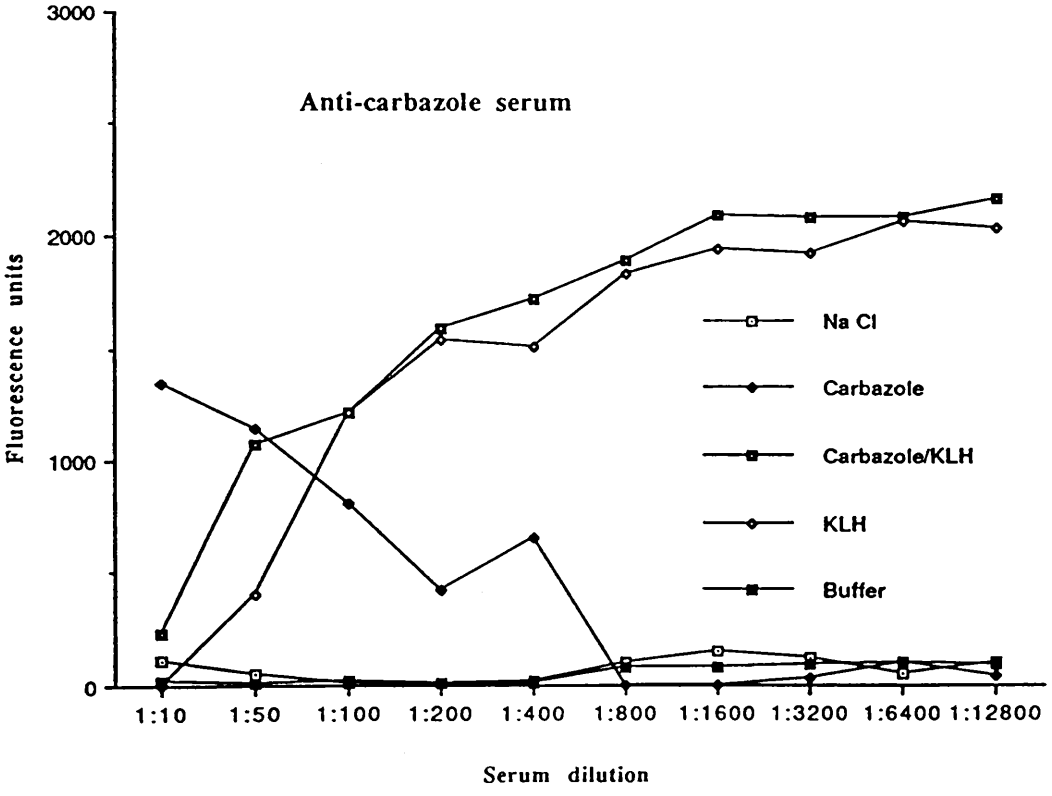


Figure 8.9 Reaction of anti-carbazole serum with sodium chloride, carbazole, carbazole/KLH, KLH and carbonate buffer.

8.4 Conclusions.

While the experiments described in this chapter are preliminary, they do indicate that it is possible to prepare antibodies against compounds of geological interest and that these antibodies may be of use in detection systems. A field assay kit using an antibody prepared as described above could be useful for the detection of a molecular maturity parameter or a compound which indicates the presence of gas or oil in the vicinity of the test site.

The work reported here has shown that it is possible to elicit an immune response to a small molecular weight compound of geological interest by coupling that compound to a protein carrier. This is the first time work of this type has been undertaken.

Chapter 9 DISCUSSION AND SUGGESTIONS FOR FUTURE WORK

9.1 Discussion

The hypothesis that molecular information extracted from the intra-crystalline material of brachiopod shells can be of use in phylogenetic studies has been supported by this study. This work showed that it is possible to generate antibodies against intra-crystalline skeletal macromolecules and that antibodies produced in this way are specific enough to recognise differences between brachiopod families.

Immunological distances can be computed which give results that are in general agreement with current taxonomy. Although in some instances conflicting results were obtained, e.g. in the case of *Argyrotheca*, an overall pattern was established by repeated experimentation. In some cases the result obtained using a mouse antiserum was different from that obtained using a rabbit antiserum, showing the value of using both in further studies.

The molecular taxonomy arrived at using sero-taxonomy is not at odds with the current morphology-based classification of terebratulide brachiopods but is rather an amplification of the previously accepted taxonomy, with redefinition of the assignments of some genera which proved to be difficult to assign using morphological characteristics.

The relationships shown by sero-taxonomy to exist between long- and short-looped brachiopods, previously thought to belong to separate families, have indicated that morphological features other than the length of the loop should be taken into account in assigning genera, and have also cast light on possible evolutionary histories which would otherwise have remained unrecognised.

The emphasis placed on the brachial loop as the main character of taxonomic importance in terebratulide brachiopods has been called into question by the work reported here. This study suggests that the distinction within the Order Terebratulida is not between long- and short-looped forms but between those long-looped forms in which a median septum is involved in loop ontogeny and those in which it is not.

From the results of this study it seems necessary to erect a new high level taxonomy of the Order Terebratulida.

The value of the molecular technique shown by this study, carried out for the first time in brachiopods, is of potential application to many other groups, particularly for those museum specimens of which no more material will ever be available. This study was undertaken using mostly Recent specimens since these are more readily available than fossil specimens, but the results of this work reveal the potential use of molecular techniques for fossil material. Work on fossil biopolymers of Plio-Pleistocene age (Collins *et al.*, in press) showed that they contained valuable systematic information detectable by appropriate immunological techniques. Older samples (4-21 Myr) were immunologically reactive but failed to satisfy the criterion of systematic specificity.

This work has indicated that the integration of morphological with molecular taxonomy would benefit both approaches to classification.

Monoclonal antibodies and hybridoma supernatants have been shown to be potentially useful in phylogenetic studies.

This study has shown that it is possible to prepare antibodies against clay samples and compounds of geological interest such as carbazole. Antibodies of this type may be of use in detecting these compounds in rock samples.

9.2 Suggestions for Future work

1. A more complete brachiopod taxonomy could be arrived at if more taxa were made available for the preparation of antisera.

2. A panel of monoclonal antibodies against a wide variety of taxa, particularly those which are difficult to assign, may further elucidate relationships between terebratulide brachiopods.

3. Using SDS-PAGE and immunoblotting procedures monoclonal antibodies could be used to identify which protein band of a given antigen was responsible for the difference between one taxon and another.

4. The development of antibodies to compounds of geological interest may have applications in commercial areas of gas or oil exploration. Using such antibodies it may be possible to prepare a field assay kit for the detection of chosen compounds. Such a system would be time-saving and cost-effective.

5. The inclusion of a wide range of fossil material for antiserum preparation and testing would lead to a better understanding of evolutionary relationships and histories.

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APPENDIX I

Duplicate readings of FELISA reactions of a panel of rabbit antisera prepared against the genera listed below, described in Chapter 6.

Mean of results minus blank reading.

Key to genera:-

D.s.	*Dallina septigera*
G.v.	*Gryphus vitreus*
K.r.	*Kraussina rubra*
L.r	*Laqueus rubellus*
L.n.	*Liothyrella neozelandica*
L.u.n.	*Liothyrella uva notocadensis* (Antiserum excluded).
M.t.	*Megerlia truncata*
M.m.	*Mercenaria mercenaria*
N.l.	*Neothyris lenticularis*
N.n.	*Notosaria nigricans*
P.p.	*Pictothyris picta*
T.r.	*Terebratulina retusa*
W.i.	*Waltonia inconspicua*

Blank	299	299																												
Dallina 5007	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.m.		N.l.		N.n.		P.p.		T.r.		W.l.	
Dilutions																										
8																										
16													385	382					2156	2038						
32													373	381					1684	1876						
64			537	758									364	367					1084	1513						
128			681	699					2017	2104	1830	1783	294	336					1280	1231						
256			523	378					1902	1951	1636	1612	209	206					811	789						
512			524	381	1752	1811			1669	1693	1247	1143			2085	2068										
1024			392	393	1619	1497			1460	1430	859	932			1721	1793							1984	1676		
2048					1199	1233	2518	2157	1197	1266	459	382			1347	1304					2241	2191	2025	2150		
4096					880	805	2183	2257							477	1014					1934	2094	1831	1788		
8192	2196	2196			453	495	2226	2235							653	598	2140	2249			1917	2006	1454	1353	2139	2226
16384	2167	2167					1848	1896									1999	2004			1683	1689	791	895	1973	1976
32768	1133	1133					1343	1601									1563	1615			1286	1426			1845	1856
65536	828	828															1141	1003							1397	1034
131072	412	412															599	739							753	866
262144	439	439																								
524288	395	395																								
1048576	388	388																								
2097152	308	308																								
4194304	216	216																								

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.l.	N.l.	N.n.	P.p.	T.r.	W.l.
Dilutions													
8													
16							85			1798			
32							78			1481			
64		349					67			1000			
128		391			1762	1508	16			957			
256		152			1628	1325	-92			501			
512		154	1483		1382	896		1775					
1024		94	1259		1146	597		1455					
2048			917	2039	933	122		1027			1917		
4096			544	1921				447			1740		
8192	1897		175	1932				327	1896		1663		
16384	1868			1573					1703		1387	1521	
32768	834			1173					1290		1057	1788	
65536	529								773			1510	
131072	113								370			1104	1883
262144	140											444	1675
524288	96												1451
1048576	89												916
2097152	9												510
4194304	-83												

Blank	194	194																								
Gryphus 802	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.l.	
Dilutions																										
8																										
16													374	370												
32													366	366												
64	355	354	1249	1249									297	305	2217	2220							374	372	381	361
128	377	380	1317	1317									122	182	2113	2169							364	370	372	371
256	382	375	956	956			1750	1576	1935	1705	1402	1319	116	99	1622	1586					943	891	378	357	367	360
512	380	385	635	635	820	919	1184	1384	1475	1616	1268	1235			1187	1165	368	373	383	386	615	567	358	349	360	358
1024	380	382	354	354	620	642	627	633	944	903	681	757			693	776	354	335	386	382	343	338	346	302	355	363
2048			362	362	341	358	383	367	562	621	472	385					300	316	366	381	384	374				
4096			377	377	362	374	380	364	359	328	385	383					166	162	380	390	383	376				
8192			361	361	358	374											116	134	340	381						
16384			326	326																						
32768			244	244																						
65536																										
131072																										
262144																										
524288																										
1048576																										
2097152																										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
Dilutions													
8													
16							178						
32							172						
64	161	1055					107	2025				179	177
128	185	1123					-42	1947				173	178
256	185	762		1469	1626	1167	-87	1410			723	174	170
512	189	441	676	1080	1352	1058		982	177	191	397	160	165
1024	187	160	437	436	730	525		541	151	190	147	130	165
2048		168	156	181	398	235			114	180	185		
4096		183	174	178	150	190			-30	191	186		
8192		167	172						-69	167			
16384		132											
32768		50											
65536													
131072													
262144													
524288													
1048576													
2097152													

Blank 104 104
Krauss 801

Dilutions	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.l.	
8																										
16													272	347					388	378						
32													305	288					389	382						
64													295	184					375	383						
128													243	163					383	346						
256	1618	1741											80	76			350	351	231	373			362	466	419	368
512	1638	1345															384	383					357	352	407	434
1024	1313	1284	1336	1279			2082	2077	1700	1790	1551	832					382	383			369	365	387	399	352	380
2048	984	896	1122	1063	2066	2066	1935	2054	1382	1389	1204	1047			1261	1351	368	351			386	383	379	383	386	377
4096	609	488	582	585	2015	2015	898	971	937	907	567	517			893	1052	167	207			392	383	365	373	386	386
8192			346	361	2508	2508	543	588	630	631	351	362			674	573					357	373				
16384			387	353	2158	2158	373	366	403	337	388	389			485	354					289	199				
32768					909	909									383	376										
65536					631	631																				
131072					390	390																				
262144					2022	2022																				
524288					1875	1875																				
1048576					508	508																				
2097152																										

Dilutions	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
8													
16							206			279			
32							193			282			
64							136			275			
128							99			261			
256	1576						-36		247	198		310	290
512	1388								280			251	317
1024	1195	1204		1976	1641	1088			279		263	289	262
2048	836	989	1962	1891	1282	1022		1202	256		281	277	278
4096	445	480	1911	831	818	438		869	83		284	265	282
8192		250	2404	462	527	253		520			261		
16384		266	2054	266	266	284		316			130		
32768			805					276					
65536			527										
131072			286										
262144			1918										
524288			1771										
1048576			404										
2097152													

LAQUEUS 1191 Dilutions	D.s.	G.v.	K.r.	L.n.	Lu.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
Blank	627 658											
8												
16						542 656			1230 1462			
32		1582 1683			2059 1849	638 746			1379 1302			
64		1456 1475			2030 2097	426 458			1266 1180			
128		942 891			1929 1644	226 288	1984 2038		1134 1085		2094 2119	
256		471 613		2375 2215	1803 1737	250 221	2258 2282		1164 975		1957 2062	
512		298 330		2248 2177	1562 1367		1719 1955			2289 2395	1692 1625	
1024			2105 2245	1393 1446			1850 1806			2405 2403	1636 1593	2057 2127
2048	2578 2448		1897 1849	1181 1238			1446 1475			2287 2266	1251 1311	2035 2058
4096	2311 2289		1472 1315	677 813				2325 2167		2395 2321		1892 1838
8192	2359 2132		991 1070					2284 2177		2311 2174		1583 1740
16384	2113 1980		617 527					1915 1944				1387 1617
32768	1632 1436							1598 1571				
65536								1301 1219				
131072												
262144												
524288												
1048576												
2097152												

LAQUEUS 1191 Dilutions	D.s.	G.v.	K.r.	L.n.	Lu.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
8												
16						-44			704			
32		990			1312	50			698			
64		823			1421	-201			581			
128		274			1144	-386	1369		467		1464	
256		-101		1653	1128	-407	1628		427		1367	
512		-329		1570	822		1195			1700	1016	
1024			1533	777			1186			1762	972	1450
2048	1871		1231	567			818			1634	639	1404
4096	1658		751	103				1604		1716		1223
8192	1603		388					1588		1600		1019
16384	1404		-71					1287				860
32768	892							942				
65536								618				
131072												
262144												
524288												
1048576												
2097152												

Blank	46	46																										
L. n. 803	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.i.	
Dilutions																										
8																										
16													386	394												
32													389	379					383	386						
64													395	400					393	404						
128							1958	2002					390	388			1393	841	391	389	771	940			783	571
256	917	824					1257	1446					392	394			1061	1325	377	381	601	760	732	913	612	502
512	715	741			1654	1496	898	928	2658	2656	2304	2189			2177	2068	808	778	378	383	680	551	1110	1077	382	395
1024	402	455	1640	1769	1402	1235	440	450	2379	2379	2094	1851			1878	1880	459	412			476	527	794	851	328	342
2048	381	382	1334	1141	870	842	382	384	1848	1848	1527	1617			1311	1361	385	378			357	364	659	621	380	373
4096	386	383	759	870	553	514			1032	1032	1141	1237			906	843							354	350		
8192			492	427	363	340			767	767	771	763			515	428										
16384			390	383					338	338																
32768									343	343																
65536									379	379																
131072									371	371																
262144									338	338																
524288																										
1048576																										
2097152																										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
Dilutions													
8													
16							344						
32							338			339			
64							352			353			
128				1934			343		1071	344	810		631
256	825			1306			347		1147	333	635	727	511
512	682		1529	765	2610	2201		2077	746	335	570	1048	343
1024	383	1659	1273	399	2333	1927		1833	390		456	777	289
2048	336	1192	810	337	1802	1526		1290	336		315	594	331
4096	339	769	488		956	1143		829				306	
8192		414	306		721	721		426					
16384		341			292								
32768					297								
65536					333								
131072					325								
262144					292								
524288													
1048576													
2097152													

Blank	376	376																												
Megerlia5053	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.i.	
Dilutions																										
8																										
16													413	418					533	1291						
32													358	353					626	389						
64													352	388					392	519						
128													394	392					395	403						
256	2315	2493					669	645					382	403			388	471	380	400	1518	1309	1561	1861	1379	1074
512	2480	2256					524	417									357	383			1082	1165	1371	1250	1046	905
1024	1754	2130			2733	2651	389	365									400	378			851	668	966	664	801	898
2048	1738	1402	1359	1549	2347	2753	372	389	1699	1755	1607	1832			2532	2532	397	393			368	442	351	410	745	634
4096	685	1035	1142	928	2205	1718	369	389	1539	1420	1494	1181			2249	2249	376	395			396	392	391	395	351	428
8192			525	598	1277	1769			842	1048	686	900			1621	1621										
16384			349	394	1304	385			580	531	531	358			1073	1073										
32768			385	393					397	389	390	397			337	337										
65536															742	742										
131072															363	363										
262144															383	383										
524288															389	389										
1048576															375	375										
2097152																										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
Dilutions													
8													
16							40			536			
32							-21			132			
64							- 6			80			
128							17			23			
256	2028			281			17		54	14	1038	1335	851
512	1992			95					-16		748	935	600
1024	1566		2316	1					13		384	439	474
2048	1194	1078	2174	5	1351	1344		2156	19		29	5	314
4096	484	659	1586	3	1104	962		1873	10		18	17	14
8192		186	1147		569	417		1245					
16384		- 5	469		180	69		697					
32768		13			17	18		-39					
65536								366					
131072								-13					
262144								7					
524288								13					
1048576								- 1					
2097152													

Blank	399	399																						
Merc 4951	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.l.	
Dilutions																								
8																								
16	409	419	423	422	399	370	398	425	456	983	412	362			1168	1292	394	395	1307	1460	371	500	397	379	403	391
32	406	417	419	369	374	418	465	432	516	354	408	404			750	690	384	388	1492	907	392	352	406	397	388	409
64	415	407	474	368	417	404	860	876	374	411	404	406			383	642	408	402	610	1060	365	361	414	400	414	390
128	375	418	365	420	417	371	528	673	375	406	397	396			355	409	394	406	1445	418	362	366	405	408	427	372
256	377	415	377	606	392	385	495	374	390	352	384	354			407	414	397	396	416	407	406	410	415	402	363	505
512																								
1024																								
2048																								
4096													1190	1190										
8192													1035	1035										
16384													833	833										
32768													541	541										
65536													373	373										
131072													423	423										
262144													421	421										
524288													375	375										
1048576													405	405										
2097152													381	381										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
Dilutions													
8													
16	15	24	-15	13	311	-12		831	-5	985	37	-11	-2
32	13	-5	-3	50	36	7		321	-23	801	-27	3	-1
64	12	22	12	469	-7	6		104	6	436	-36	8	3
128	-3	-7	-5	202	-9	-3		-17	1	533	-35	8	1
256	-3	93	-11	36	-28	-30		12	-3	13	9	10	35
512													
1024													
2048													
4096							791						
8192							636						
16384							434						
32768							142						
65536							-26						
131072							24						
262144							22						
524288							-24						
1048576							6						
2097152							-18						

Blank	367	325																												
NEOTHY 427	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.i.	
Dilutions																										
8																										
16			299	306	769	1308	1916	1952	1681	1888	372	414	780	821	722	669			1045	1437	1356	1314	339	327		
32	1571	1486	324	328	565	711	1526	1458	1635	1270	345	331	551	808	456	547	2019	2019	857	1171	1140	1059	329	330	1425	1652
64	1115	1015	333	334	327	348	888	827	1312	1331	380	382	428	587	344	337	1812	1812	806	977	633	541	329	333	1063	1296
128	488	543	327	329	328	315	443	444	967	932	358	354	321	445	362	360	1221	1221	523	1131	343	329	334	354	987	1024
256	286	309	318	317	336	336	304	311	460	476	352	348	354	358	357	358	562	562	361	589	370	358	353	360	731	768
512	330	342															368	368							532	533
1024																	318	318								
2048																	330	330								
4096																	325	325								
8192																	310	310								
16384																	281	281								
32768																										
65536																										
131072																										
262144																										
524288																										
1048576																										
2097152																										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
Dilutions													
8													
16		-44	692	1567	1438	47	454	349		895	989	-14	
32	1182	-21	292	1125	1106	-9	333	155	1673	668	753	-17	1192
64	719	-13	-9	491	975	35	161	-6	1466	545	241	-16	833
128	172	-13	-25	77	603	10	37	15	875	481	-11	-3	659
256	-49	-23	-11	-60	122	4	10	11	216	129	18	10	403
512	-11								22				186
1024									-29				
2048									-17				
4096									-22				
8192									-37				
16384									-66				
32768													
65536													
131072													
262144													
524288													
1048576													
2097152													

Blank	83	83																								
Notos 4910	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.i.	
Dilutions																										
8																										
16	408	399	407	401	374	424	1051	1099	522	573	404	403	408	407	383	395	401	364			472	519	519	662	402	400
32	404	397	405	398	360	374	765	642	370	359	406	402	390	395	359	369	404	390			348	442	498	391	382	381
64	396	402	398	403	364	382	381	364	395	380	404	395	398	391	395	384	386	395			357	383	374	365	388	372
128	393	381	387	380	400	392	405	400	401	398	385	389	353	375	399	400	402	391			402	400	402	399	230	180
256	382	361	197	190	402	402	397	403	396	401	341	276	386	388	389	386	324	388			387	392	397	395	178	191
512																			1840	1840						
1024																			1286	1286						
2048																			841	841						
4096																			434	434						
8192																			394	394						
16384																			413	413						
32768																			405	405						
65536																			393	393						
131072																			347	347						
262144																			324	324						
524288																										
1048576																										
2097152																										

	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
Dilutions													
8													
16	321	321	316	992	465	321	325	306	300		413	508	318
32	318	319	284	621	282	321	310	281	314		312	362	299
64	316	318	290	290	305	317	312	307	308		287	287	297
128	304	291	313	320	317	304	281	317	314		318	318	122
256	299	111	319	317	316	226	304	305	273		307	313	102
512										1757			
1024										1203			
2048										758			
4096										351			
8192										311			
16384										330			
32768										322			
65536										310			
131072										264			
262144										241			
524288													
1048576													
2097152													

Blank | PICTO 1192 | Dilutions

Dilutions	D.s. 92	D.s. 94	G.v.	G.v.	K.r.	K.r.	L.r.	L.r.	L.n.	L.n.	L.u.n.	L.u.n.	M.m.	M.m.	M.t.	M.t.	N.l.	N.l.	N.n.	N.n.	P.p.	P.p.	T.r.	T.r.	W.i.	W.i.
8																										
16																										
32			2089	1849									655	643					1488	1446						
64			1882	1554							2041	2185	262	262					1252	1026						
128			1551	1034							1748	1878	219	123					977	806			1772	2070		
256			1177	803					1721	1812	1462	1553	232	139	2060	2061			838	611			1570	1881		
512			690	441					1352	1450	1165	1239	150	75	1741	1881			548	373			1357	1569		
1024					1633	1491	2245	2318	1217	1377	752	1077			1450	1561					2622	2622	1158	1204	1938	1924
2048	2205	2073			1317	1338	[illegible]	2105	1089	1299					1261	1426					2453	2453	759	736	1949	1833
4096	1541	1595			1027	1221	1655	1823	889	882					1190	1260					1174	1174			1368	1570
8192	1113	972			706	922	[illegible]	1356									1475	1476			801	801			949	1030
16384	815	704			551	665	[illegible]	812									1097	1068			350	350			687	621
32768	499	573															1109	821			1254	1254				
65536																	1013	658			551	551				
131072																	942	653			472	472				
262144																					397	397				
524288																					301	301				
1048576																										
2097152																										

Dilutions	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
8													
16													
32		1875					[illegible]			1374			
64		1625				2020	[illegible]			1046			
128		1200				1720	[illegible]			799		1828	
256		897			1674	1415	[illegible]	1968		632		1633	
512		473			1308	1109	[illegible]	1718		368		1370	
1024			1469	2190	1204	822		1413			2529	1088	1838
2048	2049		1235	2013	1101			1251			2360	655	1795
4096	1475		1031	1662	793			1132			1081		1376
8192	950		721	1176					1383		708		897
16384	667		515	685					990		257		561
32768	443								872		1161		
65536									743		458		
131072									705		379		
262144											304		
524288											208		
1048576													
2097152													

Blank	83	83																																				
T.retusa 4962	D.s.		G.v.		K.r.		L.r.		L.n.		L.u.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.l.													
Dilutions																																						
8																																						
16													374	368																									
32													374	379																									
64													371	361																									
128							2443	2312					366	362							2019	2147																	
256			1136	1074	1097	1340	2396	2477	1797	1744	1455	1679	354	355	1839	1868			1315	1308	2134	2242																	
512			978	865	912	1519	2473	2360	1741	1840	1332	1262			1846	1765			1425	1109	2447	2266																	
1024			584	610	1264	1187	2438	2473	1778	1745	1119	1022			1391	1514			1012	1009	2331	2342																	
2048	986	968	336	336	834	913	2209	2142	1516	1630	706	661			1134	915	1719	1770	640	619	2287	2223																	
4096	928	1035	351	358	606	503			1139	1325	354	351			445	533	1726	1817	336	332					1352	1353													
8192	625	635															1394	1370					2301	2301	993	1068													
16384	372	331															990	1034					2592	2592	690	617													
32768	374	378															667	717					2294	2294	392	428													
65536																							1777	1777	358	368													
131072																							1467	1467															
262144																							588	588															
524288																							328	328															
1048576																							377	377															
2097152																							371	371															
																							363	363															

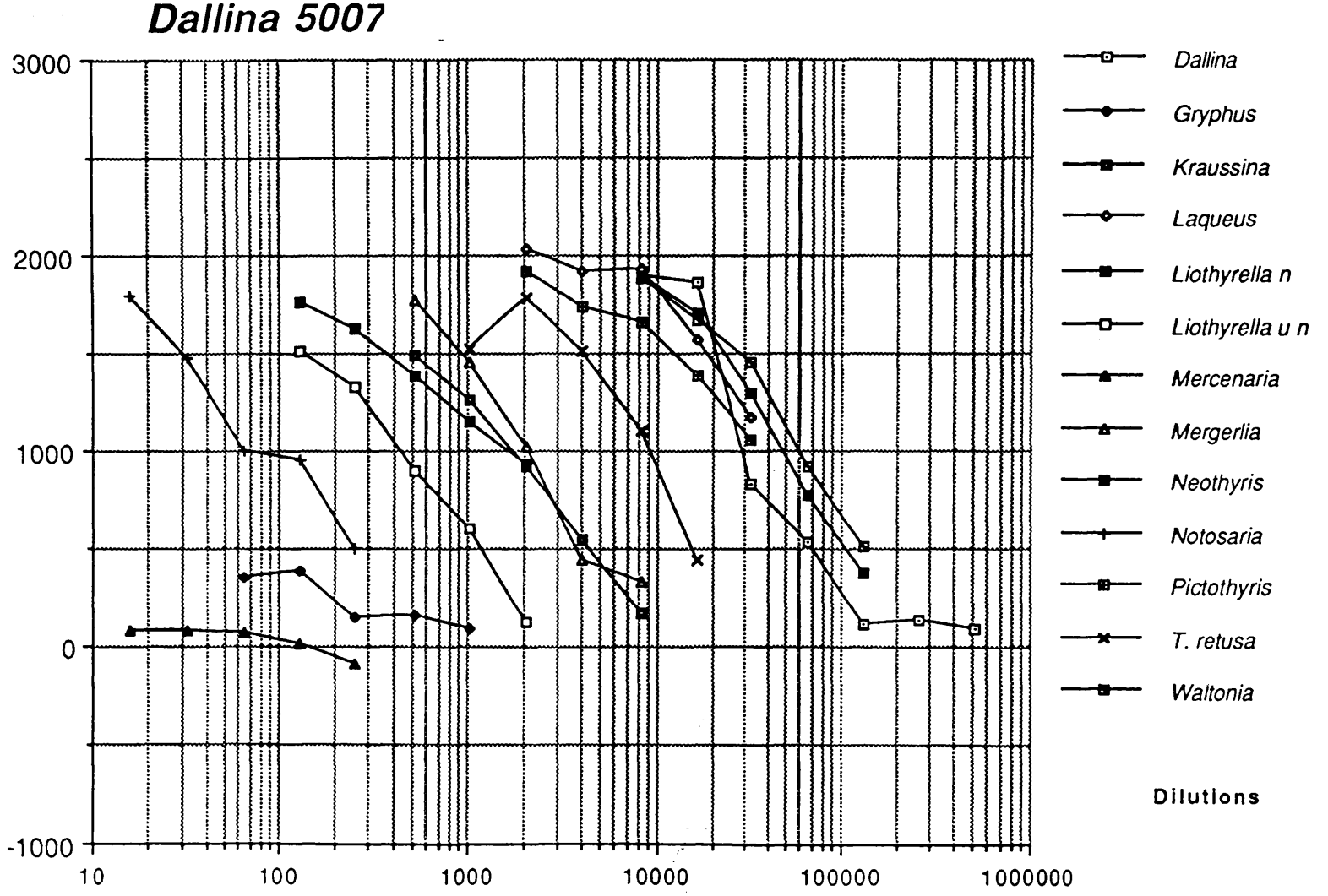
	D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.l.
Dilutions													
8													
16							288						
32							294						
64							283						
128				2295			281				2000		
256		1022	1136	2354	1688	1484	272	1771		1229	2105		
512		839	1133	2334	1708	1214		1723		1184	2274		
1024		514	1143	2373	1679	988		1370		928	2254		
2048	894	253	791	2093	1490	601		942	1662	547	2172		
4096	899	272	472		1149	270		406	1689	251			1270
8192	547								1299			2218	948
16384	269								929			2509	571
32768	293								609			2211	327
65536												1694	280
131072												1384	
262144												505	
524288												245	
1048576												294	
2097152												288	
												280	

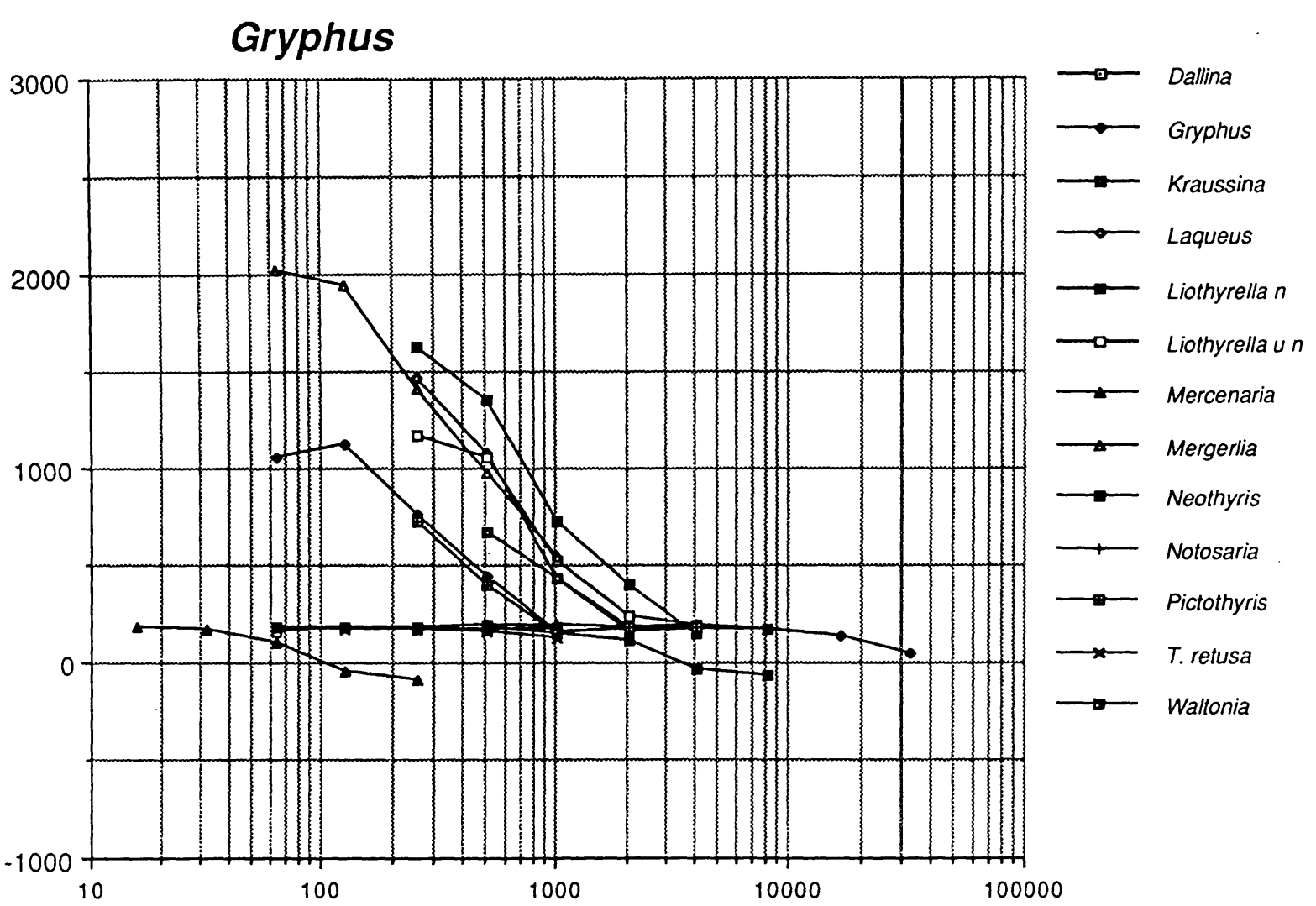
Blank	239	239																								
Waltonia5040	D.s.		G.v.		K.r.		L.r.		L.n.		M.m.		M.t.		N.l.		N.n.		P.p.		T.r.		W.i.			
Dilutions																										
8																										
16											419	416					458	432								
32											427	421					397	373								
64									730	965	381	407					418	436								
128			437	427					1265	993	355	313					491	407								
256			415	404	1868	2063			553	1012	235	212	432	462			385	395			1450	1595				
512			400	417	1804	1885			808	650			509	520							1629	1694				
1024			331	381	1508	1650	2688	2765	481	682			402	408					1602	1924	1403	1625	2015	2015		
2048	1961	2072	388	368	1164	1218	2832	2746					420	417	2333	2584			2013	1774	1031	977	1902	1902		
4096	1976	1790			923	944	2521	2720					412	435	2390	2386			1801	2109	642	772	2071	2071		
8192	2004	1889					2060	2094							2148	2020			1851	1650			2008	2008		
16384	1416	1543					1629	1760							1758	1568			1331	1377			1318	1318		
32768	1048	1116													1314	1192							1185	1185		
65536																							817	817		
131072																							619	619		
262144																							390	390		
524288																							436	436		
1048576																										
2097152																										

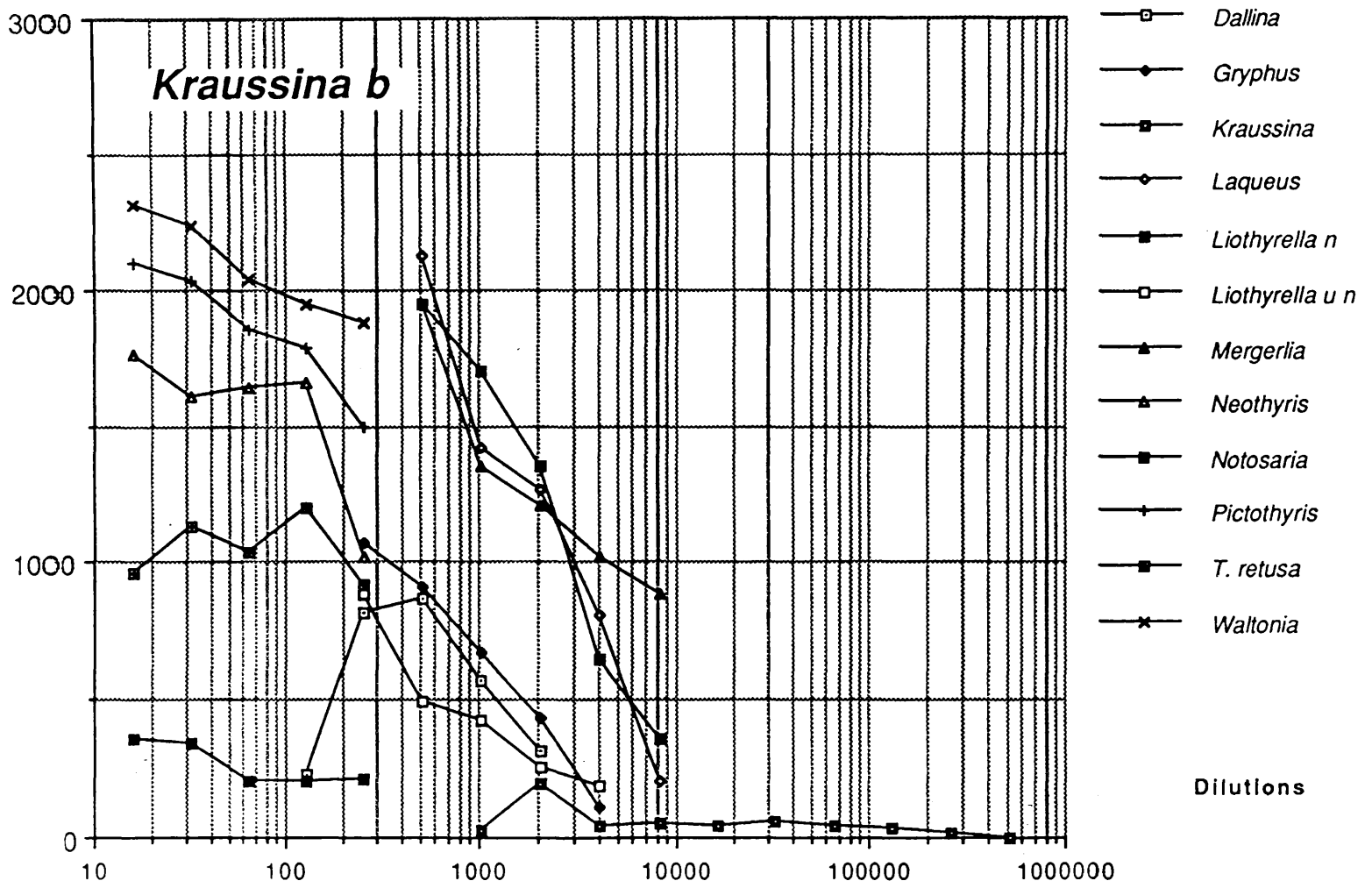
Dilutions	D.s.	G.v.	K.r.	L.r.	L.n.	M.m.	M.t.	N.l.	N.n.	W.i.
8										
16						179			206	
32						185			145	
64					609	155			183	
128		193			890	95			210	
256		171	1727		544	-16	208		151	
512		170	1606		490		276			
1024		117	1339	2488	343		166			1776
2048	1778	139	952	2550			180	2220		1663
4096	1644		695	2382			185	2149		1832
8192	1708			1838				1845		1769
16384	1241			1456				1424		1079
32768	843							1014		946
65536										578
131072										380
262144										151
524288										197
1048576										
2097152										

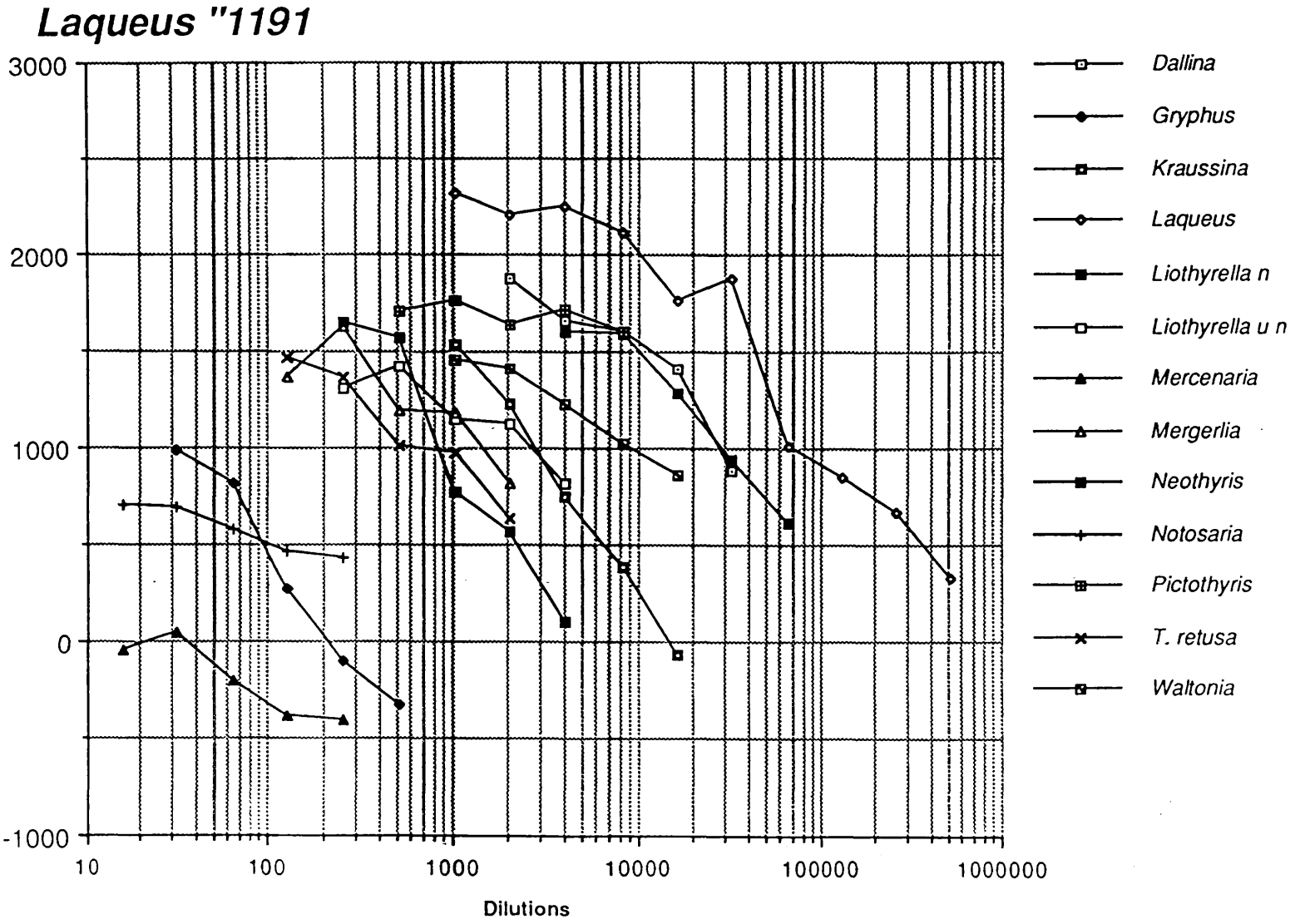
APPENDIX II

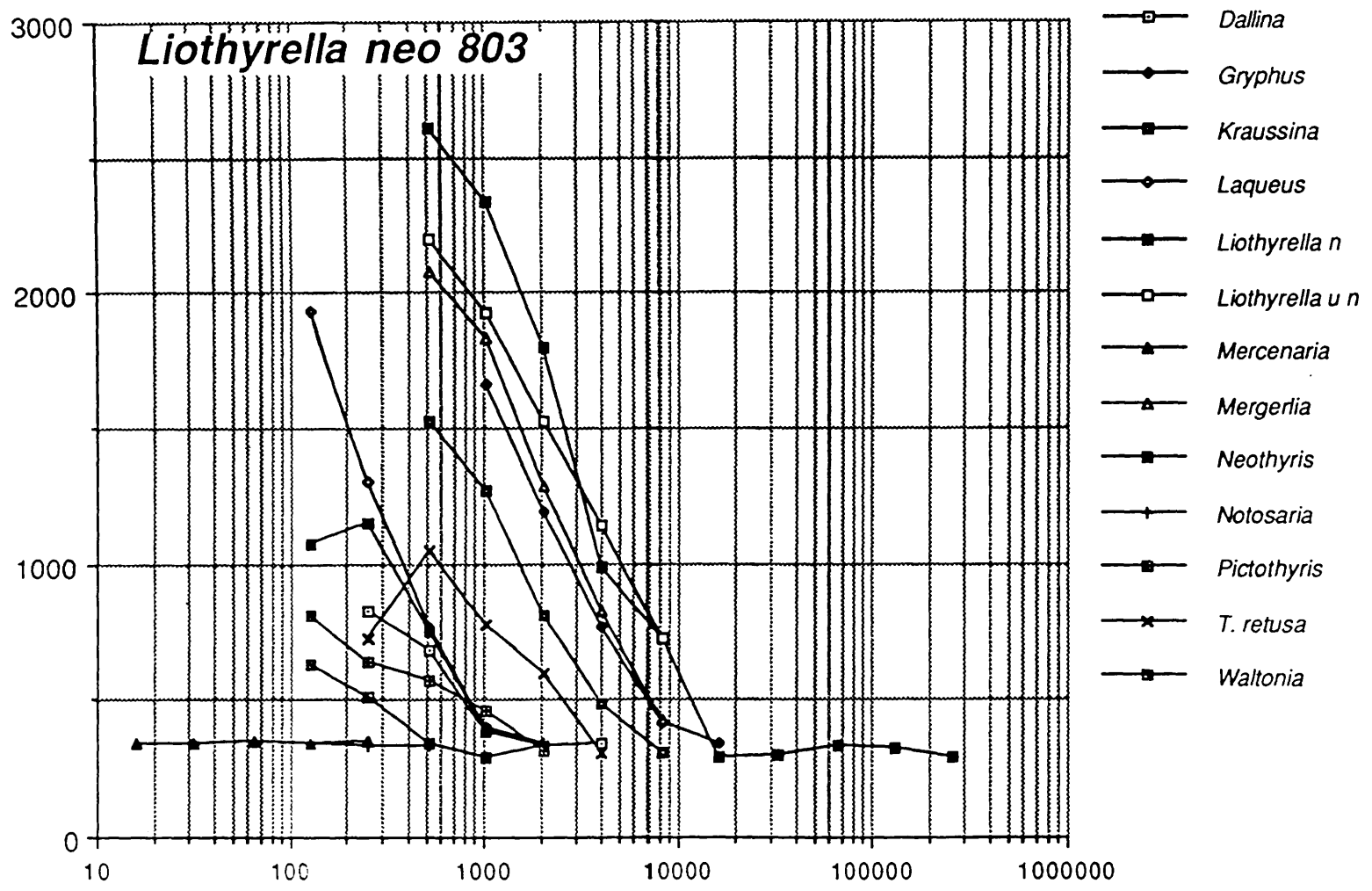
Immunological binding curves obtained by plotting the reactions of each of the rabbit antisera against the genera listed in the key to Appendix I. Data given in Appendix I. The graphs were used to obtain the measurements given in Appendix III. (See Chapter 6).


Dallina 5007
3000
2000
1000
0
-1000
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia
Dilutions

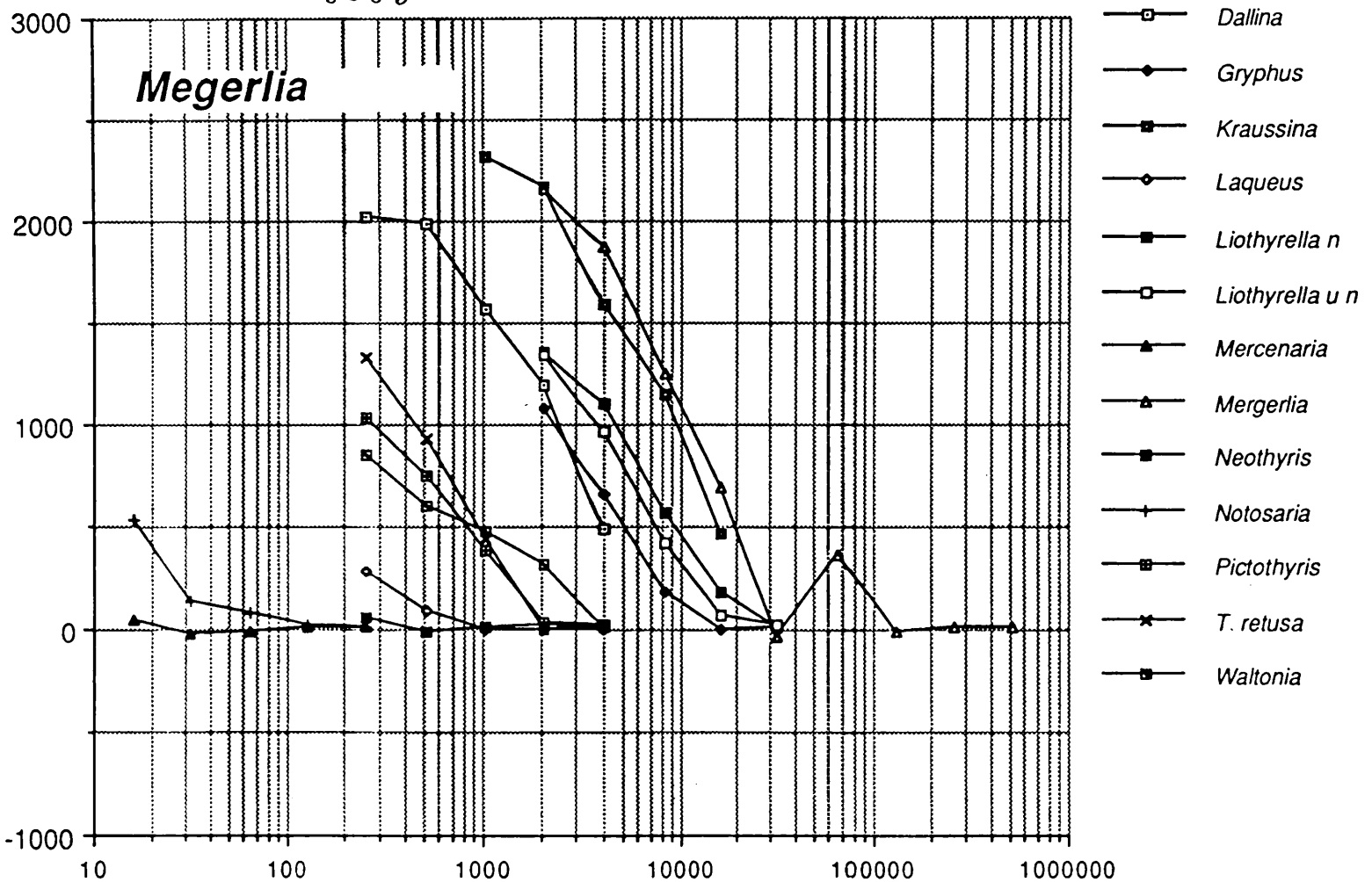

Gryphus
3000
2000
1000
0
-1000
10
100
1000
10000
100000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

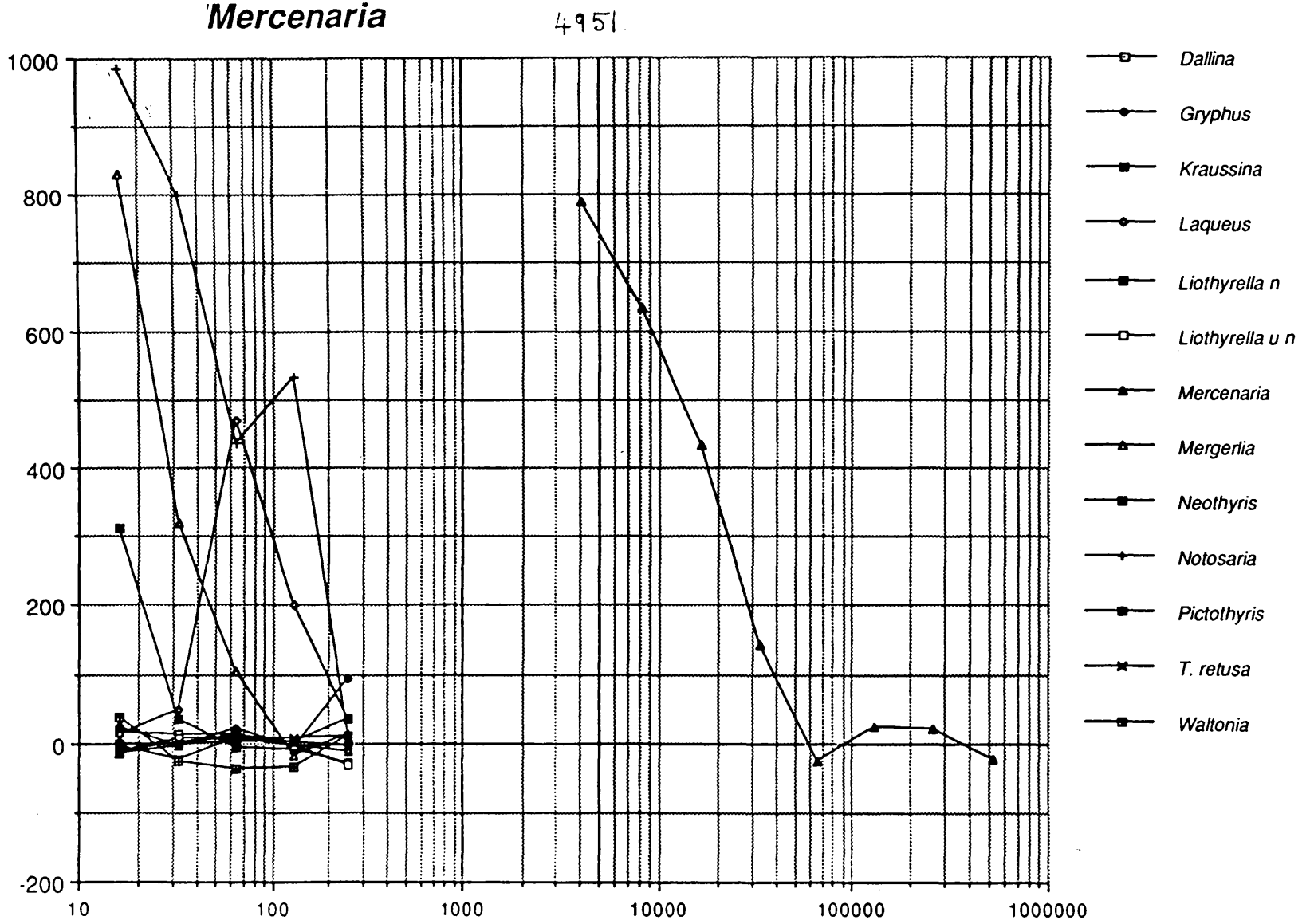

Kraussina b
3000
2000
1000
0
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia
Dilutions

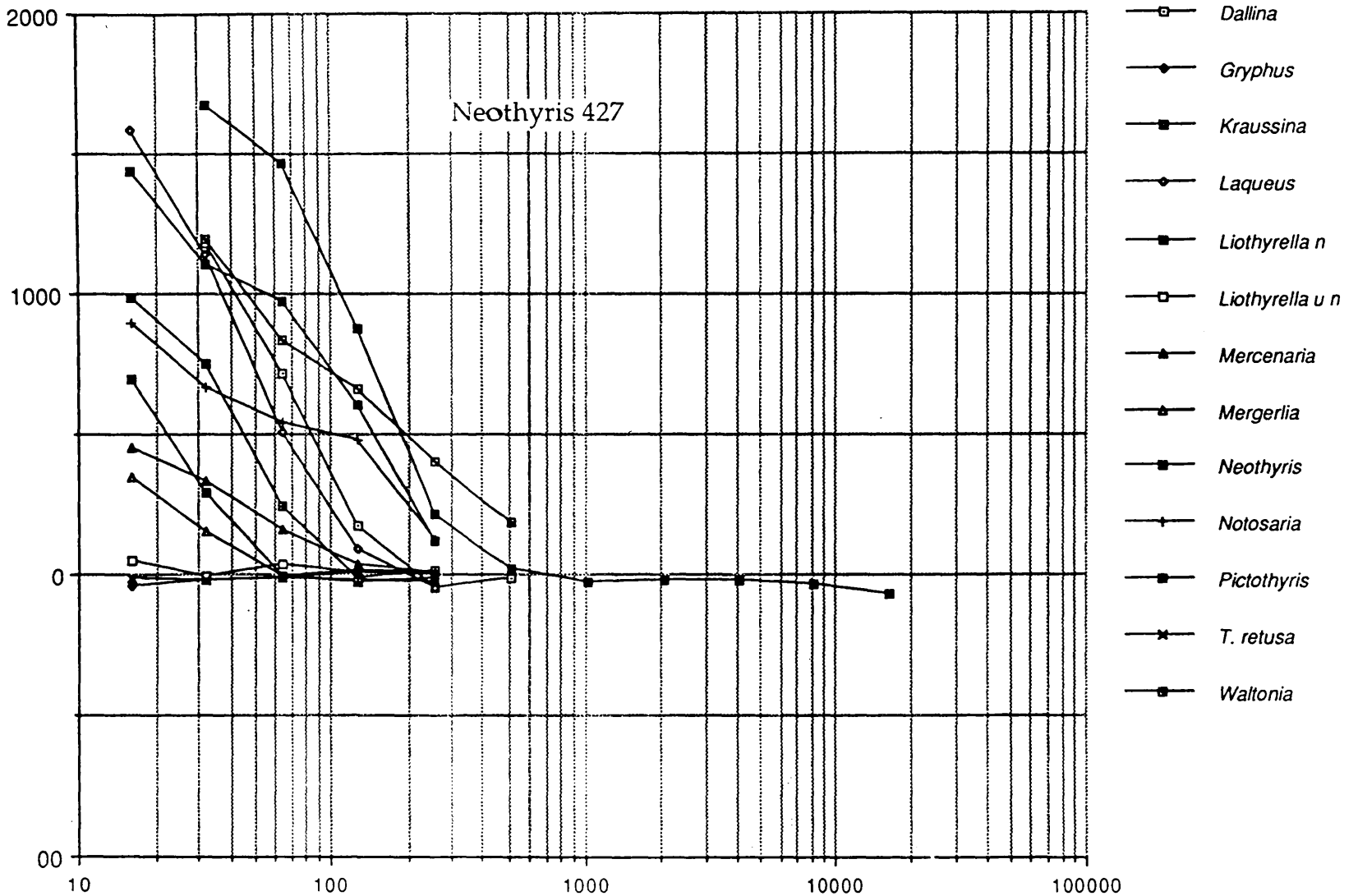

Laqueus "1191
3000
2000
1000
0
-1000
10
100
1000
10000
100000
1000000
Dilutions
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

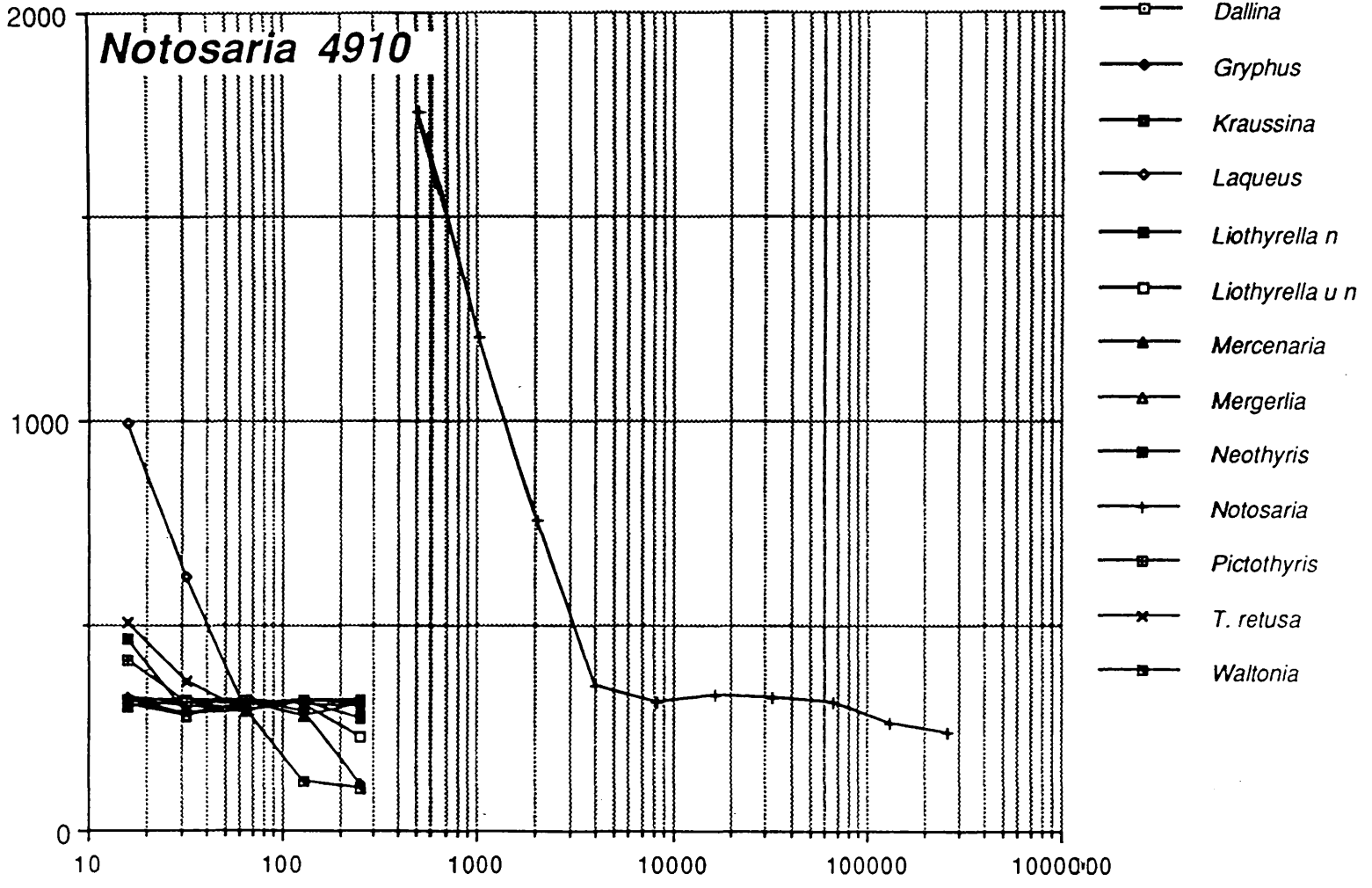

Liothyrella neo 803
3000
2000
1000
0
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

5053

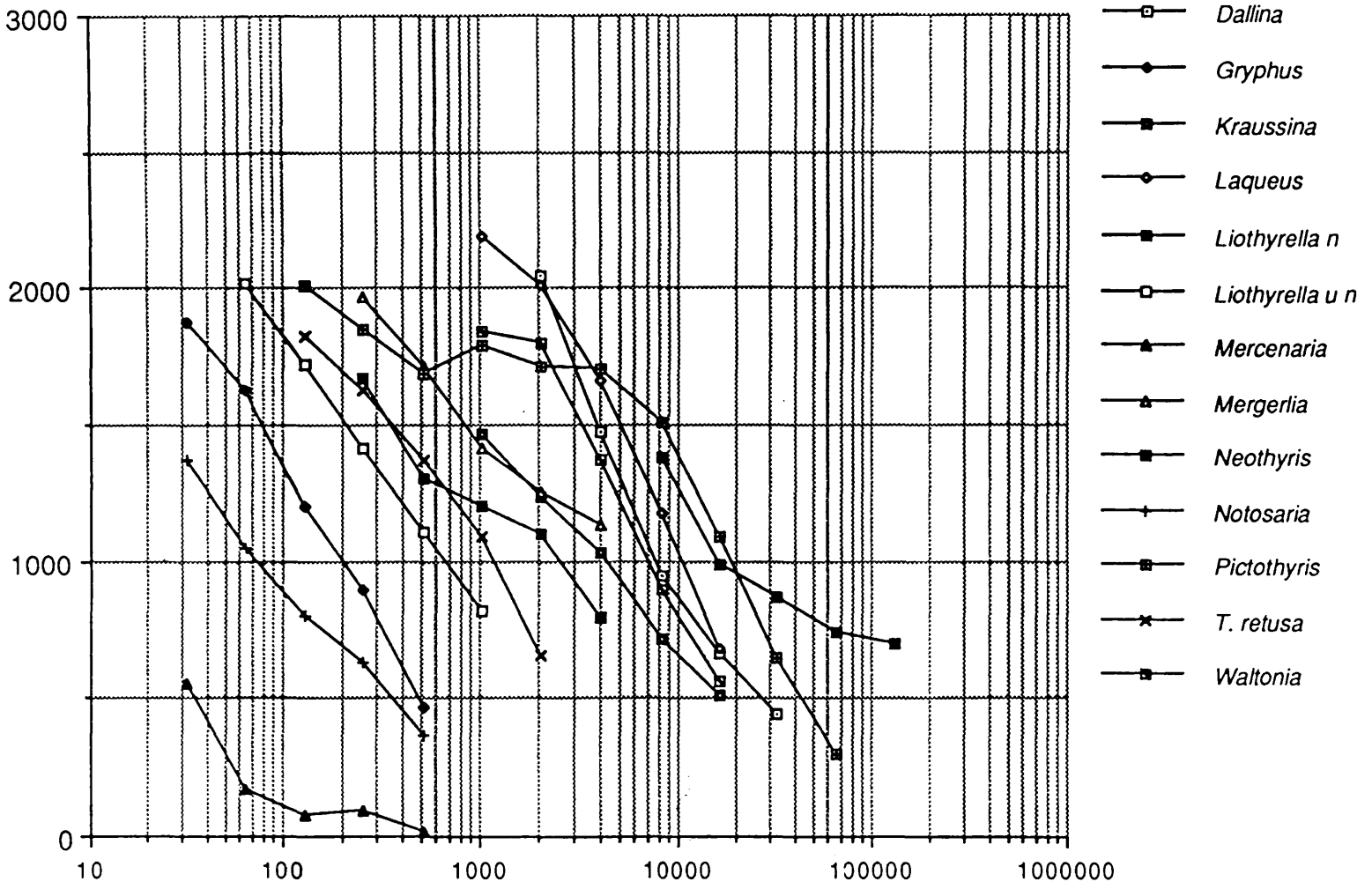

Megerlia
3000
2000
1000
0
-1000
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

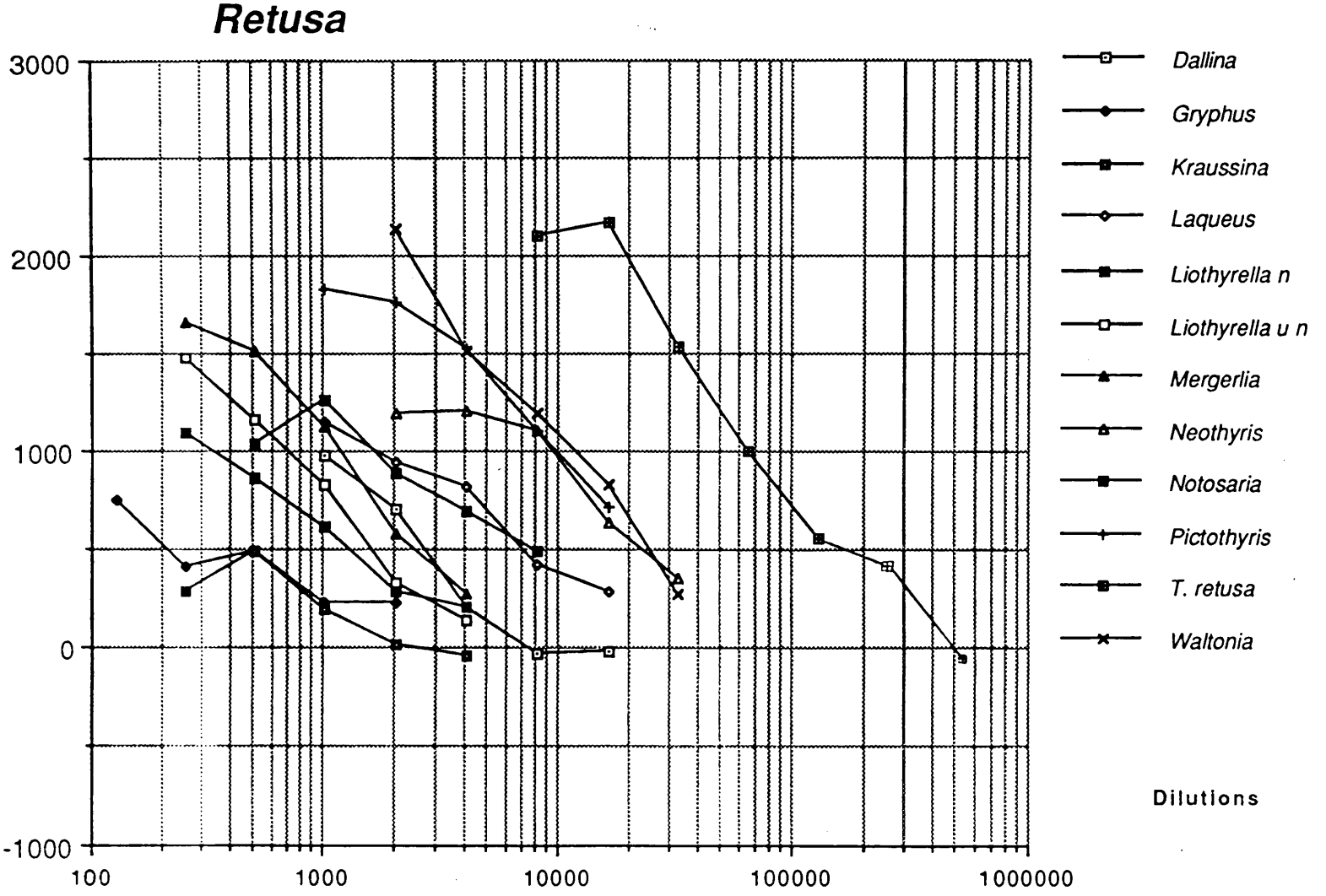

'Mercenaria
4951
1000
800
600
400
200
0
-200
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

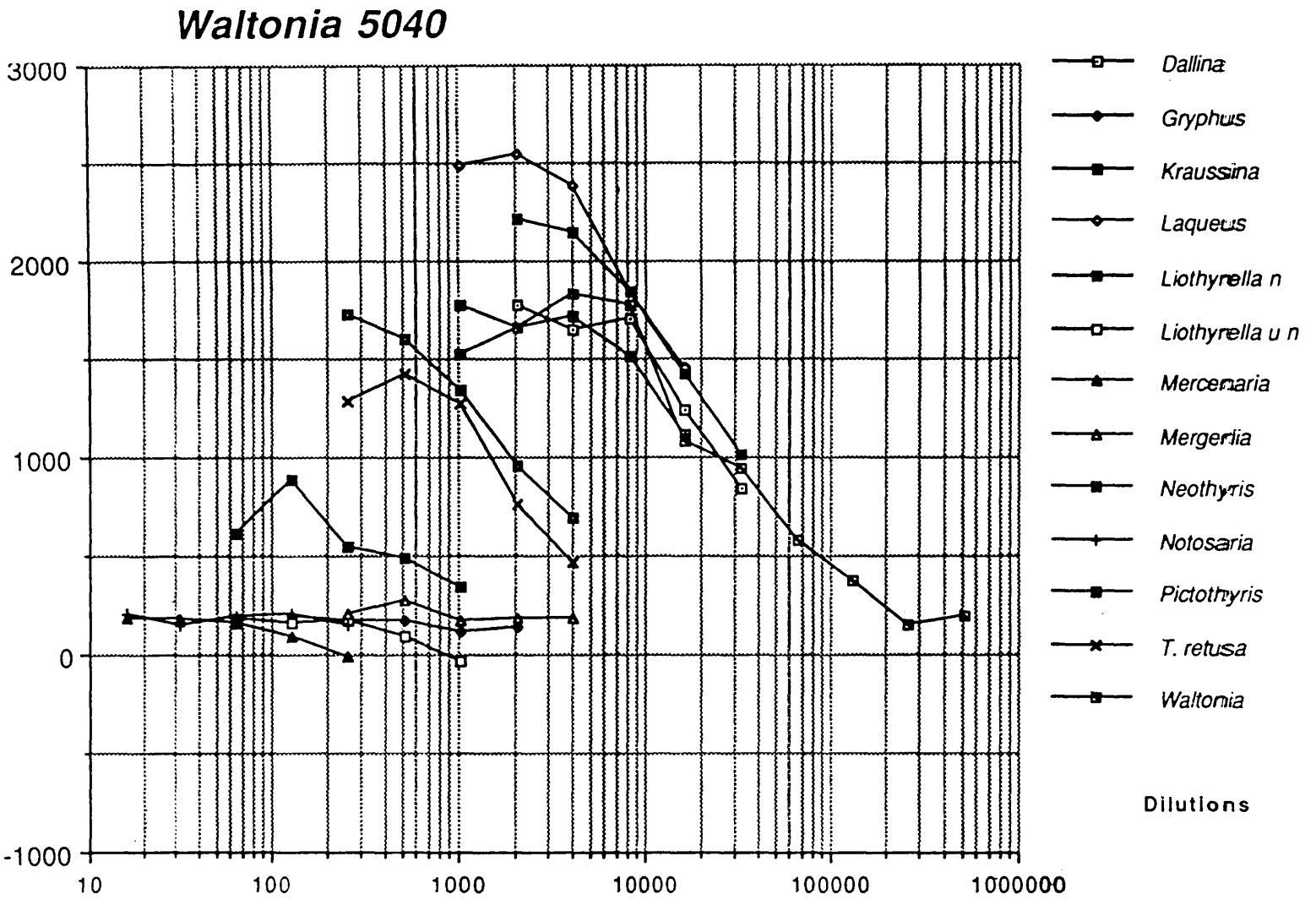

Neothyris 427
2000
1000
0
00
10
100
1000
10000
100000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia


Notosaria 4910
2000
1000
0
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia

Pictothyris with test bleed




Retusa
3000
2000
1000
0
-1000
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia
Dilutions


Waltonia 5040
3000
2000
1000
0
-1000
10
100
1000
10000
100000
1000000
Dallina
Gryphus
Kraussina
Laqueus
Liothyrella n
Liothyrella u n
Mercenaria
Mergerlia
Neothyris
Notosaria
Pictothyris
T. retusa
Waltonia
Dilutions

APPENDIX III

Measurements obtained from graphs presented in Appendix II. Values were obtained by measuring the distances between the linear part of the homologous antigen-antibody reaction curve and that of each of the curves of the reaction of that antiserum with all the other antigens and expressing this as a percentage of the homologous value. These values were used in the calculation of immunological distance in Chapter 6.

Serum Dallina 5007
Length of total X-axis 263
No of harmonics 6
Length of each harmonic 43.9

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
0	127	70.8	4	93.5	96.5	198	67.5	0	133	28	40.7	0
0	130	60.7	- 4	82.5	86	198	58.2	2	124	16	30.9	2.3
0	117	52		72	78.1	186	50.6	- 6	104	8	23	- 8
0	119	52		62	76	182	58	- 10	107	- 3	32	- 15
0		62		51	96		52	- 5				- 12
0												

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
100	0.13	2.43	81.1	0.74	0.63	0	2.89	100	0.09	23	11.8	100
100	0.11	4.13	123	1.32	1.1	0	4.71	90	0.15	43.2	19.8	88.6
100	0.22	6.53		2.28	1.66	0.01	7.02	137	0.43	73	29.9	152
100	0.19	6.53		3.86	1.85	0.01	4.76	169	0.36	117	18.6	215
100		3.86		6.88	0.65		6.53	130				188
100	0.16	4.7	102	3.02	1.18	0	5.18	125	0.26	64.1	20	149
0	0.05	1.79	29.9	2.46	0.56	0	1.65	31.4	0.16	40.9	7.46	54.6

Serum Gryphus 802
Length of total X-axis 267
No of harmonics 4
Length of each harmonic 66.8

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
79	0	- 9	- 37	- 38	- 37	120	- 31	79	79	2	79	79
	0	- 19	- 30	- 36	- 25		- 25			4		
	0	- 21	- 21	- 40	- 26					0		
	0											
	0											

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
6.574	100	136	358	364	352	1.6	291	6.57	6.57	93.3	6.57	6.57
	100	192	281	346	237		237			87.1		
	100	206	206	397	245							
	100											
	100											
6.574	100	178	282	369	278	1.6	264	6.57	6.57	90.2	6.57	6.57
####	0	37	75.8	25.9	64.2	###	38.4	###	###	4.4	###	###

Serum Kraussina 801
Length of total X-axis 272
No of harmonics 5
Length of each harmonic 54.3

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
104	76.5	0	66.6	70.8	77.9	217	60.5	116	196	82	115	130
90.4	63.1	0	51.7	59.4	62.6		49					
76.9	70	0	49.2	49	73		51					
65.2	69	0	55	49	68		49					
71		0	50	51			34					

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.t.
1.204	3.91	100	5.95	4.98	3.68	0.01	7.7	0.73	0.02	3.1	0.77	0.41
2.17	6.9	100	11.2	8.07	7.05		12.5					
3.844	5.15	100	12.4	12.5	4.54		11.5					
6.312	5.37	100	9.72	12.5	5.61		12.5					
4.936	100	100	12	11.5			23.7					
3.693	24.3	100	10.3	9.93	5.22	0.01	13.6	0.73	0.02	3.1	0.77	0.41
2.058	42.3	0	2.62	3.32	1.45	###	5.98	###	###	###	###	###

Serum Laqueus 1191
Length of total X-axis 264
No of harmonics 5
Length of each harmonic 52.8

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
43	177	73.5	0	100	152	236	101	39	218	34	124	77
36.6	180	71	0	87.5	140		106	23.5		23	113	63.6
23	194	90	0	120	125		89	20.5			112	55
15.4		94	0	116	129		101	23.5			100	48
29			0					34.5			113	48

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
15.33	0.04	4.05	100	1.28	0.14	0	1.22	18.3	0.01	22.7	0.45	3.48
20.27	0.04	4.52	100	2.2	0.22		1	35.9		36.7	0.72	6.24
36.68	0.02	1.97	100	0.53	0.43		2.06	40.9			0.76	9.09
51.09		1.66	100	0.83	0.36		1.25	35.9			1.28	12.3
28.23			100					22.2			0.72	12.3
30.32	0.03	3.05	100	1.16	0.29	0	1.38	30.6	0.01	29.7	0.79	8.69
14.16	0.01	1.45	0	0.77	0.13	###	0.47	9.81	###	9.88	0.3	3.87

Serum Liothyrella n 803
Length of total X-axis 261
No of harmonics 5
Length of each harmonic 52.2

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
73.1	18.5	36.5	59	0	19.3	139	23.5	59.6	137	89.5	46	100
64	11.6	26	57	0	12		15	62		83	45	92
71	13	27	61	0	5		15	72.5		72	40	91
61	23	31	70	0	-3		12	60.5		65	47.5	
	13	32	60	0	0		21			63		

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
3.987	44.2	20	7.42	100	42.7	0.22	35.5	7.23	0.24	1.94	13.2	1.22
5.955	60	31.8	8.11	100	58.9		51.6	6.5		2.58	13.8	1.73
4.374	56.4	30.4	6.8	100	80.2		51.6	4.09		4.19	17.2	1.81
6.797	36.3	25.5	4.57	100	114		58.9	6.95		5.7	12.3	
	56.4	24.4	7.1	100	100		39.6			6.22		
5.278	50.7	26.4	6.8	100	79.2	0.22	47.5	6.19	0.24	4.12	14.1	1.59
1.323	10	4.76	1.34	0	29.1	###	9.63	1.43	###	1.88	2.12	0.32

Serum Megerlia 5053
Length of total X-axis 262
No of harmonics 5
Length of each harmonic 52.5

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
41	36.6	0	104	19.5	29.1	172	0	108	161	84.6	77	90.5
39.4	32.5	7.4	91.8	18.6	24		0		154	77.5	72.3	81
33.6	26.8	3		11	21.8		0		139	70	68.8	68
36.3		4.7			13.2		0			62	63	55.8
							0					46

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.i.
16.54	20.1	100	1.06	42.5	27.9	0.05	100	0.87	0.09	2.44	3.41	1.88
17.74	24	72.3	1.78	44.2	34.9		100		0.12	3.33	4.19	2.86
22.38	30.8	87.7		61.7	38.4		100		0.22	4.63	4.88	5.06
20.33		81.4			56		100			6.58	6.3	8.64
							100					13.3
19.37	25	85.3	1.42	49.5	39.3	0.05	100	0.87	0.14	4.25	4.69	6.34
2.825	5.45	11.6	0.51	10.6	12	###	0	###	0.07	1.8	1.23	4.67

Serum Mercenaria 4951

Length of total X-axis	263
No of harmonics	5
Length of each harmonic	52.5

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
198	198	199	124	164	211	0	148	195	126	198	178	231
			124	185		0	150		160			
			138			0	190					
						0						
						0						

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
0.017	0.02	0.02	0.44	0.07	0.01	100	0.15	0.02	0.4	0.02	0.04	0
			0.44	0.03		100	0.14		0.09			
			0.24			100	0.02					
						100						
						100						
0.017	0.02	0.02	0.37	0.05	0.01	100	0.1	0.02	0.24	0.02	0.04	0
####	####	####	0.12	0.03	###	0	0.07	###	0.22	###	###	###

Serum Neothyris 427

Length of total X-axis	266
No of harmonics	4
Length of each harmonic	66.5

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
29	120	65.5	31	41.5	60	76.5	86.5	0	59	44.5	113	30
25		58	31.5	32	45	68	67.5	0	46.5	40		21
26		74	34.5			46.5	69	0	30	54		7
						40		0	12			-6
								0	10			-17

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
36.64	1.57	10.4	34.2	23.8	12.5	7.07	5	100	13		2	35.4
42.08		13.4	33.6	33	21.1	9.49	9.66	100	20	21.4		48.3
40.65		7.71	30.3			20	9.17	100	35.4	25		78.5
						25		100	66	15.4		123
								100	70.7			177
39.79	1.57	10.5	32.7	28.4	16.8	15.4	7.94	100	41	20.6	2	92.5
2.821	###	2.86	2.1	6.55	6.03	8.53	2.56	0	26.3	4.86	###	58.1

Serum Notosaria 4910

Length of total X-axis	268
No of harmonics	5
Length of each harmonic	53.5

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
145	145	145	104	124	126	145	145	145	0	126	122	145
			102	189					0	129	112	
									0		174	
									0			
									0			

D.s.	G.v.	K.r.	L.r.	L.n.	L.u.n.	M.m.	M.t.	N.l.	N.n.	P.p.	T.r.	W.i.
0.196	0.2	0.2	1.14	0.48	0.44	0.2	0.2	0.2	100	0.44	0.52	0.2
			1.25	0.03					100	0.4	0.81	
									100		0.06	
									100			
									100			
0.196	0.2	0.2	1.2	0.26	0.44	0.2	0.2	0.2	100	0.42	0.46	0.2
####	####	####	0.08	0.32	###	###	###	###	0	0.03	0.36	###

Serum Pictothyris 1192
Length of total X-axis 267
No of harmonics 5
Length of each harmonic 53.4

D.s.	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
11.3	98	44.4	- 2	62	78.5	160	47	-0.5	129	0	65	15.5
16.2	103	37	7	66.5	74		37	-1.5	125	0	63.7	18
114	98.7	28.8	10.6	42	69.3		25.2		119	0	59	15.7
	99	25		38					109	0	60	
		17.3							104	0		

D.s.	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
61.45	1.47	14.8	109	6.92	3.4	0.1	13.2	102	0.39	100	6.08	51.3
49.76	1.18	20.3	74	5.7	4.12		20.3	107	0.46	100	6.43	46
0.736	1.42	28.9	63.3	16.4	5.05		33.8		0.61	100	7.87	50.8
	1.4	34.1		19.5					0.91	100	7.54	
		47.5							1.16	100		
37.32	1.37	29.1	82.1	12.1	4.19	0.1	22.4	104	0.71	100	6.98	49.4
32.21	0.13	12.7	23.9	6.83	0.83	###	10.4	3.19	0.32	0	0.86	2.9

Serum T.retusa 4962
Length of total X-axis 251
No of harmonics 6
Length of each harmonic 43.5

D.s	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
70.5	120	91	59	70.5	110	207	91	53	104	75.5	0	67.5
65.5	111	86	56	65	103		86.5	48.5	96	63.5	0	58.5
	105	81	49.5		95		83	44	91	53.5	0	52
			43		90		87	38		43.5	0	58
										37.2	0	51

D.s.	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
2.395	0.17	0.81	4.4	2.4	0.3	0	0.81	6.05	0.41	1.84	100	2.81
3.121	0.28	1.05	5.16	3.2	0.43		1.03	7.67	0.62	3.47	100	5.03
	0.39	1.37	7.28		0.65		1.24	9.74	0.81	5.89	100	6.38
			10.3		0.85		1	13.4		10	100	4.64
										14	100	6.72
2.758	0.28	1.08	6.78	2.8	0.56	0	1.02	9.23	0.61	7.03	100	5.11
0.513	0.11	0.28	2.63	0.57	0.25	###	0.17	3.19	0.2	4.94	0	1.56

Serum Waltonia 5040
Length of total X-axis 263
No of harmonics 5
Length of each harmonic 52.7

D.s.	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
1	172	69.3	-3.5	130	188	221	155	-2.5	219	10	77	0
4		64.3		129				- 3		10	67	0
4.6		63.7		118							72	0
		59		115							74	0
												0

D.s.	G.v.	K.r.	L.r.	L.n.	Lu.n.	M.m.	M.t.	N.t.	N.n.	P.p.	T.r.	W.t.
95.72	0.05	4.83	117	0.34	0.03	0.01	0.11	112	0.01	64.6	3.45	100
83.95		6.01		0.36				114		64.6	5.34	100
81.78		6.17		0.57							4.29	100
		7.58		0.67							3.93	100
												100
87.15	0.05	6.15	117	0.48	0.03	0.01	0.11	113	0.01	64.6	4.25	100
7.561	###	1.13	###	0.16	###	###	###	1.74	###	0	0.8	0