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MOLECULAR SYSTEMATICS OF RECENT AND PLEISTOCENE BRACHIOPODS

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Thesis submitted for the degree of Doctor of Philosophy

Department of Geology and Applied Geology
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March 1992

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For my father, Juitsu Endo (1917-1988)

Acknowledgements

This project was funded by NERC, the Royal Society of London, and the University of Glasgow. I also gratefully acknowledge the financial support of a postgraduate scholarship from Glasgow University, the ORS award, the Ikueikai scholarship (Japan), the Systematics Association (U.K.), and the Shimadzu Foundation (Japan).

Even more than most Ph.D theses written by overseas students, this thesis owes its very existence to the help and encouragement of many people, and I would like to thank them all. Special thanks are due to Dr. Gordon B. Curry, for his supervision and constant intellectual support, and also to Dr. G. B. Curry and Mrs. Gillian Curry for all their kindness outside the department from my very first day in the U.K. I would like to thank the staff and technicians of the Department of Geology and Applied Geology, with thanks to Professor Bernard E. Leake for the use of facilities and to Mr. Roddy Morrison for technical support. Many thanks are due also to the staff and technicians of the Department of Genetics, Glasgow University, for facilitating the work on *Lingula* DNA. I am especially grateful to Dr. Howard T. Jacobs for allowing me to use his laboratory facilities, and for his excellent advice and suggestions. It is a privilege to thank Sir Alwyn Williams for his encouragement and for generously allowing me to occupy one of his visitor's offices, which was essential for the preparation of this thesis. Mrs. Sue Ogden is thanked for facilitating the work in that office.

I am grateful to Dr. Rosalind Quinn for the preparation of antisera and for her advice on immunological experiments. Many thanks to Dr. Matthew Collins and Dr. Gerard Muyzer who provided a number of brilliant ideas, technical advice, and brachiopod antisera. I would like to thank Professor Peter Westbroek, who is the source of stimulating ideas, for his encouragement. I am indebted to Dr. Maggie Cusack for her advice on every aspect of biochemical experiments, to Derek Walton for conducting amino acid analysis, and to both of them for critically reading the draft of this thesis. Thanks also to Mrs. Ikbal Al-Rikabi for her help in electrophoresis experiments, and to Stephen Ewing for some brachiopod antisera. I am grateful to Suzanne Forbes and Sandra McCormack for technical support. Thanks are due to the people in the 'Howy lab', especially Dr. Sohail Qureshi and Dr. Abdellatif El-Meziane for their patience in instructing molecular biology from scratch to a geologist.

I would like to thank Professor Itaru Hayami, Professor Kiyotaka Chinzei, Dr. Kazushige Tanabe, Dr. Katsumi Abe, and Dr. Tatsuo Oji for their encouragement and advice throughout my career both in Glasgow and in Tokyo.

At the Natural History Museum, Smithsonian Institution, U.S.A., I would like to thank Dr. Richard Grant and Dr. Rex Doescher, for their help and hospitality during the month of my working stay there, and for providing specimens for immunology. I am indebted to Dr. Tomowo Ozawa, Dr. Daphne Lee, Mr. Eiji Tsuchida, and Mr. Masahiro Kiuchi for the donation of brachiopod samples. My appreciation goes also to many other researchers who have kindly provided samples for the Molecular Palaeontology laboratory. I am grateful to Dr. David MacKinnon for the access to his unpublished translation of Russian literature, and for constructive discussions.

At the sampling trip in New Zealand, I express my gratitude to every member of the caravan brigade, Dr. G. B. Curry, Dr. Spaff Ackerley, Derek Walton, and Heather Clegg, for the memorable month.

I would like to thank my mother Shiho Endo, Mr. Kenichi Maeda, Mrs. Hagiko Maeda and my brothers for their constant support and encouragement.

Finally, very special thanks to Shuko Endo and Wakana Endo who provided me with most support and encouragement.

Declaration

The material presented in this thesis summarises the results of three years of research carried out in the Department of Geology and Applied Geology of Glasgow University under the supervision of Dr. Gordon B. Curry. This study is based on my own independent research and any previously published or unpublished results of other researchers used in this thesis have been given full acknowledgement in the text.

Kazuyoshi Endo
March 1992

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Abbreviations

Au	-absorbance unit
d_I	-immunological distance
d_I'	-quasi-immunological distance
DNA	-deoxyribonucleic acid
E 280 nm	-UV absorbance (extinction) at the wave length of 280 nm
EDTA	-ethylenediamine tetra-acetic acid
ELISA	-enzyme linked immunosorbent assay
hplc	-high performance liquid chromatography
ICZN	-International Commission on Zoological Nomenclature
Ma	-million years ago
mRNA	-messenger RNA
mtDNA	-mitochondrial DNA
MUP	-4-methylumbellifenyl phosphate, dilithium salt
MW	-molecular weight
PAGE	-polyacrylamide gel electrophoresis
PCR	-polymerase chain reaction
RFLP	-restriction fragment length polymorphism
RIA	-radioimmunoassay
RNA	-ribonucleic acid
rRNA	-ribosomal RNA
SDS	-sodium dodecylsulphate
TBAP	-tetrabutylammonium phosphate
TBS	-tris buffered saline
TFA	-trifluoroacetic acid
Tris	-tris (hydoroxymethyl) amino ethane
tRNA	-transfer RNA
Tween 20	-polyoxyethylen sorbitan monolaureate
UPGMA	-unweighted pair-group method with arithmetic mean

Units

°C	-degrees centigrade
g	-grammes
g	-centrifugal force equivalent to gravitational acceleration
h	-hours
kb	-kilo base pairs
kDa	-kilo dalton
M	-Molar
min	-minutes
ml	-millilitres
µl	-microlitres
mm	-millimetres
µm	-micrometres
nm	-nanometres
mM	-milliMolar
Myr	-million years
pmol	-picomoles
sec	-seconds
yr	-years

Three-letter abbreviation of the common amino acids

One letter code in brackets

Gly (G)	-Glycine	Cys (C)	-Cysteine
Ala (A)	-Alanine	Asn (N)	-Asparagine
Val (V)	-Valine	Gln (Q)	-Glutamine
Leu (L)	-Leucine	Tyr (Y)	-Tyrosine
Ile (I)	-Isoleucine	Trp (W)	-Tryptophan
Met (M)	-Methionine	Asp (D)	-Aspartate
Phe (F)	-Phenylalanine	Glu (E)	-Glutamate
Pro (P)	-Proline	His (H)	-Histidine
Ser (S)	-Serine	Lys (K)	-Lysine
Thr (T)	-Threonine	Arg (R)	-Arginine

Definitions of immunological terms

(After Wilson and Goulding, 1986)

Adjuvant:- A substance that increases the biosynthesis of antibody in response to antigens.

Antibody:- A protein, with the molecular properties of an immunoglobulin, capable of specific combination with the antigen that caused its production in a susceptible animal.

Antigen:- Any foreign substance that elicits an immune response (e.g. the production of specific antibody molecules) when introduced into the tissues of a susceptible animal and which is capable of combining with the specific antibody molecules produced. Antigens are usually of high molecular weight and are commonly either proteins or polysaccharides.

Antigenic determinant or epitope:- A small site on an antigen to which a complementary antibody molecule may be specifically bound through its combining site. This is usually 1 to 6 monosaccharides or amino acid residues on the surface of the antigen, not necessarily covalently linked to each other.

Antiserum:- A serum containing antibodies against a specific antigen or antigen mixture; for example anti-ovalbumin serum, or anti-sheep erythrocyte serum.

Avidity:- The net combining power of an antibody molecule with its antigen.

Clone:- A family of cells of genetically identical constitution derived asexually from a single cell by repeated division.

Monoclonal antibody:- Immunoglobulin derived from a single clone and therefore homogeneous.

Polyclonal antiserum:- An antiserum, possibly to a single antigen, containing a number of antibodies to that antigen from different plasma cell clones (all antisera are in fact polyclonal).

Summary

Enzyme linked immunosorbent assay (ELISA) of shell intracrystalline proteinaceous macromolecules has been applied to investigate the phylogenetic relationships among 53 living articulate brachiopods (Class Articulata), covering all the living superfamilies and most of the living families. One of the articulate superfamilies, the Cancellothyridoidea, has been investigated by a combined immunological and morphometric approach, with additional materials to include most of the living species of *Terebratulina*, which is one of the most prolific among the Cenozoic brachiopod genera. The immunological techniques have also been applied to the phylogenetic investigation of Pleistocene brachiopods, including 2 extinct species. Both living and fossil brachiopod shell intracrystalline macromolecules have been analysed using various biochemical techniques.

Antisera prepared against 14 living and taxonomically-diverse species allowed ordinal and superfamilial discriminations within the class, and using the more specific pre-absorbed antisera, it was possible to obtain precise species-level information with taxonomic consistency. These experiments revealed that the examined living terebratulide families belonged to one of the following four groups (expressed in provisional superfamilial denotations), which were further clustered into three major groups (provisionally considered as subordinal rank) of a trichotomous relationship: (A) Cancellothyridoidea (short-looped):- Cancellothyrididae, Chlidonophoridae; (B1) Terebratuloidea (short-looped):- Terebratulidae, Dyscoliidae; (B2).'Kraussinoidea' (long-looped):- Kraussinidae, Megathyrididae, Macandreviidae, Ecnomiosidae; (C) Terebratelloidea (long-looped):- Terebratellidae, Laqueidae, Dallinidae.

These results were compared and intercalated with both morphological data and the fossil record reaching the following phylogenetic interpretation: The ancestors of the three groups radiated in the early Devonian, and each of those gave rise to the group A, B1, and C in the Mesozoic; in the Triassic another long-looped lineage (possibly the extinct Zeilleriidae) diverged from the short-looped B1 lineage, and this long-looped stock gave rise to the Kingenidae and group B2, probably by processes of neoteny. This scenario suggests that the long loop evolved at least twice independently in the Terebratulida, and also highlights the enigmatic origin of the Cancellothyridoidea.

Among the Cancellothyridoidea, 21 living species and subspecies of *Terebratulina* were assigned into two major phylogenetic groups, which were further divided into 7 subgroups on the basis of the immunological, morphometric, and other data. Degrees of molecular variations between some *Terebratulina* species were comparable with those between families in other terebratulide superfamilies, suggesting the existence of deep branching events within the living *Terebratulina*.

Fractionations of *Terebratulina* shell intracrystalline macromolecules by various liquid chromatography techniques revealed several discrete components, including at least one proteinaceous component, most of which were antigenic. Some of these

components were revealed to have been preserved more or less intact in a Pleistocene sample, in terms of the molecular weight, amino acid composition, and antigenic properties. The fossil sample also contained degradation and condensation products.

Immunological assays on Pleistocene materials from a series of horizons indicated a progressive decay of the macromolecules through time, but the novel serial antisera concentration method demonstrated that even 1 Myr old shells contained lineage-specific molecular information, which allowed family- to species-level phylogenetic reconstructions for the extinct terebratulide species, *Kikaithyris hanzawai* and an undescribed species of *Terebratulina*.

Outline of the thesis

The first two chapters constitute the introductory part of this thesis. **Chapter 1** introduces historical aspects and some current topics of Molecular Palaeontology to outline this novel branch of science, from which the idea of this thesis originated. **Chapter 2** describes the traditional taxonomy of the phylum Brachiopoda and some previous morphological and molecular studies on brachiopods. **Chapter 3** summarises the materials and techniques utilised in this study. The three subsequent chapters deal with the studies on Recent materials. The nature of the intracrystalline macromolecules of living brachiopod shells is investigated in **Chapter 4**. The phylogenetic relationships of living brachiopods are discussed in **Chapter 5** (among the Class Articulata) and in **Chapter 6** (among the Superfamily Cancellothyridoidea), considering the evidence of immunological response, morphology, morphometry, and the fossil record. The following two chapters deal with the studies on fossil materials, with the preservation of the brachiopod intracrystalline macromolecules in fossil shells being examined in **Chapter 7**, and the phylogenetic affiliation of a Pleistocene extinct species discussed in **Chapter 8**, on the basis of both immunological and morphometric data. Finally **Chapter 9** discusses possible extensions of this project.

Chapter 1 Molecular approaches in Palaeontology

1.1 Introduction

The 'molecule' concept has dominated 20th century science. Molecular approaches have altered or enriched the way we look at materials, natural phenomena, and even life itself. Today, the molecular approach can even be extended to the study of fossil material, and is clearly becoming an important part of Palaeontology.

The primary source of molecular information lies in the outermost lithosphere. Most of the organic materials produced by living creatures are consumed through food chains, degraded by bacteria, and then recycled. However, some proportion of this organic matter is known to 'escape' these recycling processes, moving from the biosphere to the lithosphere and becoming trapped in sediments. The amount of organic carbon accumulated in such a way in the sedimentary record since the onset of photosynthesis on Earth is enormous, estimated as about 10,000 times more than that in the present biomass (Ourisson *et al.* 1984). A number of fossil remains, such as shells, bones, and leaves, contain remnants of original organic materials, and provide a relatively small, but often better preserved, subset of this vast organic reservoir in the lithosphere.

Retrieval of molecular information from the lithosphere is a difficult task, as most of these molecules are degraded, condensed, or sparsely distributed. Despite these obvious complications, the value of this kind of information, as direct molecular clues to the past, are readily apparent, and justify the effort required. Recent technological advances in analytical chemistry, organic chemistry, biochemistry, and molecular biology have allowed the recovery and identification of various kinds of biological compounds from the complex mixtures of the ancient organic materials found in rocks and fossils. The aim, both of the broad field of Molecular Palaeontology and of this thesis in particular, is to utilise such molecules to provide geological and biological information.

The other source of information for Molecular Palaeontology is the present-day biosphere. The present may be regarded and studied as one of the cross sections of geological time. It is vital to investigate molecules from living organisms to act as references for the identification of fossil molecules. There is another rationale for studying extant molecules in Palaeontology, as unlike other non-biological molecules, biomolecules have an evolutionary history as old as life itself, and biological linear polymers can, and do, carry a large quantity of information (Runnegar, 1988). This leads to a notion that evolutionary histories are 'hallmarked' in biomolecules.

Indeed, genomic DNA, revealed as the molecule of heredity, is now considered to contain three basic kinds of information: genetic information, a developmental programme, and historical information (Gehring, 1985). Historical information, reflecting organismal

evolution, primarily records the history of the molecules themselves, although it can also be extrapolated to examine the history of the host organisms (a history which is obviously of great interest to palaeontologists).

The term 'Molecular Palaeontology' was first coined by Calvin (1968) for a study of the history of life using fossil biomolecules preserved in the geological record, and later used by Doolittle (1985) in an entirely different context to denote the inference of phylogenetic relationships from living proteins. The term has since been reintroduced as a wider topic involving all aspects of the study of both fossil and living biological molecules of geological and biological interest (Runnegar, 1986; Curry, 1987a, b). Under this broad definition, Molecular Palaeontology constitutes a conglomeratic field of research, which concerns a wide range of subjects including origins of life, organismal phylogeny, molecular evolution, biomineralisation, origins of fossil fuels, diagenesis, geochronology, reconstruction of palaeoenvironments, study of global environment, etc.

In the following sections, a few, mostly biological, aspects of this extensive field are introduced. For recent compilation of knowledge in this and related disciplines consult Degens (1989), Eglinton and Curry (1991), Runnegar and Schopf (1988), and Westbroek and de Jong (1983).

1.2 Molecular fossils

The organic compounds recovered from ancient sediments and characterised as having retained historical attributes of their biological origin have variously been called molecular fossils, chemical fossils, biomarkers (biological marker compounds), or geochemical fossils. In the context of Molecular Palaeontology, the term 'molecular fossils' is preferred to denote those ancient biomolecules, and will also embrace biological molecules isolated from fossils.

Due to its importance for industrial applications, chemical characterisation of petroleum and coal started early in this century. Many authors cite the work of Treibs (1934) describing metal porphyrins, analogues of chlorophyll *a* in plants, from many crude oils and shales as the starting point of this research. Since that time, organic geochemistry has been leading the study of organic materials in Recent and ancient sediments (Eglinton and Murphy, 1969; Mackenzie *et al.*, 1982; Johns, 1986). Major candidates for molecular fossils are lipids, pigments, carbohydrates, proteins, and nucleic acids, all of which are common constituents of living cells. Because of their chemical stability in the geological environments, the derivatives of lipids and pigments, which have extremely stable hydrocarbon skeletons, have been commonly recovered and studied in the field of organic geochemistry.

The progress of this field was promoted by the developments of various analytical methods, such as chromatography techniques, and especially combined gas chromatography-mass spectrometry (GCMS). With the sophistication of these instruments, wide varieties of organic compounds have been isolated and identified from the extremely complex mixtures of organic fractions found in the sedimentary rocks. In addition to the geological interests, such as the characterisation of fossil fuels, depositional environments, and diagenetic processes, biological aspects became one of the driving motivations in organic geochemistry (Calvin, 1969) as those ancient organic compounds were increasingly linked to known molecules of the living biota.

One of the most important and dramatic impacts of organic geochemistry has been the recovery of various kinds of bacterial lipids from oils and sediments. Indeed one group of bacterial lipids, the biohopanoids, were first discovered as hydrocarbon skeletons, or hopanoids, from oils and sedimentary rocks, occurring in abundance and with remarkable ubiquity. These molecules were only subsequently identified in living organisms during a deliberate search to find their origins. It is now clear that biohopanoids are important constituents of eubacterial cell membranes (Ourisson *et al.*, 1984). Another group of bacteria, archaebacteria, have entirely different kinds of membrane lipids from those of eubacterial or eukaryotic cells, and it is suggested that these archaebacterial lipids are the major precursor molecules of many acyclic isoprenoids, commonly found in oils and sediments (Volkman and Maxwell, 1986). These findings not only led to the suggestion of a bacterial origin of oils (Ourisson *et al.*, 1984), but also suggest the use of organic geochemistry to study bacterial ecology and evolution.

Molecular fossils of the information-rich classes of biomolecules, i.e., proteins and nucleic acids, have relatively recently been recovered from fossilised hard tissues, which provide protective micro-environments for the entombed molecules, and from remains of soft tissues preserved in special environments, using biochemical, immunological, and molecular biological techniques.

1.3 Fossil proteins

Abelson (1954) reported amino acids from Palaeozoic shells, and proposed Paleobiochemistry as a study on biological compounds, especially amino acids, preserved in fossils. Subsequently, Abelson and other workers have identified fossil amino acids from various sources of different ages (e.g. Abelson, 1955; 1956; Wyckoff, 1972; Hare *et al.*, 1980).

The studies of fossil amino acids have been making contributions to Geology through the applications of racemisation of amino acids preserved in Quaternary fossils for the use as geochronometer and geothermometer, and through the studies of amino

acids in sediments and meteorites (Hare *et al.*, 1980; Zhao and Bada, 1989) for inferring terrestrial and extra-terrestrial environments.

Attempts have also been made to elucidate biological information from fossil amino acids, but, as biological information is stored in the sequence of those building blocks, the compositional data have inevitably had limited use for this purpose. The direct sequencing of fossil proteins would solve the problems, but there is only one unpublished instance known to science (reported in Muyzer *et al.*, 1984), and it is a challenging project still to be achieved (Curry, 1988).

The presence of peptides in fossil samples has been reported, based on biochemical experiments rather than direct sequencing, from bones and teeth up to 100 Myr old (Wyckoff, 1972), from molluscan shells 80 Myr old (Weiner *et al.*, 1976), from dinosaur egg shells (Krampitz *et al.*, 1977), and from Quaternary bones (Tuross *et al.*, 1980; Armstrong *et al.*, 1983).

In the 1970's, major technological advances occurred in immunochemistry, including the development of extremely sensitive techniques for assaying immunological reactions, such as ELISA and RIA (cf. Lowenstein, 1985; Johnstone and Thorpe, 1987). In 1974, immunological techniques were first applied to detect preserved antigenic determinants in fossils, using molluscan shells more than 70 Myr old (de Jong *et al.*, 1974).

Table 1.1 Immunological studies on fossil macromolecules

References	Materials	Max. age	Target molecules
de Jong *et al.* (1974)	molluscan shell	70 Myr	shell extracts
Westbroek *et al.* (1979)	molluscan shell	late Cret.*	shell extracts
van der Meide *et al.* (1980)	molluscan shell	late Cret.	shell extracts
Prager *et al.* (1980)	mammalian soft tissue	40000 yr	albumin
Lowenstein (1980a, b; 1981)	human bone	1.9 Myr	collagen, albumin
Lowenstein *et al.* (1981)	mammalian soft tissue	40000 yr	albumin
Lowenstein *et al.* (1982)	jaw of Piltdown man	Recent	collagen
Loy (1983)	prehistoric tools	6000 yr	haemoglobin
Westbroek *et al.* (1983)	molluscan shell	10000 yr	shell extracts
Muyzer *et al.* (1984)	molluscan shell	10000 yr	shell extracts
Shoshani *et al.* (1985)	muscle & bone, mammals	53000 yr	collagen, albumin
Huq *et al.* (1985)	avian bone	Pleistocene	osteocalcin
Muyzer *et al.* (1986)	microbial mats	2000 yr	bacterial debris
Rowley *et al.* (1986)	bone, aves & mammals	Miocene	collagen
Ulrich *et al.* (1987)	mammalian bone	30 Myr	osteocalcin
Rothschild and Turnbull (1987)	mammalian bone	11500 yr	treponemal antigen
Muyzer *et al.* (1988)	molluscan shell	1 Myr	shell extracts
Muyzer and Westbroek (1989)	molluscan shell	75000 yr	shell extracts
Lowenstein *et al.* (1991)	mammalian urine	20000 yr	albumin
Collins *et al.* (1991a)	brachiopod shell	2 Myr	shell extracts

*Cret. = Cretaceous

Later works indicate that this approach is remarkably effective in surveying fossil macromolecules, including proteins (**Table 1.1**).

In immunological approaches, antibodies are prepared, using usually rabbits or mice, against, in most cases, macromolecules from extant organisms homologous to the fossil molecules of interest. The degree of molecular similarity between those homologous molecules is then measured by the extent of immunological reactivities between these antibodies and the antigens isolated from fossils. Immunology has successfully been applied to detect fossil macromolecules of various ages, to solve various phylogenetic problems of recently extinct mammals (see Lowenstein, 1986 for a review), and to the study of preservation and degradation of macromolecules over geological time (Muyzer *et al.*, 1984; Muyzer, 1988; Lowenstein and Scheuenstuhl, 1991; Collins *et al.*, 1991c).

One advantage of the immunological approach is that, since the antibodies are raised against small regions of primary or three-dimentional antigenic structures (i.e. the antigenic determinants), it allows the recovery of both structural and compositional information, even from more or less degraded fossils molecules. Another advantage is that, since antibodies recognise antigenic determinants with very high specificity, they can detect target molecules out of the complex mixtures of degraded fossil compounds. Contamination is one of the major obstacles faced in the study of molecular fossils. For example, Armstrong *et al.* (1983) suggested that most of the proteins recovered from pre-Quaternary bones are of bacterial origin. Because of the specificity of antibodies, immunology can be applied even to those contaminated samples, provided the antibodies used are carefully selected.

Exploiting these features, antibodies can be used for the *in situ* localisation of target molecules in fossils (Muyzer and Westbroek, 1989). It has also been suggested that antibodies could also be utilised for the purification of fossil molecules through affinity chromatography (Ulrich *et al.*, 1987; Muyzer, 1988).

1.4 Fossil DNA

The recovery of fossil DNA had been considered hopeless until very recently, as the polymeric skeletons of DNA contain sugar groups, which are considered to be chemically unstable in diagenetic processes (Calvin, 1968). However, developments in molecular biology have opened up the possibility of studying fossil DNA.

The presence of fossil DNA was first recognised in the early 1980's (E. M. Jope, 1980). Higuchi *et al.* (1984) successfully sequenced, using ordinary molecular cloning techniques, a fragment of mitochondrial DNA isolated from Quagga samples, 140 years old, and obtained phylogenetic information of this extinct creature. Pääbo (1985) cloned and sequenced a part of human nuclear DNA from an Egyptian mummy 2400 years old.

Ancient DNA were also isolated from mammoths up to 53,000 years old (Johnson *et al.*, 1985) and from human brains 8000 years old (Doran *et al.*, 1986). Despite these novel findings, the range of application to palaeontological materials and the amount of obtainable biological information still seemed to be limited.

A major breakthrough in the study of fossil DNA was the development of PCR (polymerase chain reaction, Saiki *et al.*, 1985; Mullis and Faloona, 1987; PCR mediated by a heat resistant enzyme, Saiki *et al.*, 1988), which, with its innovative ability to amplify a region of DNA million-fold in a few hours *in vitro*, has rapidly spread to many fields of life sciences (White *et al.*, 1989).

Using PCR, it became possible to amplify a specific DNA region of particular interest, starting from virtually any amount of template DNA. It has also been pointed out that a set of template DNA with different damaged positions may work complementarily to eventually provide a complete template DNA after initial amplification cycles ("jumping PCR"; Pääbo *et al.*, 1990). Therefore PCR can be applied to ancient DNA better than ordinary cloning approaches, in which the damage sometimes cause spurious results, or makes manipulations of DNA impossible (Pääbo and Wilson, 1988; Pääbo, 1989; Pääbo *et al.*, 1989; 1990).

Pääbo *et al.* (1988) amplified and sequenced a specific region of mitochondrial DNA, a region which is polymorphic in extant human populations, from a 7000 years old human brain excavated in Florida, and concluded that the individual, from which the DNA originated, belonged to a population which is different from the two populations known to have inhabited prehistoric North America.

Subsequently, a series of successful amplifications of ancient DNA, in many cases with good biological confirmations, has been reported from, for example, Maize ears 1000 years old (Rollo *et al.*, 1988), soft tissues of a marsupial wolf 90 years old (Thomas *et al.*, 1989), human bones up to 6000 years old (Horai *et al.*, 1989; 1991; Hagelberg and Sykes, 1989; Hagelberg *et al.*, 1991; Hänni *et al.*, 1990; Hummel and Herrmann, 1991), and museum samples of various animal taxa up to 80 years old (Kocher *et al.*, 1989; Thomas *et al.*, 1990).

According to the review of ancient DNA by Pääbo *et al.* (1989), the oldest DNA confirmed as authentic is from a mammoth muscle, 40,000 years old. Pääbo *et al.* (1989) proposed Molecular Archaeology as a field of research on ancient DNA for the study of evolution of genes and populations, and on the genealogical relationships of extinct species.

In 1990, a piece of chloroplast DNA from a Miocene fossil leaf (17-20 Ma), was reported to be extracted, amplified, and sequenced (Golenberg *et al.*, 1990). This sensational result is still a matter of debate (see Golenberg, 1991; Sidow *et al.*, 1991; Pääbo and Wilson, 1991; Sykes, 1991), but if this is verified, it is considered to open up a new phase in Molecular Palaeontology.

1.5 Molecular Systematics

Two major kinds of evolutionary information may be obtained from biological molecules, in both living and fossil samples. One is the information regarding temporal change of the molecules, or molecular evolution, elucidated by comparing molecules from various creatures of known, or assumed, organismal ancestry. The other is the information regarding organismal phylogeny, deduced from comparisons of molecules between different taxa which carry them. These two are certainly closely related, but they are quite distinct and must therefore be distinguished explicitly in order to avoid circular arguments. It is the inference of the latter type of information, or 'Molecular Systematics' (Hillis and Moritz, 1990), that is the main concern of this thesis.

The development of the idea of applying molecular variations to phylogenetic reconstruction may be regarded as a by-product of the rapid advances of life sciences in the 1960's, although the pioneering immunological study greatly predates such advances (Nuttall, 1904). Early studies in this field were largely concerned with proteins. After a series of advances in manipulating nucleic acids in the 1970's, elucidation of data from nucleic acids has been gaining dominance (**Table 1.2**; see Hillis and Moritz, 1990, for an overview).

The currently used methods in molecular systematics involve isozyme electrophoresis, immunological methods, protein sequencing, DNA/DNA hybridisation, DNA restriction site analysis, nucleic acids sequencing, and analysis of chromosome structure and number (Hillis and Moritz, 1990). In addition to these methods, comparisons of metabolic and biosynthetic pathways can be used for phylogenetic inferences (Florkin, 1966; Berry and Jensen, 1988). These techniques have their own advantages and drawbacks, in terms of the range of phylogenetic applications and their cost effectiveness, and appropriate techniques must be chosen for each particular systematic problem (Hillis and Moritz, 1990).

The advantages of molecular approaches over more traditional methods include (1) the large size of the potential data sets (all heritable information of an organism is encoded in DNA), (2) very small involvement of nonheritable variations, and (3) unlimited distribution of characters in phylogenetic scale (Hillis, 1987). It has also been claimed that molecules can be utilised to date the time of divergence among organisms, because molecules evolve at constant rates (i.e. molecular clock hypothesis, Zuckerkandl and Pauling, 1962; reviewed by Wilson *et al.*, 1977; Thorpe, 1982; Nei, 1987). However, the molecular clock has been, and still is, one of the major controversies in molecular systematics (Clegg, 1990; Lewin, 1990; Melnick, 1990; Wolpoff and Thorne, 1991), and further qualifications seem to be required. Molecular Palaeontology may provide a new ground to test this hypothesis through the molecular study of organisms with excellent fossil records, such as marine invertebrates with mineralised skeletons, on which little has been studied in this light.

Table 1.2 Selected events relevant to molecular systematics

Technical events list either the first development of the technique or the first application of the technique to systematic problems.

Year	Technical	Conceptual
1900's	precipitin test (Nuttall, 1904)	
1950's	protein sequencing (Sanger, 1952)	structure of DNA (Watson and Crick, 1953) genetic distance (Sanghvi, 1953)
1960's	immunodiffusion (Goodman, 1962)	molecular clock (Zuckerkandl and Pauling, 1962)
	isozyme electrophoresis (Harris, 1966; Lewontin and Hubby, 1966) microcomplement fixation (Sarich and Wilson, 1966)	immunological distance (Sarich and Wilson, 1967)
	Edman degradation protein sequencing (Edman and Begg, 1967)	neutral theory (Kimura, 1968)
1970's	DNA hybridisation (Kohne, 1970) restriction enzymes Southern hybridisation (Southern, 1975)	modified genetic distance (Nei, 1972)
	DNA sequencing (Sanger *et al.*, 1977; Maxam and Gilbert, 1977) RIA, ELISA molecular cloning	archaebacteria (Woese and Fox, 1977)
1980's	DNA data base PCR (Saiki *et al.*, 1988)	RFLP (Botstein *et al.*, 1980)
1990's		molecular systematics labs in Natural History museums

Exploiting these advantages, molecular approaches have been bringing about a renaissance in the phylogenetic reconstruction of various organisms at every taxonomical level (see Fernholm *et al.*, 1989 for a recent summary of findings). Ribosomal RNA (rRNA), for example, has been used for the study of branching events, some of which correspond to the highest category in systematics (e.g. in bacterial evolution), as rRNA molecules retain conserved structures related to their crucial role in protein synthesis (Olsen *et al.*, 1986; Woese, 1987; Hori and Osawa, 1987; Lake, 1988). In the other extreme, some DNA sequences in mitochondrial DNA (mtDNA) evolve rapidly, and have been utilised for the study of population genealogy within single species (e.g., Cann *et al.*, 1987).

Ribosomal RNA sequencing approaches have recently been applied to the study of

metazoan evolution (5S rRNA; Ohama *et al.*, 1984; Hori and Osawa, 1987; 18S rRNA; Field *et al.*, 1988; Ghiselin, 1988; Ghiselin, 1989; Raff, 1988; Raff *et al.*, 1989; Patterson, 1989; Sogin *et al.*, 1989; Lake, 1989; 1990; 28S rRNA; Christen *et al.*, 1991). Whether or not metazoa are of monophyletic origin has been one of the main arguments (Walker, 1989; Bode and Steele, 1989; Patterson, 1989; Patterson, 1990; Lake, 1991; Barnes, 1991), and one that remains unresolved, although several results of interest to palaeontologists have already been generated by this work. For example, many metazoan groups form a very close cluster in the most phylograms, suggesting that the 'explosive' Cambrian radiation is genuine (Valentine and Erwin, 1987; McMenamin, 1988; Erwin, 1991). Bilateria and Eucoelomates are monophyletic (Raff, 1988; Ghiselin, 1988). Monophyly of Chordata, Echinodermata, Mollusca, and Arthropoda is supported by most studies, while the paraphyly of Arthropoda and Mollusca is suggested by Lake (1990). The embryological categories of deuterostomes and protostomes are supported by Patterson (1989) and Lake (1990), although a tetrachotomous relationship among the Echinodermata, Chordata, Arthoropoda, and other protostomes (Mollusca, Polychaeta, Oligochaeta, Pogonophora, Sipuncula, and Brachiopoda) is also reported (Raff, 1988; Ghiselin, 1988). Other studies (Patterson, 1989; Lake, 1990) confirm the result that Brachiopoda, the phylogenetic position of which has been embryologically thought as somewhat transitional between deuterostomes and protostomes, is closely related to the protostome mollusc-annelid group.

As the molecular approach to systematics developed and expanded, it caused conflicts and controversies with more traditional methods, such as morphological approaches. However, these conflicts now seem to be converging to a consensus that both molecular and traditional approaches have their own advantages and disadvantages, and that the coordination between these approaches is the better choice (Patterson, 1987; Hillis, 1987; Sytsma, 1990; Hillis and Moritz, 1990). This trend is indicated by the fact that major prominent Natural History museums in the world have launched molecular systematics laboratories in the last few years (Gibbons, 1991).

1.6 Conclusions

Molecular fossils and living biomolecules provide invaluable information for the study of the history of life and the Earth. Molecular approaches in palaeontology are not only fruitful for palaeontology, but also rewarding to many other related fields of biological, chemical, and geological sciences. In the biological context, for example, the molecular researches on well documented fossils may provide not only additional knowledge on the phylogeny of the organisms, but also a chance to study patterns and processes of molecular evolution directly, using the fossil record.

In the study presented in this thesis, molecular approaches have been applied to the phylogenetic reconstruction of fossil and Recent Brachiopoda in an attempt to incorporate molecular data with traditional data regarding this classical group of organisms in evolutionary Palaeontology.

Chapter 2 The Brachiopoda

2.1 Introduction

Brachiopods are solitary, marine, filter-feeding invertebrates, most of which are sessile epifaunal benthos, commonly anchored to the substratum by a fleshy stalk, or pedicle. Some burrowers and various free-living recliners are known from both living and fossil brachiopods, and a swimming life habit has been suggested for some extinct forms (Rudwick, 1970). Brachiopods constitute one of the most important phyla that can be studied by the palaeontologists. Brachiopods have bivalved chitinophosphatic or calcareous shells, and as a result are often preserved in geological history with exquisite morphological, including ultrastructural, details (Williams, 1968a). The fossil record of brachiopods extends back to the early Cambrian, subsequent to which they flourished to become one of the most dominant and diversified macrobenthos in the Palaeozoic seas. Among the lineages that survived the terminal Permo-Triassic event, the terebratulide brachiopods have undergone considerable diversification to the present-day to provide an abundant and continuous fossil record. Other lineages provide excellent examples of 'living fossils', which have experienced, as documented by the fossil record, extremely conservative morphological evolution, with the genus *Lingula* (Silurian-Recent) among the most famous. This exceptionally long and continuous recorded history is almost unrivalled, which, together with the fact that brachiopods attained remarkable diversification in form and life habit within the same body plan, while some retain their basic features for an unusually long time, makes this phylum suitable and intriguing for a study of evolutionary processes.

In the sections below, a brief overview of the phylum is presented, followed by descriptions of general morphology, classification, and phylogenetic interpretations, with an emphasis on the Order Terebratulida, to which most of the material collected for this study belong. Finally, the problems addressed by this study are considered.

A detailed account of brachiopod Palaeontology and Biology is found in the two volumes of *Treatise on Invertebrate Palaeontology* (Williams *et al.*, 1965; revision is due in 1994). Recent collections of works can be found in the proceedings of the two international conferences on Brachiopoda (Racheboeuf and Emig, 1986; MacKinnon *et al.*, 1991).

2.2 Origin of Brachiopoda

Previous studies concerning the origins of brachiopods can be summarised, according

to Williams (1965), as follows:- In 1758, Linnaeus, in his tenth edition of Systema Naturae, described brachiopod species as a member of bivalvia. Cuvier (1800-05) proposed the familial group of 'Brachiopodes', which was formalised by Duméril in 1806, to distinguish brachiopods from bivalvia, but the Brachiopoda was still considered as a member of the Mollusca. Hancock (1850) carried out a comparative studies on bryozoans, and suggested that they were closely related to both tunicates and brachiopods. This led Huxley (1853) to place brachiopods in Molluscoides with bryozoans and tunicates. Morse (1870) advocated that the Brachiopoda should be grouped with the Annelida, rather than with Mollusca. Meanwhile, Kovalevskiy (1866; 1867) demonstrated the chordate affinities of tunicates and the precise nature of phoronid development, and Nitshe (1869) separated the ectoproct and entoproct bryozoans. These findings led Caldwell (1882) to infer that ectoprocts, phoronids, brachiopods, and sipunculoids were closely related, a relationship which was later supported by Blochmann (1892) and Haeckel (1896), while Hatschek (1888) thought it most satisfactory to exclude the sipunculoids from this assemblage, and erected phylum Tentaculata, which embraced three classes, the Brachiopoda, Ectoprocta, and Phoronida. It was Hatschek's arrangement that became generally accepted in the next century, usually with the classes elevated to phyla and grouped under the designations of Tentaculata, Lophophorata (Hyman, 1959) or an emended Molluscoidea.

The origins of a taxon is always a difficult question to answer. In particular the investigation of the relationships between groups of higher categories, such as phylum and class, is difficult by using solely morphological characters of embryos and adults, because each of the divisions of this level is generally grouped up on the basis of fundamental morphological construction which is characterised by a number of features, and the morphological characters consistently shared between each group are not numerous. There are still three main areas of debate concerning the origin of the Brachiopoda.

Firstly, although the monophyly of the Brachiopoda has been assumed and widely believed (Williams *et al.*, 1965; Rudwick, 1970; Williams and Hurst, 1977), there is a polyphyletic view claiming that the shell acquisition in various early inarticulate brachiopod lineages occurred many times independently (Valentine, 1973; Wright, 1979; reviewed by Rowell, 1982 and Willmer, 1990).

Secondly, the brachiopods have been grouped with the ectoproct bryozoans and the phoronids, due to having several features in common, most notably the presence and the similar structure of a feeding organ, the lophophore, as well as shared embryological characteristics. The most popular view of the relations between the lophophorates is that the primitive phoronids gave rise to both brachiopods and bryozoans. However, there is another view that the brachiopods are ancestral to the bryozoans, which then gave rise to the phoronids (Emig, 1982; see Willmer, 1990 for a review).

Finally, and perhaps most problematically, lophophorates have been thought of as

an intermediate between protostomes and deuterostomes, on the basis of embryology, with a relationship either of the following three, (1) a link from protostomes to deuterostomes, (2) a link from deuterostomes to protostomes, or (3) a separate independent offshoot from acoelomates (Willmer, 1990). However, as discussed in the previous chapter, recent data on 18S rRNA sequences support neither of these, but indicate that brachiopods emerged from the protostome lineage well after the diversification of the protostomes and deuterostomes. The sequence data also suggests that brachiopods are closely related to Annelida, Pogonophora, and Sipuncula (Ghiselin, 1988; and Mollusca in Lake's phylogram, Lake, 1990). If this is true, it recalls Morse's contention and also the view by Gutmann *et al.* (1978), who argue on the basis of biomechanical analysis, that brachiopods are derived (through a phoronid-like stage) from metameric polychaete-like forms. These interpretations explain the presence of annelid-like setae in brachiopods. The distribution of respiratory pigments among invertebrates (Willmer, 1990) indicate a close link of Brachiopoda to Annelida, Sipuncula, and Priapulida by the presence of hemerythrin, a fact which could not be explained by the traditional schemes, but is concordant with the rRNA sequence data (e.g. Field *et al.*, 1988). However, the presence of haemoglobin in Phoronida, a fact which was thought to indicate the intermediate nature of lophophorates between protostomes and deuterostomes, poses problems; for example, to the interpretation of the 'phoronid-like immediate ancestor of brachiopods' or even to the status of 'lophophorates', if both the rRNA scheme and the orthologous evolution of hemerythrin are accepted.

Obviously the molecular data is still limited in the range of samples studied, e.g., Brachiopoda has always been represented by a single species of *Lingula*. However the accumulation of further molecular data may help to solve many of the above problems.

2.3 Main divisions of Brachiopoda

Fossil remains of brachiopods were well known in the early Middle Ages, and they were figured as early as in the late 16th century (Muir-Wood, 1955). The history of the classification of the Brachiopoda as a distinct group started with the work by Cuvier in the beginning of the 19th century. During the 19th century, many aspects of brachiopods were explored by a number of researchers, for example, their anatomy (Owen, Huxley, Hancock, Blochmann), embryology (Lacaze-Duthiers, Kovalevskiy, Morse, Muller, Shipley), and fossil record (Davidson, Hall), and in 1891, Beecher synthesised a classification for the phylum (Williams, 1965). By this time, many of the fundamental differences, such as the presence or absence of teeth and sockets, and the presence or absence of anal aperture, which divide the Brachiopoda into two major groups (inarticulates and articulates), had been discovered. Subsequently, a number of classifications have been proposed on the basis of a variety of morphological characters, and those classifications

Table 2.1 Outline of brachiopod classification in the *Treatise* (Williams *et al.*, 1965) with stratigraphic distribution

Extant taxa in bold. Abbreviations: Cam. = Cambrian, Ord. = Ordovician, Dev. = Devonian, Perm. = Permian, Trias. = Triassic, Jur. = Jurassic, Rec. = Recent, L. = Lower, M. = Middle, U. = Upper.

Class Inarticulata L.Cam.-Rec.
- **Order Lingulida L.Cam.-Rec.**
- **Order Acrotretida L.Cam.-Rec.**
- Order Obolellida L.Cam.-M.Cam.
- Order Paterinida L.Cam.-M.Ord.

Class Uncertain
- Order Kutorginida L.Cam., ?M.Cam.

Class Articulata L.Cam.-Rec.
- Order Orthida L.Cam.-U.Perm.
- Order Strophomenida L.Ord.-L.Jur.
- Order Pentamerida M.Cam.-U.Dev.
- **Order Rhynchonellida M.Ord.-Rec.**
- Order Spiriferida M.Ord.-Jur.
- **Order Terebratulida L.Dev.-Rec.**
- Order Uncertain (Suborder Dictyonellidina) M.Ord.-Perm.
- **Order Uncertain (Suborder Thecideidina) Trias.-Rec.**

were reviewed by Muir-Wood (1955), and re-synthesised in the *Treatise* (Williams *et al.*, 1965; **Table 2-1**).

The phylum is divided into two classes, that are distinguished by shell composition, muscle arrangement, development of articulation and lophophore support, presence or absence of an anus, development of pedicle, and mantle reversal during larval stages of growth (Williams and Hurst, 1977). Both classes emerge in the early Cambrian as separate groups. Rudwick (1970) transferred the Kutorginida from Class Uncertain to Class Inarticulata in view of the lack of true articulation. Cooper (1967) and Rudwick (1970) proposed the separation of Spiriferida into two orders, i.e., Spiriferida and Atrypida, which differ in the directions of the spiralium coiling, but Williams and Hurst (1977) regarded this separation unnecessary. Rudwick (1970) also advocated the replacement of Thecideidina from Order Uncertain to Order Strophomenida. Williams and Hurst (1977) proposed that it was derived from the Spiriferida (as defined in the *Treatise*), though stating that "the Thecideidina is currently the most controversial aspect of brachiopod evolution". Pajaud (1970) erected the Order Thecideida, subsequently

supported by Cooper (1988).

Indeed, taxonomy reflects the underlying phylogenetic interpretations, and in the context of this study, where Recent articulates belonging to Rhynchonellida, Terebratulida, and Thecideidina were available, the competing phylogenetic interpretation can be formulated in the following ways: either (1) Thecideidina comes as the outgroup of the other two (strophomenide origin of the Thecideidina), or (2) Rhynchonellida comes as the out group of the other two (spiriferide origin of the Thecideidina). In both views, it is assumed that the Rhynchonellida is ancestral to the Terebratulida.

2.4 Morphology of terebratulide brachiopods

2.4.1 General comments

The brachiopod shell is normally bilaterally symmetrical about the longitudinal median plane, and consists of two dissimilar valves, from one of which emerges the pedicle, and is called the pedicle (ventral) valve. The other valve is typically smaller and is referred to as the brachial (dorsal) valve. The beak region, which normally represents the first formed part of each valve, is posterior. The articulates are pre-eminently characterised by a pair of teeth in the pedicle valve which fit into a pair of sockets in the brachial valve. The shell of inarticulates is chitinophosphatic or calcareous, and that of articulates is calcareous (almost always in the form of calcite). The shells of articulates typically have triple stratified layers, and in most articulates, these layers consist of an outermost periostracum, an outer crystalline primary layer, and an inner fibrous secondary layer (Williams, 1968b). A tertiary layer is developed in some taxa (e.g., the Family Terebratulidae among terebratulide brachiopods; MacKinnon and Williams, 1974). Terebratulida is an articulate order, and is characterised by the presence of the calcareous brachidium, in the form of a loop, which supports the lophophore. The shell of terebratulides is always perforated by punctae (a condition called punctate). Terebratulides are also characterised by the existence of spicules in the mesoderms of the mantle and lophophore (Williams and Hurst, 1977).

Many of the descriptions in the following subsection are adapted from the *Treatise* (Williams *et al.*, 1965), unless otherwise stated.

2.4.2 External features

Outline of the shell:- The most common shape for terebratulides is a 'tear-drop' shape, with variations such as circular, oval, triangular, pentagonal, etc. Valves are often nearly equally convex, though the brachial valve may be planar, concave, or sulcate

posteriorly. Folding of the valves varies from rectimarginate to sulcate, plicate, and combinations of the latter two. The posterior margin of the shell is non-strophic (non parallel with hinge axis), and usually narrow, although some genera have a broad cardinal margin.

Ornament of the shell:- The shell surface is commonly smooth, although concentric growth lines, and radial ribs are sometimes conspicuous. Fine and coarse ribs are called capilla and costa, respectively. Spinose granular ornamentation develops in some taxa.

Foramen:- The foramen is a subcircular to circular perforation of shell through which the pedicle passes. The size of foramen varies from a pinhole to very large. The position of foramen relative to the apex and beak-ridges varies from hypothyridid (foramen is on the dorsal side of the beak ridges), mesothyridid (on the beak ridges), permesothyridid (almost on the apex), and epithyridid (wholly on the dorsal side of the apex). The deltidial plates may be conjunct, or disjunct; in the latter cases, the foramen is referred to as incomplete. The incurvature of the beak varies from nearly straight to strongly incurved.

Coloration of the shell:- The natural coloration of the shell is variable in intensity and pattern. The colour may be red, yellowish, warm brown, dull brown, greyish, or pure white. The pattern may be dots, sprayed streaks, radial streaks, marginal (concentric), gradational, or uniform (Hatai, 1940).

Thickness of the shell:- The thickness of the shell varies between taxa from very thin, in which case the valves are translucent, to very thick (more than 5 mm in thickness in some genera).

2.4.3 Internal features

Lophophore:- The lophophore, the brachiopod feeding organ (which may also assist in respiration), is a filamentous appendage suspended from the anterior body wall, extending into the mantle cavity. The amount of nutrition and oxygen that the lophophore can absorb may be related to the surface area of the organ. The geometric solution that many terebratulides adopt to maximise the surface area in the limited volume of mantle cavity is the *plectolophe*, which has a pair of U-shaped side arms with medially placed spirals. Some genera adopt *ptycholophe*, in which a pair of arms (or brachia) are folded into one or more lobes. A few genera have *spirolophe*, in which a pair of brachia is spirally coiled. Juveniles and some adults of certain genera have less elaborate lophophores, such as *zygolophe* (brachia consists of straight or crescentic U-shaped side arms), *schizolophe* (a pair of brachia with anteromedially indented termini), or trocholophe (lophophore disposed as ring surrounding mouth).

Loop:- The loop, the support for lophophore, is composed of secondary shell and extends anteriorly from the crura as a closed apparatus, variably disposed and generally ribbon-like, with or without supporting a septum from the floor of brachial valve. Three

major types of loop have been recognised: (1) the centronelliform loop, which is simple, variable in length, and suspended free of the valve floor, commonly bearing median vertical plate, (2) the short loop, which develops directly from the cardinalia, and (3) the long loop, which develops in connection with both the cardinalia and median septum. Typical forms and the names of the parts of the latter two types are shown in **Figure 2.1** and **Figure 2.2**. A variety of terminology has been used to denote forms of juvenile and adult loops of various taxa. A simple terminology with descriptive, rather than generic, adjectives was proposed for terebratelloid brachiopods by Richardson (1975), and is reproduced here (**Table 2.2**).

Spicules:- Some terebratulide brachiopods have spicules within the connective tissue of the mantle and lophophore. Both the density and shape of the spicules varies depending on the species.

Hinge teeth:- The hinge teeth in the pedicle valve are composed of secondary shell, and are commonly supported by a pair of variably disposed plates, also built up of secondary shell, and known as dental plates.

Dental plates:- The dental plates butress the hinge teeth of the ventral valve in some taxa. Since dental plates, if there are any, develop in the very early stages of the shell growth, the presence or absence of them is often considered to be valuable in the higher-level classification of terebratulides (Baker, 1972; Smirnova, 1984).

Table 2.2 The description of loop phases by Richardson (1975)

1. Axial phase: the formation of the septum, the hood, and the rudiments of the descending branches.

2. Annular phase: the presence of a hood in place of a ring and complete descending branches, each structure being separately attached to the septum.

3. Haptoid phase: the anterior fusion of the attachments of the ring and the descending branches.

4. Bilacunar phase: the presence of two lacunae in the dorsal segments of the band forming the ring.

5. Diploform phase: the fusion of the attachments of the ring (completely free of the septum) with the descending branch attachments to form descending branches with doubled anterior limbs.

6. Bilateral phase: the presence of two pairs of connecting bands, lateral and latero-vertical.

7. Latero-vertical phase: the presence of latero-vertical connecting bands only.

8. Trabecular phase: the presence of lateral connecting bands only.

9. Teloform phase: the absence of any connecting bands so that the loop is free of the septum.

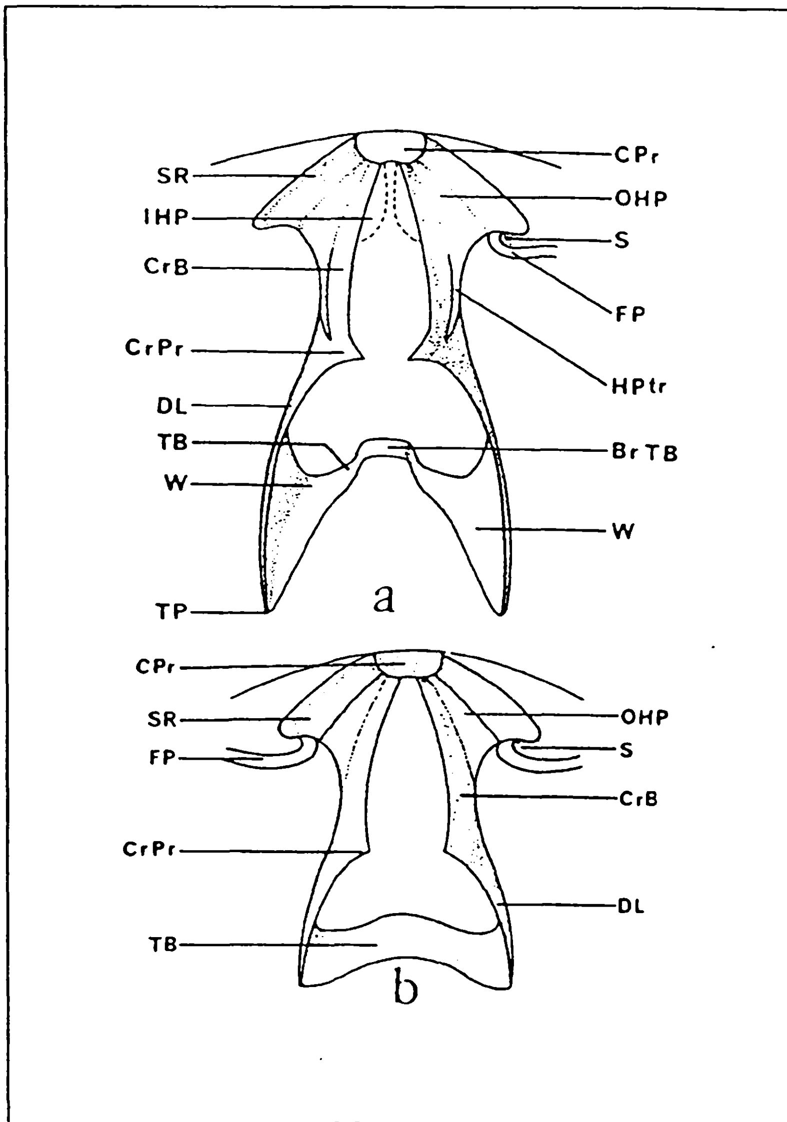


Figure 2.1 Pattern and elements of the short loop (after Cooper, 1983)

a, Jurassic terebratuloid with long terminal points; b, hypothetical view of a Recent terebratuloid. Abbreviations: BrTB = bridge of the transverse band and transverse band; CPr = cardinal porcess; CrB = crural base and crus; CrPr = crural process; DL = descending lamella; FP = fulcral plate; HPtr = outer hinge plate taper; IHP = inner hinge plate; OHP = outer hinge plate; S = socket; SR = socket ridge (inner socket ridge to some authors); TB = transverse band; TP = terminal point ; W = web.

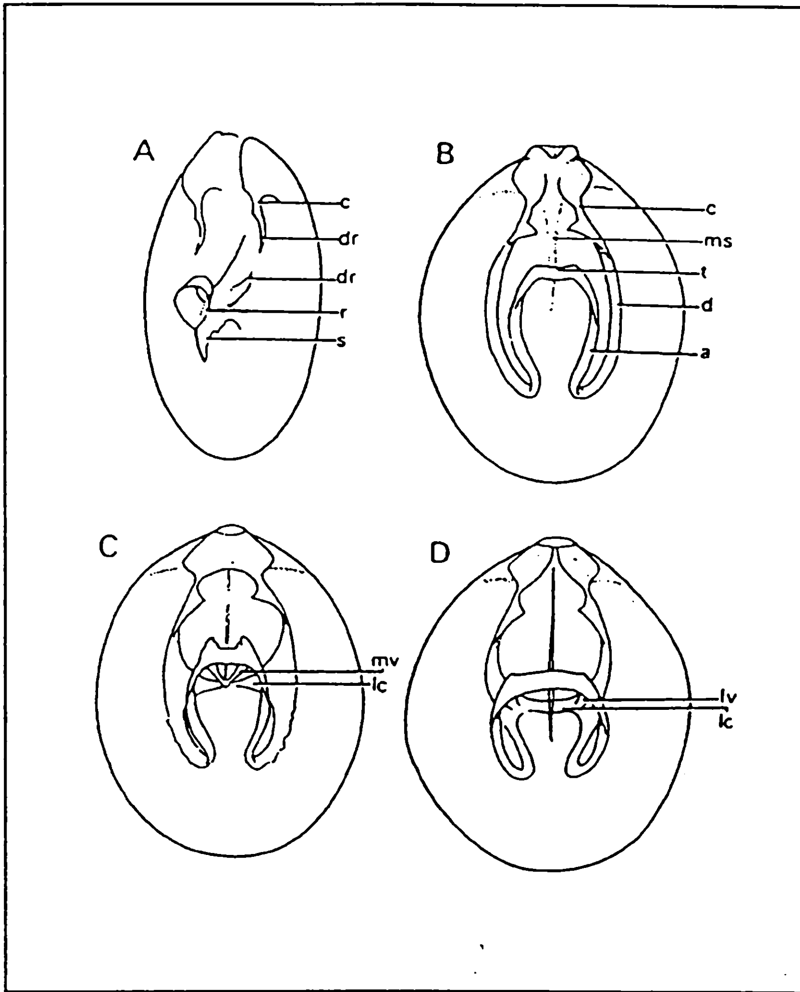


Figure 2.2 Pattern and elements of the long loop (after Richardson, 1975)

A, the loop rudiments; B, a loop free of the septum as in *Magellania insolita* (Tate); C, a loop with lateral and medio-vertical connecting bands as in *Paraldingia woodsii* (Tate); D, a loop with lateral and latero-vertical connecting bands as in *Frenulina pumila* (Tate). Abbreviations: a = ascending branches; c = crura; d = descending branches; dr = descending branch rudiments; lc = lateral connecting bands; lv = latero-vertical connecting bands; ms = median septum; mv = medio-vertical connecting bands; r = ring; s = septal pillar; t = transverse band.

Cardinalia:- The cardinalia are the outgrowths of secondary shell in the posteromedian region of the brachial valve, and are connected with articulation, support of lophophore, and muscle attachment, including, for example, the cardinal process, socket ridges, crural bases and their accessory plates (cf. **Figure 2.1**). Hinge plates are developed in many genera, and are used for the attachment of diductor muscles and dorsal pedicle muscles. Inner hinge plates are located median of crural bases and fused laterally with them. Outer hinge plates separate inner socket ridges and crural bases.

Muscles:- Adductor muscles contract to close the shell. Two adductor muscles, each dividing dorsally, are commonly present, to produce single pair of scars located between diductor impressions in pedicle valve and two pairs (anterior, posterior) in brachial valve. Diductor muscles serve to open the valves, and commonly consist of two pairs attached to the hinge plates, or cardinal process, of the brachial valve. Pedicle muscles, or adjustor muscles, are associated with the pedicle, and are attached to the hinge plates, or in the Cancellothyridoidea, to the valve floor (Cooper, 1973c).

2.4.4 Shell structure

Punctation:- Punctae accommodate caeca, extensions of outer mantle epithelium, with distal brushes (Owen and Williams, 1969). There are some variations in the density of punctation and in the profile of punctae between taxa.

Periostracum ultrastructure:- The terebratulide periostracum originates within a slot, separating the lobate and vesicular cells of the outer mantle lobe, and three fundamentally different types have been recognised (Williams and Mackay, 1978). The simplest type consists of a uniformly textured layer, which can be correlated to an undifferentiated basal layer. The second type has the basal layer and an overlying complex superstructure, which is made up with a labyrinthine structure (amalgamated structure of proteinaceous succession of vesicules and secretion droplets), covered by fibrillar proteinaceous rods. The third type consists of proteinaceous sheets secreted as a series of isoclinal folds normal to the basal layer (Williams and Mackay, 1978).

2.5 Phylogeny and taxonomy of terebratulides based on morphology

Terebratulide phylogeny has been "overwhelmingly expressed in the evolution of the loop" (Williams and Hurst, 1977, **Figure 2.3**), as has the taxonomy. In the *Treatise* (Williams *et al.*, 1965), the Terebratulida was divided into three suborders, Centronellidina, Terebratulidina, and Terebratellidina based mainly on the loop morphology, corresponding to the centronelliform loop, the short loop, and the long loop, respectively (**Table 2.3**). The Centronellidina is thought to have given rise to both the Terebratulidina and the

Terebratellidina (**Figure 2.3**) in the early Devonian. The Centronellidina became extinct at the end of the Palaeozoic, and a new superfamily from the Terebratulidina and two new superfamilies from the Terebratellidina diverged at the beginning of the Mesozoic, and one superfamily of each suborder has survived to the present (**Table 2.3**).

Since the publication of the *Treatise*, knowledge about Recent brachiopods has increased considerably, notably through the work of Cooper (1972, 1973a-c, 1975, 1977, 1981a,b, 1982, 1983). Cooper (1973c) recognised a fundamental difference in the way the pedicle muscles are attached to the dorsal shell in the short loop family Cancellothyrididae, in which the muscles are attached to the valve floor rather than to the

Table 2.3 Classification of the Order Terebratulida, with stratigraphic range, according to *Treatise*

Extant taxa in bold. Cret. = Cretaceous; Eoc. = Eocene; Mio. = Miocene; see Table 2.1 for other abbreviations.

Suborder Centronellidina L.Dev.-Perm.

  Superfamily Stringocephalacea L.Dev.-U.Perm., 4 families

Suborder Terebratulidina L.Dev.-Rec.

  Superfamily Dielasmatacea L.Dev.-U.Trias., ?L.Jur., 5 families

  Superfamily Terebratulacea U.Trias.-Rec., 8 families

    Family Terebratulidae U.Trias.-Rec.

    Family Dyscoliidae ?U.Jur., U.Cret.(Cenomanian)-Rec.

    Family Cancellothyrididae ?L.Jur.-?M.Jur., U.Jur.-Rec.

    and 5 other extinct families

Suborder Terebratellidina L.Dev.-Rec.

  Superfamily Cryptonellacea L.Dev.-Perm., 1 family

  Superfamily Zeilleriacea Trias.-L.Cret., 2 families

  Superfamily Terebratellacea U.Trias.-Rec.

    Family Megathyrididae U.Cret.-Rec.

    Family Platidiidae Eoc.-Rec.

    Family Kraussinidae Mio.-Rec.

    Family Dallinidae U.Trias.-Rec.

    Family Terebratellidae L.Cret.-Rec.

    Family Laqueidae Mio.-Rec.

    Family Uncertain Jur.-Rec.

Suborder, Superfamily, and Family Unknown, 2 genera, Eoc. & Mio.

Figure 2.3 Interpretation of terebratulide phylogeny based on the loop morphology and ontogeny (after Williams and Hurst, 1977)

Table 2.4 Classification of Recent terebratulides according to Cooper (1972 *et seq.*)

Suborder Terebratulidina
- Superfamily Terebratulacea
  - Family Terebratulidae
  - Family Dyscoliidae
- Superfamily Cancellothyridacea
  - Family Cancellothyrididae
  - Family Chlidonophoridae
  - Family Cnismatocentridae

Suborder Terebratellidina
- Superfamily Terebratellacea
  - Family Terebratellidae (included in the Dallinacea in Cooper, 1978)
  - Family Megathyrididae
  - Family Platidiidae
  - Family Kraussinidae
  - Family Thaumatosiidae
- Superfamily Dallinacea
  - Family Dallinidae
  - Family Laqueidae
  - Family Macandreviidae
  - Family Ecnomiosidae

Table 2.5 Classification of the post-Palaeozoic Terebratulida according to Smirnova (1984)

Taxa only found in Palaeozoic in italic, extant taxa in bold.

Superfamily Dielasmatoidea
- *Family Cranaenidae*
- Family Dielasmatidae
- Family Aulacothyrioideidae

Superfamily Terebratuloidea
- Family Nucleatidae
- Family Weberithyrididae
- **Family Cancellothyrididae**
- **Family Terebratulidae**
- Family Orthotomidae
- Family Gibbithyrididae

Superfamily Cryptonelloidea
- *Family Notothyrididae*
- *Family Cryptonellidae*
- *Subfamily Cryptacanthiinae*
- Family Zeilleriidae

Superfamily Dallinoidea
- Family Aulacothyropsidae
- Family Clathrithyrididae
- **Family Dallinidae**
- **Family Laqueidae**
- **Family Kingenidae**
- **Family Macandreviidae**
- **Family Diestothyrididae**

Superfamily Loboidothyroidea
- Family Loboidothyrididae
- Family Boreothyrididae
- Family Dictyothyrididae
- Family Spasskothyrididae
- Family Tegulithyrididae
- Family Chenoiothyrididae

Superfamily Terebratelloidea
- **Family Terebratellidae**
- **Family Megathyrididae**
- **Family Platidiidae**
- **Family Kraussinidae**

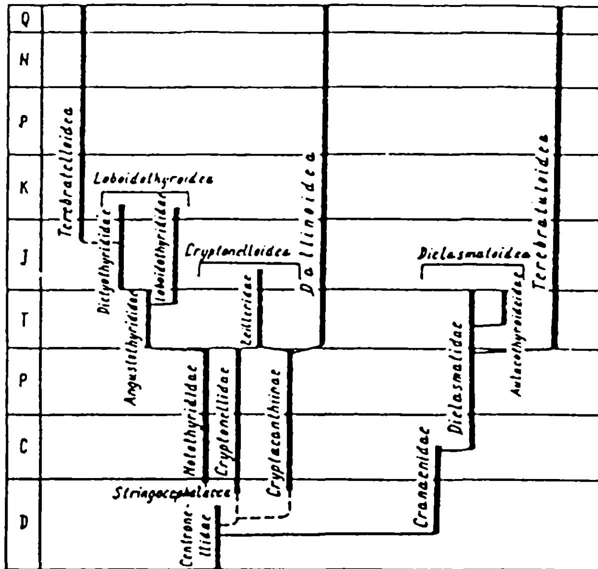


Figure 2.4 Interpretation of terebratulide phylogeny by Smirnova (1984)
Key: D = Devonian, C = Carboniferous, P = Permian, T = Triassic, J = Jurassic, K = Cretaceous, P = Palaeogene, N = Neogene, Q = Quaternary

hinge plate as in other articulates. On the basis of this muscle attachment and the characteristic features of the cardinalia, Cooper (1973c) erected a new superfamily Cancellothyridacea (**Table 2.4**). The long loop superfamily was divided into two superfamilies, the Terebratellacea and the Dallinacea (e.g., Cooper, 1979), presumably on the basis of the loop and the presence or absence of dental plates (original description of the diagnosis is not available). Richardson (1975) suggested that four families (the Kraussinidae, the Platidiidae, the Megathyrididae, and the Thaumatosiidae) are different from the Terebratellidae, the Dallinidae, and the Laqueidae in many respects, but doubted that the similarities between the former four families were sufficient to group them separately from the latter three families. Indeed, the loop patterns of the Kraussinidae, the Platidiidae, and the Megathyrididae are aberrant, and they have been thought to be neotenously derived from other terebratelloid lineages (**Figure 2.3**). Thaumatosiids are also aberrant in that they have no loop (Cooper, 1973a). Adding to these families of terebratelloid brachiopods, Zezina (1981) described another aberrant family, the Phaneroporidae, in which the ascending branches of the loop are absent. Richardson (1975) reviewed the loop development of the Terebratellidae, the Dallinidae, and the Laqueidae, and made a number of emendments, notably the enlargement of the family Laqueidae. In this arrangement, the Laqueidae include, for example, *Macandrevia*, *Diestothyris* and the Kingeninae, all of which were formerly included in the Dallinidae

(Richardson, 1975). In the latest account of the traditional classification of brachiopods, Smirnova (1984) assigned the terebratulide genera into six superfamilies (**Table 2.5; Figure 2.4**). The familial separations of the Dallinidae, Laqueidae, Macandreviidae, Kingenidae, and the new family Diestothyrididae were recognised, and they were included in the superfamily Dallinoidea, distinguishing this superfamily from another extant long-looped superfamily Terebratelloidea which embraced the Family Terebratellidae, Megathyrididae, Platidiidae, and Kraussinidae (Smirnova, 1984; **Table 2.5**).

2.6 Previous molecular studies on the Brachiopoda

Ayala *et al.* (1975), using isozyme electrophoresis, reported very low genetic variation in the terebratuloid species, *Liothyrella notorcadensis*. Hammond and Poiner (1984) revealed a higher variability in the inarticulate brachiopod, *Lingula*, using isozyme electrophoresis, and also demonstrated small genetic distances between three populations of *Lingula* from localities up to 1200 km apart, and concluded that the populations, which had been believed to be assigned to two species, belong to a single species, *L. anatina* Lamarck. Cohen *et al.* (1986, 1991) studied the genetic divergence between and within the species of the Cancellothyridoid genus, *Terebratulina*, using isozyme electrophoresis and restriction fragment analyses on mitochondrial DNA.

M. Jope (1980; and references therein) reported amino acids from various fossil and Recent brachiopod shells. Curry and Ansell (1986) demonstrated that up to 50% of the tissue mass of living adult brachiopods is situated within the shell. Subsequently, organic macromolecules situated within crystals of the fibrous secondary layer of articulate brachiopod shells were demonstrated, using immunological methods, to contain significant taxonomic information (Collins *et al.*, 1988). This initial result has been further strengthened by the immunological surveys by later studies (Curry *et al.*, 1991c, see **Appendix II-4**; Collins *et al.*, 1991b). One of the most interesting findings of these phylogenetic studies was that the division of the terebratulide brachiopods into the long loop and short loop suborders may be wrong. This result has been difficult to interpret in terms of the traditional morphological schemes, because the long-looped genera (e.g., *Kraussina*) and the short-looped genera (e.g., *Liothyrella*), which were demonstrated by immunological methods, to be closely linked, are radically different not only in the loop structure but also in a few other morphological features (see Brunton and Hiller, 1990). Collins *et al.* (1991b) proposed a new interpretation of terebratulide phylogeny based on the immunological data (**Figure 2.5**). In the later part of this thesis, the immunological view of the terebratulide phylogeny is re-examined based on the published data (**Appendix II-4**) and unpublished new data (cf. **Chapter 5**). Combined molecular and morphometric approaches have been applied to the investigation of the relationships within the brachipod genus

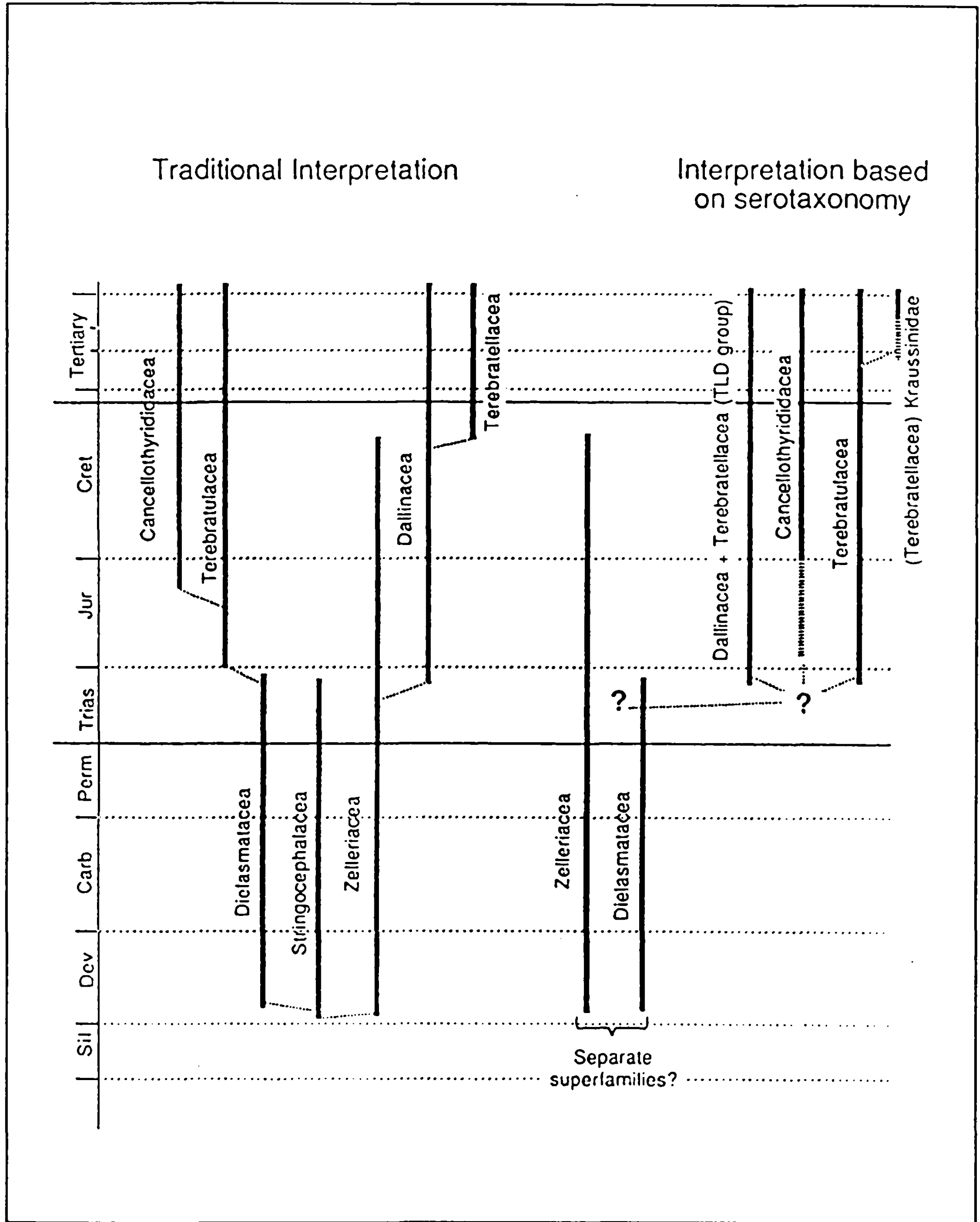


Figure 2.5 A comparison of terebratulide phylogeny interpreted from traditional and immunological data (after Collins *et al.*, 1991b)

Terebratulina (Endo and Curry, 1991, see **Appendix II-1**; Cohen *et al.*, 1991), and in the demonstration that a species of this genus, *T. retusa*, has migrated rapidly from the Iberian Peninsula to Spitzbergen, Norway in the last 10,000 years, in response to climatic change (Curry and Endo, 1991, see **Appendix II-5**).

Shell intracrystalline macromolecules are expected to be preserved even in fossil shells, because the surrounding mineral phase would protect the molecules from ground water leaching, microbial attacks, or it might stabilise the molecules to minimise structural degradation. Collins *et al.* (1991a) reported the detection of superfamily-level information from up to 2 Myr old brachiopod shells using immunological techniques. Indigenous amino acids have been recovered from fossil brachiopod shells collected from a series of horizons of the New Zealand Tertiary (Curry *et al.*, 1991b; Walton and Curry, 1991).

Biochemical characterisations of brachiopod shell intracrystalline macromolecules have demonstrated that the shell fibres of the articulate brachiopods contain proteins, lipids (Curry *et al.*, 1991b), and carbohydrates (Collins *et al.*, 1991c). Characterisation of the protein fraction has been carried out, using immunoassays, hplc, SDS-PAGE, amino acid analyses, and N-terminal amino acid sequencing (Curry *et al.*, 1991a, see **Appendix II-2**; Curry *et al.*, 1991b, see **Appendix II-3**; Cusack *et al.*, 1992). The organic fraction involved several species of protein, including a pigment associated protein, and a cell adhesion, RGD-motif, containing protein (Curry *et al.*, 1991a, b; Cusack and Curry, 1991; Cusack *et al.*, 1992).

Chapter 3 Materials and methods

3.1 Materials

3.1.1 Chemicals and equipment

Chemicals:- Methanol, acetonitrile and TFA of analytical grade were purchased from Applied Biosystems, EDTA from Boehringer Mannheim, BCL, and TBAP from Pierce. General chemicals and buffer materials were purchased from Sigma. Milli-Q™ water (Millipore) was used throughout the experimental procedures.

Ultrafiltration:- An Amicon filtration system and PM10 filters (10 kDa cutoff) was used to concentrate the macromolecules from the intracrystalline extract and remove low molecular weight material such as salts.

Immunisation:- Complete and incomplete Freund's adjuvant were purchased from Difco Laboratories, syringes and needles were from Becton Dickinson Ltd., and the Rocket syringes were supplied from Vicarey Davidson.

ELISA:- Microfluor plate reader, plate shaker-incubator, microplate washer, and black flat-bottom microtitre plates were supplied by Dynatech. The second antibody [goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase] was from Sigma, fluorescent substrate [4-methylumbelliferyl phosphate dilithium salt (MUP)] was from Boehringer Mannheim, BCL.

Liquid chromatography:- The Waters 650 Advanced Protein Purification System, a liquid chromatography system, which comprises the Waters 490 Programmable Multiwavelength Detector, the NEC Powermate 1 personal computer and the Maxima 820 Chromatography Workstation, with the Gilson FC203 fraction collector, was used for the sample injection, pumping control, gradient formation, sample collection, data acquisition, and data analysis. Gel filtration columns were from Pharmacia (Superose 6, Superose 12) and Waters (300SW). Reverse phase columns were from Pharmacia (ProRPC HR 5/2) and Waters (Delta Pak C18 300A). Anion exchange columns were from Pharmacia (Mono Q) and Waters (DEAE-5PW). Cation exchange columns were from Pharmacia (Mono S) and Waters (SP-5PW). A hydrophobic interaction column was from Waters (Phenyl 5PW)

3.1.2 Brachiopod samples

Samples of 53 living articulate brachiopod species (4 rhynchonellides, 2 thecideidines, and 47 from terebratulides; **Table 3.1**), and fossil samples of 8 terebratulide species (**Table 3.2**) were collected for this study, and samples of 64 populations of 25 living cancellothyridoid species and subspecies (**Table 3.3**) stored in the Natural History Museum of the Smithsonian Institution, U.S.A., were also utilised.

The samples of the living species comprised both wet samples preserved in 70% ethanol or 10% formalin solution with soft body tissues and samples of dried shells without soft tissues. Fragmentary shells of obvious identity were also utilised for the immunological assays. Only dried complete specimens were employed in the morphometric analyses. Most of the Pleistocene shells were collected from unconsolidated sandy sediments, and were generally well preserved with articulated valves.

Most of the living specimens from the Japanese waters were collected by the author and colleagues by dredging operations, while all others were the generous gifts or exchanges accumulated in the Molecular Palaeontology Laboratory, Glasgow University over recent years from various sources and localities around the world. Fossil specimens were collected by the author and colleagues from well documented strata and localities in Japan and New Zealand.

3.1.3 Antisera

Rabbit (New Zealand White rabbit) antisera prepared against shell macromolecules isolated from 15 different living brachiopods (**Table 3.4**), and a normal rabbit antiserum (preserum) were used in this study. Six antisera (**171, 173, 174, 1191, 1192**, and the **preserum**) were prepared in the course of this study by Dr. R. Quinn (licensed to carry out immunisation) and the author (antigen preparations and serum purifications), and all other antisera had been prepared and provided to this study by the courtesy of Dr. G. Muyzer, Dr. M. Collins, Dr. R. Quinn, and S. Ewing. See **section 3.2.2** for the method of preparation of the antisera.

Table 3.1 Living brachiopod species used in this study

Ordinal and familial denotations from Cooper (1972 *et seq.*). Abbreviations in the 'Status' column: L = living when collected and preserved wet; D = dead shells, preserved dry.

Species	Locality	Status
Rhynchonellida		
Basiliolidae		
Basiliola lucida (Gould)	Izu islands, Japan	D
Hemithyridae		
Hemithyris psittacea (Gmelin)	Otsuchi Bay, Japan	L
Notosaria nigricans (Sowerby)	Christchurch, New Zealand	L
Frieleiidae		
Frieleia halli Dall	off San Diego, California, U.S.A.	D
Terebratulida		
Terebratulidae		
Liothyrella neozelanica (Thomson)	Foveaux Strait, New Zealand	L
L. uva notorcadensis (Broderip)	Ross Island, Antarctica	L
Tichosina floridensis Cooper	off Florida, Gulf of Mexico	D
Gryphus vitreus (Born)	off Corsica, Mediterranean	L
Dyscoliidae		
Abyssothyris parva Cooper	off Jacksonville, Florida, U.S.A.	D
Cancellothyridae		
Cancellothyris australis Thomson	off Melbourne, Australia	D
Terebratulina retusa (Linnaeus)	Firth of Lorn, Scotland	L
T. septentrionalis (Couthouy)	Bay of Fundy, Canada	L
T. unguicula (Carpenter)	Friday Harbour, U.S.A.	D
T. unguicula rotundata Cooper	off Otsuchi, Japan	L
T. japonica (Sowerby)	Izu Islands, Japan	D
T. peculiaris Hatai	Izu Islands, Japan	D
T. pacifica Hatai	Kii Strait, Japan	D
T. crossei Davidson	Otsuchi Bay, Japan	L
T. reevei Dall	Sibuyan Sea, Philippines	D
T. abyssicola Adams & Reeve	off Inhambane, Mozambique	D
T. latifrons Dall	off Key West, Florida, U.S.A.	D
T. cailleti Crosse	off Pelican Island, Caribbean Sea	D
T. kiiensis Dall & Pilsbry	off Valparaiso, Chile	D
Chlidonophoridae		
Chlidonophora incerta (Davidson)	off Venezuela, Caribbean Sea	D
Dallinidae		
Dallina septigera (Loven)	Hebrides Shelf, Scotland	L
Campages basilanica Dall	Izu Islands, Japan	D
Terebratellidae		
Terebratella dorsata (Gmelin)	Strait of Magellan, Argentina	D
T. sanguinea (Leach)	New Zealand	L
Waltonia inconspicua (Sowerby)	Christchurch, New Zealand	L
Magellania macquariensis Thomson	South Pacific	D
Gyrothyris mawsoni antipodensis Foster	South Pacific	D
Neothyris lenticularis (Deshayes)	Foveaux Strait, New Zealand	L

Table 3.1 (Continued)

Species	Locality	Status
Laqueidae		
Terebratalia transversa (Sowerby)	Friday Harbor, Washington, U.S.A.	D
Coptothyris grayi (Davidson)	Japan	L
Dallinella occidentalis (Dall)	off San Diego, California, U.S.A.	D
Jolonica nipponica Yabe and Hatai	Izu Islands, Japan	D
Frenulina sanguinolenta (Gmelin)*	off Shimoda, Japan	D
Laqueus rubellus (Sowerby)	Sagami Bay, Japan	L
L. rubellus obessus Yabe & Hatai	Bungo Strait, Japan	L
L. blanfordi (Dunker)	Otsuchi Bay, Japan	D
L. quadratus Yabe & Hatai	Kii Strait, Japan	D
L. californicus (Koken)	off Santa Barbara, California, U.S.A.	D
L. californicus vancouvensis Davidson	Puget Bay, Washington, U.S.A.	D
Pictothyris picta (Dillwyn)	Sagami Bay, Japan	L
Macandreviidae		
Macandrevia cranium (Muller)	Hebridean Shelf, Scotland	D
M. africana Cooper	off Angola, South Atlantic	D
Ecnomiosidae		
Ecnomiosa sp.	Izu Islands, Japan	D
Kraussinidae		
Kraussina rubra (Pallas)	Southern Tip, South Africa	D
Megerlia truncata (Gmelin)	off Corsica, Mediterranean	D
Megathyrididae		
Megathiris detruncata (Gmelin)	off Corsica, Mediterranean	D
Argyrotheca barretiana (Davidson)	Jamaica, Caribbean Sea	D
Thecideida		
Thecideidae		
Lacazella sp.	Jamaica, Caribbean Sea	L
Thecidellina sp.	Jamaica, Caribbean Sea	L

* '*Frenulina sanguinolenta*' from off Shimoda Japan could possibly be a new species of a new genus. It very much resembles *Frenulina* externally, but the loop is different from the pattern of the typical *Frenulina* (D. MacKinnon, pers. comm.).

Table 3.2 Fossil samples used in this study

All localities are in Japan except for the localities for *Waltonia inconspicua* which are in the North Island of New Zealand. The mark '†' indicates extinct species.

Species	Formation	Locality	Age (Ma)
Waltonia inconspicua (Sowerby)	Rapanui	Wanganui	0.2
	Tainui Shellbed	Wanganui	0.4
	Lower Castlecliff	Wanganui	0.4
	Hautawa Shellbed	Parihauhau Rd.	2.2
Terebratalia coreanica (Adams & Reeve)	Kioroshi	Ohtayama	0.15
	Yabu	Semata	0.29
	Jizodo	Jizodo	0.37
	Mandano	Iwatabashi	0.50
	Sakahata	Higashihigasa	0.7-1.0
Coptothyris grayi (Davidson)	Kioroshi	Toyonari	0.15
	Yabu	Semata	0.29
	Jizodo	Atebi	0.37
	Mandano	Iwatabashi	0.50
	Sakahata	Higashihigasa	0.7-1.0
Laqueus rubellus (Sowerby)	Yabu	Ochi	0.27
	Yabu	Semata	0.27
	Jizodo	Jizodo	0.37
	Jizodo	Nishiatsu	0.37
	Jizodo	Atebi	0.37
	Jizodo	Ichinosawa	0.37
	Mandano	Iwatabashi	0.50
Pictothyris picta (Dillwyn)	Yabu	Ochi	0.27
	Yabu	Semata	0.27
	Jizodo	Jizodo	0.37
	Jizodo	Nishiatsu	0.37
	Jizodo	Atebi	0.37
	Jizodo	Ichinosawa	0.37
	Mandano	Iwatabashi	0.50
†*Kikaithyris hanzawai* (Yabe)	Wan	Kikai Island	0.08
	Naha limestone	Okinawa Island	ca. 1
Laqueus sp.	Sawane	Sado Island	ca. 1
†*Terebratulina* sp.	Sawane	Sado Island	ca. 1

Table 3.3 Samples of cancellothyridoids used for morphometry

USNM=Natural History of the United States National Museum, Washington, D.C. All samples are from living populations.

Species	Cat. No. (USNM)	Locality	Number of specimens
Cancellothyrididae			
Cancellothyris australis Thomson	110834	S.Australia	1
	332788	S.Australia	1
	318179	E.Bass Strait, Australia	8
Terebratulina crossei Davidson	342218	Japan	1
	342217	Japan	1
	110833	Enoshima, Japan	1
T. kiiensis Dall and Pilsbry	549413	Japan	1
	334758	33°13'N, 120°04W	1
	123154	Santa Cruz, California	4
	550692	Redondo Beach, Calif.	1
	385104	53°51'S, 71°36'W	2
T. hataiana Cooper	254533	Philippines	1
T.pacifica Hatai	342216	Kii, Japan	9
T.callinome Dall	238880	Philippines	13
T. photina Dall	254536	Borneo	1
T. latifrons Dall	Oregon5955	13°14'N, 60°53'W	1
	549754	07°45'N, 57°34'W	1
	P769	12°31'N, 71°41W	1
	G986	24°05'N, 80°19'W, Florida	1
	314855	Barbados	1
T. cailleti Crosse	550756	27°08'N, 84°53'W	8
	Oregon4570	23°11'N, 86°28'W	1
	A	Gulf of Mexico	2
	551210	25°00'N, 84°00'W	1
	314848	Barbados	1
T. reevei Dall	246330	Philippines	2
	334779	6°05'N, 123°14'E	1
	298312	Philippines	11
T. cavata Verco	214308	Cape Jaffa, S. Australia	1
T. meridionalis Jackson	372702	West coast, South Africa	2
T. abyssicola Adams and Reeve	Cr7.37217	South Africa	2
	187142	South Africa	1
	284193	South Africa	1
	110841	South Africa	2
	127017	South Africa	5
T. unguicula (Carpenter)	222202	Gulf of Georgia	5
	110897	Santa Cruz Island, Calif.	11
	3841178	Alaska	12
T. unguicula rotundata Cooper	110823	Kamchatka, Russia	6
	204672	Kamchatka, Russia	4
T. compressa Cooper	110844	Manazuru Zaki, Japan	1
T. valdiviae Blochmann	238829	Mindanao	8
	110843	Korea	1
	110437	Sumatra	1

Table 3.3 (continued)

Species	Cat. No. (USNM)	Locality	Number of specimens
T. retusa (Linnaeus)	173522	Skye, Scotland	12
	173564	Norway	11
	173579	Sicily	5
T. retusa emarginata (Risso)	317025	Mediterranean	1
	Exp 17	Mediterranean	1
T. septentrionalis (Couthouy)	Jenny B472	Cape Cod Light, Mass.	12
	203032	Marthas Veneyard, Mass.	12
	110869	Eastport, Maine	12
	49311	Nova Scotia	8
	266223	Cabot Strait, Greenland	1
	334751	Finmark, Norway	11
	173532	Loch Duich, Scotland	6
T. japonica (Sowerby)	563646	37°48'N, 137°18'E, Japan	3
	549344	37°48'N, 137°18'E, Japan	1
	110821	Ose Saki, Japan	1
	204687	Oki Shima, Japan	1
	110819	Oki Shima, Japan	2
T. peculiaris Hatai	563640	37°48'N, 137°18'E, Japan	2
T. hawaiensis Dall	274156	Hawaiian Islands	1
Chlidonophoridae			
Chlidonophora incerta (Davidson)	Acc.363295	Venezuela	12
Cnismatocentridae			
Cnismatocentrum sakhalinensis (Dall)	110788	Sakhalin	1
	222598	Alaska	2

Table 3.4 Antisera used in this study

Abbreviations for the type of antigens; F: extracts from the fibrous secondary layer; W: extracts from the whole shell; P: purified protein. Antisera preparations; MJC: Dr. M. J. Collins; GM: Dr. G. Muyzer; RQ: Dr. Rosalind Quinn; SE: S. Ewing; KE: K. Endo.

Serum ID No.	Name of Species from which antigens originated	Type of antigen	Titre (1/x)	Prepared by
K5038	*Notosaria nigricans* (Sowerby)	F	3000	MJC, GM
803	*Liothyrella neozelanica* (Thomson)	F	2000	SE, RQ
K5010	*L. uva notocardensis* (Broderip)	F	3000	MJC, GM
802	*Gryphus vitreus* (Born)	W	250	SE, RQ
K4962	*Terebratulina retusa* (Linnaeus)	W	40000	MJC, GM
173	*T. septentrionalis* (Couthouy)	F	3000	RQ, KE
174	*T. unguicula* (Carpenter)	F	3000	RQ, KE
171	*T. crossei* Davidson	F	400	RQ, KE
K5007	*Dallina septigera* (Loven)	F	10000	MJC, GM
K5040	*Waltonia inconspicua* (Sowerby)	F	40000	MJC, GM
427	*Neothyris lenticularis* (Deshayes)	P	100	MJC, RQ
1191	*Laqueus rubellus* (Sowerby)	F	20000	RQ, KE
1192	*Pictothyris picta* (Dillwyn)	F	5000	RQ, KE
801	*Kraussina rubra* (Pallas)	F	20000	SE, RQ
K5053	*Megerlia truncata* (Gmelin)	F	8000	MJC, GM

3.2 Methods

3.2.1 Isolation of shell intracrystalline macromolecules

(1) Shell cleaning:- Soft body tissues (if any) of brachiopods and epibionts on the shells were removed mechanically prior to incubating the specimens in an aqueous solution of bleach (10% v/v) for at least 2 days.

(2) Isolation of secondary shell fibres:- The fibrous materials of the shell secondary layer was isolated from the cleaned and agitated shells by exploiting differential precipitation rates by either a successive decanting method using two tubes ('mini-prep'), or by a recirculation method using two flasks (one for the agitation of the shells, the other for the precipitation of the shell fibres) connected by two tubes through a peristaltic pump ('maxi-prep'). The dense crystalline material of the primary layer was removed at this stage. Small amounts of bleach were allowed to remain in the preparations to completely destroy the organic matrix of the shell. In the cases of some fossil specimens, where the shell fibres were difficult to release, the whole shells were ground to powder. The 'maxi-prep' typically took a few days to complete, while the 'mini-prep' and shell powdering method took about an hour or less (depending on the number of specimens). The isolated fibres or powders were washed thoroughly to remove NaOCl (about 10 times wash and rinse) using distilled water and then lyophilised.

(3) Extraction of macromolecules from fibres:- For immunisation and analyses by liquid chromatography, organic material was extracted from the shell fibres or powders by decalcification in excess (500 ml for 20 g fibres) an aqueous solution of EDTA (20% w/v, pH 8.0) at 4°C for 48 h with continuous stirring. The decalcified material solution was concentrated and EDTA-calcium complex removed through ultra-filtration (10 kDa cutoff). The preparation was then lyophilised. Organic material extracted for biochemical analyses as well as antigen in ELISA was extracted by decalcification in an aqueous solution of EDTA (20% w/v, pH 8.0) with excess fibres (50 mg fibres in 1ml EDTA) with top-to-bottom mixing for at least 24 h, in order to keep the antigen concentration constant (saturated).

3.2.2 Immunisation protocol

Prior to immunisation, blood was obtained from New Zealand White rabbits to provide pre-immune sera for use as negative controls in ELISA.

The antigen to be injected was emulsified with an equal volume of Freund's Complete adjuvant so that 1 mg of shell macromolecules was administered in 1 ml to one

rabbit. The emulsion was injected subcutaneously into the scruff of the neck in 5 sites (Quinn, 1990). One month later the procedure was repeated using 0.5 mg of the macromolecule antigen in Freund's incomplete adjuvant. The procedure was repeated at 2 week intervals on two further occasions. Blood was collected from the ear vein 2 weeks after the fourth immunisation and tested for the presence of antibody by ELISA. For antisera against three *Terebratulina* species (Antisera **171**, **173**, **174**), another injection with 0.5 mg of macromolecule antigens (purified by gel filtration chromatography to remove EDTA) was made, and the sera were obtained 2 weeks after the fifth immunisation.

3.2.3 Isolation of serum from blood

Blood was collected in a glass universal bottle (Sterilin) and incubated at room temperature 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 50 ml centrifuge tube and centrifuged at 1500 *g* for 20 min at 4 °C. The serum was decanted off, aliquoted and stored at -20°C (Quinn, 1990).

3.2.4 Enzyme-linked immunosorbent assay (ELISA)

Antigen solution (5 μl) in 95 μl of 10 mM TBS (10 μl antigen in 90 μl TBS in some cases) was incubated in microtitre plates at 37 °C for 1.5 h using a shaker incubator, or at 4 °C without agitation overnight. The plates were rinsed three times with 0.02% (v/v) Tween 20 in TBS (TBS/Tween) using a microplate washer, and then incubated with 100 μl per well of gelatin (2%, w/v) in TBS for 1 h at 37 °C, using a shaker-incubator, to block non-specific binding sites. The plates were washed once with TBS/Tween. Antiserum (100 μl) (1st antibodies: prepared against brachiopod shell macromolecules) diluted appropriately in gelatin (0.2%, w/v) in TBS, was added and incubated at 37 °C for 1.5 h using the shaker-incubator. The plates were washed five times with TBS/Tween. Goat anti-rabbit antibody conjugated with alkaline phosphatase (2nd antibody) diluted 1:1000 in gelatin (0.2%, w/v) in TBS/Tween (100 μl per well) was added and incubated for 1.5 h using a shaker incubator. The plates were washed five times with TBS/Tween. Substrate (MUP; final concentration 0.2 mM) in the substrate buffer (100 μl per well) was added. The resulting fluorescence was measured automatically on a Microfluor reader at 0, 5, 15, 20, and 30 min, 1 and 1.5 h, the plates being kept in the dark between readings. The optimal incubation time in most cases was 30 min, but readings at different time intervals were also used when appropriate.

Buffers used in ELISA

Tris buffered saline (TBS):- Tris (10 mM); NaCl (0.9 %, w/v); pH adjusted to 7.4 with HCl.

TBS/Tween:- 0.02% (v/v) Tween 20 in TBS.
Substrate buffer:- diethanolamine (10 mM); $MgCl_2$ (1 mM); pH adjusted to 9.8 with HCl.

3.2.5 Titre determination

For each antiserum, the optimal dilution of the serum (titre) was determined by assays with a series of antiserum dilution. The lowest concentration which gives an 90-100 % reading of the maximum reaction was taken as the titre. Other than for the inhibition ELISA (see **section 3.2.7**), where the concentration which gives 50-70% of the maximum reaction (antibody-limiting concentration) was used as the titre. A pre-serum was used as the negative control for the sera.

3.2.6 ELISA with Sodium metaperiodate treated antigens

To determine the content of carbohydrate in the antigens, antigens were treated with sodium-*m*-periodate ($NaIO_4$) to oxidise OH groups in carbohydrates (Collins *et al.*, 1991c).

After the coating of the antigens on the microtitre plates, the plates were washed three times with TBS/Tween, then a aqueous solution of $NaIO_4$ (10 mM) in acetate buffer (20 mM) (100 µl) was added to each well, while 100 µl acetate buffer (20 mM) was added to the controls. The plates were incubated at room temperature for 1 h without shaking followed by a single wash with TBS/Tween and treatment with the blocking protein and the ELISA procedure continued.

Acetate buffer (20 mM, pH 5.4):- Sodium acetate (20 mM); pH adjusted to 5.4 by acetic acid.

3.2.7 Inhibition ELISA

To increase the specificity of the antisera prepared against brachiopod shell macromolecules, each antiserum was mixed with a series of antigens (EDTA shell extracts from species of interest) to remove the antibody reactivity to that particular antigen prior to use in the ELISA.

In the experiments on living species, one part of each antigen solution was diluted with nine parts TBS/Tween-gelatin (final concentration of gelatin: 0.2% w/v)-antiserum solution so that the final dilution of antiserum became the appropriate 'limiting' titre (**section 3.2.5**) for the immediate use in the subsequent ELISA experiments. Pre-absorption with 5% antigens, rather than 10%, was carried out for four living *Terebratulina* species (**Chapter 8**). The preparations with the homologous antigen and with aqueous solutions of EDTA (20%, pH 8) were also included as the controls, which were expected

to give 0% and 100% reactions, respectively. The preparations were mixed well, and incubated at 4 °C overnight. The resulting precipitations were removed by centrifugation at 4000 *g* for 20 min at 4 °C prior to applying to the ELISA plates, which had been previously prepared, and which were then analysed in the usual way (**section 3.2.4**).

In order to determine whether antigenic epitopes to each antiserum were present in the extracts from a fossil *Terebratulina* species (**Chapter 8**), pre-absorption of each antiserum prepared against *Terebratulina* species with 50 % and 100 % (v/v) antigen solution extracted from the fossil *Terebratulina* was carried out using the same procedures as described above, and the reduction (if any) in the reactivity of each pre-absorbed antiserum to the homologous antigen (extracts from extant material) assayed by ELISA.

3.2.8 Calculation of immunological reactivity

The assays in ELISA were performed at least in duplicate and often in triplicate. Negative controls for the antigens were aqueous solutions of EDTA (20% w/v, pH 8) appropriately diluted in TBS (10 mM, pH 7.4) and the EDTA extracts from a bivalve *Mercenaria* species (or *Codakia* species) diluted appropriately in TBS. Positive controls (the antigen against which the antiserum was raised) were also included in every assay. The reactivity of an antiserum to an unknown antigen was expressed as a percentage of the mean value for the positive controls. The mean EDTA-blank reading being substracted from each reading prior to the calculation. The calculated strengths of reactivity were analysed using various multivariate statistical methods, such as cluster analysis (single linkage method; cf. Sneath and Sokal, 1973) and principal component analysis (cf. Reyment *et al.*, 1984) on an Apple Macintosh computer using Odesta Corporation software, 'Datadesk Professional™'. In multivariate statistics, an analysis of relationships between variables is often referred to as an *R*-mode analysis, whilst an analysis of the relationship between the specimens of a sample is usually referred to as a *Q*-mode analysis (Reyment *et al.*, 1984). In this study (**Chapter 5**), the analysis of relationships between antisera, in a data matrix of immunological reactivity between a panel of antigens and antisera, is referred to as *R*-mode analysis, and the analysis between antigens is referred to as *Q*-mode analysis.

The 'immunological distances' (d_I) among four living *Terebratulina* species were determined from the equation: $d_I = 100 \log_{10}$I.D., where I.D. (index of dissimilarity) is the factor to which the antiserum concentration must be raised to produce the same level of reactivity with a heterologous antigen as with the homologous antigen (Sarich and Wilson, 1966; applied by Collins *et al.*, 1991b and Curry *et al.*, 1991c for the measurements by ELISA) (**Chapter 8**). I.D. was obtained graphically from semi-logarithmic binding curves plotted against a dilution series of antibody concentration, and calculated as an mean value of reciprocal experiments.

For the detection of signals from fossil macromolecules, an attempt was made to increase the first antibody concentrations with a protocol similar to the determination of the titre (**section 3.2.5**) or the 'immunological distances' (see above). From each assay on a fossil species with each antiserum, three titration (or binding) curves, one with the fossil species as the antigen, one with the positive control (homologous antigen to the antiserum), and one with the negative control (extracts from irrelevant taxa), were obtained on semi-logarithmic graphs. The extent of reactivity was calculated using the former two curves. Firstly, the factor (denoted here as I.D.'), by which the antiserum concentration was required to increase to obtain the same level of fluorescence reading for the fossil species as that of the positive control, was measured from the two binding curves. The extent of reactivity [denoted here as the 'quasi-immunological distance' (d_I'), because this value is biased by diagenetic effects on the antigenic macromolecules] was then calculated using the formula; $d_I' = 100 \times \log_{10} I.D.'$.

3.2.9 Liquid chromatography

General procedures:- A high performance liquid chromatography (hplc) system and columns of high-quality support materials were employed for the separation of macromolecules on the basis of size and shape (gel filtration), hydrophobicity (reverse phase chromatography, hydrophobic interaction chromatography), and charge (ion exchange chromatography). The hplc system allowed accurate control of the isocratic and the gradient elution, and precise collection of the eluent fractions. The monitored UV absorption data were stored in, and handled by, a NEC computer. The entire procedure was carried out at room temperature, and after a clean room became available, was performed under controlled temperature conditions of 20 °C in the resulting dust free environment.

All the buffers and solutions used as the mobile phase were filtered through a membrane with a pore size of 0.2 µm (Whatman), except for the TFA (ABI) and the TFA in acetonitrile (ABI) solutions, since these were hplc grade materials. The buffers were degassed either by ultrasonication for a few minutes prior to use or by Helium gas sparging during chromatography.

The initial amount of the sample loaded on each column varied between 10 and 100 mg, and was reconstituted using a volume of between 100 µl and 1 ml of the starting buffer. When a fraction was loaded onto a different elution system, the fraction was mixed with an equal volume of the new starting buffer. Each sample was prepared in an eppendorf tube and centrifuged at 15,000 *g* for 1 min or filtered through a syringe filter (Acrodisc 13, 0.2 µm pore size; Gelman Science) to remove any precipitants or large particles prior to loading onto the sample loop, which had a loading capacity of 250 µl or 1 ml.

Columns were equilibrated using manufacturers' instructions. The eluate was

monitored by UV absorption at 280 nm, 214 nm, and 260 nm. Separated sample fractions were automatically collected in eppendorf tubes at an accurate fraction size.

Gel filtration:- Fractionations of shell extracts, isolated both from living *Terebratulina* (**Chapters 4** and **7**), and from fossil *Terebratulina* (**Chapter 7**), on Superose 6, Superose 12, or SW300 gel filtration column were performed using Tris buffer (20 mM Tris/HCl, pH 7.5), Tris buffer containing sodium chloride (20 mM Tris/HCl, 1.5 M NaCl, pH 7.5), acetate buffer (50 mM CH_3COONa/CH_3COOH, pH 4.5), or phosphate buffer containing SDS (50 mM NaH_2PO_4/H_3PO_4, 0.2% w/v SDS, pH 6.5) as the elution buffer. Isocratic elutions were carried out usually at flow rates of 0.5 ml/min for the Superose 6 and 12, and 0.8 ml/min for the 300SW columns for 60 and 40 min respectively.

Standard protein kits (Pharmacia) were used for the molecular weight calibrations. The low molecular weight kit consisted of Dextran blue (a carbohydrate for the estimation of the void volume of the column; MW = 2,000,000), Serum albumin (MW = 67,000), Ovalbumin (MW = 43,000), Chymotrypsinogen A (MW = 25,000), and Ribonuclease (MW = 13,700). The high molecular weight kit consisted of the Dextran blue, Thyroglobulin (MW = 669,000), Ferritin (MW = 440,000), Catalase (MW = 232,000), and Aldolase (MW = 158,000). Using these standard proteins, a calibration curve was constructed for each elution system between log MW and Kd (the distribution coefficient), which was calculated by the following formula: $Kd = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of a protein, V_t is the total accessible volume, or the elution volume of a small molecule, and V_0 is the void volume, or the interparticle volume (Welling and Welling-Wester, 1989). The V_0 and V_t for Superose 12, using Tris buffer with sodium chloride (20 mM Tris/HCl, 1.5 M NaCl, pH 7.5) as the elution buffer, were estimated as 7.4 ml and 20 ml, respectively. The V_0 and V_t for 300SW using the same elution buffer were 5.5 ml and 15 ml respectively. A calibration curve for Superose 6 column is presented in **Figure 3.1**.

Reverse phase chromatography:- Intracrystalline extracts isolated from living *Terebratulina retusa* were fractionated on ProRPC HR 5/2 or Delta Pak C18-300A (Waters) column using various pairs of elution systems (**Table 3.5**). Acetonitrile was customarily used as the organic component of the mobile phase. Isopropanol was tested in one instance, but was unsuitable for routine use because of the high viscosity of isopropanol. TFA was usually added to the mobile phase to solubilise proteins in organic solvents. Addition of metal ions ($CaCl_2$, $MgCl_2$) was attempted in order to increase the stability of the proteins. A zwitterion, betaine, was added to reduce molecular interactions which result in aggregation. An ion-pairing agent, TBAP, was added in an attempt to increase the retention time for proteins carrying relatively large charges of the opposite sign (Corran, 1989). The following elution program was the most frequently used: 100% solvent A for 4 min; 0-100% solvent B (with linear gradient) in 20 min; 100% B for 4 min; 100% A for 12 min, at a flow rate of 1 ml/min.

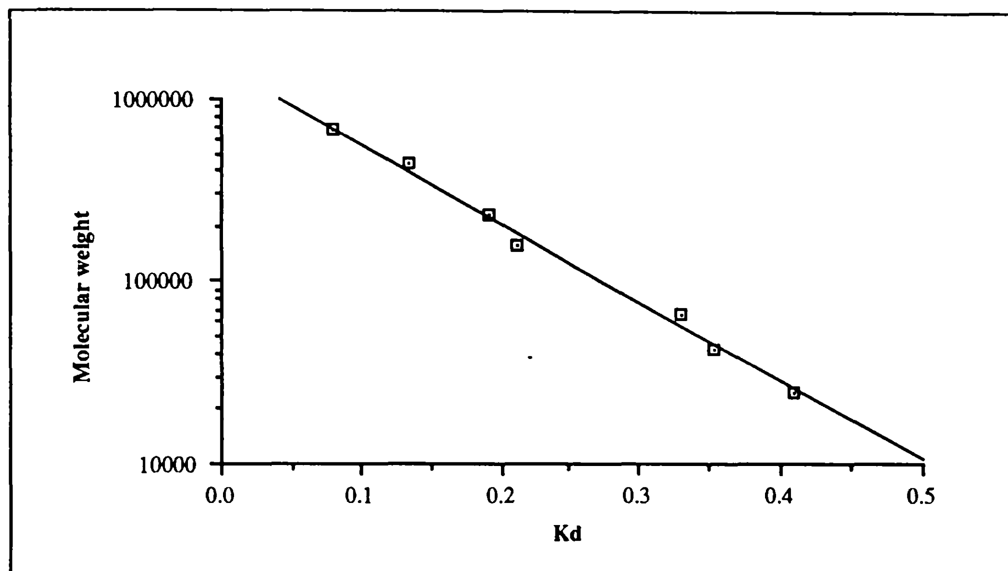


Figure 3.1 Calibration curve for Superose 6 column

A Superose 6 column was equilibrated with phosphate buffer (50 mM, pH 6.5) which contained SDS (0.2% w/v). Gel filtration size marker proteins (Pharmacia) were reconstituted with the elution buffer, boiled for 3 mins, and centrifuged for 1 min at 14000 rpm prior to loading the resulting supernatant onto the column. The proteins were eluted at a flow rate of 0.5 ml/min, and a distribution coefficent (Kd) was calculated for each standard protein (see text for method).

Table 3.5 Solutions tested as mobile phase in reverse phase chromatography

Solutions
A = 0.1% TFA; B = 0.085% TFA in 70 % acetonitrile (CH_3CN: McCN)
A = 0.1% TFA; B = 0.1% TFA in 70% McCN
A = 0.5% TFA; B = 0.5% TFA in 60% Propan-2-ol (iso-propanol)
A = 0.15% TFA, 0.1 M $CaCl_2$; B = 0.1% TFA, 0.1 M $CaCl_2$ in 60% McCN
A = 0.15% TFA, 0.1 M $MgCl_2$; B = 0.086% TFA, 86 mM $MgCl_2$ in 70% McCN
A = 0.13% TFA, 2% (w/v) betaine; B = 0.1% TFA, 2% (w/v) betaine in 70% McCN
A = 5 mM TBAP/H_3PO_4, pH 6.5; B = 5 mM TBAP in 70% McCN
A = 5 mM TBAP/H_3PO_4, pH 4.5; B = 5 mM TBAP in 70% McCN

TFA: trifluoroacetic acid; TBAP: tetrabutylammonium phosphate
Percentages are in volume per volume (v/v) unless otherwise stated.

Table 3.6 Buffers tested in anion exchange chromatography

A = Diethanolamine (50 mM, pH 8.8); B = A + 1.0 M NaCl
A = Diethanolamine (20 mM, pH 8.4); B = A + 1.0 M NaCl
A = Tris/HCl (20 mM, pH 9.0); B = Tris/HCl (20 mM, pH 6.0) + 0.8 M NaCl
A = Tris/HCl (30 mM, pH 8.5); B = A + 0.8 M NaCl
A = Tris/HCl (20 mM, pH 8.0); B = A + 1.0 M NaCl
A = Tris/HCl (20 mM, pH 7.5); B = A + 0.8 M NaCl
A = Tris/HCl (20 mM, pH 7.2); B = A + 0.5 M NaCl
A = Tris/HCl (20 mM, pH 7.0); B = A + 0.5 M NaCl
A = Trimethylamine/formic acid (20 mM, pH 4.0); B = A + 1.0 M $HCOONH_4$

Table 3.7 Buffers tested in cation exchange chromatography

A = Sodium phosphate buffer (50 mM, pH 6.0); B = A + 0.5 M NaCl
A = Sodium acetate buffer (50 mM, pH 4.8); B = A + 1.0 M NaCl
A = Formic acid (50 mM, pH 3.8); B = A + 1.0 M NaCl
A = Phosphoric acid/NaOH (50 mM, pH 2.0); B = A + 1.5 M NaCl

Ion exchange chromatography:- Ion exchange chromatography of intracrystalline extracts from living *Terebratulina retusa* were performed on Mono Q, DEAE-5PW (anion exchanger) and Mono S, SP-5PW (cation exchanger) columns. Various buffers of different pH (between 4-9 for anion exchange; between 2-6 for cation exchange chromatography) were tested for each column (**Tables 3.6, 3.7**). Gradient elutions were usually performed using the following program: 100% buffer A for 3 min; 0-100% linear increase of buffer B in 15 min; 100% buffer B for 3 min; 100% buffer A for 9 min; at a flow rate of 1.5 ml/min.

Hydrophobic interaction chromatography:- An aqueous solution of intracrystalline extracts from living *Terebratulina retusa* was applied to Phenyl 5PW column in NaH_2PO_4/H_3PO_4 (50 mM, pH 7.0), 1.7 M $(NH_4)_2SO_4$ at a flow rate of 1 ml/min. After 5 min, a 20 ml linear gradient of 1.7 to 0 M $(NH_4)_2SO_4$ in NaH_2PO_4/H_3PO_4 (50 mM, pH 7.0) was applied.

3.2.10 Amino acid analysis

Automatic amino acid analysis was carried out using the Applied Biosystems 420H system (Dupont *et al.*, 1989).

3.2.11 Morphometrics on cancellothyridoid species

Seven morphologic characters were measured on complete specimens of each species or subspecies (**Figure 3.2**). Length (*L*), width (*W*), and height (*H*) were measured to an accuracy of 0.1 mm using a dial calliper, while foramen length (*fL*), and foramen width (*fW*) were measured to an accuracy of 0.1 mm using a graticule in a binocular microscope. The number of capilla (*Ribs*) was counted in a width of 4 mm on the dorsal valve at the point 4 mm from the dorsal umbo. Shell weight (*Wt*) of dried specimens was measured to an accuracy of 0.1 g. Every variable was standardized by subtracting the mean and dividing by the standard deviation prior to multivariate analyses to eliminate scaling problems between variables. Principal component analyses were carried out on an Apple computer using Odesta Corporation software, 'Datadesk Professional™'.

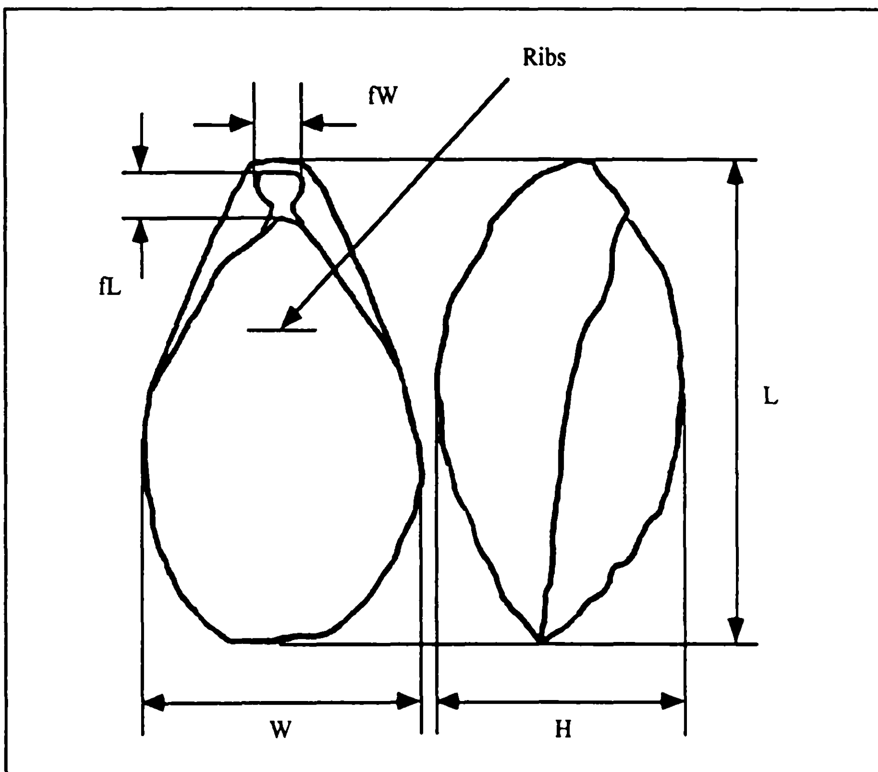


Figure 3.2 Measurements used in the morphometry of cancellothyridoid species

Abbreviations: L = shell length; W = shell width; H = shell height; fL = foramen length; fW = foramen width; Ribs = number of capilla in the indicated part of the dorsal valve (a line parallel to the hinge line, with a width of 4 mm, and with a distance of 4 mm to the dorsal umbo). The weight of the shell (Wt) was also measured.

Chapter 4 Purification of shell intracrystalline macromolecules

4.1 Introduction

When this project began, very little was known about the nature of intracrystalline macromolecules in the fibrous calcite of the brachiopod secondary shell layer, although the presence of proteins was expected on the basis of qualitative biochemical analyses, and because these molecules had a taxonomic significance when studied by immunological methods (Collins *et al.*, 1988).

Fractionation of the components of the intracrystalline shell extract was required to prepare homogeneous populations of protein for further biochemical analyses. In addition, antibodies prepared against these purified proteins should be powerful phylogenetic tools.

In the following sections, results and implications of the purification of the shell intracrystalline macromolecules from *Terebratulina retusa* (Linnaeus) using hplc (see **section 3.2.9** for method) are described. The starting materials were the crude extracts isolated by the method described in **section 3.2.1**; and thus consisted mostly of molecules larger than 10 kDa. Eluate fractions were analysed by ELISA (**section 3.2.4**) using the antiserum prepared against the whole shell extracts of *T. retusa* (K4962).

4.2 Gel filtration

Intracrystalline extracts of *T. retusa* were fractionated by gel filtration chromatography as described in **section 3.2.9**. In each elution system, consistent chromatograms were obtained with several components forming rather continuous fractionation profiles. Almost every chromatogram contained EDTA as the highest composite peak (verified by loading EDTA solution as the control), which did not show any reaction with K4962.

Several components eluting earlier than the EDTA peak were identified (**Figures 4.1, 4.2**), and most of them reacted with K4962, suggesting that these components may possibly be either carbohydrate or protein, the common antigenic macromolecules. Calibration curves generated from retention times of standard proteins were used to compare the molecular weight of components in different chromatograms (**Table 4.1**). Carbohydrate oxidation experiments using sodium-*m*-periodate (**section 3.2.6**) on the fractions indicated that peak No. 5 in **Table 4.1** was least glycosylated, and that this peak represented one of the pure proteins (**Figure 4.1**). However, this result must be taken with caution because the experiments with sodium-*m*-periodate did not always give consistent results.

Elution by acetate buffer (pH 4.5) revealed fewer components, which were further fractionated into five well-defined peaks (**Figure 4.3**). The reduction in the number of peaks, notably for the components of higher molecular weight, may be the result of a decrease in the intermolecular reactions due to the loss of surface charges of the molecules.

Table 4.1 Components detected by gel filtrations

peak ID.	Molecular weight (kDa)			ELISA*	Notes
	Superose 6 (Figure 4.1)	300 SW (Figure 4.2)	Superose 12 (Figure 4.3)		
p1	380	200	-	++	?aggregate
p2	110	140	-	++	?aggregate
p3	-	75	60	++	
p4	-	47	-	++	
p5	40	35	41	++	?protein
p6	22	27	24	+	
p7	-	-	19	+	
p8	13	13	16	-	EDTA
p9	7.7	-	-	-	EDTA

*Immunological reactivity: ++ = strong, + = weak, - = no reaction.

4.3 Reverse phase chromatography

After successful purification of a gel filtration fraction (peak 5 in Table 4.1) using the Pharmacia ProRPC column (**Figure 4.4**), it took some time to optimise conditions for the newly-acquired Delta Pak C18 column. Many of the attempts with different components in the mobile phase, such as isopropanol, betaine, and metal ions, resulted in unsatisfactory separations, but eventually a stepwise method using TBAP was developed which yielded both reproducible results and some highly purified components (as judged by the shape of the peaks, starting from the crude extracts rather than from gel filtration fractions) (**Figure 4.5**). This method involved two reverse phase chromatography procedures in tandem using different elution systems: the first step utilised 5 mM TBAP-H_3PO_4-acetonitrile (pH 4.5) system, which fractionated the macromolecules into a rather continuous spectrum with several peaks, then each peak-containing fraction was re-chromatographed using 0.1% TFA/acetonitrile system to separate several sharp discrete components (**Figure 4.5** and **Table 4.2**). On the basis of the UV absorbance ratio of the reading at 260 nm to that of 280 nm, a ratio which should be below 0.6 for pure proteins (Johnstone and Thorpe, 1987), the peak No.1 in **Table 4.2** had a ratio of 0.51, and is considered proteinaceous, and the peak No.4 in **Table 4.2** (a ratio of 0.65) is considered as a possible candidate for a protein. In most elution systems used in reverse phase chromatography, the macromolecules reactive with K4962, eluted after the gradient started, and the high peaks eluted in the void volume of the column were considered to be mostly of salt components, especially EDTA.

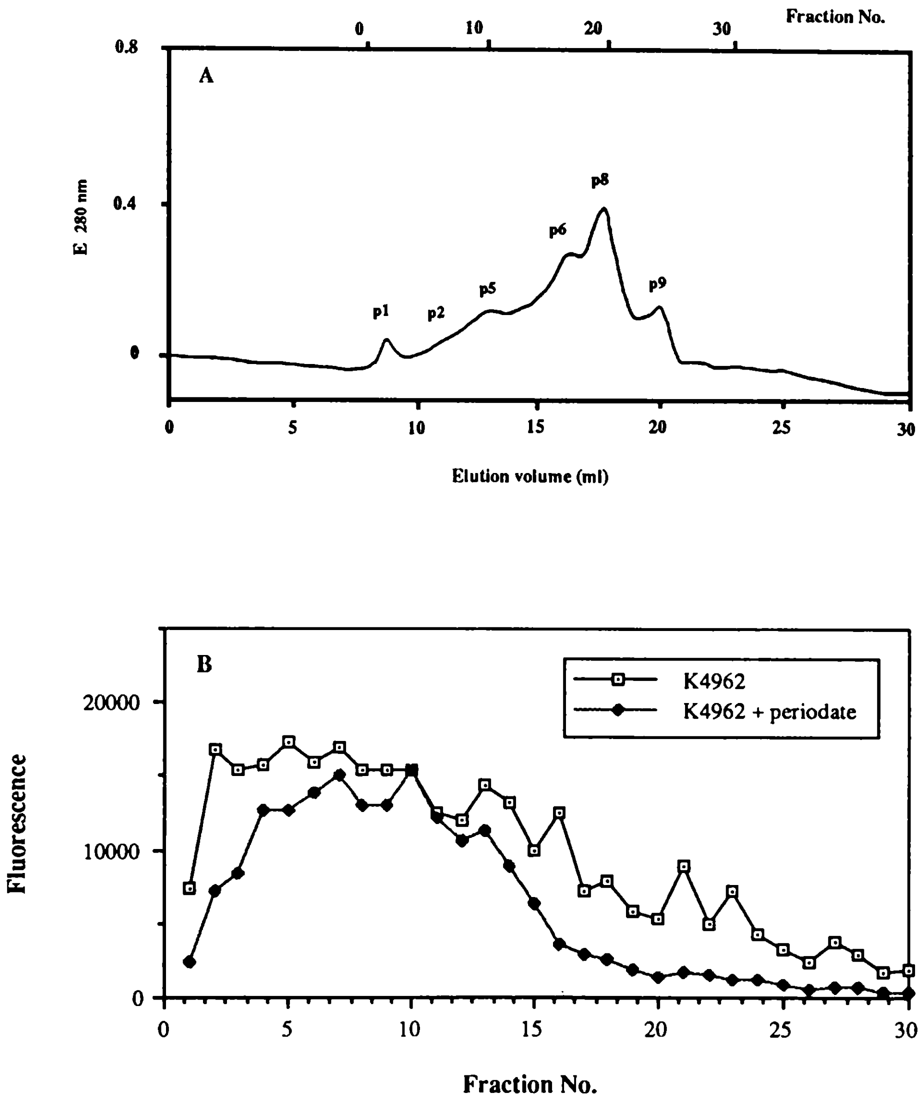


Figure 4.1 Gel filtration of shell intracrystalline molecules

A: Fractionation of the intracrystalline extract from *T. retusa* (for extraction method see **section 3.2.1**) on Superose 6 by isocratic elution with Tris buffer containing sodium chloride (10 mM Tris/HCl, 1.5 M NaCl, pH 7.5) at a flow rate of 0.5 ml/min. Fractions collected from 16 to 46 min with an interval of 1 min. For peak identities see **Table 4.1**.

B: ELISA (**section 3.2.4**) on the eluate fractions using the antiserum prepared against the whole shell extracts of *T. retusa*. Keys: K4962, without *m*-periodate treatment; K4962 + periodate, with *m*-periodate treatment (**section 3.2.6**).

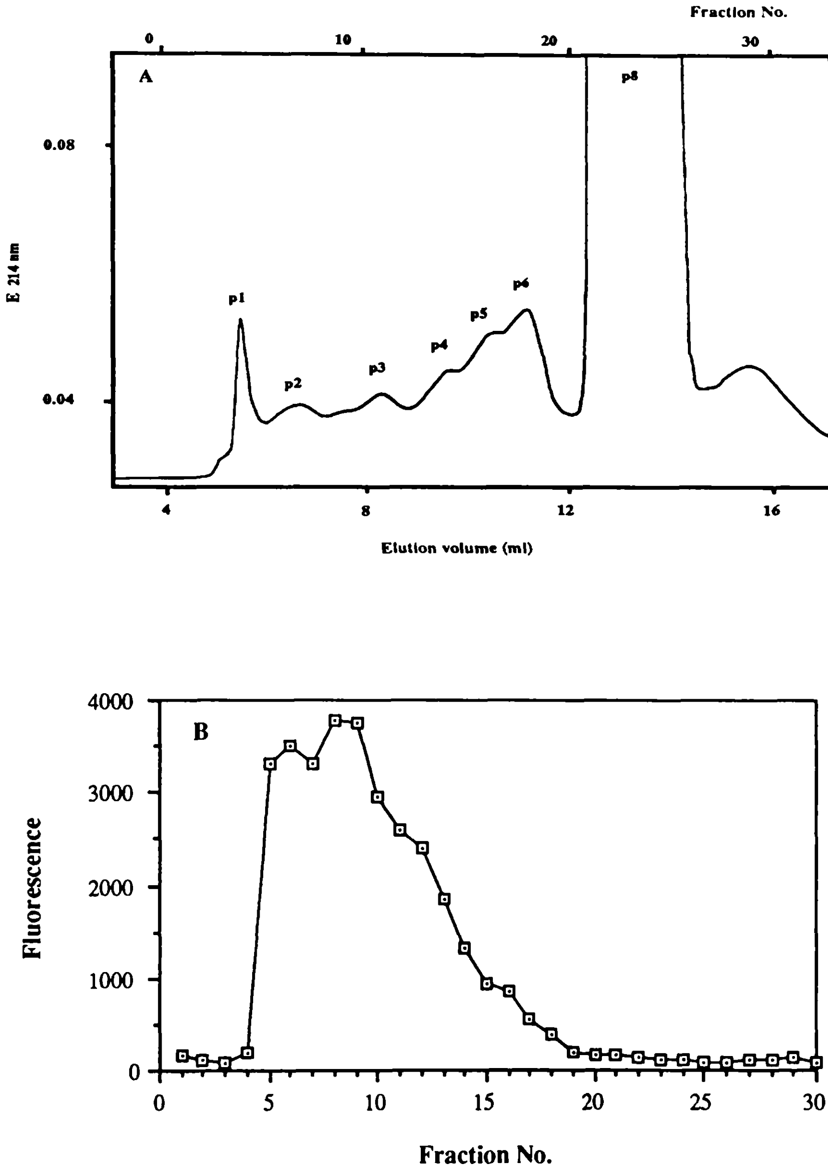


Figure 4.2 Gel filtration of shell intracrystalline molecules

A: Fractionation of the intracrystalline extract from *T. retusa* on 300SW by isocratic elution of Tris buffer containing sodium chloride (20 mM Tris/HCl, 1.5 M NaCl, pH 7.5) at a flow rate of 0.8 ml/min. Fractions collected from 5 to 20 min with an interval of 0.5 min. For peak identities see **Table 4.1**. B: ELISA on the eluate fractions using the antiserum prepared against the whole shell extracts of *T. retusa*.

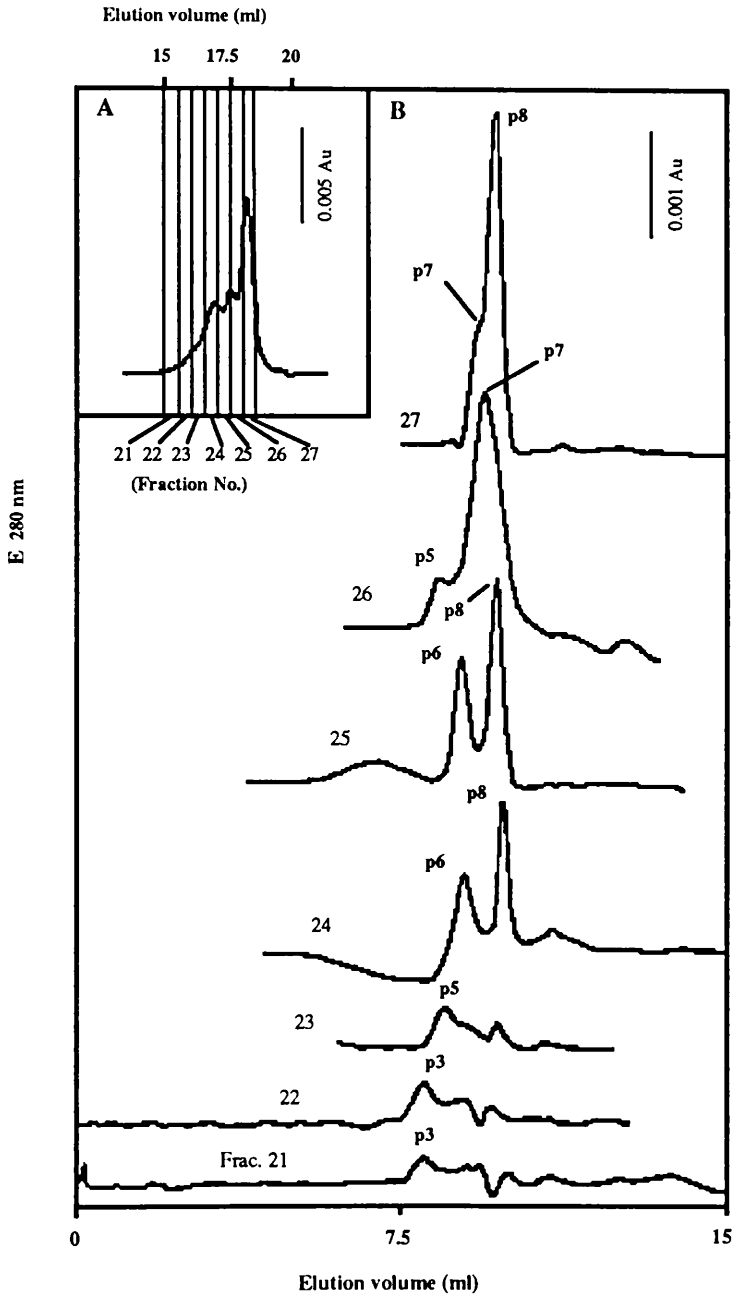


Figure 4.3 Two step gel filtration of shell intracrystalline molecules

A: Fractionation of the intracrystalline extract from *T. retusa* on Superose 12 by isocratic elution of acetate buffer (50 mM, pH 4.5) at a flow rate of 0.25 ml/min. Fractions collected at an interval of 2 min.

B: Rechromatography of fractions 21-27 in the previous run. Each fraction was rechromatographed in the same elution system on the same column as Panel A at a flow rate of 0.5 ml/min. For peak identities see **Table 4.1**.

Table 4.2 Components purified by a stepwise reverse phase chromatography (Figure 4.5) of shell macromolecules

Peak No. and Elution time indicate the peak identity number and the retention time in the second chromatography (**Figure 4.5, B-D**). Relative heights of the peaks in the second chromatography are indicated by the number of '+' marks. '260/280' denotes the ratio of the UV absorbance readings at 260 nm and 280 nm for each peak. ELISA indicates the extent of immunological reaction of each component with K4962.

Peak ID.	Elution time (min)	1st chromatography			260/280	ELISA	Notes
		Frac. 6	Frac. 9	Frac. 12			
p1	19.17		+++		0.51	+	?protein
p2	20.18	+			1.41	++	
p3	20.37			+++	0.82	++	
p4	20.75	+++	++		0.65	+++	?protein
p5	22.12	++			1.96	++	
p6	22.48		++	+++	1.35	+++	
p7	23.13	+	++		1.08	+++	
p8	25.37			++	1.47	++	

4.4 Ion exchange chromatography

A series of tests using different eluting buffers (**section 3.2.9, Tables 3.6, 3.7**) indicated that it was difficult to fractionate shell macromolecules by ion exchange chromatography under 'usual' conditions. The cation exchange column could not retain shell macromolecules, except with the elution buffer at very low pH values (i.e. pH 2). At pH 2, a few components, which showed some reaction to K4962 (**Figure 4.6, B**), eluted after the salt gradient started, but the separation was unsatisfactory and most of the immunological reaction was detected in the fractions in the void volume of the column (**Figure 4.6, A**). In anion exchange chromatography, it was possible, using neutral or basic elution buffers, to fractionate some components which had strong reactivity with K4962. However, the yields were low, and the purity of each component, judging from the shape of the peak, was unsatisfactory (**Figure 4.7**). These results were possibly caused by avid binding of the shell macromolecules to the anion exchanger. In fact, some molecules were so strongly bound to the column that they remained bound after vigorous overnight cleaning of the column with 2 M NaCl. **Figure 4.7** also indicates that some immunologically reactive molecules were not retained by the anion exchange column at pH 8.8. The

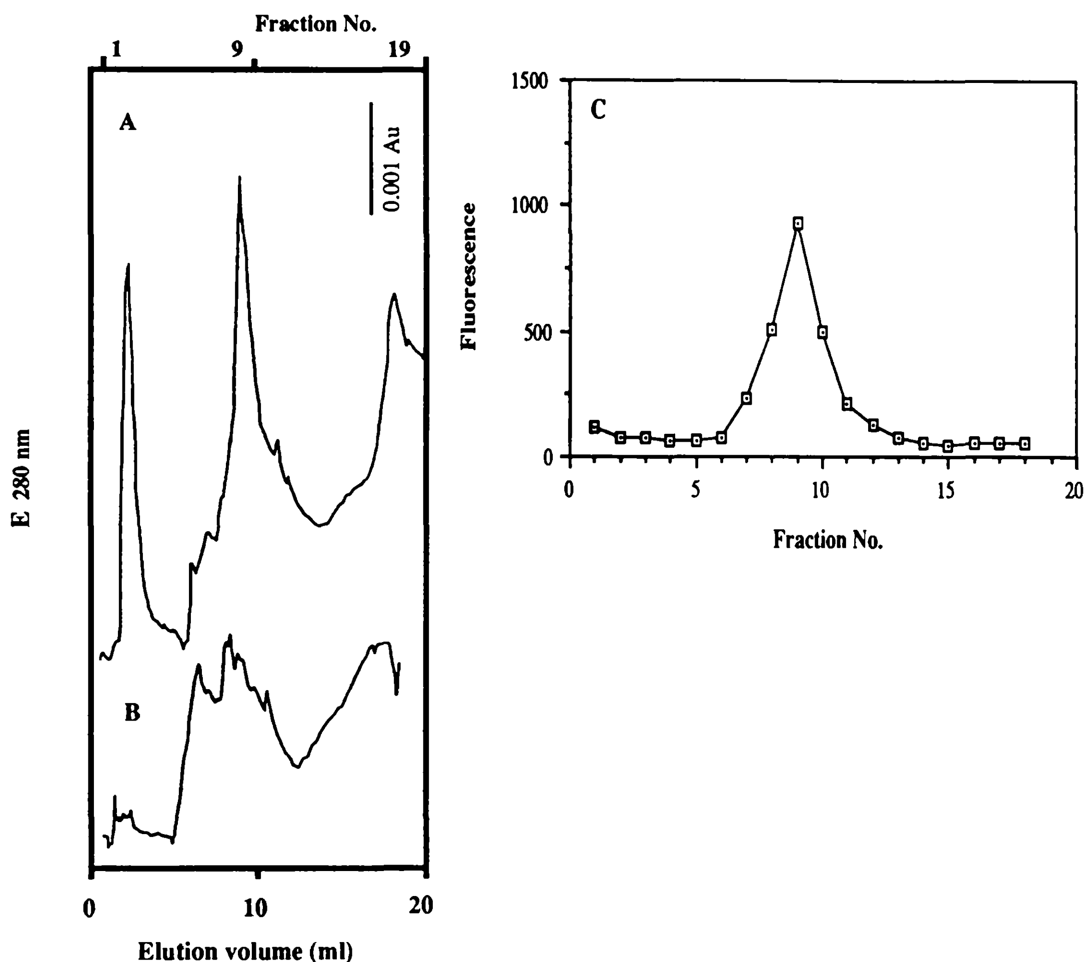


Figure 4.4 Reverse phase chromatography of a gel filtration fraction

A: Reverse-phase separation of fraction 10 in Figure 4.1 on ProRPC HR 5/2. Mobile phase, A = 0.1% (v/v) TFA, B = 70% (v/v) acetonitrile, 0.1% (v/v) TFA, flow rate, 1.0 ml/min, gradient, linear, 0-100% B over 20 min from 4 min. Fractions collected from 1 to 20 min with an interval of 1 min.

B: Blank run with the same condition as panel A.

C: ELISA on the eluate fractions of panel A.

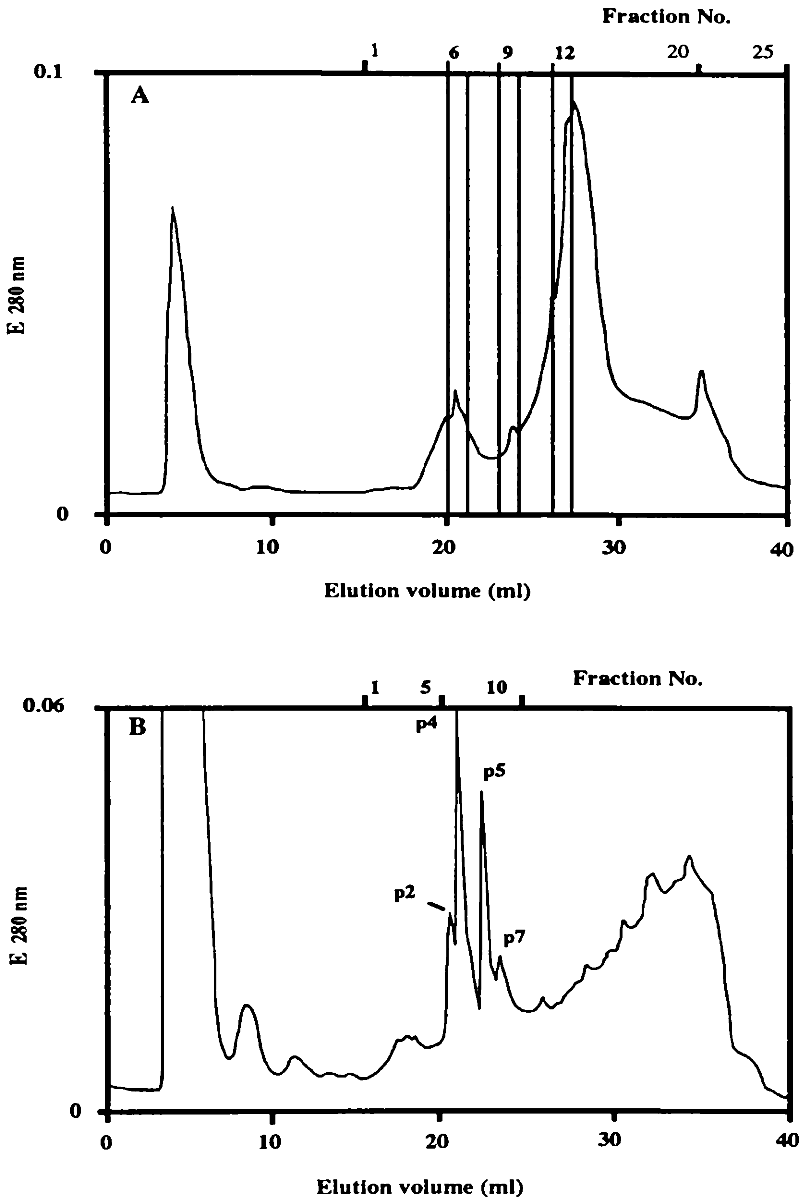


Figure 4.5 Two step reverse phase separation of shell macromolecules

A: Separation of crude shell extracts from *T. retusa* on Delta Pak C18-300A. Mobile phase, A = 5 mM TBAP, B = 70% (v/v) acetonitrile, 5 mM TBAP, flow rate, 1.0 ml/min, gradient, linear, 0-100% B over 20 min from 4 min, 100% B for 4 min, 100% A for 12 min.

B: Rechromatography of fraction 6 of panel A. Same column as panel A. Mobile phase, A = 0.1% (v/v) TFA, B = 70% (v/v) acetonitrile, 0.1% (v/v) TFA. Same gradient as panel A. For peak identities see **Table 4.3**.

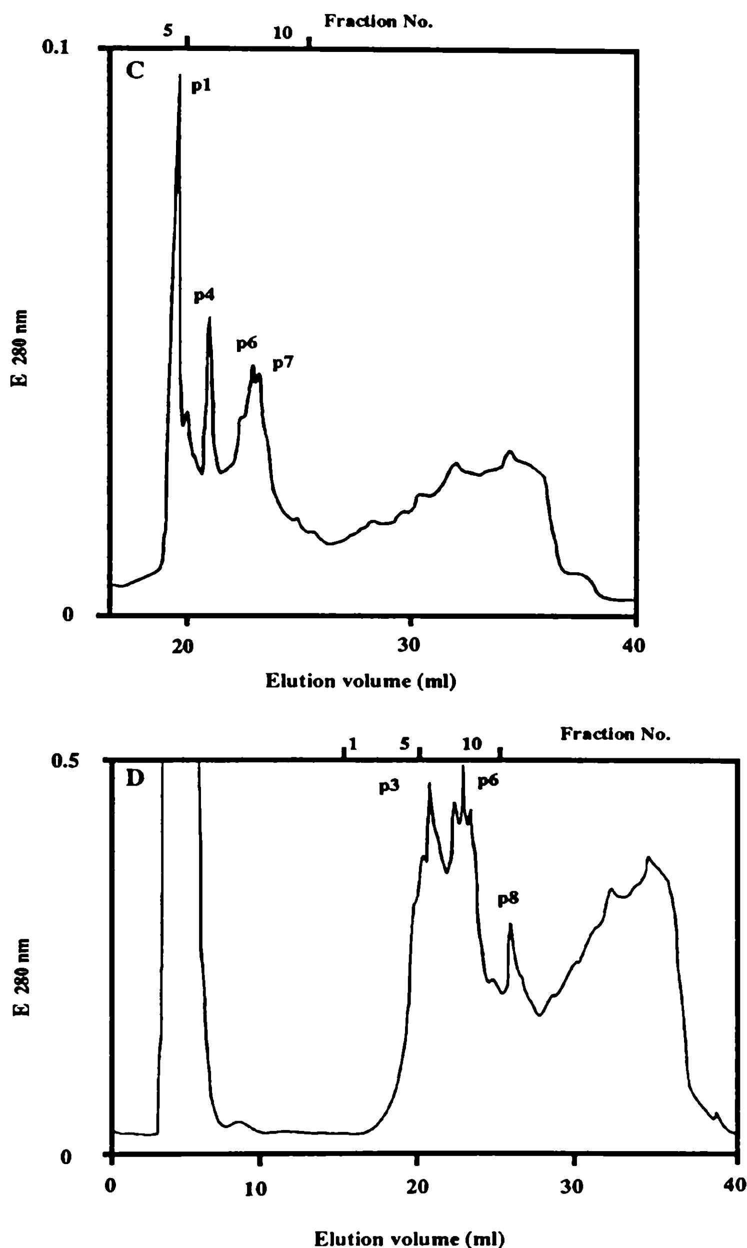
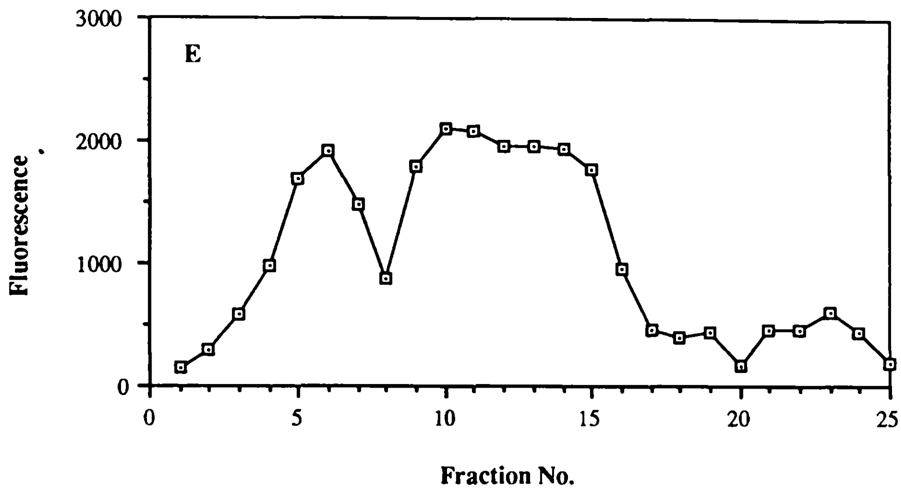


Figure 4.5 (continued)

C: Rechromatography of fraction 9 of panel A. Same column as panel A. Mobile phase, A = 0.1% (v/v) TFA, B = 70% (v/v) acetonitrile, 0.1% (v/v) TFA. Same gradient as panel A. For peak identities see **Table 4.3**.

D: Rechromatography of fraction 12 of panel A. Same column as panel A. Mobile phase, A = 0.1% (v/v) TFA, B = 70% (v/v) acetonitrile, 0.1% (v/v) TFA. Same gradient as panel A. For peak identities see **Table 4.3**.



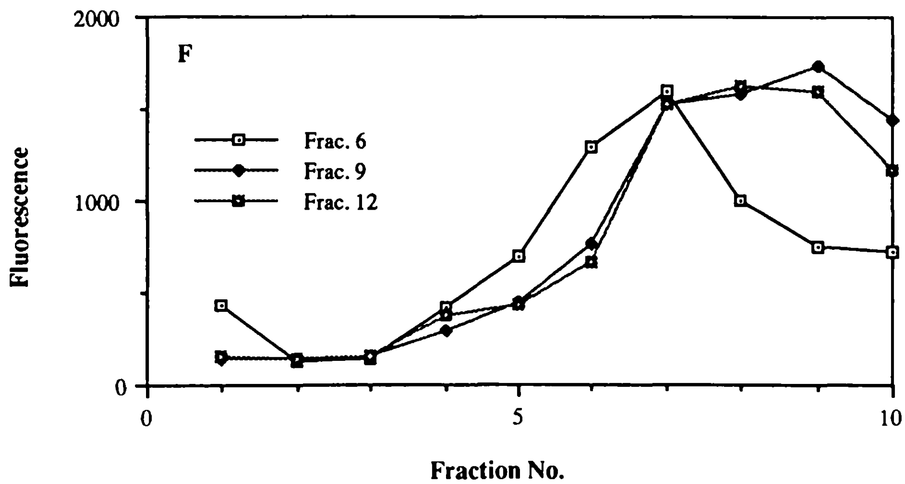


Figure 4.5 (continued)

E: ELISA on the eluate fractions of panel A using anti-*T. retusa* antiserum (K4962).

F: ELISA on the eluate fractions of panels B-D using anti-*T. retusa* antiserum (K4962).

elution-disturbing characteristics of EDTA was another problem in the anion exchange chromatography. In most elution systems, EDTA appeared in the beginning or the middle of the gradient, producing a composite broad peak, which was usually the highest of all peaks (**Figure 4.7**). Mono Q anion exchange chromatography with an acidic buffer (pH 4) yielded a better separation profile (the ionic charge capacity of Mono Q is independent of pH over the range between 2 and 12, Pharmacia, 1985). K4962-reactive macromolecules were separated into two major fractions, and several other uncharacterised components, some of which may possibly be EDTA, have been separated (**Figure 4.8**).

4.5 Hydrophobic interaction chromatography

Fractionation of intracrystalline extracts from *T. retusa* was attempted using a hydrophobic interaction column (**section 3.2.9**), but shell macromolecules were not retained by the column and eluted out before the gradient.

4.6 Discussion and conclusions

One of the initial aims of this project was to purify single proteins out of the mixtures of the shell intracrystalline macromolecules using various liquid chromatography techniques, and to prepare antibodies against each of these purified proteins in order to obtain better-defined phylogenetic and other biological information than those obtained from antibodies raised against whole mixtures of shell extracts. During the course of the experiments, it became clear that it was impossible to obtain an ample volume of high purity proteins for immunisation for each species of interest, and it was therefore unrealistic to prepare antibodies against single proteins for phylogenetic studies. The quantities of shell required were too large for many species, and this would have greatly reduced the number of taxa investigated to an unsatisfactory level. However, several components could be successfully purified, and some characteristics of those macromolecules had been discovered through these purification procedures.

SDS-PAGE is another powerful analytical and preparative technique for the study of proteins. Initial experiments with SDS-PAGE failed to separate shell macromolecules into discrete bands, but yielded a continuous bands with a 'smear' like pattern. Recently an improved method for the removal of EDTA and concentrating the crude extract has been applied (Minitan™ system, Millipore; Minicon™ system, Amicon), and it became possible to visualise discrete bands of brachiopod shell proteins in SDS-PAGE gels (M. Cusack, pers. comm.; Curry *et al.*, 1991a, b). A recent study on the intracrystalline macromolecules of *Terebratulina retusa* by SDS-PAGE stained with Coomassie Brilliant Blue indicated a prominent protein band of 30 kDa in size, together with another weaker

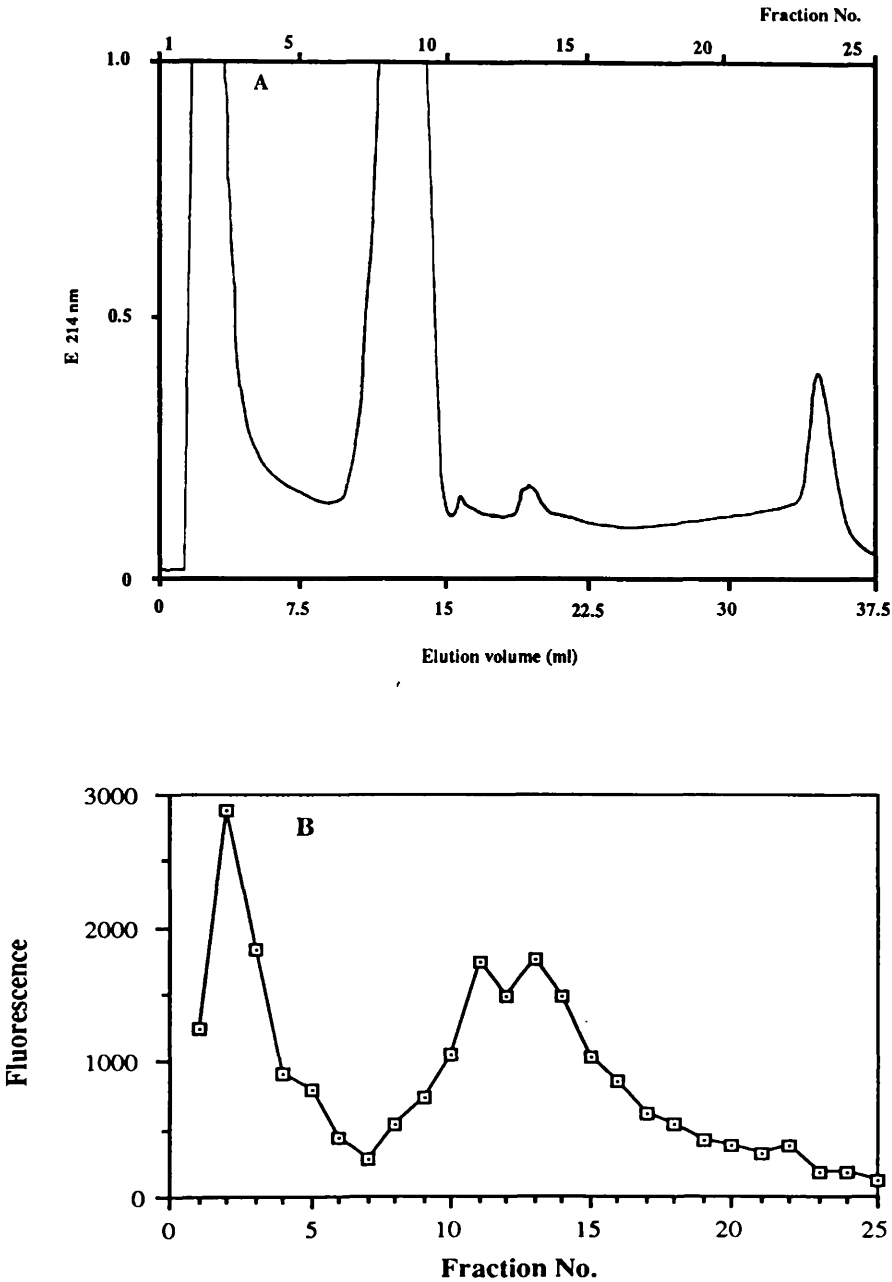


Figure 4.6 Anion exchange chromatography on shell extracts

A: Fractionation of the intracrystalline extracts from *T. retusa* on Mono Q column. Mobile phase, A = diethanolamine (50 mM, pH 8.8), B = A + 1.0 M NaCl, flow rate, 1.5 ml/min, gradient, linear, 0-100% B over 15 min from 3 min, 100% B for 3 min, 100% A for 4 min. Fractions collected from 0 to 25 min every min. Note the high EDTA peak.

B: ELISA on the eluate fractions of panel A.

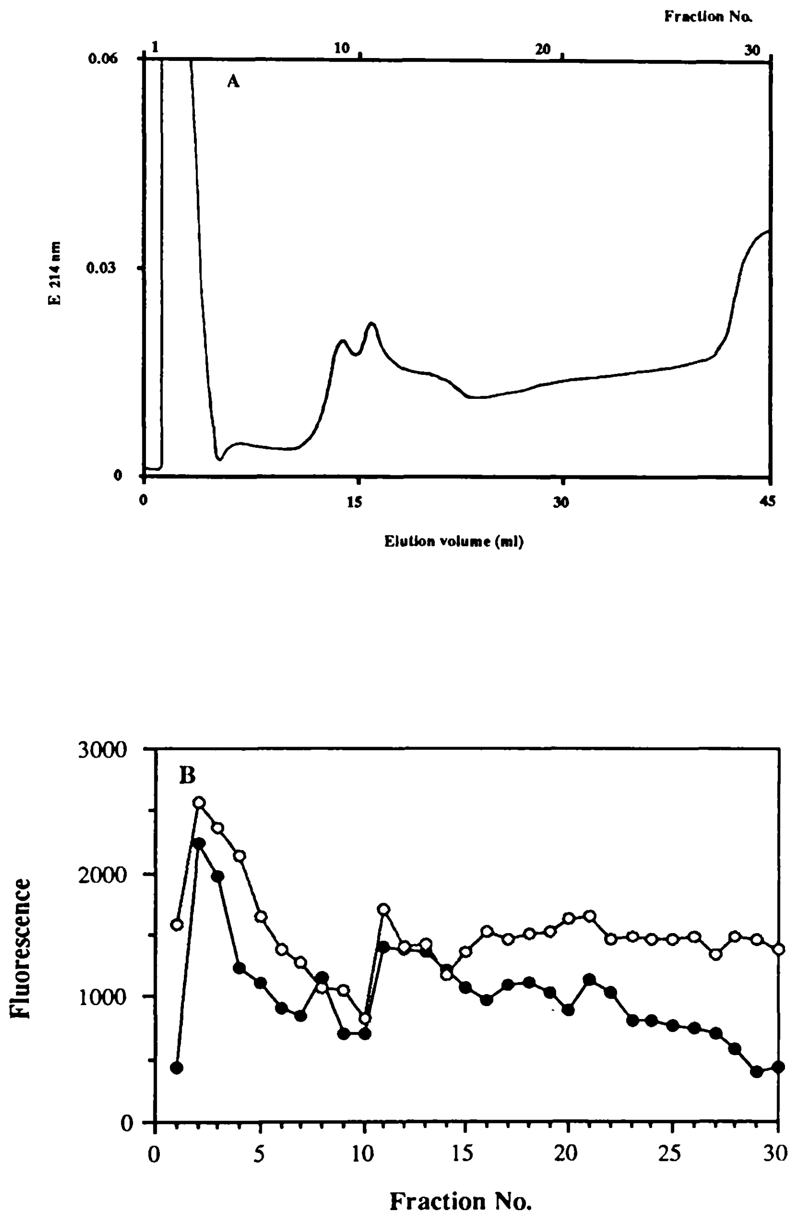


Figure 4.7 Cation exchange chromatography on shell extracts

A: Fractionation of the intracrystalline extracts from *T. retusa* on MonoS. Mobile phase, A = phosphoric acid (50 mM, pH 2.0), B = A + 1.5 M NaCl, flow rate, 1.5 ml/min, gradient, linear, 0-50% B over 20 min from 3 min, 50-100% B over 1 min, 100% B for 3 min, 100% A for 3 min. Fractions collected from 0 to 30 min every min.

B: ELISA on the eluate fractions of panel A.

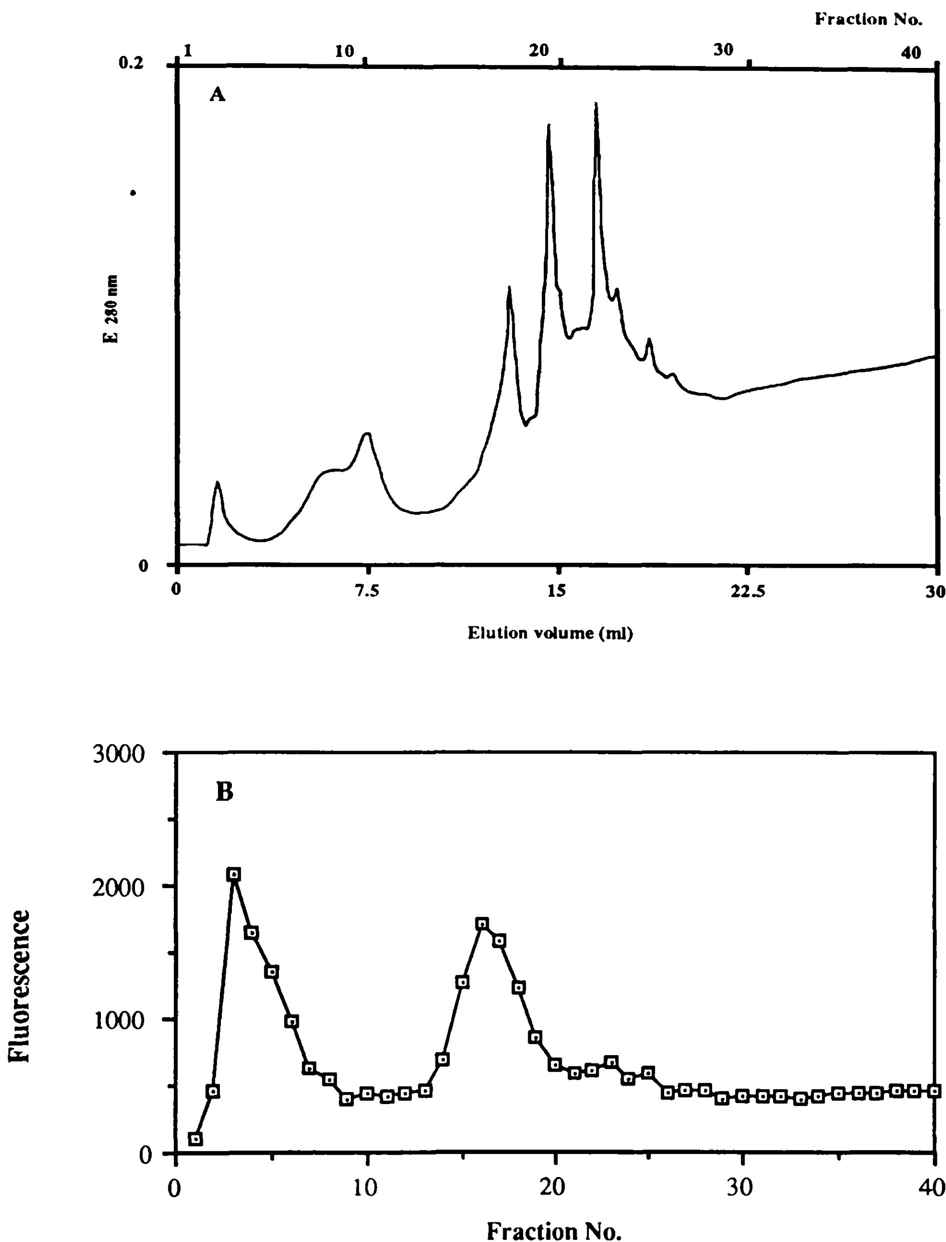


Figure 4.8 Anion exchange chromatography on shell extracts withan acidic elution buffer

A: Fractionation of the intracrystalline extracts from *T. retusa* on Mono Q column. Mobile phase, A = trimethylamine (20 mM, pH 4.0), B = A+1.0 M ammonium formate, flow rate, 1.5 ml/min, gradient, linear, 0-100% B over 15 min from 1.5 min, 100% B for 1.5 min, 100% A for 2 min. Fractions collected from 0 to 20 min every 0.5 min.

B: ELISA on the eluate fractions of panel A.

possible protein band larger than 100 kDa, which could be an aggregate product (D. Walton, pers. comm.; Curry *et al.*, 1991b).

In gel filtration, correlations of the sizes of the components between different elution systems was generally poor (**Table 4.1**), suggesting that some interactions of molecules with the solid phase material occurred. Alternatively or additionally, the shape of the molecules may complicate the separation of the molecules. It is possible that the 40 kDa component in the gel filtration (**Table 4.1**) is correlated with the 30 kDa protein in the SDS-PAGE gel, in which case, the conformation of this protein is likely to be less globular than the size marker proteins [at least less globular than the ovalubumin (MW = 43000) and chymotrypsinogen A (MW = 25000)] utilised in the gel filtration.

Various chromatography procedures revealed that the *Terebratulina* shell extract contained several discrete species of macromolecules, at least one of which is protein. These macromolecules could be separated by their size, hydrophobicity, and to some extent by their charge (**Figures 4.1-4.6**). Fractionation by gel filtration followed by reverse phase chromatography, or the tandem stepwise reverse phase chromatography, would be the best way to purify shell macromolecules using liquid chromatography. Purification by ion exchange chromatography was preferred, because the high loading capacity of the columns easily allow the scaling up of the separations, and the 'milder' condition of the elution buffers, compared with reverse phase chromatography, may keep the denaturation of proteins to a minimum. However, it was difficult to separate brachiopod shell macromolecules by ion exchange chromatography, probably because the shell macromolecules have aberrant ionic features. Considering the behaviour of the macromolecules in ion exchange chromatography, some appear to be neutral in the pH range 3.8 to 8.8, whilst others are highly acidic, and many of them still retain a negative charge on their surface at pH 4.5, and possibly even at pH 2.

No known stain effectively binds to highly acidic proteins in an electrophoresis gel (Weiner and Addadi, 1991). If this is the case, the 30 kDa protein detected by the SDS-PAGE may not be an overall acidic protein, but rather it is possible that the acidic residues are localised mostly on the surfaces of the protein folded in the native state. It is also suspected that another acidic protein, which can not readily be visualised in SDS-PAGE gels, is involved in the *Terebratulina* shell secondary layer, for example, peak 4 in **Table 4.3** is a possible candidate. The possible glycosylation of the brachiopod shell protein is another question to be answered (Curry *et al.*, 1991b; Collins *et al.*, 1991c). Amino acid and carbohydrate analyses on the highly purified components and, if any protein is involved, the sequencing of the protein (or its coding gene) are needed to answer these questions.

Immunological assays on separated components indicated that the antiserum (K4962) had been raised not only against presumed proteinaceous molecules, but also against some other kinds of macromolecules, possibly carbohydrates. Collins *et al.* (1991c) conducted bulk analyses on the secondary shell intracrystalline EDTA soluble fractions

from two brachiopod species; *Neothyris lenticularis* and *Waltonia inconspicua*, and reported that the former species contained 0.52 mg protein and 0.12 mg neutral sugar per g $CaCO_3$ with little acidic polysaccharide, and that the latter contained 0.53 mg protein and 0.21 mg neutral sugar per g $CaCO_3$. Collins *et al.* (1991c) also inferred that the antigenic determinants for the antibodies prepared against these macromolecules were "predominantly (if not exclusively) carbohydrate". It was these antisera, K4962, anti-*Neothyris*, anti-*Waltonia*, and others prepared by similar means (**Table 3.3**), that have been utilised in the phylogenetic analyses in this study. Because of these complications, i.e., the antisera were not raised against purified proteins, it was often required to pre-absorb these antisera with various antigens to destroy non-specific antibody reactivity (including presumably information-poor reactivity directed to carbohydrates) when applying these antisera to the species-level inferences of the brachiopod phylogeny.

Chapter 5 Sero-taxonomy of Recent articulate brachiopods

5.1 Introduction

Immunological assays on the organic extract from living brachiopod shells revealed that the macromolecules embedded within the fibrous calcitic biocrystals contained significant taxonomic information (Collins *et al.*, 1988). Subsequent comparative immunological investigations on these proteinaceous molecules led to a suggestion that the traditional scheme of phylogeny and taxonomy of the Terebratulida, the largest extant order of brachiopods, may require a considerable revision (Collins *et al.*, 1991b; Curry *et al.*, 1991c). The Terebratulida is characterised by the possession of the loop, an internal calcareous skeleton which in life supports the lophophore. An obvious implication of this immunological approach is that a new set of characters became available to help the reconstruction of terebratulide phylogeny, which had previously been investigated predominantly by the form and development of the loop.

Results of these pioneering studies indicated that the short-looped superfamily Cancellothyridacea (Cooper, 1973c), the short-looped Terebratulacea (Cooper, 1983), and the long-looped Terebratellacea (Muir-Wood *et al.*, 1965) were almost equidistantly related to each other, and that a subset of the Terebratellacea was more closely related to the Terebratulacea, in effect suggesting that the long loop of the living terebratulides had evolved independently at least twice. The elaborate development of the loop is undoubtedly one of the most intriguing phenomena in terebratulide evolution. The release of the loop from the prime role in taxonomy would mean that the historical, or phylogenetic, factor could be considered in the investigation of loop morphogenesis for the first time without the risk of circular arguments.

Useful as it may be, however, molecular inference of phylogeny alone, can go wrong and is best synthesised with traditional approaches (Hillis, 1987; Sytsma, 1990). Congruence between molecular and morphological schemes, which would strongly suggest that true phylogeny had been assessed, is particularly desired for brachiopod research, since most brachiopod species are represented, and often only known, as fossils, which can be studied pre-eminently by morphological methods. In other words, suitable morphological explanations are required to appreciate molecular inferences in the context of brachiopod classification when disparity between molecular and morphological methods arises. The terebratulide relationships demonstrated by the previous immunological investigations (Collins *et al.*, 1988; 1991b; Curry *et al.*, 1991c) did contain some unusual branching patterns (cf. **Figure 2.5**), such as the derivation of the second long-loop lineage, including *Kraussina*, from the short-looped Terebratulacea, an anomaly which could not readily be explained in terms of morphological evolution.

The main purpose of this chapter, therefore, is to re-examine the congruency between the traditional and molecular inferences of terebratulide phylogeny, with additional comprehensive immunological data, in an attempt to provide a new integrated interpretation. In this study, a total of 53 articulate species, belonging to 33 genera, composed of 27 terebratulides, 4 rhynchonellides, and 2 thecideidines, and covering about 40% of all known living articulate genera, and three quarters of extant families, have been screened immunologically, using 15 brachiopod antisera.

5.2 Materials and Methods

Table 3.1 lists the samples which were available to this study. Antisera were prepared against shell intracrystalline macromolecules extracted from 15 species (**Table 3.4**) using the method described in **sections 3.2.1, 3.2.2,** and **3.2.3**. Thirty-five out of the 53 species were assayed against 12 antisera (remaining antisera being the ones against three *Terebratulina* species) using ELISA (**section 3.2.4**) to assess the framework of relationships. Cancellothyridoid species, involving 12 other *Terebratulina* species and subspecies (15 in total) were assayed using ELISA with 4 antisera against different *Terebratulina* species. Subsets of the cancellothyridoid species, subsets of the 35 species in the framework study, and the remaining 6 species (5 *Laqueus* and 1 *Terebratella*) were assayed using the specific pre-absorbed antisera (inhibition ELISA, **section 3.2.7**) to study in more detail the relationships within each taxonomic group. Resulting data were processed and analysed using the method described in **section 3.2.8**. In multivariate methods, an analysis of relationships between variables is often referred to as an *R*-mode analysis, whilst an analysis of the relationship between the specimens of a sample is usually referred to as a *Q*-mode analysis

5.3 Results

The calculated reactivity scores between the 35 antigens of articulate species and the 12 brachiopod antisera are summarised as a data matrix in **Table 5.1**. The antiserum against the rhynchonellide *Notosaria* (K5038) reacted only with itself and one of the thecideidine genus *Lacazella*, and did not react with any other rhynchonellides, terebratulides, nor *Thecidellina*. The other antisera prepared against terebratulide species generally had a stronger reaction with terebratulide species than with rhynchonellides or thecideidines, although some of the antisera (e.g. K5007, K5040) showed considerable reactions with the thecideidines.

Antisera prepared against *Liothyrella*, *Gryphus*, *Kraussina*, and *Megerlia* (803,

K5010, 802, 801, K5053) showed more or less similar patterns, with strong reactions with terebratulaceans (Cooper, 1983), kraussinids, megathyridids, macandreviids (Cooper, 1973b), and an ecnomiosiid (Cooper, 1977), and little reaction with other species. The terebratulacean *Abyssothyris* and the megathyridid *Argyrotheca* yielded among the least reactive terebratulide antigens to most of the antisera, but reacted moderately with one of the antisera prepared against *Liothyrella* (803, K5010).

Antiserum prepared against *Terebratulina septentrionalis* (173) (the antisera are hereafter also denoted as 'Anti-*Terebratulina septentrionalis*', etc.) reacted strongly with *T. septentrionalis* and the other two cancellothyridaceans (Cooper, 1973c), and showed weaker reactions with a few other non-cancellothyridacean terebratulides. Cancellothyridacean antigens reacted strongly with anti-*Terebratulina* (173) and anti-*Dallina* (K5007), and moderately with anti-*Pictothyris* (1192).

Antisera prepared against *Waltonia*, *Laqueus*, and *Pictothyris* (K5040, 1191, 1192) showed similar profiles to each other with strongest reactions with terebratellids, dallinids (Richardson, 1975), and laqueids (Richardson, 1975, with the exception of Macandreviinae). Moderately strong reactions occurred with one of the *Macandrevia* species (*M. cranium*), and sporadic weaker reactions with other terebratulides. Anti-*Dallina* (K5007) was the least specific antiserum of all. However, anti-*Dallina* (K5007) hardly reacted with rhynchonellides, and did not react too strongly with terebratulaceans, kraussinids, megathyridids, macandreviids, *Ecnomiosa*, and *Lacazella*. Anti-*Neothyris* (427) serum, on the contrary, reacted very specifically, only with the terebratellid genera, *Neothyris*, *Magellania*, *Terebratella*, and *Waltonia*.

The overall pattern of reactions discriminated rhynchonellides from other articulates by the lack of significant reactions with any of the antisera, except for the self-reaction with the anti-*Notosaria* serum. Pair-wise comparisons of antigens using reactivities against each antiserum as characters (*Q*-mode bivariate analysis) revealed no indication of correlation in the reaction against each antiserum among the four rhynchonellides species, though it was indicated that *Hemithyris* had slight correlations with terebratulids, macandreviids, kraussinids, and *Lacazella*, and that *Frieleia* also had slight correlations with *Gryphus*, *Abyssothyris*, and *Argyrotheca* (**Figure 5.1**).

The pair-wise comparisons also indicated that there was a slight correlation between the two thecideidine species (**Figure 5.1**; significant level: $p < 0.1$). *Lacazella* correlated slightly with *Pictothyris* and *Hemithyris*. *Thecidellina* had some affinities with *Terebratulina*, *Chlidonophora*, dallinids, terebratellids, and laqueids (**Figure 5.1**).

Among the terebratulides, four major groups were recognised by the *Q*-mode bivariate analysis : (1) Terebratulaceans (Cooper, 1983), denoted as group *T* here, (2) Cancellothyridaceans (Cooper, 1973c), group *C*, (3) a group of dallinids, terebratellids, and laqueids (Richardson, 1975 with Macandreviinae being subtracted from the laqueids), group *D*, and (4) a group of kraussinids, megathyridids, macandreviids (Cooper, 1973b),

and an ecnomiosiid (Cooper, 1977), group *K* (**Figure 5.1**). Although *Megathiris* was indicated to be included in group *K*, another megathyridid *Argyrotheca* could not be assigned to any of these groups by this bivariate analysis. Strong and consistent correlations between groups *T* and *K* were indicated. Weak affinities between groups *C* and *D*, and a single instance of weak correlation between members of groups *D* and *K* were also observed (**Figure 5.1**). The strong correlations between terebratulids and kraussinids were also demonstrated by the *R*-mode bivariate comparisons of each antiserum (**Table 5.2**).

The *Q*-mode multivariate analysis (principal component analysis) further confirmed these groupings, with groups *T* and *K* being very closely plotted, and this bundle was well apart from the plots of group *D* or from another bundle of group *C* plots, though *Argyrotheca* and *Abyssothyris* plotted rather closer to the latter group (**Figure 5.2**). Rhynchonellides and thecideidines also occupied more or less close places with group *C* in the plane defined by the first two latent vectors. However, the third axis pulled the rhynchonellides well apart from group *C*, and the thecideidines filled the space between rhynchonellides and terebratulides in the three dimensional plots (**Figure 5.2**).

While major groups of terebratulides were elucidated by analysing these results of the assays carried out using the crude antisera, these data were generally insufficient for the inference of relationships *within* each group. More specific antisera were required to obtain a finer-grain picture of phylogeny, and the pre-absorption technique was employed for this purpose. Cluster analysis was employed to fractionate the species into major groups, and each major group was re-analysed using the data of the assays with pre-absorbed antisera (**Figure 5.3**).

The assays on the species belonging to groups *T* and *K* using pre-absorbed antisera clearly discriminated these two groups (**Table 5.3**), a discrimination that was shown by the cluster analysis (**Figure 5.3**), but was not absolutely explicit in the principal component analysis (**Figure 5.2**) because of the close relationship between these two groups. These assays also indicated that the two *Liothyrella* species and two kraussinid species were coherent groups, and also discriminated each of the four as distinct species (**Table 5.3, Figure 5.3**). Two *Macandrevia* species behaved more or less differently, and formed a cluster with a longer branch length than that of the two kraussinid species in the cluster analysis (**Figure 5.3**).

Assays on species of *D* group using the pre-absorbed antisera clearly separated the 'laqueids' of the northern hemisphere and terebratellids of the southern hemisphere, but failed to detect the 'dallinids' (*Dallina* and *Campages*) as a coherent group, with *Dallina* closely linked with the terebratellids and *Campages* well apart from all others (**Table 5.4, Figure 5.3**). This could be an experimental artifact as the anti-*Dallina* serum yielded rather high non-specific bindings (**Table 5.1**). More work is required to determine the relationships of the dallinids to the other two families. The 'laqueids' involved *Laqueus*, *Pictothyris*, *Jolonica*, *Terebratalia*, and *Dallinella* (**Table 5.5, Figure 5.4**), together with '*Frenulina*'

Table 5.1 Matrix of immunological reactivities for living articulate brachiopods

Reactions with homologous antigens as 100, reactions with negative controls (bivalve *Mercenaria*) as 0. Negative readings as 0. Mean values of duplicate experiments. Asterisk denotes 10-20% variation between the duplicate readings, other figures contained less than 10 % variations. For antiserum identities see **Table 3.4**, for antigen identities see **Table 3.1**; *1 = *L. neozelanica*, *2 = *L. uva notocardensis*, *3 = *T. septentrionalis*, *4 = *T. dorsata*, *5 = *L. rubellus*, *6 = *M. cranium*, *7 = *M. africana*.

Antigens \ Antisera	**5038** Not	**803** Lio	**5010** Lio	**802** Gry	**173** Ter	**5007** Dal	**5040** Wal	**427** Neo	**1191** Laq	**1192** Pic	**801** Kra	**5053** Mer
Rhynchonellida												
1. Basiliola	0	0	1	3	*0	0	9	0	0	0	12	4
2. Hemithyris	0	10	7	44	0	20	7	0	10	0	12	2
3. Notosaria	**100**	0	3	5	*0	15	8	1	0	1	11	1
4. Frieleia	0	9	4	0	*0	0	9	1	0	0	0	5
Terebratulacea												
5. Liothyrella*1	0	**100**	96	134	19	71	16	1	10	41	39	86
6. Liothyrella*2	0	102	**100**	136	0	64	9	0	10	7	49	89
7. Tichosina	0	92	94	119	0	0	8	1	7	0	42	68
8. Gryphus	0	82	80	**100**	2	0	2	1	6	0	22	40
9. Abyssothyris	0	17	47	0	0	1	9	1	9	12	11	11
Cancellothyridacea												
10. Cancellothyris	0	48	9	0	102	104	17	0	7	40	13	8
11. Terebratulina*3	0	0	5	0	**100**	116	9	0	7	20	10	5
12. Chlidonophora	0	4	3	0	90	103	17	0	6	*41	12	7
Terebratellacea												
13. Dallina	0	0	7	22	1	**100**	92	1	80	97	16	44
14. Campages	0	3	6	0	41	108	96	1	75	98	*19	7
15. Terebratella*4	0	8	7	0	0	106	98	70	78	100	22	63
16. Waltonia	0	0	7	11	0	105	**100**	17	88	103	10	7
17. Magellania	0	0	6	0	0	100	95	73	81	101	10	14
18. Gyrothyris	0	5	7	0	0	104	100	2	85	102	12	8
19. Neothyris	0	3	6	0	0	103	96	**100**	84	103	13	5
20. Terebratalia	0	0	7	43	0	113	82	1	78	99	10	9
21. Coptothyris	0	0	6	11	67	111	87	0	85	102	12	8
22. Dallinella	0	0	3	0	61	108	94	1	77	105	12	7
23. Jolonica	0	8	8	34	0	112	93	0	86	101	11	7
24. Frenulina	0	5	7	27	41	112	92	1	85	100	12	8
25. Laqueus*5	0	*4	7	104	0	104	90	1	**100**	101	12	5
26. Pictothyris	0	8	7	21	0	108	95	0	88	**100**	12	8
27. Macandrevia*6	0	31	79	105	0	81	58	1	74	86	92	100
28. Macandrevia*7	0	66	74	*79	0	86	20	1	19	37	*51	74
29. Ecnomiosa	0	60	89	53	0	86	9	1	10	0	65	85
30. Kraussina	0	67	70	94	0	78	27	1	9	27	**100**	88
31. Megerlia	0	73	82	124	0	74	9	1	9	4	73	**100**
32. Megathiris	0	33	49	30	0	*84	*10	1	29	57	50	79
33. Argyrotheca	0	47	6	0	0	1	6	1	11	0	13	7
Thecideidina												
34. Lacazella	29	1	3	*47	0	*45	*20	1	11	1	11	8
35. Thecidellina	0	12	6	40	0	105	55	0	10	32	11	3

Table 5.2 ***R*-mode analysis of the immunological data matrix**

Correlation coefficient between reactivity values for each pair of the antisera (between each pair of columns in the **Table 5.1**) is tabulated; asterisks indicate significant correlations ($p < 0.001$). For antiserum identities, see **Table 3.4**.

	803	5010	802	173	5007	5040	427	1191	1192	801	5053
5038	-0.105	-0.156	-0.118	-0.105	-0.276	-0.195	-0.066	-0.220	-0.245	-0.125	-0.172
803		*0.879	*0.784	-0.180	-0.357	-0.595	-0.224	-0.538	-0.542	*0.623	*0.738
5010			*0.824	-0.272	-0.259	-0.490	-0.199	-0.400	-0.414	*0.792	*0.879
802				-0.332	-0.157	-0.322	-0.272	-0.233	-0.280	*0.668	*0.729
173					0.376	-0.018	-0.166	-0.054	0.105	-0.238	-0.288
5007						*0.699	0.215	*0.659	*0.778	-0.301	-0.321
5040							0.403	*0.959	*0.950	-0.301	-0.321
427								0.359	0.366	-0.137	-0.062
1191									*0.958	-0.226	-0.237
1192										-0.211	-0.216
801											*0.899

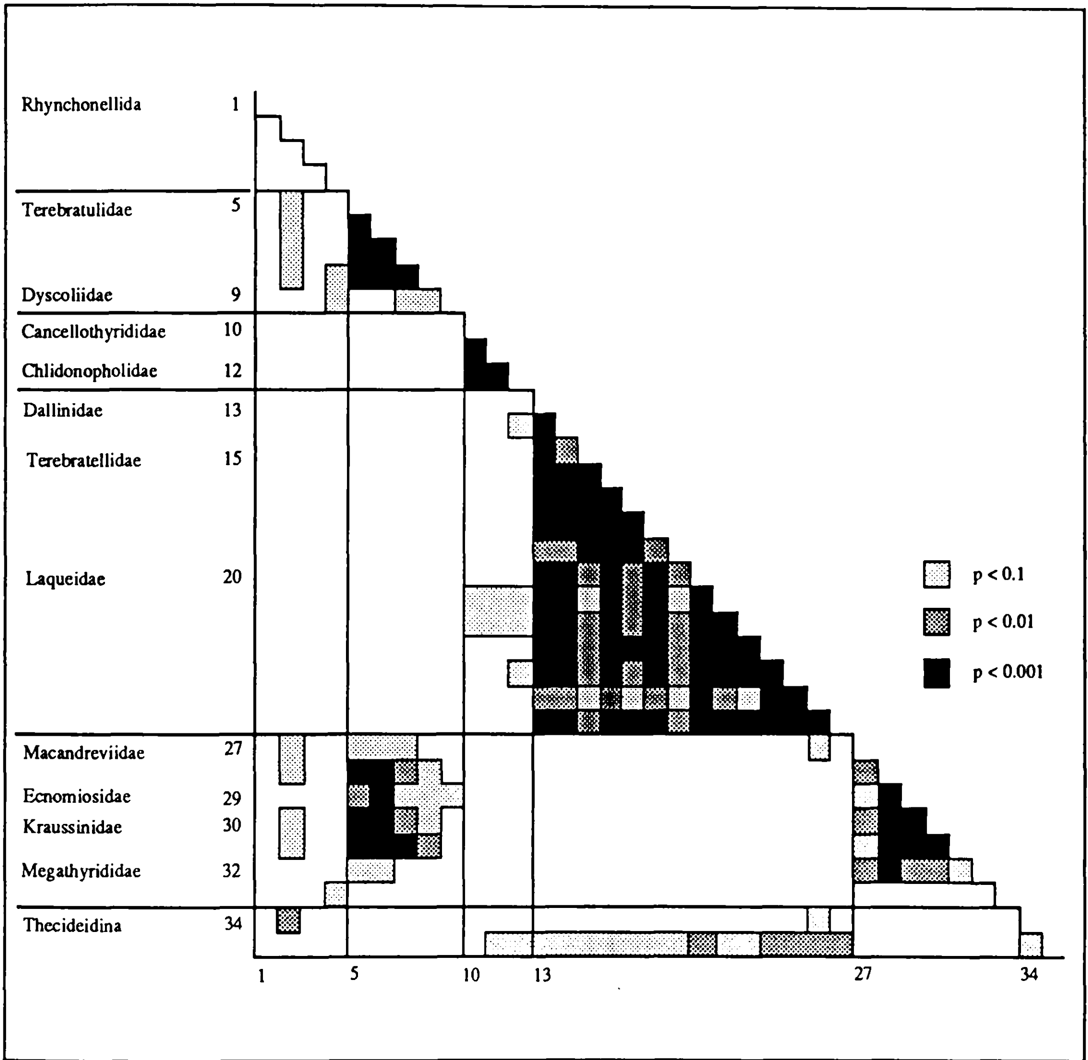


Figure 5.1 ***Q*-mode bivariate comparisons of immunological reactivity data**

Correlation coefficients for each pair of antigens (rows in **Table5.1**) were calculated, and positive correlations ($p < 0.1$) are indicated with their significance levels. Numbers indicate the species (antigen) identity numbers in**Table 5.1**.

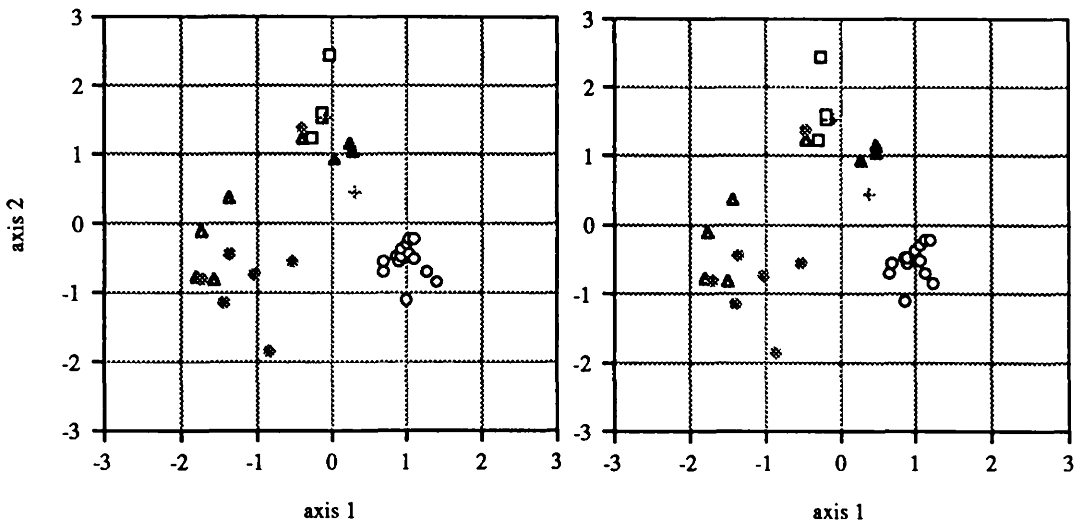


Figure 5.2 Stereo pair of diagram illustrating the results of principal component analysis

Separations along the first three latent vectors of the principal component analysis on the data in **Table 5.1**. The third axis is vertical to axes 1 and 2. Key: squares, rhynchonellides; crosses, thecideidines; filled triangles, Cancellothyrididae and Chlidonophoridae; open triangles, Terebratulidae and Dyscoliidae; open circles, Terebratellidae, Laqueidae, and Dallinidae; filled circles, Kraussinidae, Megathyrididae, Macandreviidae, and Ecnomiosidae.

Table 5.3 Inhibition ELISA on Terebratulidae, Macandreviidae, Ecnomiosidae, Kraussinidae and Megathyrididae

Reactions on homologous antigens with the non-preabsorbed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments. Each column (antiserum pre-absorbed by different antigen) can be compared to each other, and higher figures indicate less inhibition by the pre-absorption with the antigen, suggesting a more distant relationship between the species of that pre-absorbed antigen and the species against which the antiserum was prepared. Each row (antigen) can be compared to each other, and higher figures indicate more reactions with the specific pre-absorbed antisera, suggesting a closer relationship between the species of the assayed antigen and the species against which the antiserum was prepared (see next page).

Antigens	Antisera (pre-absorbed against)									
	-Ln	-Lu	-Gv	-Tf	-Md	-Mc	-Ma	-Ec	-Kr	-Mt
Anti-Liothyrella (803)										
Liothyrella neozelanica (Ln)	0	0	21	19	100	91	89	100	85	88
Liothyrella uva (Lu)	0	0	0	94	100	100	87	100	87	45
Gryphus vitreus (Gv)	0	0	0	3	92	97	22	74	79	88
Tichosina floridensis (tf)	0	0	16	19	91	90	80	91	70	86
Megathiris detruncata (Md)	0	0	2	0	3	0	3	0	1	0
Macandrevia cranium (Mc)	0	0	7	0	10	0	10	0	8	0
Macandrevia africana (Ma)	0	0	1	0	71	56	7	82	11	55
Ecnomiosa sp. (Ec)	0	0	1	0	6	5	6	21	2	0
Kraussina rubra (Kr)	0	0	0	0	61	56	40	83	1	25
Megerlia truncata (Mt)	0	0	0	0	36	56	43	69	9	6
Anti-Gryphus (802)										
Liothyrella neozelanica	0	0	0	0	100	98	49	100	62	58
Liothyrella uva	0	0	0	0	96	81	55	95	50	55
Gryphus vitreus	0	0	0	8	89	90	0	0	66	76
Tichosina floridensis	0	0	0	0	89	97	70	100	52	78
Megathiris detruncata	0	0	0	0	63	53	27	79	3	5
Macandrevia cranium	0	0	0	0	59	0	37	64	26	0
Macandrevia africana	0	0	0	0	81	58	42	77	19	6
Ecnomiosa sp.	0	0	0	0	70	52	39	73	7	2
Kraussina rubra	0	0	0	0	100	69	68	100	29	36
Megerlia truncata	0	0	0	0	83	23	47	93	33	0
Anti-Kraussina (801)										
Liothyrella neozelanica	2	0	2	0	0	0	0	0	0	0
Liothyrella uva	14	0	5	0	9	0	8	3	0	0
Gryphus vitreus	3	0	2	0	3	0	0	0	0	0
Tichosina floridensis	2	0	3	1	5	1	4	3	0	0
Megathiris detruncata	1	1	0	0	4	2	1	3	0	3
Macandrevia cranium	75	41	60	51	0	0	0	0	0	0
Macandrevia africana	18	8	15	28	0	0	0	2	0	0
Ecnomiosa sp.	20	20	31	23	2	1	2	1	0	1
Kraussina rubra	83	93	94	91	56	22	63	79	0	79
Megerlia truncata	41	26	49	56	6	0	4	6	0	0
Anti-Megerlia (K5053)										
Liothyrella neozelanica	9	21	48	46	36	51	54	68	39	0
Liothyrella uva	31	0	38	15	31	0	32	22	11	0
Gryphus vitreus	0	0	5	6	6	0	0	0	0	0
Tichosina floridensis	10	0	15	23	24	11	35	45	0	0
Megathiris detruncata	56	58	63	72	2	13	37	21	3	0
Macandrevia cranium	69	46	70	76	13	0	15	18	0	0
Macandrevia africana	69	54	86	82	43	0	24	41	11	0
Ecnomiosa sp.	79	67	84	79	20	9	36	31	0	0
Kraussina rubra	80	69	83	85	37	26	57	46	7	0
Megerlia truncata	85	80	90	89	61	67	72	79	58	0

Table 5.4 Inhibition ELISA on Terebratellidae, Dallinidae, and Laqueidae

Reactions on homologous antigens with the non-preabsorbed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments.

Antigens	Antisera (pre-absorbed against)									
	-Nl	-Td	-Ts	-Wi	-Mm	-Gm	-Ds	-Cb	-Lr	-Do
Anti-Dallina (K5007)										
Neothyris lenticularis (Nl)	0	0	0	16	8	10	0	66	60	49
Terebratella dorsata (Td)	0	0	5	27	10	21	0	70	64	59
Terebratella sanguinea (Ts)	0	0	0	0	8	3	0	69	0	53
Waltonia inconspicua (Wi)	0	0	0	6	6	5	0	69	59	53
Magellania macquariensis (Mm)	0	0	0	14	6	15	0	64	62	49
Gyrothyris mawsoni (Gm)	0	0	0	6	7	10	0	63	58	50
Dallina septigera (Ds)	0	0	7	25	10	26	0	69	69	56
Campages basilanica (Cb)	0	0	0	10	4	9	0	60	53	37
Laqueus rubellus (Lr)	0	0	0	0	4	7	0	62	33	27
Dallinella occidentalis (Do)	0	0	0	0	8	4	0	64	43	34
Anti-Waltonia (K5040)										
Neothyris lenticularis	0	20	21	0	9	0	0	41	3	0
Terebratella dorsata	0	8	2	0	13	10	7	41	14	27
Terebratella sanguinea	0	29	48	0	11	0	0	57	0	29
Waltonia inconspicua	0	24	8	0	18	8	13	55	20	26
Magellania macquariensis	0	14	6	0	9	0	5	47	6	12
Gyrothyris mawsoni	4	30	12	0	19	0	0	36	12	24
Dallina septigera	0	9	28	0	5	0	0	56	14	18
Campages basilanica	2	1	0	0	0	0	0	0	0	0
Laqueus rubellus	0	21	9	0	12	0	0	39	0	0
Dallinella occidentalis	0	21	1	0	17	0	0	28	0	0
Anti-Neothyris (427)										
Neothyris lenticularis	0	33	83	75	32	58	65	82	100	90
Terebratella dorsata	0	0	68	75	0	79	45	76	93	98
Terebratella sanguinea	0	0	0	93	0	22	1	39	65	50
Waltonia inconspicua	0	7	44	11	13	54	50	64	57	32
Magellania macquariensis	0	7	69	77	0	73	48	76	87	72
Gyrothyris mawsoni	0	1	0	0	4	1	2	15	28	17
Dallina septigera	0	0	21	53	0	40	4	57	62	69
Campages basilanica	0	4	0	0	0	0	0	0	26	7
Laqueus rubellus	0	0	0	0	0	6	0	1	0	8
Dallinella occidentalis	0	0	15	0	0	29	23	9	16	5
Anti-Laqueus (1191)										
Neothyris lenticularis	0	9	0	0	7	10	8	37	0	17
Terebratella dorsata	0	2	0	0	4	5	6	35	0	15
Terebratella sanguinea	0	22	0	0	4	2	2	34	0	21
Waltonia inconspicua	0	10	0	0	6	6	4	41	0	23
Magellania macquariensis	0	0	0	0	0	2	3	31	0	11
Gyrothyris mawsoni	0	0	1	0	1	2	2	27	0	11
Dallina septigera	0	1	0	0	5	4	3	32	0	16
Campages basilanica	0	0	0	0	0	0	1	4	0	0
Laqueus rubellus	59	77	78	80	79	75	81	81	0	69
Dallinella occidentalis	34	38	38	41	40	44	49	53	0	23

Table 5.5 Inhibition ELISA on laqueid species

Reactions on homologous antigens with the non-preabsorbed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments.

Antigens	Antisera (pre-absorbed against)				
	-Lr	**-Pp**	**-Jn**	**-Do**	**-Tt**
Anti-Laqueus (1191)					
Laqueus rubellus (Lr)	0	12	19	46	23
Pictothyris picta (Pp)	0	0	0	8	0
Jolonica nipponica (Jn)	0	0	0	3	0
Dallinella occidentalis (Do)	0	0	0	0	0
Terebratalia transversa (Tt)	0	0	0	1	0
Anti-Pictothyris (1192)					
Laqueus rubellus	1	0	0	12	2
Pictothyris picta	0	0	0	13	1
Jolonica nipponica	1	0	1	0	1
Dallinella occidentalis	1	0	1	1	0
Terebratalia transversa	0	0	1	1	1

Table 5.6 Inhibition ELISA on *Laqueus, Pictothyris* and *Jolonica*

Reactions on homologous antigens with the non-preabsorbed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments.

Antigens	Antisera (pre-absorbed against)							
	-Lr	-Lo	-Lq	-Lb	-Lc	-Lv	-Pp	-Jn
Anti-Laqueus (1191)								
Laqueus rubellus (Lr)	0	22	69	60	67	78	41	37
L. rubellus obessus (Lo)	0	22	49	46	54	61	56	47
L. quadratus (Lq)	0	11	24	13	39	35	33	22
L. blanfordi (Lb)	0	26	41	42	50	55	38	29
L. californicus (Lc)	0	9	19	8	28	27	21	26
L. calif. vancouvensis (Lv)	0	3	14	1	18	21	22	13
Pictothyris picta (Pp)	0	17	46	35	50	50	6	18
Jolonica nipponica (Jn)	0	2	29	22	25	30	9	0

Table 5.7 Immunological reactions with cancellothyridoid species

Reactions with homologous antigens as 100, reactions with negative controls (bivalve *Codakia*) as 0. For details of the antisera and antigens, see **Tables 3.1** and **3.4**. Antisera were not pre-absorbed with antigens. Mean values of duplicate experiments.

Antigens \ Antisera	**K4962** (ret)	**173** (sep)	**174** (ung)	**171** (cro)
Terebratulina retusa	**100**	90	87	83
T. septentrionalis	102	**100**	97	105
T. unguicula	93	87	**100**	77
T. unguicula rotundata	93	90	102	61
T japonica	106	89	93	95
T. peculiaris	107	95	98	99
T. pacifica	56	81	78	61
T. crossei	59	75	66	**100**
T. reevei	56	29	72	51
T. abyssicola	75	44	76	11
T. latifrons	73	36	92	67
T. cailleti	92	63	93	29
T. kiiensis	46	66	77	96
Chlidonophora incerta	99	86	102	73
Cancellothyris australis	113	94	105	60

Table 5.8 Inhibition ELISA on *Terebratulina* species

Reactions between homologous antigens with the non-preabsobed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments.

Antigens	Antisera (pre-absorbed against)							
	-re	-se	-un	-ro	-ja	-pe	-pa	-cr
Anti-T. retusa (K4962)								
Terebratulina retusa (re)	0	7	22	25	16	0	33	47
T. septentrionalis (se)	0	0	14	22	1	0	15	18
T. unguicula (un)	0	4	0	0	5	4	40	50
T. unguicula rotundata (ro)	0	5	0	0	1	2	39	24
T. japonica (ja)	0	7	10	5	0	6	36	44
T. peculiaris (pe)	0	13	19	18	3	11	42	53
T. pacifica (pa)	0	0	11	12	6	3	0	11
T. crossei (cr)	0	0	0	3	0	2	0	0
Anti-T. septentrionalis (173)								
Terebratulina retusa	5	0	32	29	14	40	57	60
T. septentrionalis	0	0	27	37	19	26	52	69
T. unguicula	8	0	20	34	22	35	64	83
T. unguicula rotundata	8	0	24	31	12	31	56	78
T. japonica	10	0	40	48	10	33	59	73
T. peculiaris	0	0	38	38	12	13	65	84
T. pacifica	10	0	33	35	3	8	19	50
T. crossei	11	0	21	35	0	0	16	38
Anti-T. unguicula (174)								
Terebratulina retusa	20	31	0	1	5	9	76	95
T. septentrionalis	37	27	0	1	2	19	75	79
T. unguicula	45	45	0	0	47	40	70	86
T. unguicula rotundata	45	43	0	3	42	48	80	87
T. japonica	50	41	0	2	13	15	78	92
T. peculiaris	32	31	0	0	13	8	77	81
T. pacifica	17	7	0	1	0	1	27	59
T. crossei	21	5	0	2	13	15	43	14
Anti-T.crossei (171)								
Terebratulina retusa	0	0	87	97	0	0	0	0
T. septentrionalis	0	0	50	80	4	0	19	0
T. unguicula	23	12	8	41	18	43	76	0
T. unguicula rotundata	9	0	0	21	25	15	46	0
T. japonica	0	0	62	70	0	0	0	0
T. peculiaris	0	0	85	76	19	0	4	0
T. pacifica	0	0	70	92	0	0	0	0
T. crossei	32	27	72	88	47	19	31	0

Table 5.9 Inhibition ELISA on *Terebratulina*, *Cancellothyris*, and *Chlidonophora*

Reactions on homologous antigens with the non-preabsorbed antisera as 100, with the antisera pre-absorbed by the homologous antigens as 0. Mean values of duplicate experiments.

Antigens	Antisera (pre-absorbed against)				
	-Tr	-Tu	-Tc	-Ca	-Ci
Anti-T. retusa (K4962)					
Terebratulina retusa (Tr)	0	68	68	69	60
T. unguicula (Tu)	0	2	40	14	9
T. crossei (Tc)	0	0	0	1	0
Cancellothyris australis (Ca)	0	19	45	26	26
Chlidonophora incerta (Ci)	0	1	29	2	0
Anti-T. unguicula (174)					
Terebratulina retusa	0	0	63	0	0
T. unguicula	10	0	84	8	24
T. crossei	26	0	16	2	11
Cancellothyris australis	21	0	72	7	14
Chlidonophora incerta	7	0	66	0	0
Anti-T. crossei (171)					
Terebratulina retusa	0	84	0	60	46
T. unguicula	0	9	0	50	52
T. crossei	63	91	0	88	81
Cancellothyris australis	0	67	0	13	10
Chlidonophora incerta	13	29	0	54	9

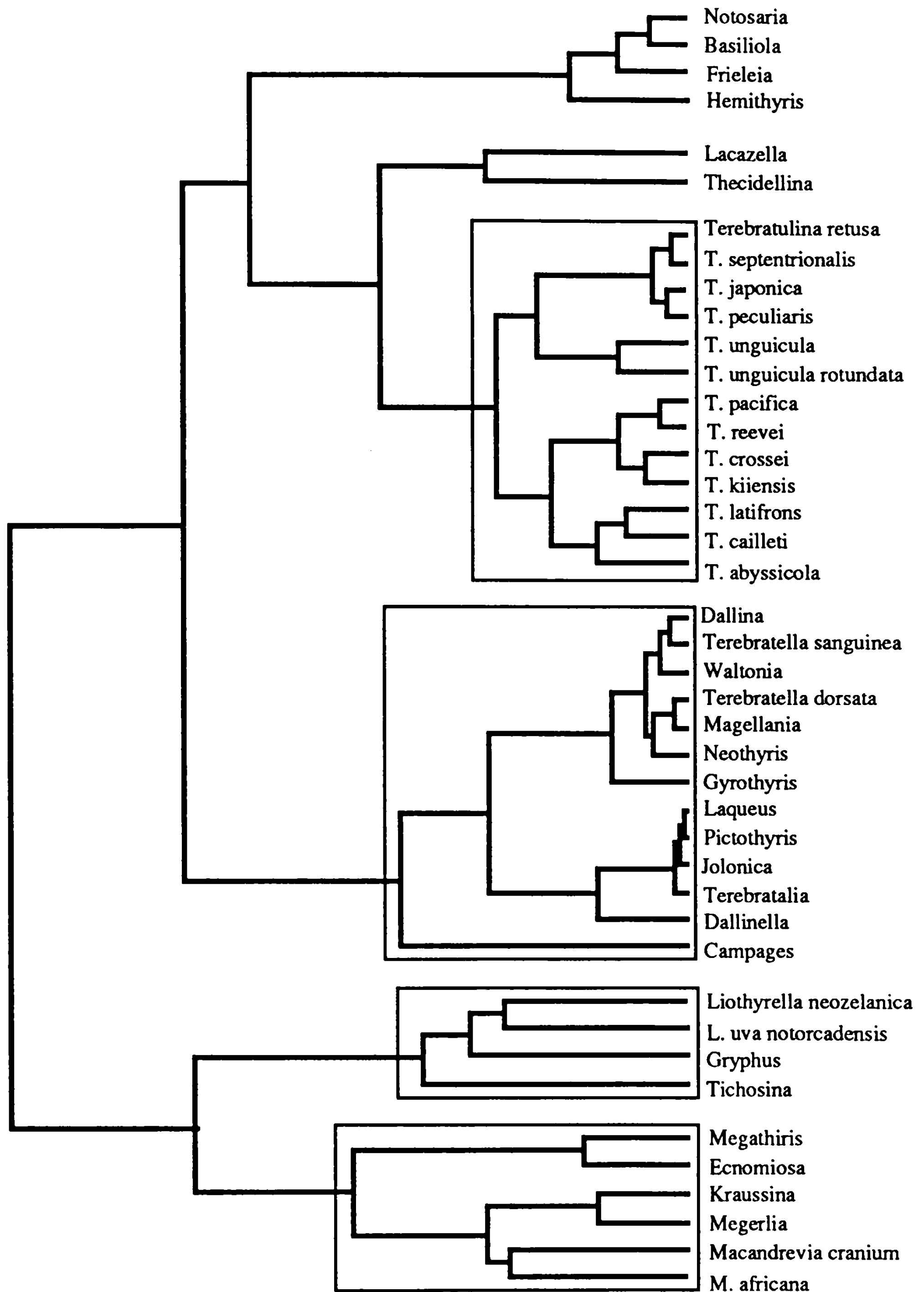


Figure 5.3 Cluster analyses based on immunological reactivity data

Overall pattern based on the single-linkage analysis on the data in **Table 5.1**. The relationship within each box based on separate analysis on the inhibition data tabulated in Tables 3, 4 and 8. Note that this is not a phylogenetic interpretation, but a representation of the structure of the immunological data.

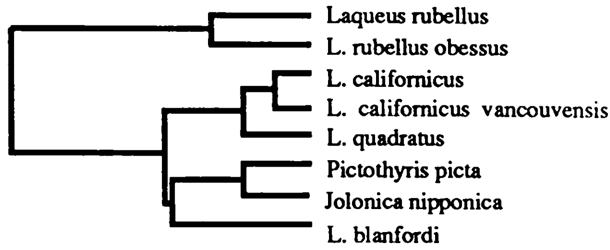


Figure 5.4 Cluster analysis of the inhibition data on laqueid species

Single-linkage analysis from the data in **Table 5.6**.

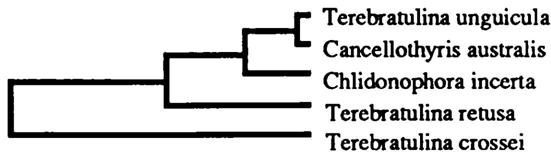


Figure 5.5 Cluster analysis of the inhibition data on *Terebratulina*, *Cancellothyris* and *Chlidonophora*

Single-linkage analysis from the data in **Table 5.9**.

and *Coptothyris* (**Figure 6.3**), supporting the assignment of Richardson (1975) of *Coptothyris*, *Dallinella*, *Terebratalia*, *Frenulina*, and *Jolonica* in the Laqueidae. Experiments on the genus *Laqueus* (**Table 5.6, Figure 5.5**) indicated the close relationship between the two Northwest American species, but failed to clearly discriminate the genus from other laqueid genera. A possible interpretation of this result is that one of the *Laqueus* stocks gave rise both to *Pictothyris* and to Jolonica. Among the terebratellids, *Neothyris*, *Magellania*, and *Terebratella dorsata* form a cluster, and the genus *Terebratella* is a coherent group (**Figures 5.3, 5.4**).

Antisera prepared against four *Terebratulina* species, even without pre-absorption treatment, detected a considerable amount of molecular variations among species of *Terebratulina*, variations that were larger than those among the group *D* families (**Table 5.7**). *Terebratulina* was divided into two major groups, one comprising *T. retusa*, *T. septentrionalis*, *T. japonica*, *T. peculiaris*, *T. unguicula*, and *T. unguicula rotundata*, and the other group comprising *T. pacifica*, *T. reevei*, *T. crossei*, *T. kiiensis*, *T. latifrons*, *T. cailleti*, and *T. abyssicola* (**Figure 5.3**). The former group was further divided into two subclusters, one being *retusa-septentrionalis-japonica-peculiaris* group and the other being *unguicula-unguicula rotundata* group, a pattern which was also supported by the assays with pre-absorbed antisera (**Table 5.8**). The relationships among the latter group of the major two groups were poorly characterised because anti-*T. crossei* was the only antiserum directed to the species belonging to this cluster. This antiserum indicated that *T. crossei* was most closely related to *T. kiiensis* and very distantly related to *T. abyssicola* (**Table 5.7**). The assays with pre-absorbed antisera demonstrated a large molecular variation between *T. crossei* and *T. pacifica* (**Table 5.8**). These facts suggested the involvement of at least three deeply branched lineages in this second major group of *Terebratulina*. Both of the other cancellothyridacean genera assayed, namely *Cancellothyris* and *Chlidonophora*, were more closely related to one of the two major lineages of *Terebratulina* (**Table 5.7**). The assays with pre-absorbed antisera demonstrated that these genera were most closely related with the *T. unguicula* - *T. unguicula rotundata* lineage (**Table 5.9, Figure 5.6**). This result suggests that diversifications of the ancestors of the living *Terebratulina* occurred before *Chlidonophora* or *Cancellothyris* diverged from *Terebratulina* stocks.

5.4 Discussion

5.4.1 Nature of the immunological data

For any molecular inference of phylogeny, it is essential to obtain samples of good quality, and often quantity, for the isolation of the target molecules. This is one of the

factors limiting systematic molecular surveys for brachiopods, since most of the living brachiopods constitute a rare element among marine macrobenthos, having patchy distribution under deeper waters, and usually available only through dredging operations.

The immunological investigation of the shell intracrystalline macromolecules was ideally suited in this regard because these molecules were remarkably resistant to degradation due to the protective surrounding minerals. Therefore, the extensive museum collections, however preserved (in formalin, alcohol, or even dried), could be utilised for the survey. It is even possible to study macromolecules preserved in fossil shells by the immunological methods (de Jong *et al.*, 1974; Westbroek *et al.*, 1979; Muyzer *et al.*, 1986), as exemplified by recent work on Plio-Pleistocene brachiopods (Collins *et al.*, 1991a). The immunological approach was also beneficial for the molecular inference of brachiopod phylogeny because only a small amount of each sample was required for the immunoassays, which enabled the molecular study of rare species, available only as single shell fragments. This point may be illustrated by the fact that a quarter of a gram of shell fibres, which is equivalent to the amount obtained from a single adult specimen of a common *Terebratulina* species, provides sufficient antigen for 500 assays, while the duplicate experiments for one species against 15 different antisera requires only 30 assays. In these respects, the immunological approach can only be rivalled by the sequencing of nucleic acids via polymerase chain reaction (PCR; Saiki *et al.*, 1987), which made it possible to elucidate molecular information from museum collections (Kocher *et al.*, 1989), or from archaeological samples (Pääbo *et al.*, 1989). Even compared to this approach, the immunological approaches still have some advantages for the study of brachiopods, especially when the dead shells without soft tissues are the only available source of samples, since no nucleic acids have yet been reported from dried brachiopod shells.

Various biochemical experiments have demonstrated that the brachiopod secondary shell fibrous calcite contains proteins, lipids (Curry *et al.*, 1991b), and neutral carbohydrates (Collins *et al.*, 1991c). The brachiopod intracrystalline proteins have been partially characterised by amino acid analysis, gel electrophoresis, liquid chromatography, and N-terminal amino acid sequencing (Curry *et al.*, 1991a, b; Collins *et al.*, 1991c; Cusack *et al.*, 1992). Curry *et al.* (1991b) reported that at least three different proteins of discrete sizes (47 kDa, 16 kDa, and 6.5 kDa) are involved in the terebratellid *Neothyris lenticularis* (Deshayes) and at least one protein (30 kDa) in the cancellothyridid *Terebratulina retusa* (Linnaeus). The amino acid sequence of these proteins had no significant similarity with known proteins (Curry *et al.*, 1991a), however, one is responsible for shell colour (Curry *et al.*, 1991b; Cusack *et al.*, 1992), and another contains the amino acid sequence (RGD) recognised as a cell adhesion motif (Cusack and Curry, 1991). The extent of glycosylation in the brachiopod intracrystalline proteins is a matter to be determined. Some indirect evidence suggests that these proteins are heavily glycosylated (Collins *et al.*, 1991c). However, the fact that amino acid sequences could be determined by the Edman

degradation method for the first twenty amino acid sequences of the "10.5 kDa protein" and the first ten sequences of the "47 kDa protein" (Curry *et al.*, 1991a) indicates that at least these residues are not glycolylated. Curry *et al.* (1991b) reported no galactosamine or glucosamine from the brachiopod shell fibre extracts, so typical glycosylation structures are unlikely for these proteins.

The antibodies utilised in this study and previous studies (Collins *et al.*, 1988; Collins *et al.*, 1991b; Curry *et al.*, 1991c) were prepared against crude extracts from the fibrous secondary layer of the shell or from the whole shell powder, except for the anti-*Neothyris* serum which was prepared against a purified protein (Collins *et al.*, 1991b). Immunological assays using these sera on the liquid chromatography fractions of purified shell extracts indicated that these antisera were directed not only against proteins but also against carbohydrates (Collins *et al.*, 1991c; K. Endo, unpublished data; **Chapter 4**). Pre-absorption of the antisera with various antigens was, therefore, required to remove these non-specific reactivities to obtain species-specific reactions. Many of the non-specific, or non-systematic, reactions in the assays carried out using the crude antisera (**Table 5.1**) may be explained by these cross-reactions with the carbohydrate units in the antigenic determinants.

A second cause for spurious patterns of immunological reactions, in addition to the non-specific binding of the antibodies, is the variation in the antigen concentration. The amount of antigen molecule assayed in ELISA was controlled by dissolving an exact amount of dried shell fibres from each species in a specific amount and concentration of EDTA solution. If a certain species contained less molecules per fibre weight than others, then this species should give systematically weaker reactions. This seemed to be the case for *Gryphus vitreus* (**Table 5.1**), since this genus develops a prominent tertiary shell layer, which made the isolation of the secondary shell fibres difficult, thus the same amount of the whole shell powder rather than fibres, effectively less fibres per solution, was used as the antigen.

In this study, antigens isolated from all the available species were assayed against 15 brachiopod antisera using a fixed optimal titre for each antiserum. Reciprocity is, therefore, limited to the 15 antigens to which the antisera were prepared. Nonetheless, the pattern of reaction to the 15 different antisera can provide clues to examine the consistency of the reactions for each antigen. In the 'immunological reactivity' data, it is difficult to tell how distantly taxa are related to each other, especially when no significant immunological reaction is detected.

The 'immunological distancing' approach (Collins *et al.*, 1991b; Curry *et al.*, 1991c) is suitable for these cases, since this approach involves the serial increase of the antibody concentration to boost the reactivity with a distantly related species to the level of the self-reaction to calculate the required factor of the antibody concentration. This approach also provides robust evaluations of molecular similarities because it involves reciprocal measurements using the series of antibody dilutions, rather than a fixed titre, for each pair

of species. The immunological distance was first defined for the reactivity measured by the quantitative microcomplement fixation of a purified protein (Sarich and Wilson, 1967), and this distance was linearly related to the proportion of different amino acids between the compared sequences in many proteins (see Nei, 1987). However, the validity of applying the 'immunological distance' to evaluate time of divergence between brachiopod taxa (Collins *et al.*, 1991b) is dubious, leaving aside the validity of the 'molecular clock', because each of the brachiopod antisera was directed against carbohydrates as well as proteins, and these antisera were demonstrated to be highly variable in the degree of specificity (cf. **Table 5.1**). Therefore, similar values of immunological distances determined for brachiopods could be indicating, very roughly, similar times of divergence, but it is unlikely that these values are linearly related to absolute time.

Methods commonly used to relate taxa by their molecular variations involve either the comparison of shared features, or the calculation of quantifiable degree of relatedness, both of which usually generate phylograms on the basis of computerised parsimony analysis. The latter method often presumes a uniform rate of molecular evolution (i.e. the molecular clock), while the former does not imply rates or mechanisms of evolution. The immunological data presented in this study were subjected to the former method, regarding the reactivity to each antiserum as a discontinuous character state. The relationships are best visualised in the form of a tree, which, in this study, generated by cluster analysis. However, a molecular phylogram is sometimes misleading because no known clustering method for systematics is free from any procedural or biological problems. It is always better, therefore, to check the raw data manually by a method within the capacity of human brain.

5.4.2 Framework of the articulate serotaxonomy

The overall pattern of the immunological reactions was consistent by itself and with previous studies (Collins *et al.*, 1988; 1991b; Curry *et al.*, 1991c). The pattern of immunological reactions also indicated remarkable correspondence with morphological classification, proving the significance of the immunological inference of brachiopod phylogeny, while the critical disagreements with traditional terebratulide classifications suggested by the original study (Collins *et al.*, 1988) were also confirmed.

Immunological data indicated that every rhynchonellide species investigated in this study was distantly related to the terebratulide species (**Table 5.1**), which is concordant with the ordinal morphological separation of these groups. No immunological clue was obtained to associate the rhynchonellide species as a coherent group, however, there was also no evidence to disprove it.

There were some indications that the thecideidines were more closely related to the

terebratulides than to the rhynchonellides (**Table 5.1, Figure 5.1**). The affinity of this suborder to other brachiopods has been, and perhaps still is, problematic, having been assigned to such a wide variety of taxa as the Terebratulida, Spiriferida, and several strophomenide taxa (see Baker, 1990 for review). The immunological data provide only limited evidence for the affinity, since no antisera against this group were available and the pattern of immunological reaction was not perfectly consistent. The results of the assays with anti-*Dallina*, anti-*Waltonia*, and anti-*Gryphus* sera (**Table 5.1**) does not provide any support for the strophomenide origin. On the basis of the low levels of reaction with other terebratulide antisera, the spiriferide origin may be preferred as adopted by Baker (1990), although again it is not conclusive immunologically. Affinity between the two studied thecideidine species was only weakly indicated (**Figure 5.1**), a fact which may correspond with the palaeontological evidence that these two lineages were separated as far back as in the Jurassic time (Williams and Hurst, 1977).

There is strong evidence to support the three-fold division of the living terebratulides proposed by the previous immunological investigations (Collins *et al.*, 1991b; Curry *et al.*, 1991c). One group is the short-looped Cancellothyridacea (Cooper, 1973c), the second group comprises two distinct subgroups; the short-looped Terebratulacea (Cooper, 1983) and a group of the long-looped families Ecnomiosidae (Cooper, 1977), Macandreviidae (Cooper, 1973b), Megathyrididae, and Kraussinidae, and the third group includes the typical long-looped species of Dallinidae, Laqueidae (Richardson, 1975; except for *Macandrevia*), and Terebratellidae (**Table 5.1; Figures 5.1-5.3**).

Affinities between these three main groups were not assessed in this study. However, the 'immunological distancing' approach indicated that these groups were almost equidistantly related to each other with the rhynchonellides as the clear outgroup (Collins *et al.*, 1991b; Curry *et al.*, 1991c).

To summarise the immunological view of the living articulate relationships, a possible, and tentative, scheme of classification is indicated in **Table 5.10**. From the serotaxonomical viewpoint, living terebratulides can be divided into three suborders, the 'Cancellothyrididina', 'Terebratellidina', and 'Terebratulidina'. The first two suborders include one superfamily each, the Cancellothyridoidea (Cooper, 1973c) and 'Terebratelloidea'. The third suborder can be divided into two superfamilies, the Terebratuloidea (Cooper, 1983), and 'Kraussinoidea'. Division of the Recent and Tertiary Terebratellidina (Muir-Wood *et al.*, 1965) into two superfamilies, the Terebratellacea and Dallinacea, was introduced by Cooper (1978, 1979, 1981a, 1981b) and Smirnova (1984) and, in some parts, the former corresponds with the 'Kraussinoidea' and the latter with the 'Terebratelloidea' of this study. In order to avoid confusion, especially in the assignment of the Terebratellidae, Macandreviidae, and Ecnomiosidae, the denotations shown in **Table 5.10** have been provisionally proposed. The morphological and phylogenetic interpretations and implications of this scheme are discussed below.

Table 5.10 Systematic hierarchy suggested by serotaxonomy

Only families investigated in this study are included. Numbers in parentheses indicate numbers of species screened for each family. Categorical designations after Ager *et al.* (1965) for rhynchonellides, Baker (1990) for thecideidines, Cooper (1973c) for cancellothyridoids, Cooper (1983) for terebratuloids, Cooper (1977) for the ecnomiosid, Cooper (1973b) for macandreviids, Muir-Wood *et al.* (1965) for kraussinids and megathyridids, and Richardson (1975) for dallinids and terebratellids. The three terebratulide suborders, Superfamily 'Kraussinoidea', Superfamily 'Terebratelloidea', and the family 'Laqueidae' are provisionally emended or proposed in this study. '-OIDEA' is added to the superfamily stem as the preferred suffix according to the ICZN recommendation 29a (Ride *et al.*, 1985). See Appendix III for stylised loop morphology of selected genera.

- Class Articulata
  - Order Rhynchonellida
    - Superfamily Rhynchonelloidea
      - Family Basiliolidae (1)
      - Family Hemithyridae (2)
      - Family Frieleidae (1)
  - Order Spiriferida
    - Suborder Thecideidina
      - Superfamily Thecideoidea
        - Family Thecidellinidae (1)
        - Family Thecideidae (1)
  - Order Terebratulida
    - Suborder 'Cancellothyrididina'
      - Superfamily Cancellothyridoidea
        - Family Cancellothyridae (14)
        - Family Chlidonophoridae (1)
    - Suborder 'Terebratulidina'
      - Superfamily Terebratuloidea
        - Family Terebratulidae (5)
        - Family Dyscoliidae (1)
      - Superfamily 'Kraussinoidea'
        - Family Economiosidae (1)
        - Family Macandreviidae (2)
        - Family Megathyrididae (2)
        - Family Kraussinidae (2)
    - Suborder 'Terebratellidina'
      - Superfamily 'Terebratelloidea'
        - Family Dallinidae (2)
        - Family Terebratellidae (6)
        - Family 'Laqueidae' (12)

5.4.3 Interpretation of the immunological data

Immunological data suggest that the long loop of the living terebratulides may have evolved at least twice on separate occasions. This result is not as inconsistent with the morphological data as might first appear. Significantly, every one of the second long-looped terebratulide taxa recognised in this study, kraussinids, megathyridids, *Macandrevia*, and *Ecnomiosa*, has characteristic 'aberrant' morphological features which distinguish it from the other 'typical' long-looped terebratulides (namely, the dallinids, terebratellids, and 'laqueids'). The kraussinids and megathyridids have a simpler structure of the lophophore and loop, which is believed to be neotenously derived from the typical long-looped stocks (Williams and Hurst, 1977). The adult loop of *Macandrevia* is not particularly aberrant, as the cardinalia have very characteristic features which are reflected in the various assignment of this genus to the Dallinidae (Muir-Wood *et al.*, 1965), Macandreviidae (Cooper, 1973b), and Laqueidae (Richardson, 1975). The adult loop of *Ecnomiosa*, on the other hand, is also of long-looped type, at least in its anterior part. Posteriorly, however, the mode of loop development is characteristic, and its cardinalia are also unique. Cooper (1977) considered that "the loop of this brachiopod is so unusual as to set the genus apart from all others known", and erected the new family Ecnomiosidae for this genus, a family which was later assigned to the Dallinacea (Cooper, 1981a).

Another major finding of immunological investigations was that the Cancellothyridacea (Cooper, 1973c) was almost equidistantly related to both a subset of the Terebratellacea (Muir-Wood *et al.*, 1965) and the Terebratulacea (Cooper, 1983) to which another subset of the Terebratellacea was linked (Collins *et al.*, 1991b; Curry *et al.*, 1991c). From the evidence of the earliest fossil records of these three major taxa and the families to which the second long-looped species belong, Collins *et al.* (1991b) and Curry *et al.* (1991c) interpreted that the last common ancestor of all living terebratulides diverged in the Triassic, and Collins *et al.* (1991b) further suggested that the second long-looped lineage diverged at earliest in the Cretaceous from the terebratuloid stocks (cf. **Figure 2.5**). Curry *et al.* (1991c) suggested that the long loop of *Macandrevia* and *Ecnomiosa* evolved convergently. These interpretations have self-consistencies, however, considering the fact the Terebratulacea had already been morphologically established in the Cretaceous time (Cooper, 1983), the numbers of simultaneous mutations required to derive the megathyridids from terebratulaceans in the Cretaceous are incredibly large, as Brunton and Hiller (1990) have pointed out, and the relationships among the second long-looped species were virtually unknown.

The questions are, therefore, how can the species of the second long-looped terebratulides recognised in this study be related to each other (the morphological affinities among the other long-looped group, dallinids, terebratellids, and 'laqueids', may scarcely be refuted; see Richardson, 1975), and how could the second long-looped

lineage be derived from the short-looped Terebratulacea? The key seems to lie in the morphology of *Ecnomiosa* and *Macandrevia*.

The specimen of *Ecnomiosa* used in the immunological assay was one of two adult specimens collected from a locality in Japanese waters. It was a dead, though fresh, complete articulated shell (the other being alive when collected) which allowed unambiguous observations of both the internal features, including the loop, and the external features; these provided unequivocal confirmation of the identity of the genus. The size, the extent of sulcation, and the geographic distribution suggest that these individuals constitute a new species. Two other species are known, one each from the Caribbean Sea and the Indian Ocean, and for these species, the loop development and other morphological characters have been illustrated and described (Cooper, 1973a, 1981a).

The adult loop pattern of *Ecnomiosa* is characterised by the presence of medio-vertical connecting bands and the absence of the lateral connecting bands (in the loop terminology of Richardson, 1975; cf. **Appendix III**). This is certainly an advanced phase of the bilacunar phase of the Laqueidae *sensu* Richardson (1975), as the loop of a younger stage retains the lateral connecting bands (the bilacunar phase; see Cooper, 1973a; 1981). Richardson (1975) lists no genus with the adult loop pattern of *Ecnomiosa*, but lists the genera with the adult loop of the bilacunar pattern as *Aldingia*, *Jolonica*, *Kingena*, and *Paraldingia*. Out of these genera, *Aldingia* and *Paraldingia* have strikingly similar internal and external morphologies with *Ecnomiosa*. Features such as shell outline, prominent concentric growth lines, tendencies to sulcation, cardinalia organisation, and, of course, the loop pattern are all similar. Indeed, the only major morphological features distinguishing the former two genera from *Ecnomiosa* appears to be the presence or absence of the lateral connecting bands and the extent of calcifications in the cardinalia and the loop. *Aldingia* had been assigned in the Kraussinidae or the subfamily which formerly embraced the kraussinid genera (Thomson,1927; Muir-Wood *et al.*, 1965), until Richardson (1975) reassigned it to the Laqueidae. The genus *Paraldingia* was erected by Richardson (1973) for the species which differ from *Aldingia* in possessing the dental plates, more excavated and thinner cardinalia, and thicker, broader, and spinous loop elements. The presence of dental plates suggests an affinity of *Ecnomiosa* more with *Paraldingia* than with *Aldingia*.

The prominent spinosity of the ascending and descending branches of the juvenile loop is another major feature of *Ecnomiosa* morphology (see Cooper, 1981a). This is the very feature that is observed also in *Macandrevia* and almost completely lacking, except for the only slightly developed spinous outgrowths from the septum (Richardson, 1975), in the species of the other long-looped terebratulide group (terebratellids, 'laqueids', and dallinids) recognised in this study. Although Elliot (1953) noted the spiny nature of many juvenile and adult dallinid loops, which was cited by Atkins (1959) in the study of *Macandrevia*, it may be noteworthy that *Macandrevia* was then considered as one of the

most typical dallinids, and as far as the then considered dallinid species used in this study, except for *Macandrevia* are concerned (e.g., *Dallina, Dallinella, Frenulina*: figured in Thomson, 1927), the spinous outgrowths are distinguishably trivial compared with those of *Macandrevia* or *Ecnomiosa.*

Another unusual juvenile feature of *Ecnomiosa* is the welding of the descending lamellae to the valve floor during the early growth stages, when only descending branches and the septal pillar are developed (Cooper, 1981a). The junction of the descending branches to the valve floor is also observed in the adults of *Argyrotheca, Megathiris,* and *Gwynia* (all megathyridids), some fossil *Magadina* from New Zealand (D. MacKinnon, pers. comm.), and a Jurassic genus *Zellania,* whose familial allocation in the Terebratellacea was uncertain (Muir-Wood *et al.,* 1965). No welding is observed in any other terebratulide species included in this study.

The loop development of *Macandrevia* is well documented (Friele, 1877; Elliott, 1953; Atkins, 1959; Richardson, 1975), and the basic features are certainly those of the typical long-looped species except for the spinous projections, and the very early abortion of the connection between the loop and the septum for a dallinid species as noted by Smirnova (1984). The cardinalia, on the other hand, are unique, with the inner hinge plates sloping to join the valve floor separately. This feature is considered to reflect the unusual organisation of the muscles of *Macandrevia,* especially the dorsal adjustors (Cooper, 1973b).

The mode of attachment of the dorsal adjustors to the dorsal valve could possibly constitute a uniting character, which may not be exclusive, however, as in the *Ecnomiosa-Macandrevia*-kraussinids-megathyridids cluster, the adult dorsal adjustor attachment sites appear to be confined to either the inner hinge plates, or the valve floor (or both for *Macandrevia*). In the species of the terebratellid-'laqueid'-dallinid cluster, the adult dorsal adjustors do not attach to the valve floor, but to parts of the cardinalia, notably the outer and inner hinge plates. The development of the outer hinge plates is known in *Ecnomiosa,* but they are described as narrow (Cooper, 1977), and the function for the muscle attachment is doubtful.

From the discussions above, it may be appreciated that the two long-looped genera, *Ecnomiosa* and *Macandrevia* form a coherent group, and that species of the other two families, the kraussinids and megathyridids, may well have been derived neotenously from this long-looped group, both families possibly separately. This interpretation, however, is still inadequate in the light of the fossil record and the affinity of this group to the Terebratulacea. Megathyridids and kraussinids are known from the Upper Cretaceous and Miocene, respectively (Muir-Wood *et al.,* 1965), while *Macandrevia* is known from the Miocene (Muir-Wood *et al.,* 1965), and no fossil is known for *Ecnomiosa.* From the distribution in both sides of the Atlantic and both sides of the American continents in present-day seas, Thomson (1927) suggested that the geological history of

Macandrevia should extend back at least to the Eocene. Living *Macandrevia cranium* occurs in shallow, cold waters, but most species, including *M. cranium*, range down to 1000-4000 metres (Cooper, 1975). This abyssal distribution might explain the lack of fossil record for this genus. The geographical distribution of *Ecnomiosa* also suggests a long history of this genus. Assuming the close relationship among *Ecnomiosa, Aldingia,* and *Paraldingia*, the record of this group extends back to the Upper Eocene, since *Aldingia* and *Paraldingia* are known from the Upper Eocene to Recent and the Upper Eocene to the Lower Miocene, respectively (Richardson, 1973). However, this range is still not able to explain the occurrence of megathyridids in the Cretaceous. In order to answer this question and the other question (relationship with the Terebratulacea), radical, but reasonable, ideas are introduced below.

From the pattern and the spinous nature of the loop, it is not impossible to link *Ecnomiosa* with the *Kingena* and, by analogy, the Kingenidae, which is known from the Upper Jurassic to Cretaceous (Owen, 1970); the occurrence of *Kingena* from the Eocene was refuted by this paper), or to the Recent, adopting the view of Smirnova (1984) who included *Aldingia* and *Paraldingia* in the Kingenidae. Furthermore, from the spinous loop and the supposed general similarity in the way the dorsal adjustor muscles attach to the dorsal valve, it may not be so wild a speculation to associate these genera with the Zeilleriacea. Despite the great difficulty in studying loop development using fossils, two major independent lineages in the long-looped terebratulides, the Terebratellacea and Zeilleriacea, considered as a monophyletic group, have long been recognised in the Mesozoic fauna mainly on the basis of the characters of the loop (Muir-Wood *et al.*, 1965). A more morphological similarity between these two superfamilies was suggested by Richardson (1975), and further specifically addressed by Elliott (1976). Dagis (1972) included the family Zeilleriidae in the superfamily Dallinacea. Baker (1972) demonstrated that the Jurassic *Zeilleria leckenbyi*, a 'typical' zeilleriid species, involved the septal pillar in its loop development, a character often attributed to the Terebratellacea, and he insisted that the possession of spinose ascending and descending elements is of prime importance for the inference of the zeilleriacean ancestry.

The best palaeontological solution appears to be to postulate that the second long-looped lineage recognised in the immunological study derived from the Terebratulacea through the Zeilleriacea in the Triassic times, when the Terebratulacea and Zeilleriacea started to be established, and had a variable morphological plasticity. The fact that the 'Zeilleriacea' includes two lineages, those which involve a septal pillar in the loop development, and those in which the loop development takes place without the pillar (Baker, 1972; Elliott, 1976), does accord with the hypothesis to link the long-looped 'kraussinoids' (**Table 5.10**) which involve the septum in loop development and the short-looped terebratuloids which do not. Baker (1972) observed the "resemblance between the early ascending lamellae of *Z. leckenbyi* and the two divergent plates which constitute the

early development of the loop of *Kraussina*", an observation which fits perfectly with the interpretation that *Kraussina* originated from one of the descendants of the zeilleriids. Baker (1972) denied, however, the possibility of the neotenous evolution on the basis of the presence or absence of the dental plates, a character which was regarded as important. Morphological similarity between zeilleriaceans and *Kingena* has been pointed out by Muir-Wood *et al.* (1965). Baker (1972) also reported the similarity of the juvenile loop pattern of *Zeilleria leckenbyi* with the adult loop pattern of *Kingena*, *Zittelina* (Kingenidae; Owen, 1970), and *Trigonellina* (Dallinidae; Muir-Wood *et al.*, 1965). Smirnova (1984) noted the similarity between zeilleriids and *Macandrevia* in an aspect of the loop development. Smirnova (1984) also considered dental plates to be of high taxonomic value in terebratulide classification, and included kraussinids in the superfamily Terebratelloidea (**Table 2.5**) which generally lacks dental plates, and considered that this superfamily is distantly related to the other extant long-looped superfamily Dallinoidea, or to the extinct Zeilleriidae, both of which generally possess dental plates (**Figure 2.4**).

Dental plates do appear to constitute a fairly invariable character at family level or even higher classification. Although undoubtedly important, this character may not always be reliable at the higher classifications. Among the dallinids, juvenile *Dallina* has dental plates, but the adults have none, and *Campages* does not have dental plates (Muir-Wood *et al.*, 1965). The two closely related species, *Aldingia* and *Paraldingia*, can be distinguished by the presence or absence of the dental plates according to Richardson (1973). From the serotaxonomic viewpoint, the terebratellids (without dental plates) and 'laqueids' (with dental plates) are considered to be closely related (**Table 5.1**), and the separation of these families at the superfamily level was not supported. Dental plates buttress teeth, so they can be interpreted as functioning to strengthen the articulation between the valves. These structures may be advantageous in nonstrophic brachiopods, such as most terebratulides, but in certain terebratulide taxa with a strophic tendency, such as megathyridids and kraussinids, dental plates may well have degenerated secondarily by the loss of functional constraints, since posterior edges of the shell, being more or less parallel to the hinge line, would provide some support for articulation. These morphological changes could well have occurred independently (or convergently) in these taxa.

Under this new interpretation of the immunological data, the three major lineages, the 'cancellothyrididines', 'terebratulidines', and 'terebratellidines' recognised in this study (**Table 5.10**), is deduced to have diverged *before* the early Mesozoic time, when the 'terebratulidines' (**Table 5.10**) are expected to have separated into two superfamilies. It may be natural to consider that the divergence of the ancestors of the two major groups, the 'terebratellidines' and 'terebratulidines', took place in the early Devonian times as have traditionally been believed (Muir-Wood *et al.*, 1965; Williams and Hurst, 1977; Smirnova, 1984). Affinity of these two post-Palaeozoic groups (Triassic - Recent) to the Palaeozoic forms requires palaeontological examination of late Palaeozoic and early

Mesozoic terebratulide fossils. The former group may have derived from the terebratellidine Cryptonellidae (Muir-Wood *et al.*, 1965), or one of the stocks of the Cryptonelloidea (Smirnova, 1984). The latter may have diverged from the Cranaenidae or one of the other dielasmatoid stocks (Muir-Wood *et al.*, 1965; Cooper, 1983; Smirnova, 1984), or from one of the groups which have hitherto been considered as ancestral to the Zeilleriidae, such as the subfamily Cryptacanthiinae (Smirnova, 1984). The ancestor of the remaining major lineage, 'cancellothyrididines' (**Table 5.10**), is also expected to have radiated at a similar time in the Devonian from the other two major lineages, on the basis of the immunological distances (Collins *et al.*, 1991b; Curry *et al.*, 1991c), albeit only at first order approximation. Direct evidence is lacking, however, since the widely accepted earliest record of the cancellothyridoids is the Upper Jurassic (Muir-Wood *et al.*, 1965), and even considering *Pseudokingena*, which Cooper (1983) suggested as a member of cancellothyridoids, the oldest record is Lower Jurassic (Muir-Wood *et al.*, 1965).

The morphology of this group is noteworthy. Thomson (1926) separated the subfamily Cancellothyrinae from the other Tertiary and Recent short-looped terebratulid subfamily Terebratulinae due to differences in the type of cardinalia, a separation which was followed by Muir-Wood (1955) and Muir-Wood *et al.* (1965), in which the Cancellothyrinae (Thomson, 1926) was recognised as the family Cancellothyrididae in the superfamily Terebratulacea. The absence of dental plates in the cancellothyridid cardinalia, reflecting the unusual attachment of the dorsal pedicle adjustor muscles on the valve floor, was regarded by Cooper (1973c) as so significant as to raise the family to the superfamily status. Another characteristic feature of this superfamily is the development of the flattened ears on each side of the umbo of the dorsal valve (Davidson, 1886-1888; Cooper, 1973c). The shell structure of the cancellothyridoid *Terebratulina* is also unique in that the antler-shaped punctae are developed (Davidson, 1886). This kind of punctation is known from some spiriferides (Dagis, 1972) and some orthides (Williams *et al.*, 1965), but not from other terebratulides. This superfamily is also unusual in that it embraces a genus (*Eucalathis*) with the spirolophous lophophore, only one other instance being known so far among terebratulides is *Leptothyrella* of the Phaneroporidae (Zezina, 1981). The outward direction of the filaments (cirri) up to the schizolophous stage (centrifugal position), a character which had long been used (e.g., Thomson, 1927; Allan, 1940; Muir-Wood, 1955) to separate terebratulaceans from terebratellaceans (which have an inward direction, or centripetal position), could possibly be the character which is dominantly attributed to the 'cancellothyrididines'. The only other instance of this structure described among terebratulides being the terebratulacean genus *Dyscolia* (Thomson, 1927).

In the light of the information from serotaxonomy, cancellothyridoids are clearly separated not only from the 'terebratellidines' but also from the 'terebratulidines', a fact which appears to be supported by the unusual morphology of the cancellothyridoids. The origin of the Cancellothyridoidea is, however, still enigmatic.

5.4.4 Terebratulide evolution

A possible scenario of the terebratulide evolution inferred by the immunological data can be summarised as follows (cf. **Figure 5.6**): the last common ancestor of all living terebratulides radiated in the early Devonian, and three major lineages survived to the present, each of which gave rise to the long-looped 'terebratelloids' and short-looped 'terebratuloids' in the Triassic, and the short-looped cancellothyridoids in the Jurassic. The second lineage also gave rise to another long-looped lineage, the zeilleriids in the Triassic from which the kingenids derived in the Jurassic. The zeilleriid-kingenid stock later experienced major diversifications, often involving neotenous evolutions, to produce such living descendants as megathyridids, kraussinids, macandreviids, and ecnomiosiids. The first major long-looped group also diverged considerably, to embrace such living families as the Dallinidae, Terebratellidae, and Laqueidae. In comparison, the other two short-looped lineages, the terebratuloids and cancellothyridoids, have changed little morphologically since late Jurassic, when both groups were fully established. The most morphologically conservative genus has been the cancellothyridoid *Terebratulina* (Jurassic to Recent), although it contains a considerable amount of molecular variation among living species, suggesting the involvement of deep branching events in establishing the present diversity, with the deepest one probably in the Cretaceous.

5.5 Conclusions

Despite the lack of knowledge of such important taxa as platidiids, thaumatosiids (Cooper, 1973a), phaneroporiids (Zezina, 1981), diestothyriids (Smirnova, 1984), etc., the immunological data provided major implications for terebratulide classification, most notably at the superfamily and suborder levels. The immunological view does not play havoc with the traditional schemes and approaches, but urges further morphological investigations of both Recent and fossil materials.

A number of new terebratulide taxa appeared in the early Mesozoic times (cf. Smirnova, 1984; **Figure 2.4**), possibly due to the bottle-neck effect after the terminal Permo-Triassic event. It was suggested, based on immunological data, that a single Palaeozoic lineage gave rise to the ancestral stocks of all living terebratulides during this early Mesozoic radiation (Collins *et al.*, 1991b; **Figure 2.5**). But the interpretation of the extended immunological data obtained in this study suggests that at least three major Palaeozoic terebratulide lineages survived the mass extinction to leave offsprings until present (**Figure 5.6**). The affinities between the Palaeozoic and post-Palaeozoic terebratulides, however, remained open to palaeontological investigation of the late Palaeozoic and early Mesozoic forms. The hypothesis, that the second extant long-looped

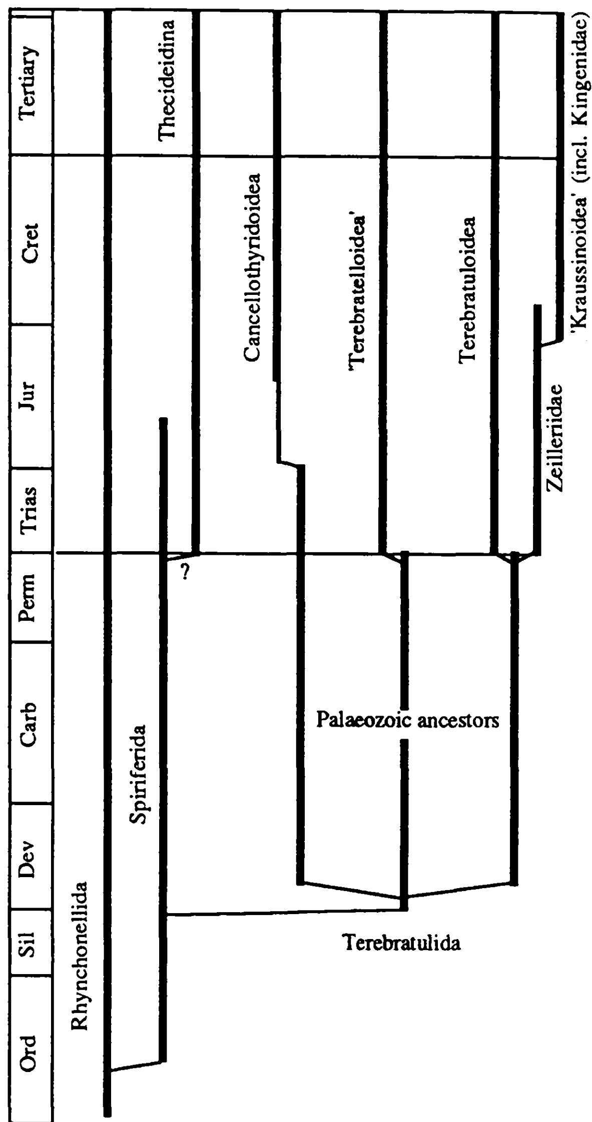


Figure 5.6 Articulate brachiopod phylogeny in the light of the re-interpreted serotaxonomic data

lineage (the 'Kraussinoidea') diverged from the short-looped Terebratuloidea *via* the Zeilleriidae and Kingenidae, may be tested by rigorous examinations of the morphology (in particular, development of the loop, cardinalia, and dental plates) of relevant Mesozoic fossils.

The molecular inferences also need to be reinforced by other molecules and techniques, and the sequencing of nucleic acids would be the next logical target.

Chapter 6 Relationships among the living Cancellothyridoidea

6.1 Introduction

Despite an enigmatic origin, as highlighted in the previous chapter, once established, *Terebratulina* flourished to become one of the most successful of all Mesozoic-Recent brachiopods (Baker, 1972). The genus is one of the most diverse and abundant taxa in the Cenozoic (Hatai, 1940; Cooper, 1973c), with the longest recorded history among the living articulate brachiopod genera (Doescher, 1981).

It may not be a coincidence that this genus embraces the species, *T. retusa* (Linnaeus), which is perhaps the most thoroughly or carefully studied of all brachiopod species so far, being an abundant Northeast Atlantic element studied by many malacologists since as early as 1767 (Davidson, 1886). Nevertheless, its species boundaries have often been controversial, even for the best documented *T. retusa*, and little is known about the interspecific relationships on a larger scale, almost certainly reflecting the remarkable evolutionary history of this genus which has experienced repeated speciations with very subtle morphological changes.

Herein, as an extension of the previous studies (Endo and Curry, 1991, **Appendix II-1**; **Chapter 5**), the genealogy among the living *Terebratulina* and related cancellothyridoid populations are examined in more detail. The data embraces not only immunological studies as described in the published paper (Endo and Curry, 1991) and in the previous chapter, but also additional morphometric analyses, carried out on most of the species which could not be included in the immunological study.

6.2 Materials and methods

The extensive collections of the living cancellothyridoid samples stored in the Natural History Museum, Smithsonian Institution, Washington, D. C., was utilised for the morphometric study (**Table 3.3**). All three families of this superfamily (Cooper, 1973c) were included, and most of the known living species of *Terebratulina* have been measured and analysed, the exceptions being *T. radula* Hedley from Australia, *T. kyusyuensis* Yabe and Hatai from Japan, an undescribed species from New Zealand, and a few other unnamed species (such as *Terebratulina* sp. of Cooper, 1973a). The sample of *T. radula* (USNM 335706) contained only small specimens with shell lengths of about 2 mm, which were too small for the measurements. The other two species were not represented in the Smithsonian collection, though two single valves of the New Zealand species were kindly provided by Dr. D. Lee, which allowed observations of morphology and immunological assays.

Measurements, multivariate analyses, and the supplementary immunological assays for the New Zealand species were carried out by the methods described in **Chapter 3**. Seven morphological characters, shell length (*L*), width (*W*), height (*H*), foramen length (*fL*), and width (*fW*), number of capilla (*Ribs*), and shell weight (Wt), were measured on a total of 259 complete specimens. Most of the specimens represented later adult growth stages for each species. The raw data of the measurements are tabulated in **Appendix I**.

6.3 Results

Table 6.1 shows the seven latent vectors obtained from the principal component analysis carried out on the specimens measured for the seven morphological characters. From the signs and the relative magnitude of the elements of each vector, it was possible to interpret the morphological variations reflected in each vector.

The first vector, which accounted for more than three quarters of total variability detected, was interpreted as the 'shell size' axis, because all elements of the vector had similar values of the same sign except for the value for the number of ribs, which had the same sign but much smaller absolute values (**Table 6.1**). In contrast to the previous study (**Appendix II-1**), this axis may have some taxonomic value, because the measured sample consisted of specimens of similar growth stages.

The second vector, which accounted for a little over 15% of the variability, predominantly reflected the variations in the number of ribs, and was interpreted as the 'rib number' axis. The third vector, accounted for 3% variation, had the largest absolute values of the same sign for both the foramen length and width, while other elements had values of the opposite sign; therefore, this was interpreted as the 'relative foramen size' axis. The fourth vector accounted for 2% variation, and had the largest absolute value for the shell weight element, while other large values were for shell length and width elements with opposite signs, thus interpreted as the 'relative shell weight' axis.

In similar ways, the remaining three vectors, accounting for 1.2% of the variation in total, were interpreted as reflecting the shell profile (shell width *vs* shell height) for the fifth vector, foramen size (foramen length *vs* foramen width) for the sixth vector, and predominantly shell shape variations for the seventh vector.

Table 6.2 indicates the compiled scores for each population along each axis. The first four vectors demonstrated generally better separations between populations than the last three vectors, which more or less revealed the presence of large variations within each population.

The first axis (shell size axis) separated the populations into very large species (*Cnismatocentrum sakhalinensis*, *T. crossei*, *T. kiiensis*, and *T. hataiana*), large species (*Cancellothyris australis*, *T. pacifica*, *T. callinome*, and *T. unguicula*), small species (*T. latifrons*, *T. cailleti*, and *T. reevei*), and other species of intermediate sizes.

The second axis (rib number axis) clearly separated the very finely ribbed *Cancellothyris*, and *Cnismatocentrum sakhalinensis* and *T. photina* (which had no recognisable ribs on that part of the shell where the rib numbers were counted), from all other species. *T. meridionalis*, *T. hawaiensis*, and *T. septentrionalis* (except for the Scottish sample, USNM 173532, which is discussed later), were characterised by much finer ribs, while *T. kiiensis*, *T. cailleti*, *T. unguicula rotundata* were revealed to have coarser ribs.

The third axis (foramen size axis) indicated that *T. compressa*, and some individuals of *T. unguicula* had a relatively large foramen, while *Cancellothyris*, *T. hataiana*, and *T. callinome* had a relatively small foramen. *T. kiiensis* has populations with foramen sizes ranging from large (the Southeast Pacific population: 385104) through intermediate (the Northeast Pacific populations: 550692, 123154) to small (the Northwest Pacific populations: 334758, 549413), a result which suggests the presence of a circum-Pacific cline, at least in this character (**Table 6.2**). This axis also indicated that *Chlidonophora incerta*, *T. latifrons*, *T. crossei*, and *T. pacifica* had smaller foramen.

The fourth axis (shell weight axis) separated the heavier species, *T. latifrons*, *T. cailleti*, *T. reevei*, and the *Cnismatocentrum* from others, while *T. photina*, *T. callinome*, *T. pacifica*, and *T. unguicula rotundata* were shown to have lighter shell weight for their size.

Among the remaining axes, the fifth axis highlighted *T. compressa*, *T. valdiviae*, and *Chlidonophora* for their invariably wider than high shell profile, and *Cancellothyris*, *T. callinome*, *T. pacifica*, *T. photina*, and *T. cailleti* for their higher than wide shell profile. The sixth axis revealed that *T. crossei* and *T. hataiana* had an invariably longer than wide foramen shape, and some individuals of *T. pacifica* and *T. callinome* also had an elongate foramen, and that *T. kiiensis* and *T. hawaiensis* had, mostly invariably, a compressed foramen shape. The seventh axis indicated that *T. photina* had longer than thick shell profile for the genus.

Table 6.1 Latent vectors of the principal component analysis on cancellothyridoid samples

Elements of each vector in reference to the measured characters, and the percentage of the variations accounted by each vector (var. %) are shown.

	V1	V2	V3	V4	V5	V6	V7
L	-0.418	-0.054	0.223	-0.329	-0.071	-0.344	0.735
W	-0.410	-0.093	0.199	-0.477	0.623	0.257	-0.319
H	-0.413	-0.042	0.383	-0.083	-0.695	0.049	-0.435
Ribs	-0.010	-0.965	0.154	0.131	0.162	0.010	-0.044
fL	-0.408	-0.062	-0.586	0.062	0.069	-0.616	-0.313
fW	-0.408	-0.105	-0.541	0.074	-0.168	0.658	0.249
Wt	-0.391	-0.203	0.322	0.794	0.256	0.002	0.076
var. %	78.5	15.2	3.0	2.0	0.8	0.2	0.2

Table 6.2 Component scores along each latent vector for each cancellothyridoid population

For the locality and the number of specimens of each population see **Table 3.3**. For each population for each vector, the symbols indicate the presence of the following individual(s).

+++ : with a positive value greater than 2
++ : with a positive value greater than 1
+ : with a positive value
- : with a negative value
-- : with a negative value less than 1
--- : with a negative value less than 2

Combinations of both plus and minus symbols indicate that the sample contains more than one individual with opposite signs of the indicated values,but otherwise indicates that the sample contains only the individual(s) with the indicated sign.

Species	Population	V1	V2	V3	V4	V5	V6	V7
Chlidonophora incerta	363295	++	+-	++	+	++	++-	--
Cnismatocentrum sakhalinensis	110788	---	+++	+	++	++	--	+
	222598	---	+++	+-	++	-	++-	+-
Cancellothyris australis	110834	--	---	+	+++	--	+	--
	332788	+	---	++	+++	---	--	-
	318179	--	---	+++	+-	+---	+++-	+++--
Terebratulina crossei	342218	---	-	+	-	+	-	-
	342217	---	+	++	+++	+	---	+++
	110833	---	-	+	--	++	-	+
T. kiiensis	385104	---	+	--	+++	+-	+++	+
	550692	-	++	-	--	+	++	-
	123154	---	++	+++-	---	+++	+++-	+--
	334758	---	++	++	+	-	++	---
	549413	---	++	+++	+++	+++	+	++
T. hataiana	254533	---	-	+++	+	-	---	+
T.pacifica	342216	+--	+-	++-	--	---	++---	++--
T.callinome	238880	+--	+-	+++	---	+---	+---	++--
T. photina	254536	+	+++	-	---	--	+	+++
T. cavata	214308	++	+	+	+	-	-	++
T. meridionalis	372702	+	--	+	+-	++	+	-
T. abyssicola	37217	++	+	+	+	-	++	-
	187142	+	+	-	+	-	+	+
	284193	+	--	--	++	-	-	---
	110841	+	+	--	+-	-	+	---
	127017	+	+-	--	+	+--	+--	+--
T. latifrons	5955	+	-	++	+	+	-	-
	549754	++	+	+	+	-	-	-
	P769	+	-	+	+	+	++	-
	G986	++	-	++	++	++	-	-
	314855	++	-	+	+	+	+	-

Table 6.2 (continued)

Species	Population	V1	V2	V3	V4	V5	V6	V7
T. cailleti	550756	++	++	+	++	- -	++-	++
	4570	++	+	-	+	-	+	+
	A	++	++	+	+	-	+-	+
	551210	+	++	-	+	- -	+	-
	314848	++	+	++	+	-	+	+
T. reevei	246330	++	+	+	++	-	+	+-
	334779	+	+	-	++	-	-	-
	298312	+	+-	+-	++	+-	+-	+-
T. unguicula	222202	-	+-	+-	- -	++	+- - -	++ - -
	110897	+- -	+-	- -	++ -	++ - -	++ - -	+- -
	3841178	+- -	+-	- -	++	++ - -	+- -	+- - -
T. unguicula rotundata	110823 (&204672)	+-	++	+-	- -	++ -	++ - -	+- - -
T. compressa	110844	-	-	- - -	-	+++	-	++
T. valdiviae	238829	++ -	+-	+- -	+-	+++	+- -	++
	110843	+	+	++	-	++	- -	-
	110437	+	+-	-	-	++	- -	+- -
T. retusa	173522	+-	++ -	++ -	- -	+- -	+- -	+- - -
	173564	+-	+-	+-	+-	+-	++ -	+- -
	173579	+-	++ -	+-	+-	+-	+- -	++
T. retusa emarginata	317025 (& Exp.17)	++	+	+-	++	+-	-	+
T. septentrionalis	B472	+-	-	- -	+-	+-	++- -	++ -
	203032	+	- -	+- -	+-	+-	+++-	++ - -
	110869	+-	- -	- -	+-	+- -	++ -	+- -
	49311	++ -	- -	+- -	+-	+-	++- -	++ -
	266223	+	-	+	-	++	+	++
	334751	+-	- -	+-	+-	++ -	+++-	+-
	173532	+-	++ -	++ - -	-	+-	++ -	++ -
T. japonica	563646 (&549344)	++	-	+- -	++	+-	+- - -	+-
	110821	+	-	+	-	+	-	++
	204687	-	-	- -	+	- -	+++	+
	110819	+-	-	+	+-	+-	-	+
T. peculiaris	563640	+-	+-	-	+-	-	- -	-
T. hawaiensis	274156	-	- -	-	-	+	++	+

6.4 Discussion

6.4.1 Subgroups of the living *Terebratulina*

Based on the results of the morphometry and other lines of evidence, such as the immunological data, qualitative morphologic characters, and geographic distribution, living *Terebratulina* can be separated into six groups (**Table 6.3**).

The morphometric analyses primarily fractionated the species by the size of the adult shells, which is one of the most conspicuous morphologic characters, into the very large species (Groups A and B), very small species (Group E), and the species of intermediate sizes, separation that was largely supported by the immunological data (**Figure 5.3**). Among the intermediate sized species, morphometric data further separated the distinctive species, *T. photina* (Group C) from all others. The immunological data predicted the existence of at least two major lineages in the rest of the intermediate species, a division which was considered to be reasonable on the basis of other morphological data and the geographic distribution, and these species were assigned to either Group D or Group F.

Both of the group A species, *T. crossei* and *T. kiiensis*, occur on both sides of the North Pacific (Dall, 1920), and *T. kiiensis* also occurs in the Southeast Pacific (Cooper, 1982). Many authors consider that these species were very closely related (Dall, 1920; Hatai, 1940; Cooper, 1982), and this is confirmed by the immunological assays on *T. crossei* from Japan and *T. kiiensis* from Chile (**Figure 5.3; Table 6.3**). The morphological difference between the two is a more circular outline in *T. kiiensis*; but the presence of intermediate forms has been suggested by Hatai (1940) for fossil and Recent Japanese materials, and by Cooper (1982) for American living material, which he suggested was a new species (Cooper, 1982). On the basis of the morphometric data (**Table 6.2**), these two species appeared to be separated by number of ribs (axis 2) and foramen shape (axis 6), although the examination of the intermediate forms is required to confirm the separation. The two species also share an important morphologic character, the disjunct crural processes (cf. Cooper, 1973c; 1982), which is usually employed to separate families in the cancellothyridoid classification (Cooper, 1973c).

There is also little doubt about the affinity between *T. pacifica* and *T. callinome* (Group B), which have very similar morphology. The morphometric analysis (**Table 6.2**) revealed that these species shared characteristic features, such as a relatively small foramen (axis 3), light shell weight for size (axis 4), and a more convex than wide shell profile (axis 5). One of the few separating morphologic features between the Japanese and the Philippine species is the width of the ribs (which are characteristically flattened in these species) compared to the width of the interspaces, as described by Hatai (1940). Individuals of the remaining large *Terebratulina* species, *T. hataiana* from the Philippines,

have similar costellation and striation as *T. callinome* or *T. pacifica*, and have the conjunct crural processes (Cooper, 1973c), and therefore, may better be allocated in this group rather than in Group A.

Dall (1920) suggested affinity between *T. crossei* (Group A) and *T. callinome* (Group B), which at that time included individuals of *T. pacifica*. It is possible, in terms of the morphometric data, to infer that all these large species are closely related, but the immunological data (antiserum against *T. crossei*, serum 171) suggested that *T. crossei* and *T. pacifica* were distantly related (**Table 6.3**).

T. photina from Borneo (Group C) is discriminated from all other *Terebratulina* species by fine and faint striation which can only be seen under a lens (Dall, 1920). The obsolescent ornament suggests a degeneration of the flattened costellae of *T. callinome*, or *T. pacifica*, and the relatively light shell weight for size as revealed by the morphometric data (**Table 6.2**) does suggest the affinity of *T. photina* to these species. However, the relationships are not conclusive, as immunological data is lacking. Cooper (1973c) suggested an affinity of this species with *T. meridionalis* from South Africa. *T. kyusyuensis* Yabe and Hatai, a living species from Japan, is said to have similar morphology with *T. photina* (Hatai, 1940), and may be the most closely related.

The Southern hemisphere species (Group D) may be characterised by the medium adult shell size and the less elevated, or weak ribs, as in *T. abyssicola* and *T. cavata*, or the posteriorly strong and fine ribs which become evanescent anteriorly, as in *T. meridionalis* (cf. Cooper, 1973c) and in the *Terebratulina* species from New Zealand. The immunological data indicated that *T. abyssicola* from South Africa and the undescribed species from New Zealand were only distantly related to the species against which the antisera were prepared (Group A and Group F). Although these data do not confirm the congruency of this group, both the geographic distribution and the rib morphology suggests that the species of this group are closely related.

The affinity amongst the group of the small sized species (Group E) is strengthened by the fact that the adult shell of all these species has a ridge, or carination, which is very characteristic and unusual in *Terebratulina* (Cooper, 1973c). The ribs of all these species are strong and beaded (Cooper, 1973b, c). The immunological data supported a close relationship among this group (though the evidence is weaker for *T. reevei*), and suggested a closer relationship of this group to the Group F (**Table 6.3**). The geographic distribution of *T. latifrons* and *T. cailleti* in the Caribbean and the Gulf of Mexico and *T. reevei* in Philippines suggests a wide geographic distribution of the ancestral species before the formation of the isthmus between the North and South American Continents in the Pliocene (Lessios, 1979).

The Group F species are characterised by medium size, the possession of large foramen relative to size (axis 3, **Table 6.2**), and the development of elevated, or strong, radial ribs. The immunological data most convincingly supported the coherency of this group (**Table 6.3**).

Table 6.3 Divisions of the living genus *Terebratulina*

Symbols for morphometry results as in **Table 6.2**, except that the involvement of individuals with opposite signs in a species is indicated by a single symbol '+-'. The first four axes are shown. Immunological data from **Table 5.7**, except for the new data for *T.* sp. from New Zealand. Key: +++++: 100% reactivity; ++++: >90%; +++: >80%; ++ >70%; +: 0-70%; n.d. : no data. For the identities of antisera see **Table 3.4**.

Species	Distribution	Morphometry				Immunology			
		V1	V2	V3	V4	4967	173	174	171
'Supergroup 1'									
Group A									
T. crossei	Pacific	- - -	+-	++	+-	+	++	+	+++++
T. kiiensis		- - -	++	+-	+-	+	+	++	++++
Group B									
T. hataiana	N.Pacific	- - -	-	+++	+	n.d.	n.d.	n.d.	n.d.
T. pacifica		+-	+-	+-	- -	+	+++	++	+
T. callinome		+-	+-	+++	- - -	n.d.	n.d.	n.d.	n.d.
Group C									
T. photina	Pacific	+	+++	-	- - -	n.d.	n.d.	n.d.	n.d.
Group D									
T. meridionalis	S.Africa	+	- -	+	+-	n.d.	n.d.	n.d.	n.d.
T. abyssicola		++	+-	+-	+-	++	+	++	+
T. cavata	Australia	++	+	+	+	n.d.	n.d.	n.d.	n.d.
T. sp.	New Zealand	n.d.	n.d.	n.d.	n.d.	++	+	++	+
'Supergroup 2'									
Group E									
T. latifrons	Caribbean	++	+-	++	++	++	+	++++	+
T. cailleti		++	++	+-	++	++++	+	++++	+
T. reevei	Philippines	++	+-	+-	++	+	+	++	+
Group F									
Subgroup F1									
T. unguicula	N.Pacific	+-	+-	+-	+-	++++	+++	+++++	++
T. u. rotundata		+-	++	+-	- -	++++	++++	+++++	+
T. valdiviae		+-	+-	+-	+-	n.d.	n.d.	n.d.	n.d.
T. compressa		-	-	- - -	-	n.d.	n.d.	n.d.	n.d.
Subgroup F2									
T. retusa	N.Atlantic	+-	+-	+-	+-	+++++	++++	+++	+++
T. r. emarginata		++	+	+-	++	n.d.	n.d.	n.d.	n.d.
T. septentrionalis		+-	- -	+-	+-	+++++	+++++	++++	+++++
T. japonica	N.Pacific	+-	-	+-	+-	+++++	+++	++++	++++
T. peculiaris		+-	+-	-	+-	+++++	++++	++++	++++
T. hawaiensis		-	- -	-	-	n.d.	n.d.	n.d.	n.d.

The distinction among the species of this group have been problematic. Dall (1920) noted that it was often extremely difficult to separate *T. retusa*, *T. septentrionalis*, and *T. unguicula* merely on the basis of the shell, though Blochmann (1908) demonstrated that these species are distinct on the basis of the morphology of their spicules. The morphometric data indicated that the number of ribs was a useful character to infer relationships at this level (**Table 6.2**, axis 2). It clearly separated the two North Atlantic species, *T. retusa* and *T. septentrionalis* (**Figure 6.1**), a separation which was also supported by immunological (**Chapter 5**) and other molecular data (Curry and Endo, 1991; Cohen *et al.*, 1991), confirming the importance of this character. On this basis, it can be concluded that the sample (USNM173532) from Scotland labelled as *T. septentrionalis* has been misidentified and is in fact *T. retusa* (cf. **Table 6.2**).

The immunological data explicitly separated *T. unguicula* (and its subspecies) from *T. retusa*, *T. septentrionalis*, *T. japonica*, and *T. peculiaris*, (**Appendix II-1**; **Chapters 5**; **Table 6.3**), which allows the separation of this group into two Subgroups F1 and F2.

The similarity between *T. valdiviae* and *T. compressa* was indicated by the morphometric data (**Table 6.2**; axes 5 and 7) as expected by the fact that *T. compressa* was identified by Dall as *T. valdiviae* (Cooper, 1973c). The number of ribs of these species were in the range closest to *T. unguicula* (**Figure 6.1**), and these species therefore were provisionally included in Subgroup F1, an allocation which follows the suggestion by Dall that *T. valdiviae* most strongly resembles *T. unguicula* (Dall, 1920). Similarly, *T. hawaiensis*, which had a fine ribbing comparable with *T. septentrionalis* (**Figure 6.1**), was included in Subgroup F2.

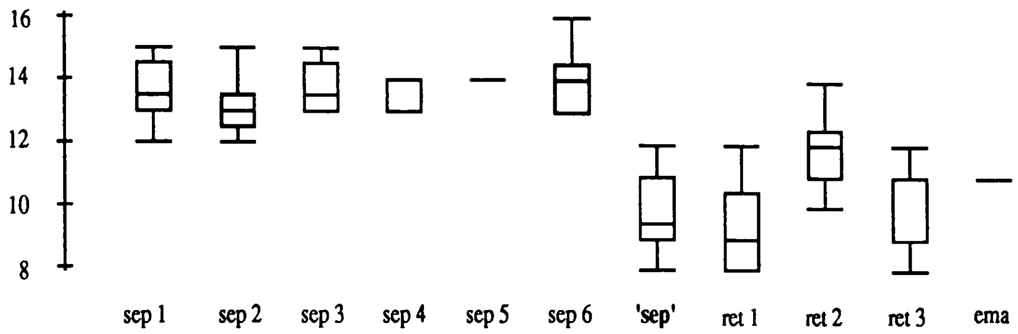
6.4.2 Phylogenetic relationships between the subgroups of *Terebratulina*

The immunological data divided the living *Terebratulina* into two major groups (**Appendix II-1**; species allocation revised in this study: **Table 6.3**). One group ('Supergroup 1') includes the Groups A, B, C, and D, and the other group ('Supergroup 2') comprises of the Groups E and F (**Table 6.3**). The monophyly of the latter major group is supported by the immunological data, but the relationships among the former group is equivocal in terms of the immunological evidence. The immunological data indicated that the species of the Supergroup 1 were very distantly related to the Group F species (Supergroup 2), and that the Groups B and D are distantly related to Group A, but did not provide clues regarding the relationships amongst Groups B, C, and D. Supergroup 1 may well be monophyletic, but it could also be paraphyletic.

Morphologically, Supergroup 2 is characterised by the medium to small shell size and the possession of strong ribs, whilst Supergroup 1 generally has weaker ribs and a medium to large shell size. Since one of the earliest species of *Terebratulina*, the Jurassic *T. silicea* (Quenstedt), had strong ribs (cf. Cooper, 1973c), and weaker ribs did not evolve

Figure 6.1 Comparison of rib width of living *Terebratulina* populations

The number of ribs was counted by the method described in **section 3.2.11**. Keys: sep = *T. septentrionalis*, 1, Massachusetts; 2, Maine; 3, Nova Scotia; 4, Cape Cod; 5, Greenland; 6, Norway; 'sep' = USNM173532; ret = *T. retusa*, 1, Norway; 2, Skye, 3, Sicily; ema = *T. retusa emarginata*; jap = *T. japonica*; pec = *T. peculiaris*; haw = *T. hawaiensis*; ung = *T. unguicula*, 1, Georgia; 2, California; 3, Alaska; rot = *T. unguicula rotundata*; com = *T. compressa*; val = *T. valdiviae*; cro = *T. crossei*; kii = *T. kiiensis*; hat = *T. hataiana*; cal = *T. callinome*; pac = *T. pacifica*; lat = *T. latifrons*; cai = *T. cailleti*; ree = *T. reevei*; cav = *T. cavata*; mer = *T. meridionalis*; aby = *T. abyssicola*. The extent of the main body of the data is depicted by the two 'whiskers' extending from the top and bottom of the outlined central box. The box depicts the data between the 25th and the 75th percentiles. The horizontal line across the box marks the median. Extreme data values are plotted individually with a circle (see next page).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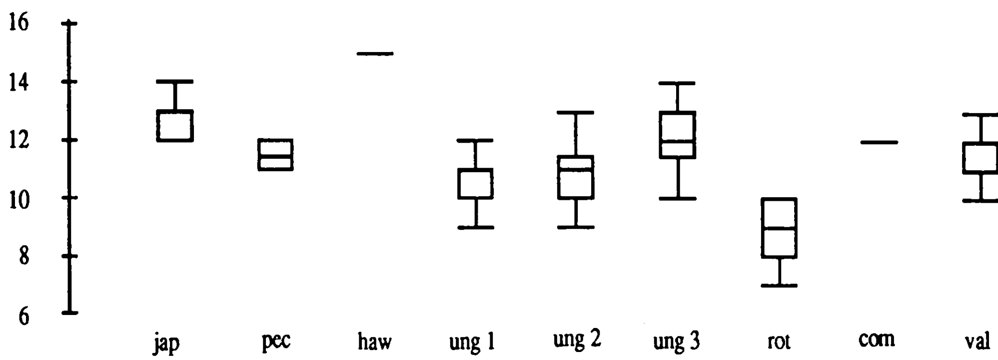
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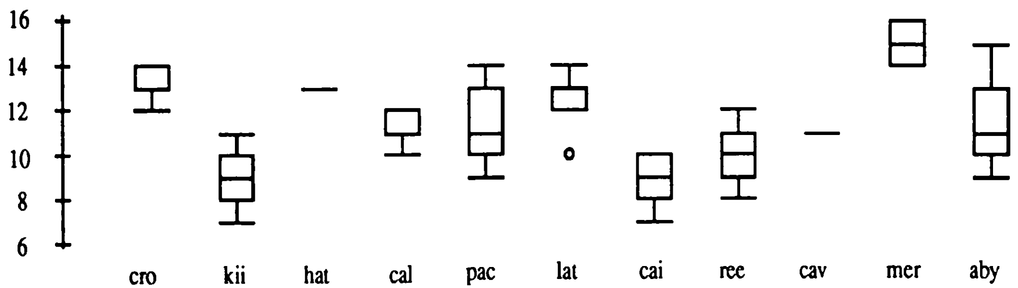
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until late Cretaceous (cf. **Appendix II-1**), it is possible to argue that Supergroup 1 is characterised by a shared derived character, and therefore is likely to be monophyletic. However, there is no guarantee that the smooth ornamentation did not evolve more than once.

6.4.3 Affinity of *Cnismatocentrum*, *Cancellothyris* and *Chlidonophora* to the *Terebratulina* species

Morphometric data separated *Cnismatocentrum* from all the other species investigated on the basis of the very large size (axis 1, **Table 6.2**), the ribs (axis 2) (which could not be counted as in *T. photina*), and the relatively heavy shell for the size (axis 4). These results suggested a close relationship of this genus to the Group A species of *Terebratulina*, namely, *T. crossei* and *T. kiiensis*.

Cancellothyris was also separated from all the *Terebratulina* species by very fine ribs (axis 2). The relative foramen size was also revealed to be characteristically small (axis 3), as expected from the conjunct delthyrium which characterise the genus. The geographic distribution in the Southern hemisphere implies that this genus is closely related to the Group D species, such as *T. abyssicola*, as suggested by Brunton and Hiller (1990). However, the immunological data indicated that *Cancellothyris australis* was most closely related to Group F species, especially *T. unguicula* (cf. **Chapter 5**). This relationship does not easily explain the geographic distribution, but the possession of a wider loop in both species does suggest an affinity between these species.

Chlidonophora has a morphology within that of the range of the Group F species (**Table 6.2**). This result is comformable with the mode of ribbing of this genus, and with the immunological data which indicated a close ancestry of *Chlidonophora* with *T. unguicula* (**Chapter 5**). Cooper (1973a) doubted the occurrence of this genus in the Cretaceous. The immunological data appear to support this, as the data indicated that the divergence of *Chlidonophora* from Group F lineage was later than the divergence between the Group E and Group F species (**Chapter 5**). This branching occurred considerably later than the Cretaceous, accepting the conclusion that the divergence between the two Supergroups of *Terebratulina* (**Table 6.3**) occurred no earlier than in the Cretaceous.

6.5 Conclusions

The morphometric analysis alone has limitations for the inference of the cancellothyridoid phylogeny because only a limited number of the external morphologic characters, some of which may be prone to convergent evolution, were taken into account. The immunological data, on the other hand, provided more or less reliable clues of the

relationships, but the data was not available for all the species. However, the integration of morphometric and immunologic data provides important new data on phylogeny, and it is obvious that much more work of this type is required to confirm relationships among this challenging and intriguing taxon.

Chapter 7 Preservation of intracrystalline macromolecules in fossil brachiopod shells

7.1 Introduction

Skeletal macromolecules can be preserved for a remarkably long time (**Chapter 1**; e.g., de Jong *et al.*, 1974; Weiner *et al.*, 1976), in contrast to the normal life span of most other biological macromolecules, presumably due to the protection against physical, chemical, and biological degradation forces provided by the surrounding, or closely associated, biocrystals. In this regard, the molecules entombed within the mineral phase, or the intra-crystalline macromolecules, are expected to have a better fossilisation potential than the inter-crystalline, or matrix, macromolecules, although current knowledge regarding intracrystalline macromolecules is sparse, since their presence and importance as candidates for molecular fossils have only recently been recognised (Towe, 1980; Curry, 1988; Collins *et al.*, 1988).

In this study, intracrystalline macromolecules isolated from Pleistocene articulate brachiopod shells, which are composed of diagenetically stable low magnesium calcite, have been investigated by biochemical and immunological methods, in an attempt to evaluate the extent of degradation in the Pleistocene material, and to estimate the taxonomic value of the macromolecules of this age, a potential which is considered in more detail in the next chapter.

Previous studies have indicated that living brachiopod shells contain proteins, lipids and carbohydrates within the fibrous calcite of the secondary layer (Curry *et al.*, 1991b; Collins *et al.*, 1991c; **Chapter 4**). Through immunological investigations, it has also been demonstrated that these macromolecules contain significant taxonomic information for living taxa (Collins *et al.*, 1988; 1991b; Curry *et al.*, 1991c; **Chapters 5-6**), and that these pieces of information were preserved, if not completely, in Plio-Pleistocene brachiopod shells (Collins *et al.*, 1991a).

7.2 Materials and methods

7.2.1 Materials

Fossil samples utilised in this study are listed in **Table 3.2**, and details of the samples of the living reference species can be found in **Table 3.1**. The shell intracrystalline macromolecules of an undescribed species of fossil *Terebratulina* from the Sawane Formation, Japan (ca. 1 Ma) and living *Terebratulina unguicula* were analysed by gel

filtration hplc, amino acid analysis and immunological assays. Samples of a terebratellid (*Waltonia inconspicua*) and five laqueids (*Terebratalia coreanica*, *Coptothyris grayi*, *Laqueus rubellus*, *Pictothyris picta*, and *Kikaithyris hanzawai*) collected from various localities of both well-dated shellbeds and the Recent seas (except for the extinct *K. hanzawai*) were analysed by immunological methods.

Anti-*Terebratulina unguicula* (174), anti-*Waltonia* (K5040), anti-*Laqueus* (1191), and anti-*Pictothyris* (1192) antisera were utilised in the immunological assays (cf. **Table 3.4**).

7.2.2 Methods

Shell secondary fibres were isolated and decalcified to release their intracrystalline macromolecules by the methods described in **Chapter 3**. For hplc analyses, a volume of 10 ml of the EDTA-extract from each of the living and fossil *Terebratulina* preparations was dialysed (MW cut off: 10 kDa) against 500 ml of Milli-Q™ water over-night with three changes of water, in order to remove EDTA. The resulting sample was concentrated in a vacuum centrifuge (GyroVap; Howe) to a volume of 1 ml each, and 400 µl of this was mixed with an equal volume of the elution buffer for gel filtration analysis.

Gel filtration was carried out using the Superose 6 column (Pharmacia), which had been equilibrated with sodium phosphate buffer (50 mM, pH 6.5) which contained SDS (0.2% w/v). Each sample of the shell extracts and size marker proteins (high and low molecular weight standards; Pharmacia), which had been mixed with the elution buffer, was boiled for 3 mins, and centrifuged for 1 min at 14000 rpm prior to loading onto the column. The fossil sample was analysed first, then the living sample, followed by the standard proteins, with complete washing elutions between each run. Elution was carried out at a flow rate of 0.5 ml/min. Other hplc conditions and methods were as described in **section 3.2.9**.

After gel filtration, a volume of 100 µl each of the peak containing fractions of both living and fossil samples was analysed by ELISA (**section 3.2.4**) using the anti-*T. unguicula* antiserum with a higher concentration of the antiserum (1: 500) than the usual working titre (1: 3000). The elution buffer for the gel filtration (100 µl) was used as the negative control for the immunological assays. The remaining 400 µl each of the peak fractions was dialysed against Milli-Q™ water overnight, re-concentrated to 400 µl by the GyroVap, and 20 µl of each preparation was subjected to amino acid analysis (**section 3.2.10**). Cluster analyses on the amino acid composition data were carried out using the software 'Datadesk professional™' on an Apple Macintosh computer.

Immunological assays on the terebratellid and laqueid fossils were carried out by ELISA, using anti-*Waltonia*, anti-*Laqueus*, and anti-*Pictothyris* antisera, with the serial antisera concentration method (**section 3.2.8**). The extent of the immunological reactions was expressed by the 'quasi-immunological distances' (**section 3.2.8**).

7.3 Results and discussion

7.3.1 Intracrystalline macromolecules of Recent *Terebratulina unguicula*

Gel filtration chromatography fractionated the intracrystalline macromolecules of the living and fossil *Terebratulina* into several components, largely by their sizes, rather than by both molecular size and shape, since denaturing elution buffer was employed (**Figures 7.1, 7.2**). The size of each component was estimated by calibrating with the standard proteins (**Figure 3.1**). The fractions, which contained a major component in either the living or fossil sample, were subjected to amino acid analyses and immunological assays (**Table 7.1**).

The chromatogram of the living *Terebratulina* (**Figure 7.1**) indicated the elution of a component at the void volume of the column (peak 1; fraction 3), followed by components of rather undefined profiles (peaks 3, and 4; fractions 11 and 13), then the highest sharp peaks (peaks 5 and 6; fractions 19 and 21), and a higher composite peak after the highest peaks (peak 7; fraction 25). Fraction 6 (peak 2) was included in the analyses as the fossil sample had a peak at that elution volume.

Amino acid analysis indicated that fractions 6, 11, 13, and 19 contained larger amounts of amino acids, with particularly high concentrations in fractions 13 and 19 (**Table 7.1; Figure 7.3**). Fractions 19 and 21 contained a considerable amount of EDTA, whose presence was confirmed by the amino acid analysis, where the EDTA peak coeluted with the proline peak (D. Walton, pers. comm.; **Table 7.1**). Considering the size of the components in these fractions (37-48 kDa; **Table 7.1**) and the much smaller size of EDTA molecules (MW = 292.25), it is expected that the EDTA molecules were forming complexes by themselves, or with the shell macromolecules, possibly *via* the calcium ions which the EDTA chelates. Fractions 3 and 25, which had low levels of amino acids and EDTA, are interpreted as being composed mainly of carbohydrates or other unknown molecules. All the fractions of the living sample were immunologically reactive, although this was rather low in the larger components (**Table 7.1**), indicating that the antisera were not generated specifically against a particular kind of molecule, but probably generated against both proteins and carbohydrates (cf. Collins *et al.*, 1991c).

The amino acids in fractions 6, 11, 13, and 19, which represent the major proteinaceous fractions, had a strikingly similar pattern of residual composition (**Figure 7.3**). This pattern was characteristic for brachiopod intracrystalline proteins (D. Walton, pers. comm.), with glycine as the predominant amino acid, high levels of acidic residues (aspartic acid and glutamic acid), and low levels of basic amino acids (lysine and arginine).

The strongly similar amino acid composition among the major proteinaceous components of different sizes allows four different interpretations.

(1) It can be interpreted as indicating different grades of glycosylation to the same sequence of peptides, as suggested by Collins *et al.* (1991c) for different brachiopod intracrystalline proteins.
(2) Since the similarity in amino acid composition does not necessarily mean a similarity in the amino acid sequence, these components could represent different proteins.
(3) It is possible to interpret smaller components as the subunits of a single large protein, which have similar amino acid compositions to each other.
(4) The larger components could have been formed by the aggregation of single small proteins (peak 5; 48 kDa).

Among these interpretations, (4) seems to be most plausible, considering the result that the intracrystalline macromolecules of the closely related species, *Terebratulina retusa*, yielded a single prominent protein band (30 kDa in size) in the SDS-PAGE gel (D. Walton, pers. comm.; Curry *et al.*, 1991b; cf **Chapter 4**). The considerably larger estimated size of the peak 5, compared to the 30 kDa protein, could be explained by the incorporation of EDTA molecules, which were not completely removed by the dialysis (**Table 7.1**). However, evidence to confirm this interpretation is lacking, and further characterisation is obviously required for *Terebratulina* intracrystalline proteins.

7.3.2 Preservation of intracrystalline macromolecules in the Pleistocene *Terebratulina* species

The gel filtration profile of the fossil *Terebratulina* (**Figure 7.2**) had similar features to that of the living *T. unguicula*, with the void volume component (peak 1'; fraction 3), the highest peaks at almost the same elution volumes as *T. unguicula* (peaks 5', 6'; fractions 19, 21), followed by a high composite peak (peak 7'; fraction 25). Between peak 1' and peak 5' were two discernible peaks, one is unique in the fossil sample (peak 2'; fraction 6). The other has a comparable size to peak 3 in *T. unguicula* (peak 3'; fraction 11), with a more defined peak shape in the fossil sample. Peak 4 of the living sample was absent, although the corresponding fraction (fraction 13; peak 4') was included in the subsequent analyses.

Amino acid analyses revealed that fractions 6 , 11, 13, 19, and 21 contained a considerable amount of amino acids, and fractions 6, 19, and 21 were particularly abundant in amino acids (**Table 7.1**). The EDTA content was high in fraction 21, as in *T. unguicula*, but was not high in fraction 19, unlike the living sample. This could indicate a loss of the calcium ion binding property in the fossil molecules, which enabled an easier removal of the chelating agent.

Amino acid composition data indicated that fractions 11, 13, and 19 had similar amino acid composition profiles as the major proteinaceous components of living *T. unguicula* (**Figure 7.3**), a similarity which was confirmed by cluster analysis (**Figure 7.4**).

Immunological assays revealed that fraction 19 (peak 5'; 45 kDa), which yielded the highest level of amino acids among the three, contained significant immunological reactivities (**Table 7.1**), suggesting that this component has been preserved more or less intact in terms of molecular size, amino acid composition and antigenic properties.

The remaining amino acid rich fractions, fraction 6 (peak 2') and fraction 21 (peak 6'), on the other hand, had different compositions from those with the typical brachiopod composition (**Figure 7.3**), as also indicated by the cluster analysis (**Figure 7.4**). Since these proteinaceous components (peaks 2' and 6') were not found in the living *T. unguicula*, and each amino acid residue was more or less evenly represented in these components, they are interpreted to be the condensation products, or comprised of amino acids from degraded shell proteins. As peak 2' had immunological reactivity, it is expected that original pieces of shell macromolecules were involved in the formation of this component. The material in peak 6' produced no significant immunological reaction suggesting that the determinants had decayed. It is also possible that this proteinaceous component was only present in the original shell of this fossil species.

The immunological response from the fractions with low amino acid concentration (fractions 3 and 25) may indicate the preservation of the antigenic properties of carbohydrates.

Table 7.1 Characterisation of gel filtration fractions of fossil and living *Terebratulina*
Extent of immunological reaction is expressed as the fluorescence value (raw data). The elution buffer for gel filtration was used as the negative control for the immunological assays (blank). The upper row in each data set is for the living *T. unguicula*, and the lower row for the fossil *Terebratulina* species from Japan. Asterisk (*) indicates that the amino acid content value does not include Proline content. Peaks 2 and 4' were not discernible in the chromatograms, but these names were used for the convenience of discussion.

Fraction No.		**3**	**6**	**11**	**13**	**19**	**21**	**25**	**Blank**
Peak ID. Number	Living	1	(2)	3	4	5	6	7	
	Fossil	1'	2'	3'	(4')	5'	6'	7'	
Molecular Weight (kDa)	Living	1500	(700)	260	150	48	37	14	
	Fossil	1500	700	240	(150)	45	34	15	
Amino Acid content (pmol)	Living	61	96	144	354	*375	*108	62	
	Fossil	24	478	160	125	*344	*447	18	
Proline/EDTA content (pmol)	Living	0	4	6	13	102	470	0	
	Fossil	0	37	7	5	15	229	0	
Immunological reaction	Living	408	1441	2044	2257	2182	2370	2035	206
	Fossil	407	489	217	220	466	224	946	206

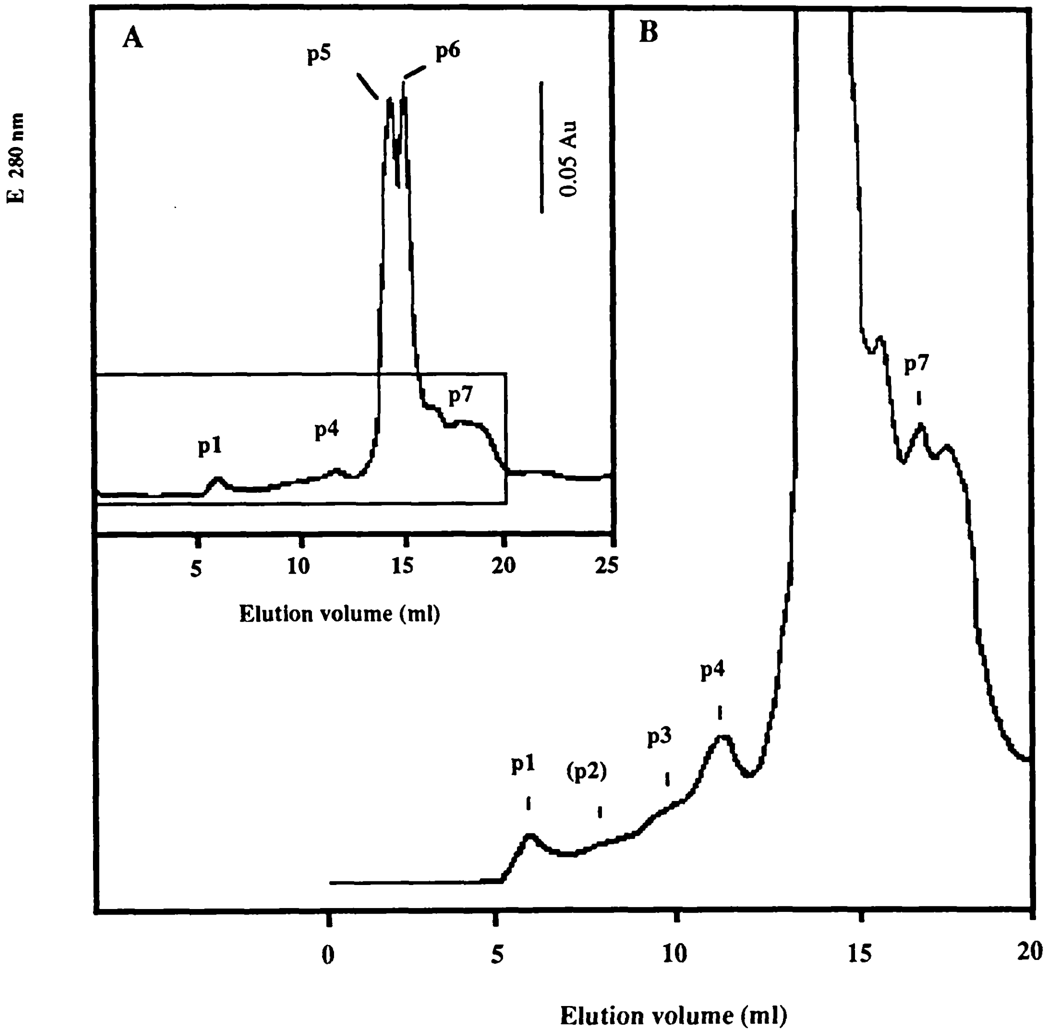


Figure 7.1 Gel filtration of living *Terebratulina* shell extracts

A: Fractionation of the intracrystalline macromolecules isolated from living *Terebratulina unguicula* on Superose 6 column eluted with phosphate buffer (50 mM, pH 6.5) containing SDS (0.2 % w/v) at a flow rate of 0.5 ml/ min.

B: Details of the boxed area of panel A. For peak identities see **Table 7.1.**

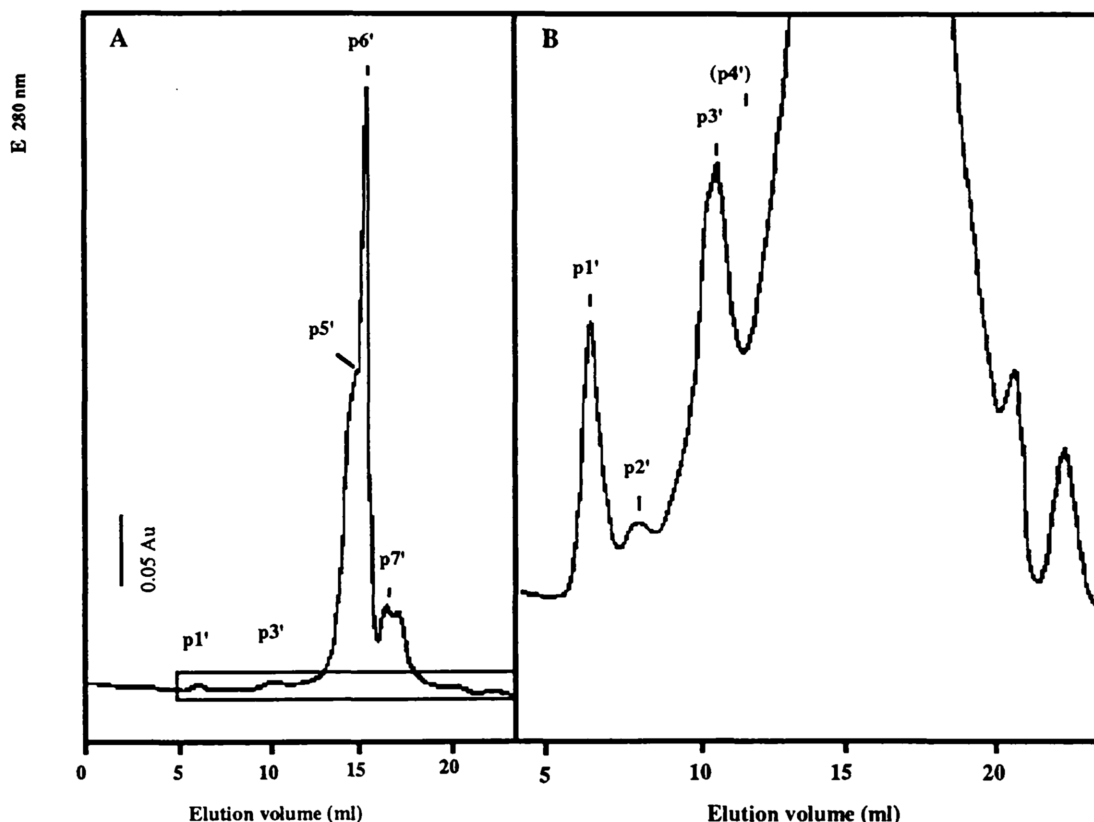
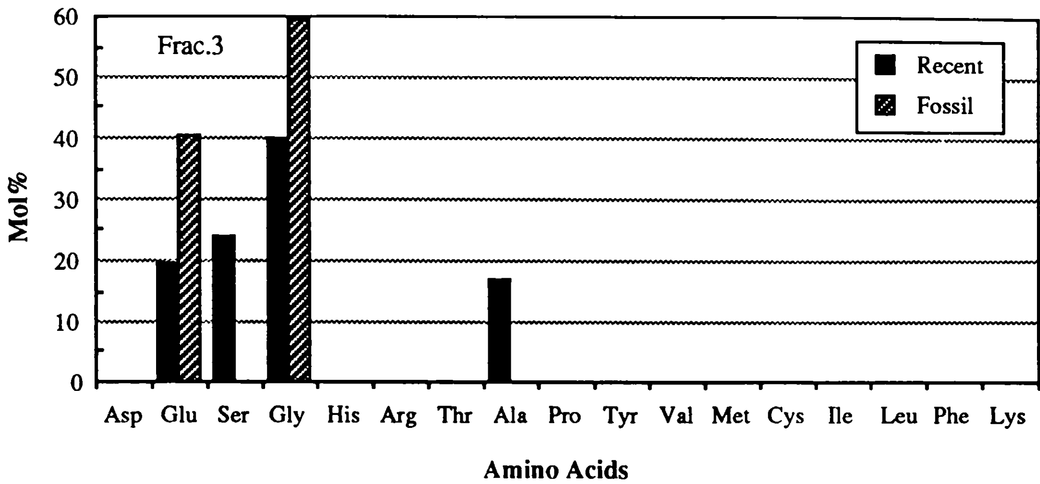


Figure 7.2 Gel filtration of fossil *Terebratulina* shell extracts

A: Fractionation of the intracrystalline macromolecules extracted from a 1 Ma *Terebratulina* species on Superose 6 column, eluted with phosphate buffer (50 mM, pH 6.5) containing SDS (0.2% w/v) at a flow rate of 0.5 ml/ min.

B: Details of the boxed area of panel A. For peak identities see **Table 7.1.**



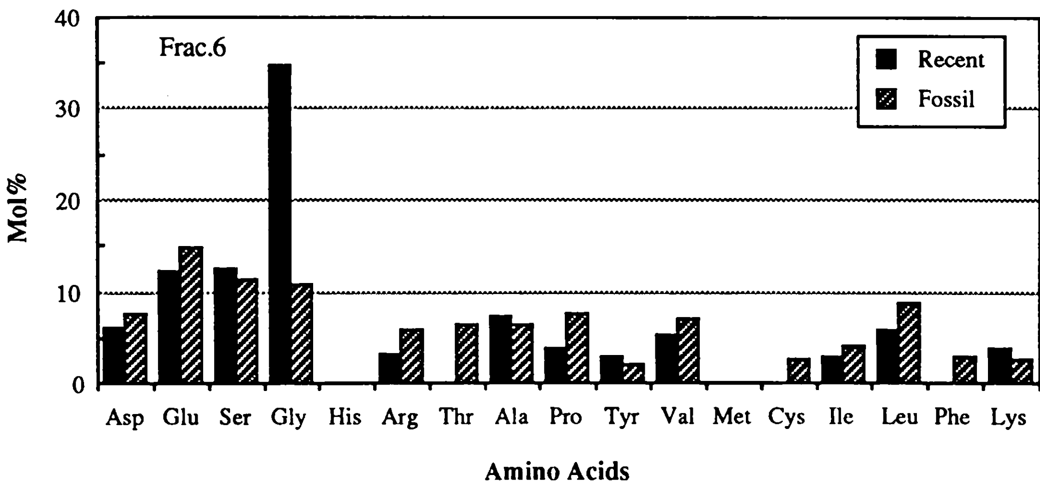
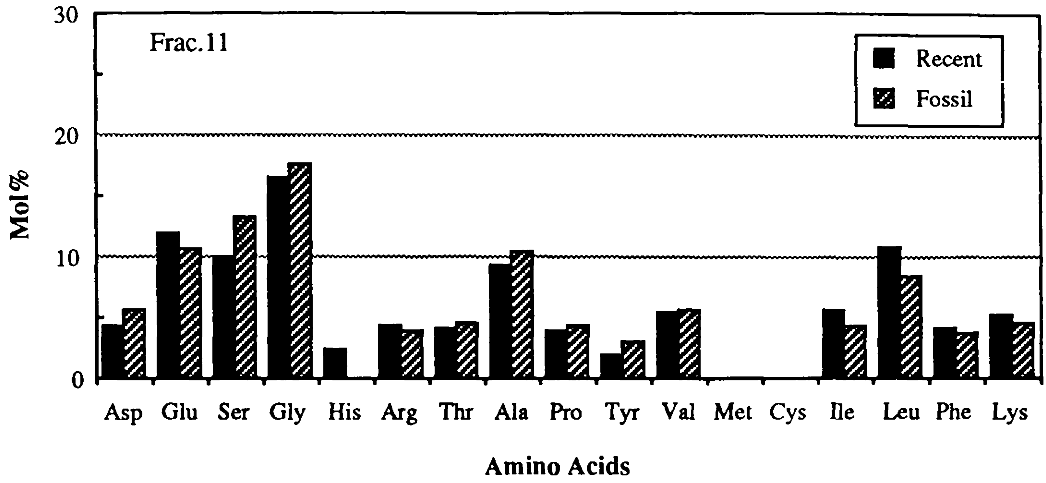
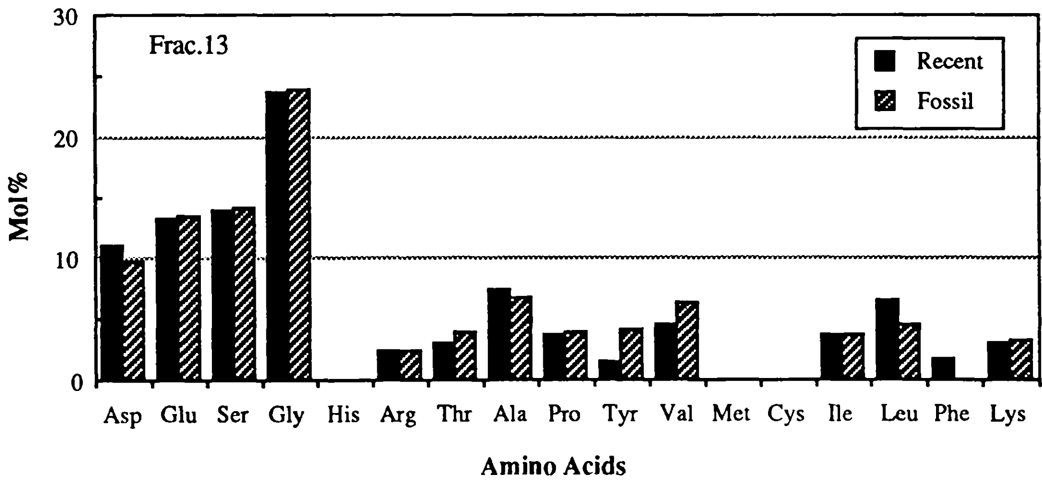


Figure 7.3 Amino acid composition of gel filtration fractions

Gel filtration fractions of the intracrystalline extracts of both living and fossil samples (cf. **Figures 7.1** and **7.2**; **Table 7.1**) were dialysed and reconcentrated (see **section 7.2.2** for method) prior to the amino acid analysis (**section 3.2.10**). Molar percentage of each amino acid residue (see page xiv for abbreviation) for each fraction is shown. Key: Recent = living *T. unguicula*, Fossil = fossil *Terebratulina* species.





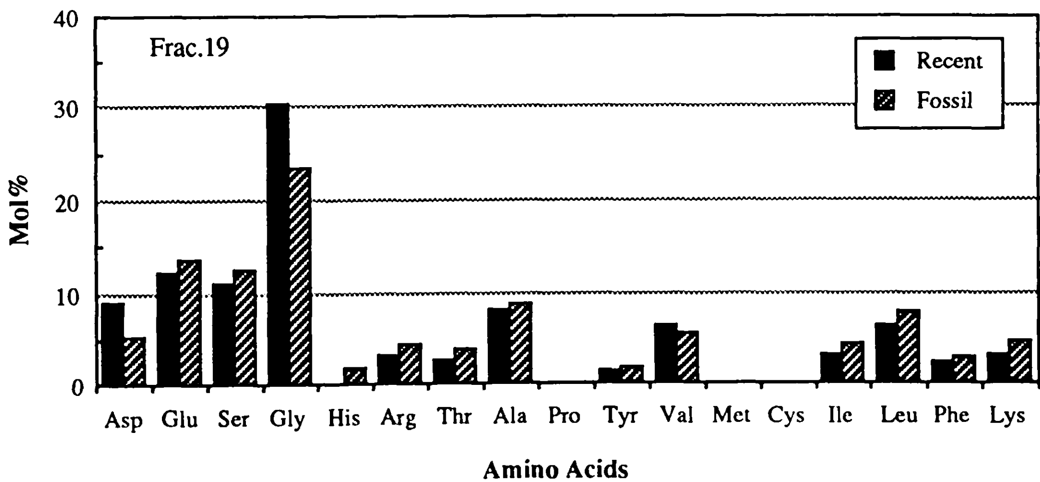
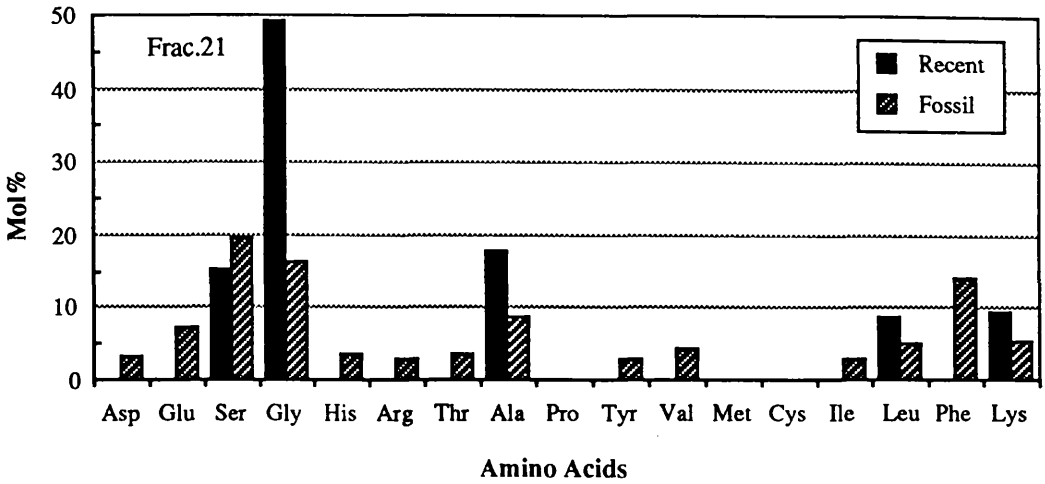


Figure 7.3 (continued)



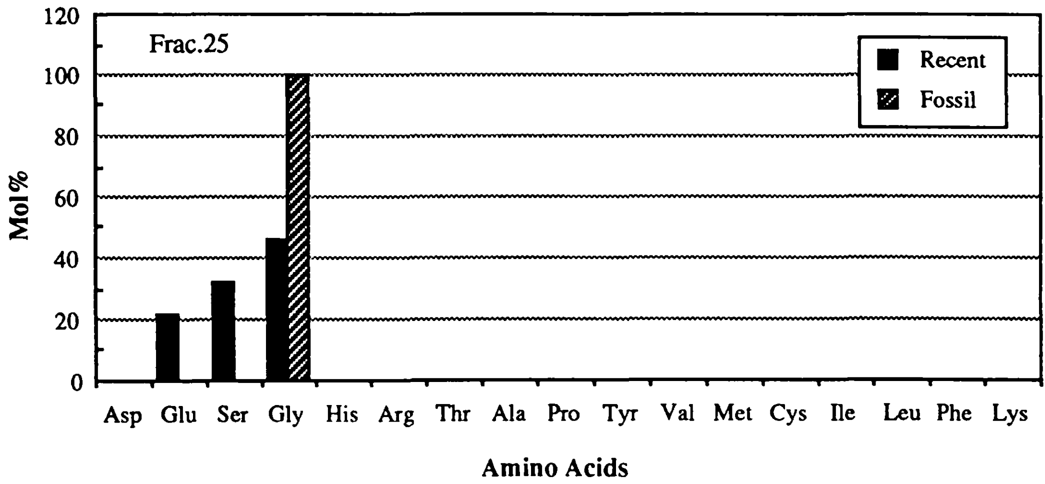


Figure 7.3 (continued)

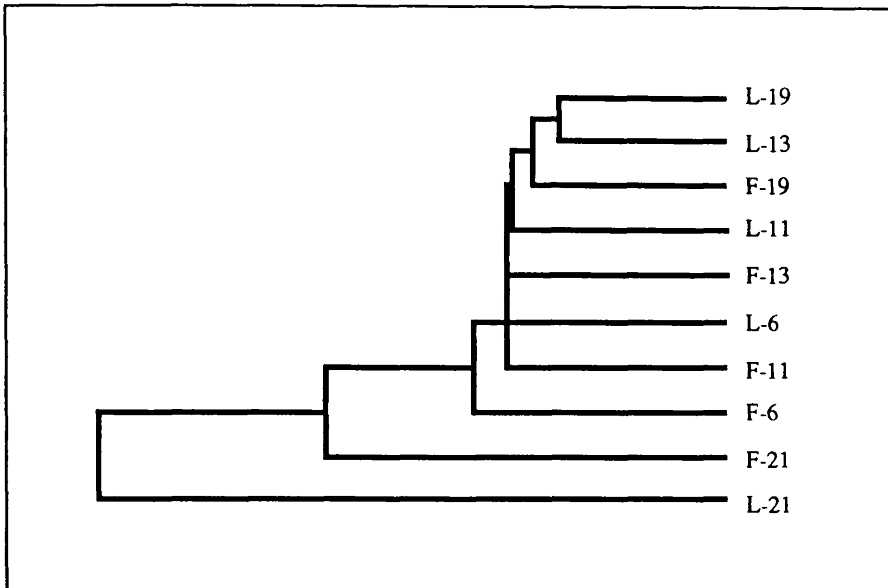


Figure 7.4 Cluster analysis on amino acid composition data

Gel filtration fractions (**Table 7.1**) of both living and fossil samples were clustered using the amino acid composition data by single linkage method (Sneath and Sokal, 1973). Percentage of each amino acid residue (**Figure 7.3**) was regarded as the variable. Key: L-6 = fraction 6 of the living sample, F-6 = fraction 6 of the fossil sample, etc.

7.3.3 Immunological responses from Pleistocene brachiopods

Figure 7.5 indicates the immunological reactions of homologous antisera against fossil *Laqueus*, *Pictothyris*, and *Waltonia*, as measured by 'quasi-immunological distances' (d_I'), showing a progressive decrease of the immunological response in each species with increasing time. The antiserum concentration required to obtain the same level of reaction as the living specimens for 0.4 Myr old *Waltonia* was about 1000-fold (d_I' = ca. 300), while the factors with *Laqueus* and *Pictothyris* fossils of comparable ages were about 100 (d_I' = ca. 200). These decreases in reactivity are too large to be caused by molecular evolution during the Pleistocene, and are largely attributed to the diagenesis of the macromolecules in each species. The observed variations in the immunological reactivity between these species are considered to be due to the differences in the preservation of the macromolecules (diagenetic factors) and the differences in the properties of the antisera (antiserum factors).

Diagenetic factors may involve temperature, moisture, pH, and other environmental conditions of the fossil sites, the shell structure, shell thickness, and the vulnerability of the antigenic determinants to diagenesis, etc. The antisera factors may include the specificity, avidity, and titre of the antisera. In the assays with heterologous antisera, the phylogenetic factor, which reflects the molecular variations due to the phylogenetic differences, is expected to contribute to the variations in the immunological reactions from fossils, and it is this factor which must be isolated from others to obtain biological information.

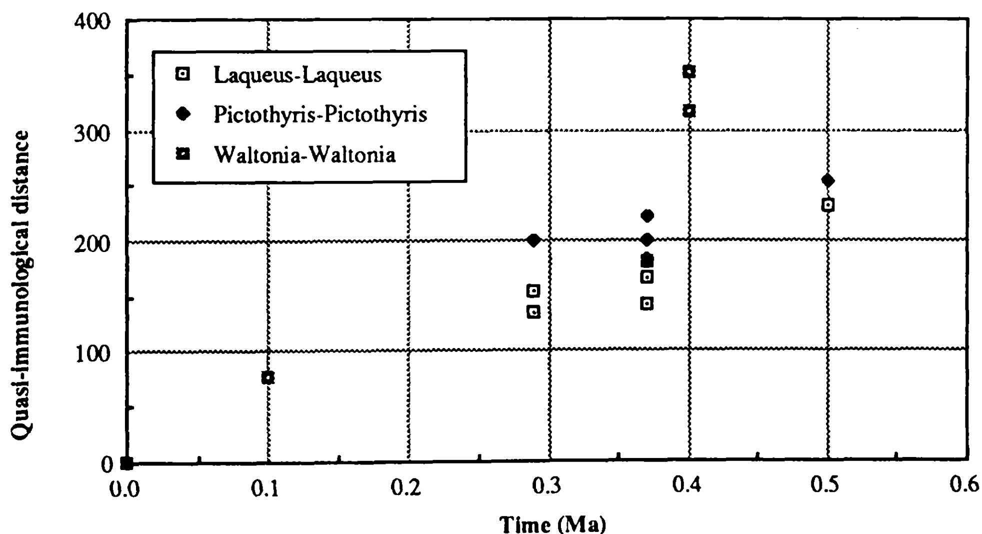


Figure 7.5 Immunological reactions from fossil shells with homologous antisera
Mean values of duplicate experiments.

Assays using anti-*Laqueus* serum against fossil *Laqueus*, *Pictothyris*, *Terebratalia*, and *Coptothyris* of different ages indicated a general pattern in which *Terebratalia* reacted strongest, followed by *Pictothyris* and *Coptothyris*, then the homologous *Laqueus* (**Figure 7.6, A**). This pattern of reactions is not reflecting phylogeny in any sense, since *Laqueus* is more closely related to *Pictothyris* than to *Terebratalia* or *Coptothyris*, both morphologically (Hatai, 1940) and immunologically (**Chapter 5**).

Since all these fossils were collected from closely located sites of similar environments, the pattern cannot be explained by the different conditions of each of the fossil sites. A good linear, or curvilinear, trend in the decrease of the immunological response in each species also suggests the absence of extreme variations of the physicochemical conditions in these fossil localities through time. The most realistic interpretation of this reactivity pattern seems to be that the differences in shell size and the thickness of the shell test, especially the thickness of the cardinalia, resulted in the differential diagenesis in each species, because, in general, *Terebratalia* had the largest and thickest shell while *Laqueus* had the smallest and thinnest.

The weaker reactions from fossil *Waltonia* (**Figure 7.5**) can be explained on the same ground, because *Waltonia* is smaller and thinner than *Laqueus*, although it is also possible that other unknown factors were different between the laqueid localities (Japan) and the *Waltonia* localities (New Zealand).

Anti-*Pictothyris* antisera detected (rightly in terms of the phylogenetic relationships) stronger reactions with *Pictothyris* fossils than with co-occuring *Laqueus* fossils (**Figure 7.6, B**). However, as *Pictothyris* has more or less thicker shells than *Laqueus* does, whether or not the observed pattern was the true reflection of the phylogenetic variations could not be determined.

These results suggest that the use of a single antiserum to screen a panel of fossil samples may not be fruitful for taxonomy at lower levels, because the reactions detected may be vastly biased by various diagenetic factors, including the effects of the shell size and thickness, which would complicate the interpretation of the data for species collected even from the same shellbed.

Assays on single samples against a panel of different antisera may not be so limited in elucidating fine-grained phylogenetic information, because this approach compares the antigen molecules which have experienced the same diagenetic histories, eliminating the effects which come from the differences in the various diagenetic factors. Assays on *Laqueus* fossils using anti-*Laqueus*, anti-*Pictothyris*, and anti-*Waltonia*, demonstrated that the immunological reactions were weaker with the anti-*Waltonia* serum than with the other two antisera (**Figure 7.7, A**). This pattern is consistent with the taxonomic scheme, because *Laqueus* and *Pictothyris* belong to the Laqueidae and *Waltonia* belongs to the Terebratellidae. The levels of the reactions with the anti-*Waltonia* serum on the *Laqueus* fossils were considerably lower than the self-reactions on the *Waltonia* fossils of similar

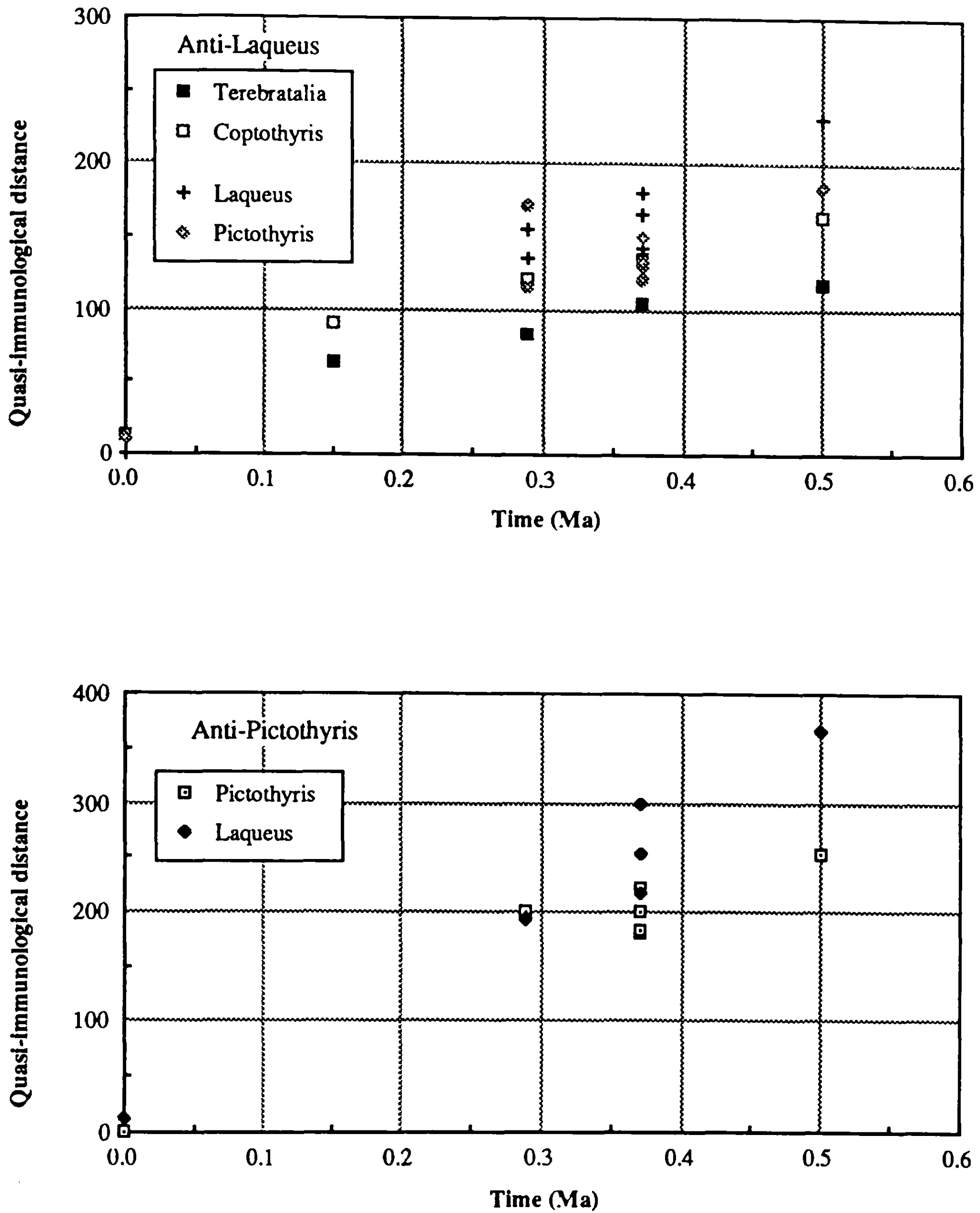


Figure 7.6 Immunological reactions from fossil laqueid species

Upper panel: anti-*Laqueus* antiserum, Lower panel: anti-*Pictothyris* antiserum. Mean values of duplicate experiments.

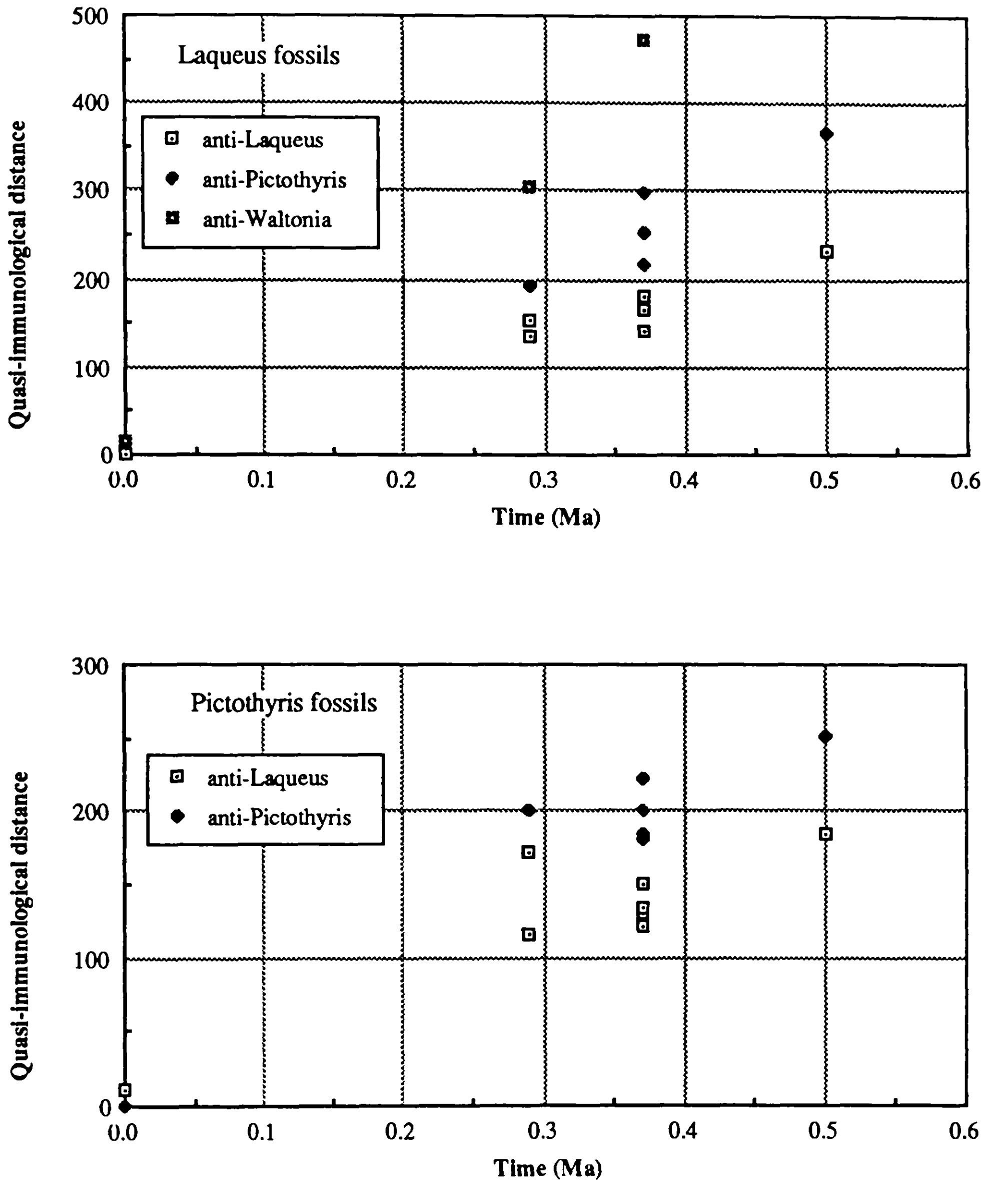


Figure 7.7 Immunological reactions from *Laqueus* and *Pictothyris* fossils

Upper panel: fossil *Laqueus rubellus*, Lower panel: fossil *Pictothyris picta*. Mean values of duplicate experiments.

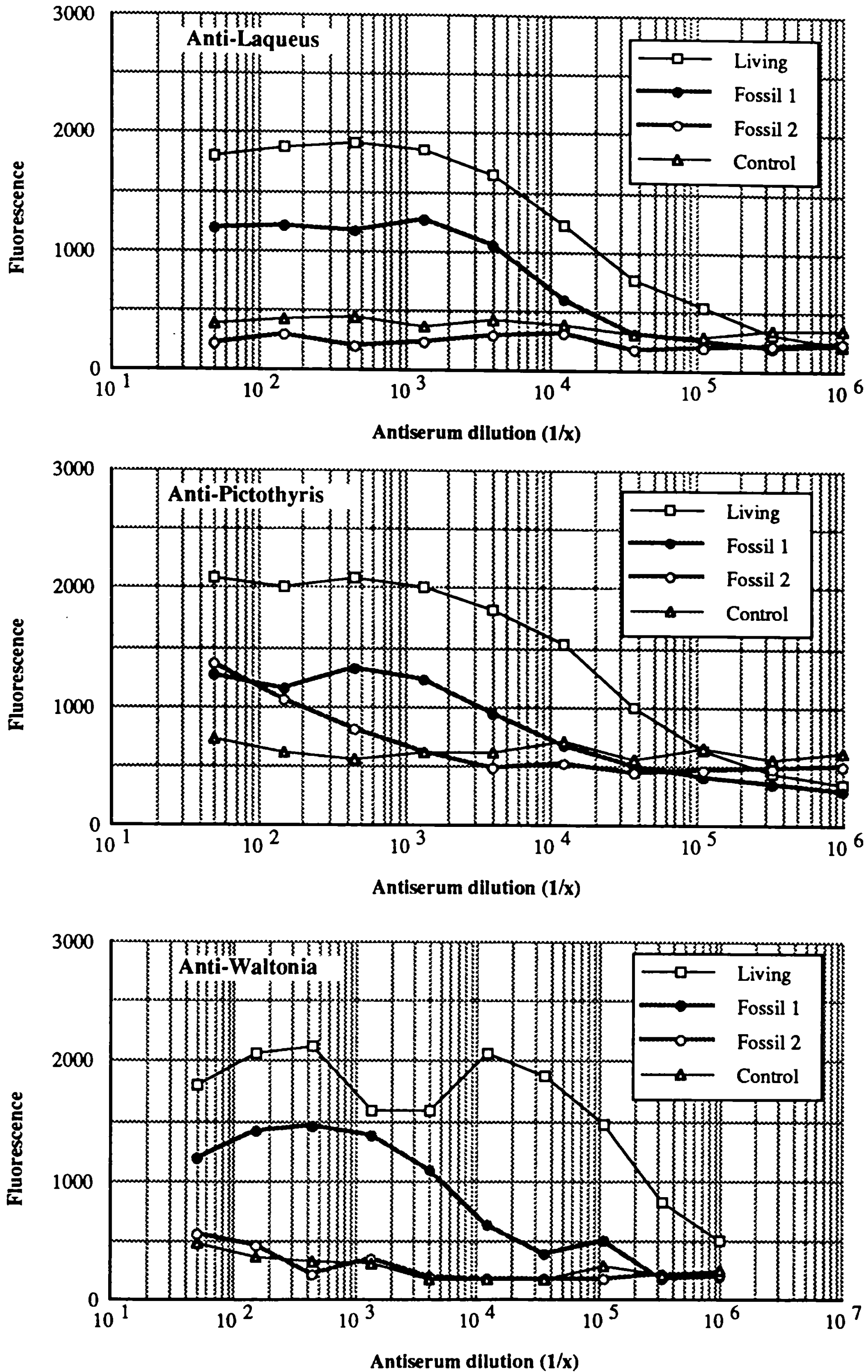


Figure 7.8 Immunological reactions from fossil *Kikaithyris*

Key: Fossil 1 = *Kikaithyris hanzawai* from the Wan Formation (0.08 Ma), Fossil 2 = *K. hanzawai* from the Naha limestone (ca. 1 Ma). Mean values of duplicate experiments.

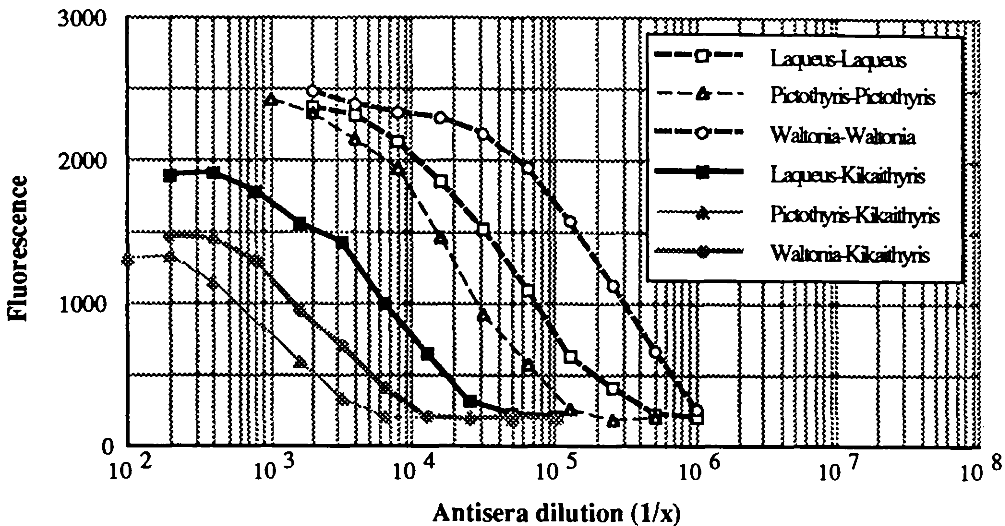


Figure 7.9 Binding curves for fossil *Kikaithyris* (Wan Formation)

Different set of assays from that of **Figure 7.8.** Mean values of duplicate experiments.

age (cf. **Figure 7.5**), despite the fact that *Laqueus* shells are larger and thicker than *Waltonia* shells (thus stronger reactions with the *Laqueus* fossils could have been observed). The weaker reactions with the anti-*Waltonia* serum, therefore, may best be interpreted as resulting from the phylogenetic differences between *Laqueus* and *Waltonia*, indicating the preservation of lineage-specific information in the *Laqueus* fossils.

Assays on the *Laqueus* fossils suggested that even the closely related genera (*Laqueus* and *Pictothyris*) had been correctly discriminated (**Figure 7.7, A**). Assays on *Pictothyris* fossils, however, invariably indicated stronger reactions with the anti-*Laqueus* antiserum than with the anti-*Pictothyris* antiserum (**Figure 7.7, B**), indicating that the anti-*Laqueus* serum was generally more reactive with fossil macromolecules than the anti-*Pictothyris* serum. The reason for the higher binding with anti-*Laqueus* antiserum is not clear, but it could be related to the optimal working titres of the antisera, which appear to be the only major difference between the anti-*Laqueus* (titre: 1:20,000) and anti-*Pictothyris* (1:5000) sera. Specificities of these sera appear to be similar, as judged from the reactivity patterns of these sera against a range of living species (**Chapter 5**: **Table 5.1**). The anti-*Waltonia* serum had the highest titre of the three (1:40,000) and appears to be less specific than the other two (cf. **Table 5.1**), therefore, as far as these known features are concerned, the weak self-reactions with the *Waltonia* fossils can not be explained

by the antiserum factors.

The above results have demonstrate that the use of a panel of antisera can elucidate lineage-specific information from fossil materials, and, obviously, this approach is most useful for the phylogenetic inference of extinct forms.

Kikaithyris hanzawai is a laqueid brachiopod from Japan which occurs in sediments as young as 80,000 years old, but is not found in the present fauna. This species is characterised by large size and thickened cardinalia, therefore, good preservation of intracrystalline molecules had been anticipated. Two samples of different ages (80,000 years old and about 1 Myr old) of this fossil species were tested against three antisera; anti-*Laqueus*, anti-*Pictothyris*, and anti-*Waltonia*, and respective averaged d_I' values (quasi-immunological distances) of 68, 105, 170 were calculated for the younger sample (**Figures 7.8, 7.9**). These figures also demonstrate negligible level of reaction with the negative controls, assuring the absence of non-specific noise caused by antisera concentration. The lower level of reactions with the anti-*Waltonia* serum confirmed the affinity of *Kikaithyris* to the laqueid species. Morphologically, this species is most closely related to *Pictothyris* (Hatai, 1940), therefore, genus-level information could not be obtained from this sample, as was expected from the above experiments. Surprisingly, however, the older sample indicated the positive reaction only with the anti-*Pictothyris* antiserum (**Figure 7.9**), suggesting that the genus-specific information has somehow been preserved in the early Pleistocene shells.

7.4 Conclusions

The major biological goals of characterising macromolecules preserved in fossils are two-fold: to estimate phylogeny of extinct organisms, and to trace molecular evolution using the fossil record. The immunological experiments carried out in this study demonstrated that the former purpose is attainable for the Pleistocene brachiopods, at least for family-level inferences, using the shell intracrystalline macromolecules. With a remarkable fossil record, and the presumed important roles of their intracrystalline proteins (Curry *et al.*, 1991b; Cusack and Curry, 1991), the articulate brachiopods also provide a potentially prosperous and intriguing opportunity for the latter purpose, although more complete biochemical characterisation, including sequencing of fossil peptides, would be required for this purpose.

The indication of the preservation of more or less intact components in the early Pleistocene *Terebratulina*, and the fact that the amino acid analysis was possible on these components using the hplc-based analyser, do suggest that it may be possible to obtain sequence information on fossil peptides using an automatic protein sequencer (e.g. ABI 477A) at least for Pleistocene proteins.

Chapter 8 An immunological investigation of the phylogeny of an extinct Pleistocene *Terebratulina* species

8.1 Introduction

Molecular techniques have provided new perspectives to the systematics of many living organisms (**Chapter 1**; e.g. Fernholm *et al.*, 1989). The molecular approach is certainly useful when morphological information is scarce or equivocal, for example, when comparing organisms of simple morphology, or comparing between highly diversified forms. For the study of fossil organisms, where information regarding soft part morphology, embryology, or ethology, is usually wanting, biomolecular data would have particular importance, if it could be determined.

It has been demonstrated during the last quarter-century that various kinds of organic compounds, including proteins and nucleic acids, are preserved in fossil remains, and that the preserved polymeric structures in these fossil macromolecules do yield taxonomic information (**Chapter 1; sections 1.3** and **1.4**). The sequencing of fossil nucleic acids has provided perhaps the most detailed genetic information recovered from the molecular fossils (e.g., Higuchi *et al.* 1984; Pääbo *et al.* 1988; Horai *et al.*, 1989; Golenberg *et al.* 1990). This approach is not only helpful for phylogenetic reconstruction, but may also be auspicious for tracing molecular evolution directly using the fossil record (Pääbo *et al.*, 1989). However, the isolation of fossil nucleic acids has only been possible from more or less exceptionally preserved fossils, and no nucleic acids have so far been reported from the more common type of fossils, namely shells. An alternative approach is to study the skeletal macromolecules, usually including proteins, which are tightly associated with, and preserved within, the mineral phase of bones, teeth, and the shelly skeletons of invertebrates.

As these preserved molecules are generally in the form of complex mixtures of degradation products of various grades, and also because there is always a chance of microbial and human contamination, the preservation of original macromolecules is often verified by biological probes, such as antibodies and oligonucleotides, which recognise and bind to the specific parts of the biopolymers. Sequencing of purified biopolymers is a more plausible way of characterising these molecular fossils. However, this approach is not yet available for fossil proteins. Instead, the use of immunological methods has been successfully applied, not only to detect original skeletal macromolecules, but also to obtain biological information from the fossil molecules (e.g., de Jong *et al.* 1974; Westbroek *et al.* 1979; Lowenstein 1980; Ulrich *et al.*, 1987; Muyzer *et al.*, 1988; Collins *et al.*, 1991a). The underlying idea is that the relative intensity of immunological reactions detected from fossil macromolecules can be extrapolated to the relative affinity of the fossil organisms

to their living relatives (see Lowenstein, 1985).

For obvious reasons, it is much more difficult to obtain immunological signals from molecules isolated from fossils than from living organisms. Major techniques which have been devised to detect, or increase, the signals from fossil macromolecules, include the extensive concentration of the fossil antigen molecules (e.g., de Jong *et al.*, 1974), the use of detection methods involving radioactive chemicals (e.g., Lowenstein, 1980; Collins *et al.*, 1991a), and the pre-absorption of the antisera with fossil antigens to determine the extent of inhibition in the immunoassays (e.g., Muyzer *et al.*, 1988). In this study, the inhibition method and another new technique, the systematic increase in the concentration of the first antisera in solid-phase immunoassays, have been applied to shell intracrystalline macromolecules isolated from an extinct Pleistocene (ca. 1 Ma) species of *Terebratulina*, in order to assess its phylogenetic relationships to other congeneric living species.

Terebratulina contains at least one intracrystalline protein (Curry *et al.*, 1991b), which is immunogenic, and provides serotaxonomic information in living species (Collins *et al.*, 1988; Endo and Curry, 1991, **Appendix II-1; Chapters 5-6**). Various biochemical analyses, such as liquid chromatography, amino acid analysis and immunological assays, demonstrated that the shells of the fossil *Terebratulina* species investigated herein contained an immunologically reactive proteinaceous component, which had similar molecular weight and amino acid composition to that of the living counterpart, among components of supposed degradation and aggregation products, suggesting that a portion of the intracrystalline protein had been preserved more or less intact within the shells (**Chapter 7**).

8.2 Materials and methods

8.2.1 Samples

Specimens of an undescribed extinct species of *Terebratulina* were collected by sieving from an unconsolidated sandy shell bed of the Sawane Formation (Pleistocene, ca. 1 Ma), Japan. Shells were well preserved, with articulated valves and occasionally with intact loops. The mode of occurrence of the fossils indicated that these shells had been considerably transported before deposition, and the ornaments on the shell surface were often torn and obscured. From the composition of the co-occuring molluscs, this species is inferred to have been a warm-water inhabitant (Endo, 1986). Antisera prepared against four living *Terebratulina* species from each side of the Atlantic and the Pacific (*T. retusa*, *T. septentrionalis*, *T. unguicula*, and *T. crossei*) (171, 173, 174, and K4962; **Table 3.4**) were utilised for immunological assays. For the morphometric comparisons, specimens belonging to 22 living species and subspecies of the genus were measured using the

samples stored in the Natural History Museum of the Smithsonian Institution, Washington, D.C.(**Table 3.3**). Samples from Skye, Maine, and California were used as the representatives of *T. retusa*, *T. septentrionalis*, and *T. unguicula*, respectively. In other species, the samples from more than one locality were treated as a single sample.

8.2.2 Methods

Extraction of the brachiopod shell intracrystalline macromolecules, preparation of the antisera, and immunological assays were carried out by the methods described in **Chapter 3**. The inhibition assays were carried out using the method described in **section 3.2.7**. The 'immunological distances' (d_I) among living species and the 'quasi-immunological distances (d_I') between fossil and living species were determined using the method described in **section 3.2.8**.

Morphometrical analyses on fossil and living species were carried out using the measurements and methods as described in **section 3.2.11** and **Chapter 6** (see **Appendix I** for raw data). Weight of the shells was not included as a character in this study since the fossil valves contained sediments which could only be completely removed by destroying the shells.

8.3 Morphometry

Principal component analyses on the living and fossil species, using only the *Terebratulina* samples and omitting the shell weight, revealed essentially the same overall patterns obtained in **Chapter 6**, except that the axis which counted for the variations in the shell weight (axis 4 in **Table 6.1**) was absent (data not shown). The first three latent vectors correlated with the first three axes in **Table 6.1**, and the fourth, fifth, and sixth vectors corresponded with the axes 5, 6, and 7 of **Table 6.1**, indicating that the same interpretations apply to these latent vectors as described in **section 6.3**. The scores along each vector for the fossil species indicated that the species was most closely related to Group F (**Table 6.3**) species (data not shown).

A monovariate comparison of the number of ribs among the Group F and the fossil species (**Figure 8.1**), a comparison which was demonstrated to be useful to infer relationships at this level (**Chapter 6**), suggested that the closest relative of the fossil species might have been one of the following species; *T. unguicula*, *T. valdiviae*, *T. compressa* of the Group F1 or *T. septentrionalis*, *T. japonica*, *T. peculiaris* of the Group F2 (cf. **Table 6.3**).

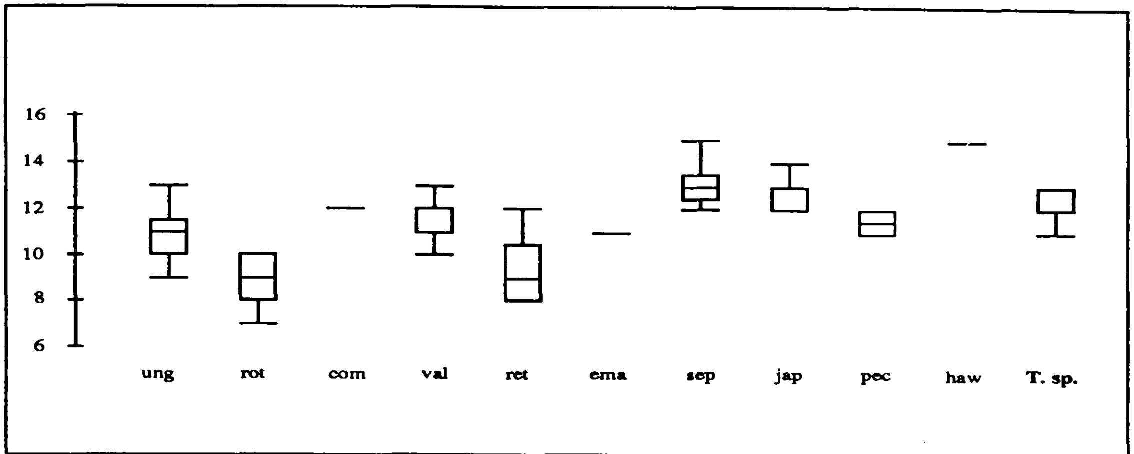


Figure 8.1 Comparison of rib width between the fossil and living *Terebratulina* species

Number of ribs were counted by the method described in **section 3.2.11**. Key: ung = *T. unguicula*; rot = *T. unguicula rotundata*; com = *T. compressa*; val = *T. valdiviae*; ret = *T. retusa*; ema = *T. retusa emarginata*; sep = *T. septentrionalis*; jap = *T. japonica*; pec = *T. peculiaris*; haw = *T. hawaiensis*; *T.* sp. = the undescribed fossil species from Japan.

8.4 Results of the immunological assays

The pre-absorbed antisera were sufficiently specific to discriminate precisely between individual living *Terebratulina* species (**Table 8.1**). The highest inhibition of each antiserum occurred with homologous crude extracts, demonstrating that the antibodies detected systematic variations in their target molecules. The overall pattern of inhibition was also consistent, yielding a clear and unequivocal phylogenetic reconstruction among the four living species. *T. retusa* and *T. septentrionalis* are the most closely related followed by *T. unguicula* and then *T. crossei*. These relationships were confirmed by the determined 'immunological distances' among them (**Table 8.2**).

Pre-absorption of each antiserum, using intracrystalline molecular extracts from fossils, significantly reduced the reactivity of each serum, confirming that immunological determinants were indeed preserved (**Figure 8.2**). Furthermore, the extent of inhibition varied considerably for each antiserum, with anti-*T. unguicula* showing the greatest inhibition, followed by anti-*T. retusa*, and least inhibition with anti-*T. septentrionalis* and anti-*T. crossei* antisera. Serial concentrations of crude antibodies also revealed the

presence of fossil antigens (**Figure 8.3**), which allowed the calculation of the quasi-immunological distances for *T. unguicula* (mean d_I' = 190) and for *T. retusa* (mean d_I' = 320). For *T. septentrionalis* and *T. crossei*, positive reactions were not observed, but the quasi-immunological distances were estimated for both species to be over 300 (**Figure 8.3, Table 8.2**). For each assay with concentrated antibodies, little immunological reaction was observed in the negative control (**Figure 8.3**), confirming that the positive reactions with fossil *Terebratulina* were not an experimental artifact caused by the antisera concentration or by any other operation.

The immunological results, both inhibition and serial antibody concentration assays, strongly suggested that the Japanese fossil species was most closely related to *T. unguicula* among the four congeneric living species studied (**Figure 8.4**).

Table 8.1 Inhibition ELISA on living *Terebratulina* species

Reactivity is expressed as the value of self-reaction compared to the values with no pre-absorption (as 100) and with pre-absorption against the homologous antigens (as 0). Mean values of duplicate experiments.

Pre-absorbed antigen	Antiserum			
	K4962 (ret)	173 (sep)	174 (ung)	171 (cro)
T. retusa	0	0	45	32
T. septentrionalis	7	0	45	27
T. unguicula	22	27	0	72
T. crossei	47	69	86	0

Table 8.2 Immunological distances and quasi-immunological distances among living and fossil *Terebratulina* species

	T. retusa	*T. sep*	*T. ung*	*T. cro*
T. septentrionalis (*T. sep*)	5			
T. unguicula (*T. ung*)	16	12		
T. crossei (*T. cro*)	47	23	56	
T. sp. (fossil)	320	>300	190	>300

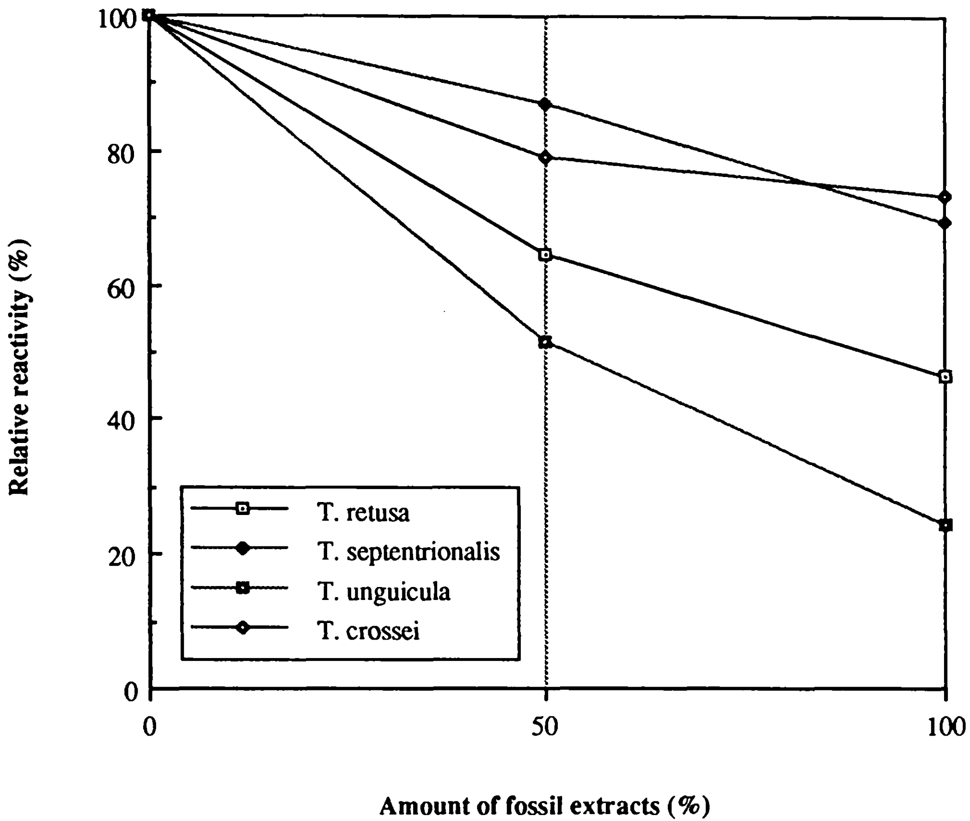


Figure 8.2 Inhibition of immunological reaction by organic extracts from Pleistocene *Terebratulina* species

Reactivity was calculated as in **Table 8.1**. Each antiserum prepared against *Terebratulina* species was pre-absorbed with 50 % and 100 % (v/v) antigen solution extracted from the fossil *Terebratulina*, and the reactivity compared with the reaction using the non-preabsorbed antisera (0% fossil extract). The highest inhibition was observed for the anti-*T. **unguicula*** serum, indicating that the fossil species contained preserved antigenic determinants which had the highest overall affinity with the shell macromolecules of *T. **unguicula***.

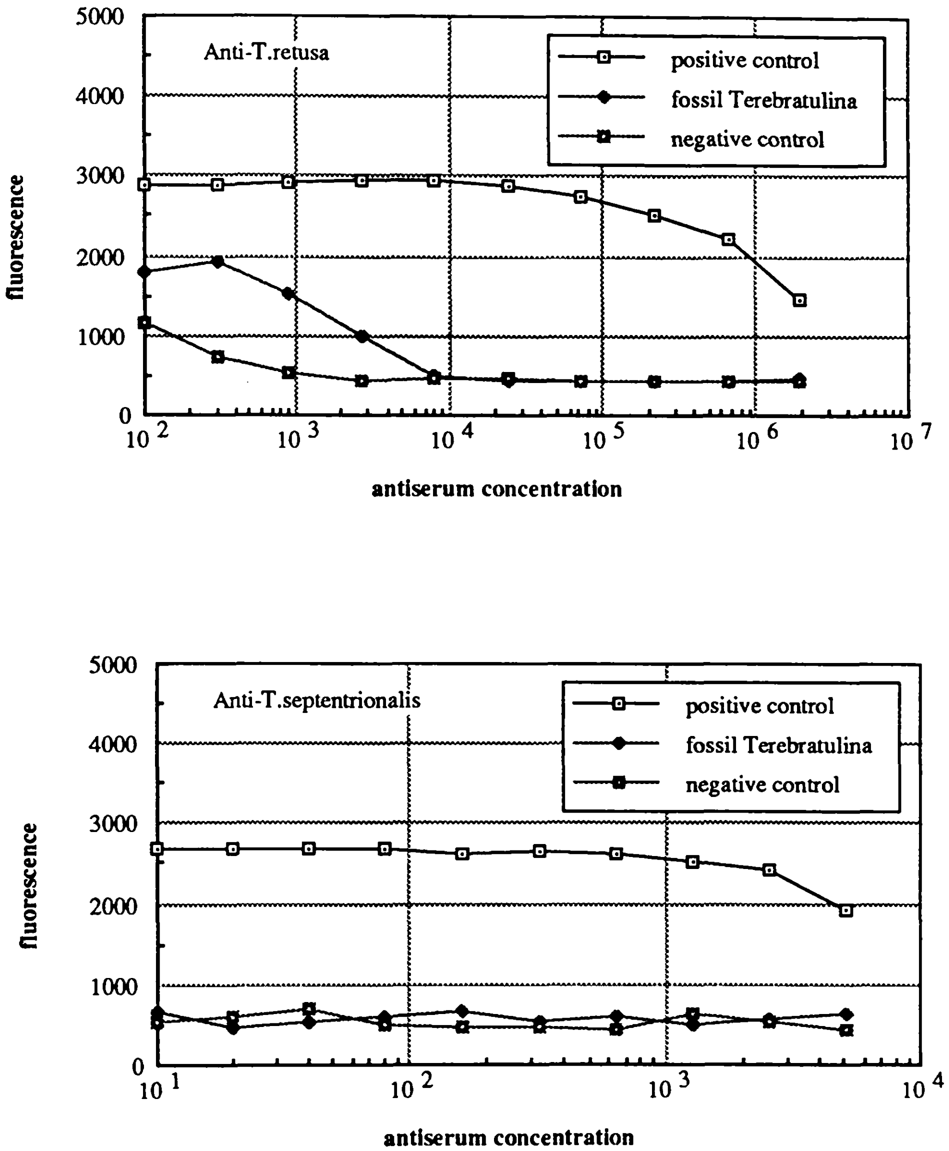
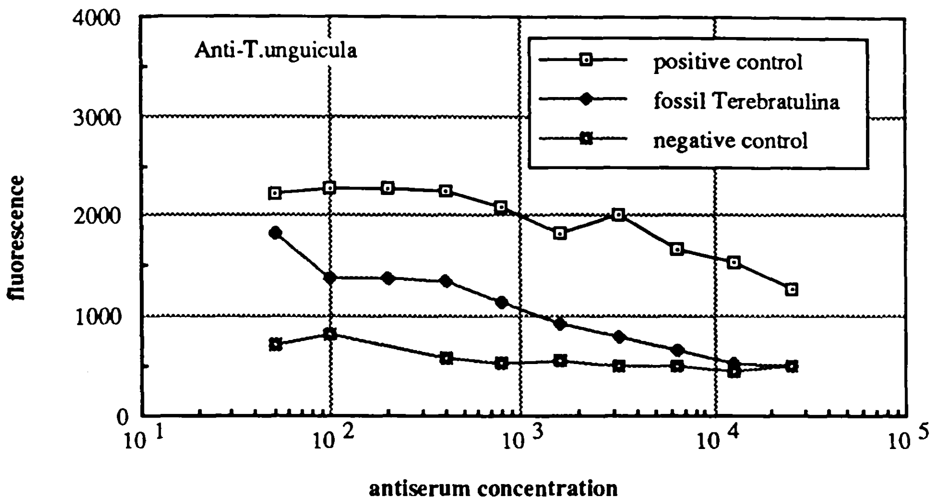


Figure 8.3 Immunological binding curves of fossil *Terebratulina* shell extracts with antisera prepared against four living species

Crude shell extracts from an irrelevant brachiopod, *Laqueus* sp.,collected from the same shellbed, were used as negative controls. Quasi-immunological distances were determined from the linear portions of the binding curves obtained from the assays with the antisera prepared against *T. retusa* and *T. unguicula.*



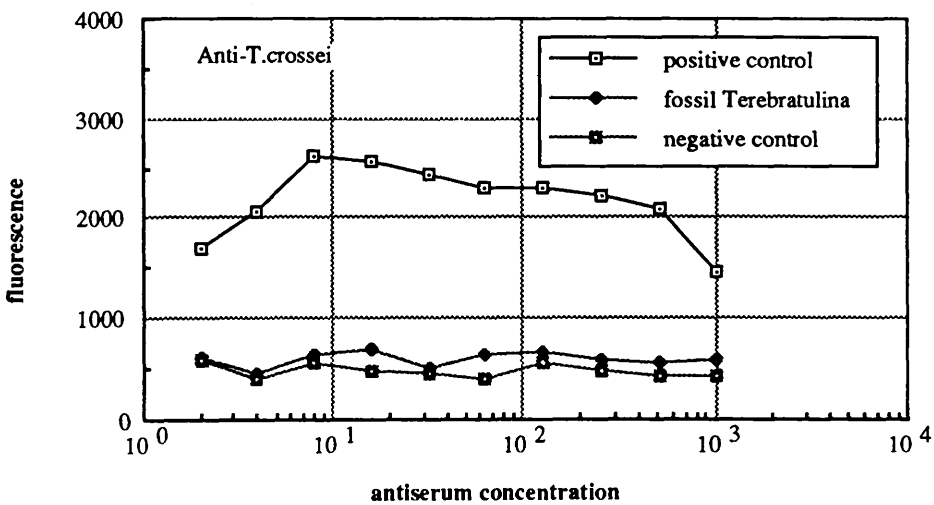


Figure 8.3 (continued)

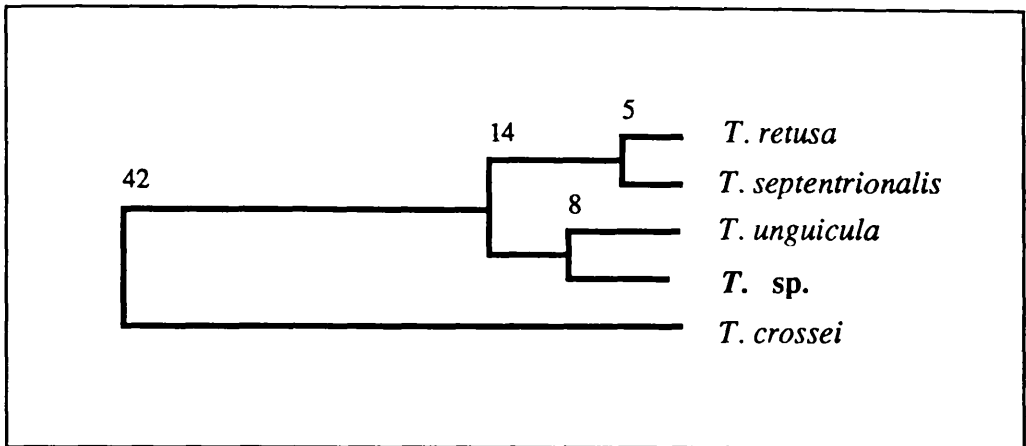


Figure 8.4 Phylogram of *Terebratulina* including the extinct species

Four living species were analysed first using UPGMA clustering (Sneath and Sokal, 1973) based on the data in **Table 8.2**. The fossil species was affiliated to the cluster based on the method described in the text (**section 8.6**). Numbers denote averaged immunological distances for the living species, and the distance of the proportionated value for the fossil species. Calibrating the divergence time between *T. crossei* and other living species as 65 Ma (1 d_I = 1.55 Ma: based on the first occurrence of smooth ribbed *Terebratulina* in the Cretaceous; see Endo and Curry, 1991, **Appendix II-1**; **Chapter 6**), and assuming the 'molecular clock', the divergence time between *T. retusa* and *T. septentrionalis* is estimated as 8 Ma, between *T. retusa-T. septentrionalis* and *T. unguicula* as 22 Ma. Applying the estimation method using quasi-immunological distances, the divergence time between *T. unguicula* and the extinct Japanese species can be estimated as 12 Ma.

8.5 Discussion

There is ample reason to believe that the immunological responses obtained from the fossil *Terebratulina* are taxonomically significant.

After pre-absorption treatment, the antisera utilised in this study, and other similarly prepared brachiopod antisera, can discriminate living species, and the resulting immunological picture of brachiopod phylogeny is morphologically consistent (**Table 8.1**; **Chapter 5**). It has also been indicated that these antisera can rightly assign Plio-Pleistocene samples, of well documented species, to their correct categorical groups, although the specificity was reduced to the superfamily-level for the Pliocene terebratulides using dot blot assays (Collins *et al.*, 1991a) or to the family-level for the Pleistocene laqueid species using the same methods utilised in this study (**Chapter 7**). Considering the fact that the extent of inter-species molecular variability (detected immunologically) among living *Terebratulina* species was comparable with that of inter-familial variations in other terebratulide superfamilies (**Chapter 5**), suggesting that some *Terebratulina* 'species' have a long history comparable with other 'families', it is not surprising that species-level information could be detected from Pleistocene *Terebratulina* fossils.

In addition, the immunological results obtained in this study revealed a general agreement with the morphometric results in that the Japanese fossil species was more closely related to the group of species to which *T. unguicula* and *T. retusa* belong than to the group to which *T. crossei* belongs. The question is, "How likely is it that the fossil species is more closely related to *T. unguicula* than to *T. retusa*, as the immunological data indicated?"

A complication in interpreting the immunological data in this study is that each brachiopod antisera was not prepared against a pure protein but against a mixture which also included carbohydrates (**Chapter 4**). If the anti-*T. unguicula* antiserum had been generated principally against carbohydrate or protein epitopes, whichever is more resistant to diagenesis, than the extent in the anti-*T. retusa* antiserum, then the immunological data would be considered as spurious. However, the immunological assays using these antisera on the gel filtration chromatography fractions of living *Terebratulina* shell extracts indicated no major difference in the specificity of both antisera to each separated components, suggesting that this was not the case (cf. **Chapter 4** and **Chapter 7**). Lineage specificity of each antiserum is another potential problem, however, there is no indication that anti-*T. unguicula* antiserum is particularly less specific than anti-*T. retusa* antiserum (**Table 8.1**). Finally, the titre of the antisera could possibly affect the extent of binding to the fossil epitopes. But, anti-*T. unguicula* antiserum has lower titre (1:3000) than anti-*T. retusa* antiserum (titre: 1:40,000).

On the above grounds, it may be concluded that the variations in the extent of the immunological responses for each antiserum from the fossil *Terebratulina* species is

reflecting the lineage-specific variations of the original macromolecules, and that the fossil species is more closely related to *T. unguicula* than to *T. retusa*. Taking the morphometric data into account, the closest living relative of the fossil species is considered to be either *T. compressa*, *T. valdiviae*, or *T. unguicula*. Among these species, *T. valdiviae* seems to be most probable, considering the adult shell size and the relative foramen size. *T. unguicula* is known from cold waters of the northern Pacific, while the other two are warm-water dwellers in the western Pacific (*T. valdiviae* is even known from tropical waters). The warm-water habitat of the Japanese fossil species strengthens this argument.

8.6 Estimation of the divergence time using 'quasi-immunological distances'

Immunological distances provide a measure of gross molecular dissimilarity, while quasi-immunological distances provide an equivalent measure for the partially degraded remains of such molecules in fossil shells. The immunological distance measured by the microcomplement fixation method on a purified protein is linearly related to the differences in the number of the amino acid substitutions (cf. Nei, 1987), and assuming the molecular clock, the distance has been applied to estimate the divergence time of the proteins and, by analogy, the organisms which carry them, despite the presumed innate inaccuracy of these measurements (Nei, 1977; 1987). Application of the same principle would be possible for living brachiopods, provided that antisera uniformly prepared against purified proteins are obtained. Such an application might even be possible for the fossil brachiopods, although any inaccuracy will obviously be exacerbated. In the following paragraphs, a model is proposed, which may be useful for the first-order estimation of the divergence time for a fossil lineage using quasi-immunological distances.

The underlying supposition is that the diagenetic breakdown of molecules will not distort the relative dissimilarity detected by antibodies because degradation does not depend on molecular composition (i.e., the ratio of d_I values measured by anti-A and anti-B sera for a particular taxon C, would be approximately similar whether the shell macromolecules were intact or degraded). This relationship is expressed by the equation $d_I(A,C)/d_I(B,C) = d_I'(A,C)/d_I'(B,C)$, where $d_I'(X,Y)$ is the quasi-immunological distance between species X and Y, and $d_I(X,Y)$ is the 'true' immunological distance between these species.

By adopting this relationship between d_I and d_I', the ratio of d_I values between two living species and a fossil species can be inferred when the respective quasi-immunological distances have been determined, even though the value of d_I cannot be calculated for fossil species. This ratio is sufficient to estimate the divergence time for extinct species (**Figure. 8.5**). The relationship between immunological distance (d_I) and the divergence time (t)

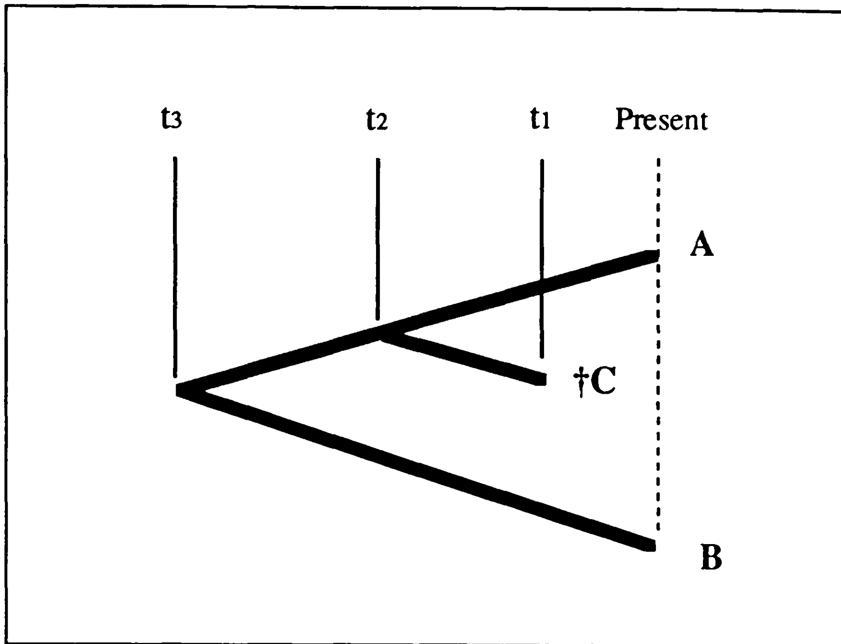


Figure 8.5 Estimation of divergence time using quasi-immunological distances
See text for explanation.

is written as $t = c \times d_I$, where c is a constant (Sarich and Wilson 1967; Nei 1987). The relationships between living species A, B and a fossil species C are then written as $t_2 + (t_2 - t_1) = c \times d_I(A,C)$ and $t_3 + (t_3 - t_1) = c \times d_I(B,C)$, where t_1 is the age of the fossil, t_2 and t_3 are the divergence time of C from A and B respectively, $d_I(X,Y)$ denotes the immunological distance between species X and Y. For the relationships between d_I and d_I' (quasi-immunological distances) among species A, B and C, $d_I(A,C)/d_I(B,C) = d_I'(A,C)/d_I'(B,C)$. From the above equations, the divergence time of the fossil species C from species A (t_2) is: $t_2 = \{d_I'(A,C)/d_I'(B,C)\}\ t_3 + 1/2\ \{1 - d_I'(A,C)/d_I'(B,C)\}\ t_1$.

Applying this method to the available data, the divergence time between *T. unguicula* and the fossil species was estimated as 12 Ma (**Figure 8.4**).

8.7 Conclusions

Both inhibition and serial antibody concentration immunoassays demonstrated that species-level molecular information was preserved within the shell crystals of an extinct 1 Myr old *Terebratulina* species. The immunological data was concordant with the results

of shell morphometry, and provided additional finer-grained information regarding the relationship between the fossil and living species.

The ability to calculate 'quasi-immunological distances' for the 1 Myr old species suggests that the technique has considerable value in unravelling the phylogenetic relationships of Pleistocene taxa which are difficult or impossible to assign to known lineages. With a set of high quality antisera (high specificity, avidity, titre, etc.) prepared in a uniform manner, it might be possible to further extrapolate the 'quasi-immunological distances' to the first-order estimation of the divergence time between extinct taxa and related extant taxa.

As intracrystalline macromolecules are believed to be ubiquitous in biocrystals, this method can readily be applied to other fossil groups.

Chapter 9 General discussion and suggestions for further study

9.1 Serotaxonomy of living brachiopods

Immunological data has yielded radical new ideas regarding higher-order phylogenetic relationships within the Terebratulida. The immunological approach was also applied, with reasonable success, to the inference of lower-order relationships, down to species-level, using the more elaborate inhibition method.

The three-fold division of the living terebratulides, and the convergent evolution of the two long-looped lineages, were the two major findings of the immunological study. These results can be tested by extending the immunological survey to include such taxa as *Platidia, Nipponithyris, Diestothyris, Aldingia, Anakinetica, Bouchardia, Gwynia, Dyscolia, Cnismatocentrum*, etc., or, for more independent tests, by studying other sets of proteins or nucleic acids of living brachiopods. Morphological studies are also important, and Palaeozoic and early Mesozoic ancestors of these groups also remain open to palaeontological examinations.

Among the cancellothyridoids, which constituted one of the three major groups of the living Terebratulida, the genus *Terebratulina* contains several species groups as demonstrated by the combination of immunological and morphometric data. The relationships between, and within, these species groups were revealed when both the immunological and morphometric data were available, although, obviously, four antisera were not enough to study more than 20 species of the living *Terebratulina*. More work, both molecular and morphological, is required to further clarify the relationships.

The morphometric analysis was effective in uncovering the morphological traits of each *Terebratulina* species and characterising each species group, but was not powerful enough to provide detailed information about the relationships between these groups. This was perhaps due to the inadequate selection of the morphological characters, which were represented by 7 measurements of mostly adult external characters. Morphological characters of the juveniles would be expected to provide valuable information about phylogeny, although it is extremely difficult, as far as visible external characters are concerned, to distinguish the juvenile *Terebratulina* species. The morphology of the protegulum, and early development of the shell, especially the development of the radial ornaments, could be informative, if there are any variations at these growth stages, and these characters can be studied by scanning electron microscopy, possibly using adult shells, since the early growth stages are preserved in the apical areas of the dorsal valves. The development of the loop, its morphological changes and timing, about which very little is known for this group, could also be informative, albeit the fact that the adult loop statistics were not very successful in discriminating species (*T.japonica, T.peculiaris*, and

T. pacifica; data not shown).

The phylogenetic relationships of other cancellothyridoid genera to *Terebratulina*, and the affinity of the cancellothyridoids to other Palaeozoic, Triassic, and Jurassic brachiopods, also require further investigation.

The close relationship between the Terebratuloidea and the group of long-looped families (the Kraussinidae, Megathyrididae, Macandreviidae, and Ecnomiosidae), two groups which constitute a second major group of the Terebratulida, was one of the most intriguing findings of the immunological study. Confirmation of this relationship by other molecular techniques is desired. This relationship is explainable in terms of morphological schemes and the fossil record, as discussed in **Chapter 5**. However, a more detailed examination is needed, especially regarding the early loop development, the cardinalia, and the dental plates, of the relevant taxa in the Mesozoic and Palaeozoic.

Concerning the loop development, the emergence of the septum, or the loop supporting element attached to the valve floor, in the zeilleriids, or in other possible ancestors of the long-looped living species of this group, is of particular importance. In the cardinalia, the elements to which the dorsal adjustors attached are of interest, because in the living species of this group, the adjustors attach to the outer hinge plates in one extreme (Terebratuloidea), and to the valve floor in the other end (Kraussinidae). The evolution of the dental plates in this group is still to be explained, being absent in terebratuloids, megathyridids, and kraussinids, and present in macandreviids, and ecnomiosiids. Palaeozoic ancestors of these groups also need to be re-examined.

The relationship of the dallinids to the laqueids and terebratellids are problematic, although these three families are closely related, to constitute the remaining one of the three major groups of Terebratulida. The assignment of these families into two separate superfamilies based on the presence (Dallinidae, Laqueidae) or absence (Terebratellidae) of the dental plates was not supported, and the evolution of the dental plates is a question that remains to be answered, as it is in the other long-looped lineage. The diagnoses of the Dallinidae and the Laqueidae need to be amended, and the assignments of the Mesozoic forms to these families also require re-examination. Relationships among the Laqueidae and the Terebratellidae were revealed to some extent, but not for the Dallinidae. As these are a prolific group as a whole, perhaps the largest group of the three major groups in the extant seas, more detailed work is required for these three families.

Conclusive data could not be obtained regarding the taxonomic position of thecideidines in the brachiopods, because no antiserum was prepared against this group. When the antisera are made available, it might be possible to test the affinity with the Terebratulida, the possibility of which was suggested by the results with the anti-terebratulide antisera. However, the study of more slowly evolving molecules than the shell macromolecules would be better suited for the inference of the thecideidine origin, so as to include appropriate outgroups to compare (see discussions below).

Relationships concerning the order Rhynchonellida were left almost unrevealed, because anti-terebratulide antisera were hardly reactive with rhynchonellides, and the anti-*Notosaria* serum did not react with any of the assayed rhynchonellides except for *Notosaria* itself. Since the anti-*Notosaria* antiserum was prepared in the same procedures as most other brachiopod sera, the specificity of this serum is likely to reflect vast differences in the shell molecules of this species from those of any other investigated species, including other rhynchonellides, rather than reflecting an experimental artifact.

The brachiopod antisera did not react either with inarticulate brachiopods (*Neocrania* and *Lingula*; data not shown), or with bivalve molluscs. These results with rhynchonellides, inarticulates and bivalves suggest the limit of the application of the brachiopod intracrystalline macromolecules for the investigation of phylogeny, a limit which may result from too fast a rate of evolution for the inference of these levels of ancestry.

9.2 Mitochondrial DNA of *Lingula*

As an extension of the immunological investigation of the shell macromolecules, the sequencing of nucleic acids is better suited for several reasons. (1) Nucleic acid sequencing provides much more fine-grained data than the immunological approach. (2) Many homologous genes or DNA sequences have a wide range of distribution in organisms, and evolve at different rates, making it possible to study any level of relationship by selecting suitable pieces of sequence. For example, ribosomal DNAs/RNAs are distributed in any organisms on earth, and have been utilised for the phylogenetic inferences between kingdoms and between phyla (**Chapter 1**). (3) The sequence data are also advantageous because they are accumulative, and the use of existing and growing databases would immensely widen the scope of the study. Recent developments in molecular biology, especially the advent of DNA sequencing methods and the *in vitro* DNA amplifying technique (PCR), made it possible to rapidly obtain DNA sequence data from very small amounts of tissue (cf. **Chapter 1**).

Mitochondrial DNA (mtDNA) has increasingly been utilised for the study of animal phylogeny, because animal mtDNA exhibits no detectable recombination of DNA sequences and contains little non-coding DNA, both of which complicate the interpretations in evolutionary study, and mtDNA is often inherited maternally, effectively in a haploid manner, making the analyses simple, and also because it is easily isolated in a high copy number, and being a small, well-defined circular molecule, is easy to map by simple restriction site analysis (Jacobs, 1989MS).

Mitochondrial DNA is useful for phylogenetic studies across a huge range of divergence times (Jacobs, 1989MS). Substitution rate for each gene, or region, of mitochondrial genomes is considerably different, making it possible to provide information

on very recent relationships between populations in a species (e.g., Cann *et al.*, 1987) to more ancient relationships at family/order level (e.g., Kocher *et al.*, 1989), or ancestry at class/phylum level by comparing the more conserved regions of mtDNA-encoded polypeptides (Jacobs, 1989MS). The gene order and organisation of mitochondrial genomes (genetic code, tRNA structures, etc.) is strikingly conserved (Jacobs *et al.*, 1988), and would give useful information on the relationships at phylum level or even at higher ranks (Jacobs, 1989MS).

The study of nucleotide sequences and gene organisation of *Lingula anatina* (Lamarck) mitochondrial DNA has been begun by the author, and it is hoped to be completed in the next few years. *Lingula* was chosen among the living brachiopods, because it has an extraordinary history as a living fossil, of which evolution at the molecular level is intriguing in the evolutionary biological sense, and its species boundary is problematic and interesting in relation to the larval ecology (Emig and Hammond, 1981; Hammond and Poiner, 1984), and also because it has large amounts of tissue when compared to other brachiopods, especially the muscles and the pedicle, due to the burrowing habit, a fact which is advantageous in a practical sense for the molecular cloning experiments.

Complete sequences of mitochondrial genomes are available for human (Anderson *et al.*, 1981), rat (Gadaleta *et al.*, 1989), mouse (Bibb *et al.*, 1981), cow (Anderson *et al.*, 1982), frog (*Xenopus*; Roe *et al.*, 1985), sea urchin (Jacobs *et al.*, 1988), fly (*Drosophila*; Clary and Wolstenholme, 1985), and nematode (cited in Wolstenholme *et al.*, 1987), but no complete sequence has been reported for brachiopods or for any of the marine protostome invertebrates.

The complete sequencing of the *Lingula* mitochondrial genome is required for three reasons. (1) It would be helpful to predict, or select, informative regions of the genome for each particular phylogenetic problem of different levels within the phylum, and facilitate the construction of oligonucleotide primers for the PCR, through comparisons with the known sequences of other genomes. (2) It would allow the inference of the relationships with other phyla by comparing the organisation of the genome. (3) Preliminary experiments indicated that *Lingula* had a very large mitochondrial genome size (around 27 kb), suggesting an unusual organisation of the genome, which, when fully sequenced, may give insights into the evolution of the mitochondrial genomes themselves, or into the mechanisms of mitochondrial gene expression and its regulation.

9.3 Characterisation of living brachiopod intracrystalline proteins

It is now becoming certain that brachiopod shell intracrystalline proteins have certain functions. It is, therefore, of interest to fully characterise these proteins, not only

for obtaining detailed phylogenetic information, but also for understanding their biological roles. Major obstacles for the characterisation appear to be the purification of homogeneous protein populations from the mixture in the shell extracts and EDTA. Further optimisations of the liquid chromatography conditions can be useful. As it seemed rather wild to apply crude extracts directly onto the 'hi-tech' analytical instrument, perhaps some preparative treatments, or fractionations, before the hplc step would be helpful.

SDS-PAGE appears to be the most successful method for purifying shell proteins so far. Preparation of monoclonal antibodies against these electrophoretically separated proteins, followed by the affinity chromatography with these antibodies as the ligands, would be an ideal way of obtaining large quantities of highly purified intact proteins from the crude shell extracts. The primary sequence of thus purified proteins can be determined by Edman degradation sequencer, and the secondary structure by circular dichroism spectroscopy. Monoclonal, or polyclonal, antibodies against single proteins may also be useful for the localisation, or *in situ* hybridisation, of the shell proteins within the crystals, and the information about the distribution might give insights into the function of these proteins, and the expression of the genes which code for them.

These intracrystalline proteins can also be studied at the DNA or RNA level. Because some amino acid sequences of the N-terminus have been determined for some of these proteins, it is possible to predict the DNA sequences which would code for the first sequences of these proteins from the knowledge of the genetic code, and appropriate oligonucleotide sequences can be chemically synthesised, although more than one oligonucleotide would be prepared and tested for each protein, since most amino acids are encoded by more than one codon.

These synthesised oligonucleotides can be used as the probes to find the DNA fragments which contain the genes coding for the particular protein. The total DNA may be digested by different restriction enzymes, then the restriction fragments separated by gel electrophoresis in two lanes (one for each enzyme treated sample), and half of the gel, which contain one of each sample, is transferred to a membrane and screened by the radiolabelled probes. The portion of the remaining half of the gel, a portion which includes the band of the longest nucleotide length among the positive bands in the autoradiogram, may be cut out from the gel, DNA fragments isolated and cloned using appropriate methods, then the resulting clones screened by the same probes (cf. Sambrook *et al.*, 1989).

Alternatively, the synthesised oligonucleotides can be used as the upstream primer, with the other primer being oligo (dT), for the polymerase chain reaction to amplify the transcribed messenger RNA (mRNA) for the protein (cf. Kawasaki, 1990).

These cloned DNA sequences, or amplified mRNA sequences may easily be sequenced, and the amino acid sequences of the protein can be determined from the RNA sequences using the genetic code, and the gene structures would be inferred from the DNA sequences.

9.4 Study of fossil intracrystalline proteins

Immunological assays confirmed the preservation of original proteinaceous antigenic determinants, which were of taxonomic value in Pleistocene materials. The level of immunological responses, as measured by the 'quasi-immunological distances', suggested that the applicable samples of this approach would not be much older than 1 Myr old. However, what has not been attempted in this study is the combined approach, that is, to use the serial antisera concentration method on highly concentrated antigens with radiolabelled antibodies, and this approach would considerably widen the range of application to older samples. To obtain accurate phylogenetic information from fossil proteins, it is necessary to prepare antisera against single proteins isolated from a series of taxa, and these antisera are desired to be as uniform as possible in quality (specificity, titre, avidity, etc.) with very high working titres.

Sequencing of fossil peptides would be one of the ultimate goals of the fossil protein study, and may be possible for brachiopod intracrystalline proteins by a well-organised approach. It will certainly require time, effort, and funding, and is perhaps too costly to complete just for the phylogenetic inferences of recently extinct brachiopod species. However, it would be realistic and worthwhile to attempt it for the purpose of tracing molecular evolution in the last few million years or so, using a series of samples of the same species, for example, the thick shelled *Neothyris*, *Terebratalia*, or *Pictothyris*, collected from a range of different horizons.

To obtain any meaningful result, it is necessary to have information about the substitution rates of the intracrystalline proteins, by comparing homologous living proteins between species, and to choose, if any, reasonably quickly evolving proteins, before attempting the work on fossils. The amino acid sequence of the extant target protein of the target species must also be determined prior to the fossil study, so that any of the peptides recovered from fossils can be compared. Purification of the fossil peptides would be the highest hurdle to overcome, and liquid chromatography and SDS-PAGE currently offer the best opportunities. The samples should be highly concentrated, and chemical or enzymatic cleavages of the molecules may be utilised to cleave particular segments of the proteins. In either method, the separated peptides must be identified using the antiserum, preferably monoclonal antibodies, prepared against the target protein. The isolated peptides may be sequenced using the Edman degradation sequencer, or might better be sequenced using mass spectrometry (Barinaga, 1989; Biemann, 1989), if available, so as to identify the chemical modifications which may have occurred to the fossil peptides.

9.5 Concluding remarks

There appears to be no doubt that the integrated molecular and traditional approach is fruitful in evolutionary biology. Molecular techniques will increasingly lead to insights into phylogenetic history of life on earth, while these molecular data would not make sense without the knowledge of traditional schemes, for example, no molecules can discriminate species without *a priori* discrimination of the species. In other words, identifications of species, or taxa, always precede the identifications of molecules, illustrating the importance of traditional taxonomy in molecular studies.

Traditional morphological and palaeontological data are also considered essential in the study of molecular evolution for similar reasons. For example, the calibration of the 'molecular clock', or the validation of the clock itself, may only be possible with the fossil data, and the use of molecular fossils, such as fossil DNA and fossil proteins, as the direct evidence of molecular evolution would require, in practice, some knowledge of palaeontology for the sampling of the source materials.

One of the aims of this thesis was to exemplify the importance of this *methodological* integration in evolutionary biology. It is hoped that this integrated approach will eventually lead to the *conceptual* integration to fill the vast gap between molecule and morphology. As an extension of the brachiopod research, the evolution of metazoa would make one of the most interesting and important themes.

It is becoming clear that significant evolutionary change of multicellular organisms can arise from systematic alterations of the genes that control temporal patterns (such as the heterochronic genes reported from a nematode; Ambros and Horvitz, 1984), or the genes that control spatial patterns (such as the homeotic genes reported from various animals; McGinnis, 1985; Gehring and Hiromi, 1986) in development. The former changes should be responsible for many phylogenetic phenomena, morphologically well-known as heterochrony (Gould, 1977), such as the evolution of the terebratulide loop. Such a phenomenon could also have been responsible for the establishment of the most body plans of the metazoa in the early Cambrian (cf. Valentine, 1990; Erwin, 1991).

The study of the molecular basis of the metazoan morphogenesis seems to be one of the most challenging in current molecular biology, almost certainly due to the complexity of the phenomenon. To put it emphatically, "we animals are the most complicated things in the known universe" (Dawkins, 1986). It is expected that the cooperation among palaeontology, evolutionary biology, and molecular biology facilitates a better understanding of the genetic mechanisms for this complex phenomenon, based on the dynamics of molecular evolution, phylogenetic history, and the history of life on Earth.

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Appendix I Morphometric data of the cancellothyridoid samples

Abbreviations:- USNM = Natural History of the United States National Museum, Washington, D.C., L = shell length (in mm); W = shell width (in mm), H = shell height (in mm), Ribs = number of ribs, fL = foramen length (in mm), fW = foramen width (in mm), Wt = shell weight (in g).

Key to species names:- Chl = *Chlidonophora incerta*, Cni = *Cnismatocentrum sakhalinensis*, Can = *Cancellothyris australis*, T.cro = *Terebratulina crossei*, T.kii = *T. kiiensis*, T.hat = *T. hataiana*; T.pac = *T. pacifica*, T.cal = *T. callinome*, T.pho = *T. photina*, T.lat = *T. latifrons*, T.cai = *T. cailleti*, T.ree = *T. reevei*; T.cav = *T. cavata*; T.mer = *T. meridionalis*; T.aby = *T. abyssicola*, T.ung = *T. unguicula*, T.rot = *T. unguicula rotundata*; T.val = *T. valdiviae*; T.com = *T. compressa*, T.ret = *T. retusa*, T.ema = *T. retusa emarginata*, T.sep = *T. septentrionalis*, T.jap = *T. japonica*; T.pec = *T. peculiaris*; T.haw = *T. hawaiensis*; T. sp. sawane = undescribed *Terebratulina* species from the Sawane Formation (cf. **Chapters 7** and **8**).

For details of measurement see **section 3.2.11**. For details of each sample, see **Tables 3.2** and **3.3**. For results of morphometric analysis, see **Chapters 6** and **8**.

species	USNM	L	W	H	Ribs	fL	fW	Wt
Chl	363295	9	8.8	4.55	11	0.9	0.9	0.1471
Chl	363295	9.15	9.45	4.4	11	1	0.9	0.1275
Chl	363295	9.7	9.5	4.2	11	0.9	0.8	0.1467
Chl	363295	9.7	9.8	4.2	11	0.9	0.9	0.1124
Chl	363295	9.2	9.25	4	12	0.8	0.9	0.1127
Chl	363295	9.25	9	4.3	10	0.9	0.9	0.1135
Chl	363295	9.15	9.15	4.25	12	0.9	0.9	0.1429
Chl	363295	8.25	8.15	3.7	12	0.8	0.7	0.1025
Chl	363295	9.2	8.8	4.05	10	0.7	0.7	0.1159
Chl	363295	9.2	8.4	4.1	12	0.8	0.8	0.1225
Chl	363295	9.35	9.2	4.4	10	0.9	0.8	0.1357
Chl	363295	9.25	9.4	4.5	13	0.8	0.7	0.1443
Cni	110788	40.5	34.9	20.1	0	3.6	3.4	3.774
Cni	222598	35.05	30.6	17.7	0	3.4	3.3	2.7166
Cni	222598	34.1	30.2	18.8	0	3	3.1	2.8088
Can	110834	25.5	18.2	15.4	20	2.5	2.6	1.2291
Can	332788	31.2	17.60	18.7	19	2.9	2.9	2.0291
Can	318179	19.65	13.25	8.9	20	1.7	1.8	0.2506
Can	318179	22.9	13.95	9.8	21	1.5	1.7	0.2587
Can	318179	25	16.15	11.65	17	2	2.4	0.5747
Can	318179	24.45	16.8	11.6	16	2	2	0.5675
Can	318179	27.85	17.25	13.8	23	1.8	2.1	0.6451
Can	318179	27	19.05	16.2	21	1.8	2	0.9561
Can	318179	29.55	18.6	16.15	20	1.8	2.1	0.992
Can	318179	29.95	19.4	15.35	23	2.1	2.3	0.825
T.cro	342218	35.65	28.45	17.25	14	3	3	1.4862
T.cro	342217	49.2	33.25	23.2	12	4	4	3.9333
T.cro	110833	31.2	24.95	13.6	14	2.6	2.5	1.1
T.kii	385104	41	33.7	19.6	9	3.8	3.9	2.5847
T.kii	550692	23.8	22	10.9	8	2.2	2.3	0.8749
T.kii	385104	40.3	32.2	20.65	11	4.1	4.4	2.5986
T.kii	334758	39.95	36.35	23.3	9	3.5	3.5	2.493
T.kii	549413	44.65	39.3	20.5	10	3.5	3.5	3.4276
T.kii	123154	23.3	21.6	9.15	7	2.1	2	0.4693
T.kii	123154	33.3	30.95	14.85	8	2.4	2.5	1.3999
T.kii	123154	33.75	29.6	15.9	9	2.3	2.5	1.4961
T.kii	123154	38.2	37	18.1	10	2.7	2.6	2.1358
T.hat	254533	44.85	32.9	22.7	13	3.2	3.1	2.3687
T.pac	342216	15.8	11.4	7.5	13	1.4	1.3	0.1926
T.pac	342216	17.65	12.7	8	11	1.8	1.8	0.2395
T.pac	342216	20.95	16.5	9.6	9	1.8	2	0.3969
T.pac	342216	21.4	14.5	9.4	14	1.8	1.6	0.3584
T.pac	342216	22	15.8	10.3	11	2	2	0.4209
T.pac	342216	25.55	16.4	11.9	12	2	1.7	0.6011
T.pac	342216	26.75	19.2	13.65	14	2.2	2.2	0.724
T.pac	342216	28.7	20.3	16.3	10	2.2	2.2	0.9249
T.pac	342216	27.5	18.8	14	9	2.3	2.5	0.7732
T.cal	238880	12.8	9.9	4.75	12	1.1	1.1	0.0781
T.cal	238880	14.5	11.85	5.7	12	1.1	1.1	0.1544
T.cal	238880	21.2	16.15	9.75	10	1.6	1.6	0.3752
T.cal	238880	25.8	20.05	12.05	11	1.7	1.7	0.5868
T.cal	238880	24.95	18.45	10.2	12	1.8	1.8	0.4141
T.cal	238880	24.8	19.75	11.45	12	1.9	2	0.4428

species	USNM	L	W	H	Ribs	fL	fW	Wt
T.cal	238880	26.4	20.3	12.2	11	1.9	1.9	0.5995
T.cal	238880	26.95	21.85	12.65	10	2	2	0.657
T.cal	238880	27.1	20.7	13.5	10	2.1	1.8	0.6715
T.cal	238880	26.15	22.5	11.75	11	2	1.9	0.5643
T.cal	238880	28.1	23.45	14	11	2.1	2.1	0.6751
T.cal	238880	29.35	22.2	15.35	11	2.1	2.2	0.7807
T.cal	238880	32.05	24.35	15.1	11	2	2.1	0.9315
T.pho	254536	15.4	13.2	6.3	0	1.1	1.2	0.1685
T.lat	Or 5955	12.6	10.3	5.4	13	1.1	1	0.1369
T.lat	549754	7.85	6.3	3.75	10	0.8	0.7	0.0531
T.lat	p769	10.45	9	4.95	12	1.1	1.2	0.1703
T.lat	g986	8.65	6.9	3.45	14	0.8	0.7	0.05
T.lat	314855	10.1	8.6	4.4	12	1	1	0.0828
T.cai	550756	6.45	4.55	3	8	0.7	0.7	0.0309
T.cai	550756	7.1	5	3.15	9	0.7	0.7	0.0419
T.cai	550756	7.35	5.9	3.7	10	0.7	0.8	0.0591
T.cai	550756	8.3	5.85	3.9	8	0.8	0.9	0.0643
T.cai	550756	8.4	5.5	4.25	7	0.8	0.8	0.0784
T.cai	550756	8.85	7.1	4.25	9	0.9	0.9	0.0909
T.cai	550756	8.9	7	3.95	8	0.9	1	0.0827
T.cai	550756	11.4	8.3	5.25	9	1	1.1	0.1658
T.cai	Or 4570	9.5	7.5	3.8	10	1	1.1	0.086
T.cai	A	9.95	7.7	4.75	10	0.9	0.9	0.1113
T.cai	A	9.8	7.45	4.45	8	0.9	0.9	0.0992
T.cai	551210	10.75	8.5	5.5	7	1.1	1.1	0.1641
T.cai	314848	9.05	7.4	3.95	10	0.7	0.7	0.0605
T.ree	246330	4.95	3.85	2.25	9	0.7	0.7	0.0169
T.ree	246330	6.4	5.75	3.1	9	0.8	0.8	0.0463
T.ree	334779	7.05	5.1	3.15	11	1	0.9	0.0349
T.ree	298312	13.6	10.8	7	10	1.5	1.4	0.3224
T.ree	298312	6.95	5.65	3.05	9	0.8	0.8	0.04
T.ree	298312	8.65	6.55	4.3	9	0.8	0.7	0.0976
T.ree	298312	7.9	6.7	3.45	10	0.9	0.8	0.052
T.ree	298312	8.05	7.2	4	8	0.9	0.9	0.0798
T.ree	298312	9	6.9	4.2	11	0.9	0.9	0.0786
T.ree	298312	8.45	7.35	3.75	10	1	1	0.0798
T.ree	298312	8.75	7.75	4.1	11	1.1	1	0.0829
T.ree	298312	8.9	7.65	4.2	12	1	1	0.126
T.ree	298312	9.85	7.75	4.4	8	1	1	0.1144
T.ree	298312	10.25	8.4	4.4	10	1	1.1	0.111
T.cav	214308	11.35	8.3	4.5	11	0.9	0.9	0.0542
T.mer	372702	13.6	11.55	5.4	16	1.3	1.3	0.1033
T.mer	372702	14.9	13	6.75	14	1.3	1.3	0.1358
T.aby	Cr.7.37217	7	6.8	3.4	9	0.7	0.8	0.0327
T.aby	187142	9.7	7	4.7	11	1.2	1.3	0.0921
T.aby	284193	12.05	9.3	6.45	15	1.8	1.7	0.1831
T.aby	110841	12.5	11.75	6.1	10	1.8	1.7	0.2767
T.aby	110841	15	12.95	7.75	10	1.7	1.7	0.2735
T.aby	127017	11.7	8.5	5.5	13	1.5	1.4	0.1264
T.aby	127017	12.1	9.6	5.55	13	1.7	1.7	0.1682
T.aby	127017	13.8	11.2	6.4	12	1.5	1.3	0.1979
T.aby	127017	16	10.8	7.6	10	1.7	1.8	0.2959
T.aby	127017	15.35	12.1	8.4	11	1.9	2.1	0.3770

species	USNM	L	W	H	Ribs	fL	fW	Wt
T.ung	222202	19.8	17.8	8.1	12	2.2	1.8	0.5837
T.ung	222202	21.3	18.35	8.05	11	2	2	0.4328
T.ung	222202	22	18.4	9.1	10	2.2	1.8	0.6082
T.ung	222202	23.15	19.2	10	11	2	1.8	0.6654
T.ung	222202	24	19.55	9.15	9	2	2	0.6359
T.ung	110897	15.65	12.7	6.9	9	1.7	1.5	0.2718
T.ung	110897	18.15	15	7.6	13	2	1.8	0.4238
T.ung	110897	19.4	15.35	9.15	9	2.1	2.1	0.4987
T.ung	110897	19.95	17.1	8.7	12	2.4	2.5	0.6751
T.ung	110897	20.25	17.05	9.35	11	2.2	2.2	0.6673
T.ung	110897	23.25	18.15	10.2	10	2.5	2.5	0.8478
T.ung	110897	25.2	18.65	12.45	12	3	2.7	1.1992
T.ung	110897	24.05	19.8	10.55	11	2.5	2.4	1.2453
T.ung	3841178	15.6	12.4	6.7	13	1.6	1.6	0.309
T.ung	3841178	16.95	13.7	7.65	12	1.8	1.8	0.4427
T.ung	3841178	18.45	13.95	9.1	11	2	2	0.6348
T.ung	3841178	18.4	14.55	8.2	12	1.9	1.7	0.4897
T.ung	3841178	18.1	14.15	9.35	11	1.9	1.8	0.5568
T.ung	3841178	18.7	13.8	8.4	13	1.9	1.9	0.4206
T.ung	3841178	20.5	13.45	9.5	12	2.1	2	0.5456
T.ung	3841178	18.55	15.45	9.25	13	2	2	0.6061
T.ung	3841178	19.3	17.35	9.3	14	2.2	2.1	0.6739
T.ung	3841178	19.5	15.4	10.2	12	2.2	2.2	0.7869
T.ung	3841178	21.5	16	10	12	2	2	0.6973
T.ung	3841178	23.8	19	13.15	10	2.5	2.3	1.451
T.rot	110823	15.5	13.5	6.1	8	1.4	1.4	0.235
T.rot	110823	16.55	14	6.8	8	1.6	1.5	0.2834
T.rot	110823	18.15	15.6	7.65	7	2	1.8	0.39
T.rot	110823	17.4	16.15	7.9	10	1.7	1.7	0.3337
T.rot	110823	18.95	17.8	7.4	9	1.9	1.7	0.3878
T.rot	204672	20	19.15	9.9	8	1.7	1.7	0.6768
T.rot	204672	18.45	17.6	7.5	9	1.7	1.5	0.3568
T.rot	204672	18.25	18.45	9.4	10	1.8	1.8	0.5783
T.rot	204672	19	18.6	8.8	10	1.9	1.7	0.4635
T.rot	110823a	20.2	19.5	9.4	10	1.8	1.7	0.6245
T.val	238829	9.25	7.15	3.15	12	1	0.9	0.0492
T.val	238829	11.6	8.55	3.7	12	1.2	1.1	0.085
T.val	238829	12.35	9.9	4.1	11	1.1	1	0.132
T.val	238829	11.9	10	4.3	12	1.1	1.1	0.1328
T.val	238829	14.35	11.85	5	11	1.2	1.3	0.1946
T.val	238829	18.4	14.9	7.05	11	2	1.9	0.4426
T.val	238829	18	16.5	5.9	11	2	1.9	0.4207
T.val	238829	19.4	16.2	7.45	13	2	1.8	0.5308
T.val	110843	16.3	14.75	7.1	11	1.4	1.2	0.3929
T.val	110437	14.75	13.2	5.8	12	1.7	1.4	0.2524
T.val	110437	15.65	13	5.85	10	1.6	1.5	0.2615
T.com	110844	20.9	18.3	6.2	12	2.3	2.2	0.5545
T.ret	173564	15	12.9	7.4	8	1.6	1.5	0.2762
T.ret	173564	15.55	13.2	6.85	10	1.4	1.4	0.3083
T.ret	173564	17.3	14.3	7.3	12	1.5	1.3	0.3132
T.ret	173564	17	14.6	7.7	9	1.3	1.3	0.37
T.ret	173564	16.75	14.15	7	11	1.4	1.4	0.3258
T.ret	173564	18.45	16.45	8.3	8	1.6	1.5	0.4507

species	USNM	L	W	H	Ribs	fL	fW	Wt
T.ret	173564	18.8	14.95	8.65	9	1.5	1.4	0.5199
T.ret	173564	18.5	15.3	8.65	11	1.4	1.3	0.5598
T.ret	173564	21.9	19.3	10.05	8	1.7	1.7	0.6563
T.ret	173564	21.85	18.3	10.4	8	1.7	1.7	0.7173
T.ret	173564	23	19.6	13.15	8	2	1.9	0.9821
T.ret	173522	14.55	12.45	6.4	12	1.4	1.3	0.321
T.ret	173522	15.15	12.35	7.3	14	1.6	1.6	0.358
T.ret	173522	18.25	14.7	8.35	11	1.6	1.6	0.6117
T.ret	173522	12.4	10.95	5.85	11	1.3	1.3	0.2432
T.ret	173522	20.85	17.55	11.2	12	2.3	2.2	0.901
T.ret	173522	16.65	14.3	7.7	13	1.7	1.7	0.4442
T.ret	173522	18.55	15	9.1	12	1.8	1.7	0.6977
T.ret	173522	16.15	13.95	6.95	12	1.6	1.6	0.414
T.ret	173522	18.05	14.7	9.3	12	1.7	1.7	0.5597
T.ret	173522	20.45	17.1	10.45	13	1.8	1.8	0.6727
T.ret	173522	13.4	12	6.4	10	1.5	1.5	0.3182
T.ret	173522	20.45	16.8	10.1	11	1.8	1.9	0.7885
T.ret	173579	15.65	12.3	6.2	8	1.4	1.4	0.2445
T.ret	173579	14.95	12.4	5.3	9	1.3	1.3	0.1789
T.ret	173579	16.55	11.65	7.3	12	1.3	1.3	0.2569
T.ret	173579	16.7	13	6.1	11	1.3	1.2	0.1873
T.ret	173579	22.6	16.15	10.3	11	2.1	2.1	0.786
T.ema	317025	9.9	7.25	4.2	11	1.2	1.1	0.08
T.ema	Exp 17	6	4.2	2.3	11	0.6	0.6	0.0123
T.sep	110869	16.4	13.8	6.75	12	1.5	1.6	0.2579
T.sep	110869	18.2	14.2	7.85	12	1.7	1.7	0.3246
T.sep	110869	16.5	13.3	8.2	13	2	2	0.2868
T.sep	110869	21.35	17.1	10	13	2.1	2.2	0.5842
T.sep	110869	19.1	13.8	9	13	2	2	0.3728
T.sep	110869	19.2	14.15	8.35	14	2.1	2.1	0.3311
T.sep	110869	19.8	14.8	9.4	13	2.1	2.2	0.6251
T.sep	110869	20.7	17.2	9.55	13	2	2.1	0.6294
T.sep	110869	20.85	16	9.05	13	2	2	0.3935
T.sep	110869	21.15	16.55	10.25	12	2.1	2.2	0.4917
T.sep	110869	21.85	17.55	9.6	15	2.1	2.1	0.5
T.sep	110869	23.55	17.9	10.9	15	2.2	2.2	0.6583
T.sep	203032	14.5	11.9	6.8	15	1.6	1.6	0.2237
T.sep	203032	15.65	11.4	6.2	14	1.5	1.5	0.1838
T.sep	203032	16.4	11.3	6.9	13	1.7	1.9	0.206
T.sep	203032	14.75	13.35	7.45	13	1.5	1.6	0.26
T.sep	203032	16.1	11.65	6.2	15	1.5	1.5	0.1853
T.sep	203032	15.35	13.1	6.45	12	1.5	1.5	0.2161
T.sep	203032	17.9	12.5	7.45	14	1.7	1.9	0.2444
T.sep	203032	16.1	13.9	7.65	13	1.5	1.7	0.3071
T.sep	203032	17.25	13.85	7.8	14	1.8	1.7	0.2605
T.sep	203032	16.55	14.1	7.8	13	1.5	1.8	0.3475
T.sep	303032	18.35	13.6	8.35	15	1.8	1.9	0.3513
T.sep	203032	17.75	14.5	8.1	12	1.7	1.7	0.3319
T.sep	49311	11.1	7.6	4.4	14	1.2	1.1	0.0735
T.sep	49311	16.1	12.1	6.95	15	1.7	1.7	0.2173
T.sep	49311	17	13.2	6.7	13	1.7	1.8	0.2458
T.sep	49311	18.45	13.4	8.75	15	1.9	1.8	0.3287
T.sep	49311	17.1	13.15	7.2	14	1.8	1.8	0.2441

species	USNM	L	W	H	Ribs	fL	fW	Wt
T.sep	49311	18.25	13.25	7.65	13	1.7	1.7	0.2693
T.sep	49311	20.75	16.9	8.4	13	2.2	1.9	0.3812
T.sep	49311	23.3	18.05	10.9	13	2.2	2.4	0.5977
T.sep	472 bt	19.7	14.45	8.2	13	2	2	0.3433
T.sep	472 bt	17.3	14.7	7.3	14	2.1	2.1	0.279
T.sep	472 bt	19.1	14.6	7.75	14	1.8	1.8	0.3124
T.sep	472 bt	18.35	14.2	7.55	13	1.9	1.9	0.3109
T.sep	472 bt	18.3	15.5	7.55	13	2	2.1	0.3255
T.sep	472 bt	21.35	15.4	8.45	14	2.3	2.2	0.3727
T.sep	472 bt	21.6	15.9	8.95	14	2.1	2.2	0.4556
T.sep	472 bt	21.3	16.25	8.15	13	2.1	2.2	0.391
T.sep	472 bt	21.9	16.3	9	14	2.3	2.3	0.5657
T.sep	472 bt	21.4	15.8	9.05	14	2.2	2.3	0.454
T.sep	472 bt	20.95	16.55	8.5	14	2.3	2.1	0.3909
T.sep	472 bt	21.95	17.5	9.4	14	2	2	0.4219
T.sep	266223	14.85	12.3	5.3	14	1.2	1.3	0.1253
T.sep	334751	17.8	13.05	7.4	13	1.8	1.8	0.2842
T.sep	334751	17.95	14.1	8	13	1.8	1.7	0.3688
T.sep	334751	18.5	13.9	7.5	13	1.8	1.8	0.3297
T.sep	334751	19.15	14.55	8.95	14	1.9	2	0.4108
T.sep	334751	19.45	16.5	8.7	14	2	2.2	0.54
T.sep	334751	21.5	17.05	9.15	14	2	2	0.441
T.sep	334751	20.9	17.05	9.4	13	2.1	2.1	0.6394
T.sep	334751	21.2	17.3	8.9	16	2	2.1	0.5517
T.sep	334751	22	17.9	10.2	16	2.1	2.4	0.6324
T.sep	334751	22.8	17.1	10.5	13	2.2	2.2	0.6843
T.sep	334751	26.5	19.25	11.9	15	2.3	2.3	0.9703
T.sep?	173532	17.2	13.95	7.6	12	1.6	1.5	0.4215
T.sep?	173532	19.55	15.65	9.15	8	2	2	0.6637
T.sep?	173532	20.6	17	9.35	9	1.8	1.8	0.7609
T.sep?	173532	21.8	17.3	9.9	9	2	1.9	0.83
T.sep?	173532	22.35	16.85	9.7	11	1.6	1.6	0.7289
T.sep?	173532	22.9	19.2	11.8	10	1.7	1.8	0.9025
T.jap	563646	8.4	6.1	3.85	12	0.9	1	0.0658
T.jap	563646	14.3	10.15	5.5	13	1.8	1.5	0.2037
T.jap	563646	16.7	12.3	7.7	12	1.9	1.7	0.4
T.jap	549344	18.2	13.3	7	13	1.9	1.8	0.3855
T.jap	110821	19	13.8	7.75	14	1.5	1.5	0.3139
T.jap	204687	23.15	16.8	11.65	12	2.2	2.5	0.7454
T.jap	110819	20.4	15.15	9.65	13	1.8	1.8	0.4729
T.jap	110819	12.8	9.55	5.4	13	1.2	1.1	0.1364
T.pec	563640	16.1	12.3	7.25	11	1.7	1.6	0.3685
T.pec	563640	19.05	14.8	8.85	12	2	1.8	0.4861
T.haw	274156	18.95	15.1	7.95	15	1.9	2.1	0.3746

species	L	W	H	Ribs	fL	fW
T.sp sawane	9.85	7.6	3.8	13	1	1
T.sp sawane	10.6	8.4	4.25	12	1.2	1.1
T.sp sawane	10.2	7.85	4.1	12	1.1	1.1
T.sp sawane	10.9	8.9	4.3	13	1.3	1.3
T.sp sawane	11	8.95	3.95	12	1	1.1
T.sp sawane	11.2	9.25	4.55	13	1.3	1.2
T.sp sawane	11	8.8	4.05	13	1	1
T.sp sawane	12	9.6	4.7	13	1.1	1.4
T.sp sawane	12.3	9.9	4.6	13	1.1	1.1
T.sp sawane	12.3	9.9	4.3	12	1.2	1.1
T.sp sawane	12.85	10.25	5.05	11	1.3	1.3
T.sp sawane	13.65	11.05	5.3	12	1.2	1.3
T.sp sawane	13.65	10.7	5.7	11	1.3	1.2
T.sp sawane	14.55	11.8	5.55	12	1.2	1.3
T.sp sawane	15.2	11.8	6.1	13	1.2	1.3

Appendix II Publications

Appendix II-1

Endo, K. and Curry, G. B. (1991) Molecular and morphological taxonomy of a Recent brachiopod genus Terebratulina. *In* D. I. MacKinnon, D. E. Lee and J. D. Campbell (eds.), *Brachiopods through time* (pp. 101-108). Rotterdam: A. A. Balkema.

Appendix II-2

Curry, G. B., Cusack, M., Endo, K., Walton, D. and Quinn, R. (1991a) Intracrystalline molecules from brachiopods. *In* S. Suga and H. Nakahara (eds.), *Mechanisms and phylogeny of mineralization in biological systems* (pp. 35-39). Tokyo: Springer-Verlag.

Appendix II-3

Curry, G. B., Cusack, M., Walton, D., Endo, K., Clegg, H., Abbott, G. and Armstrong, H. (1991b) Biogeochemistry of brachiopod intracrystalline molecules. *Phil. Trans. R. Soc. Lond. B*, **333**, 359-366.

Appendix II-4

Curry, G. B., Quinn, R., Collins, M. J., Endo, K., Ewing, S., Muyzer, G. and Westbroek, P. (1991c) Immunological responses from brachiopod skeletal macromolecules; a new technique for assessing taxonomic relationships using shells. *Lethaia*, **24**, 399-407.

Appendix II-5

Curry, G. B. and Endo, K. (1991) Migration of brachiopod species in the North Atlantic in response to Holocene climatic change. *Geology*, **19**, 1101-1103.

Brachiopods through time, MacKinnon, Lee & Campbell (eds) © 1990 Balkema, Rotterdam. ISBN 90 6191 160 5

Molecular and morphological taxonomy of a Recent brachiopod genus Terebratulina

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ABSTRACT: The taxonomy of twelve species and one subspecies of the widely distributed living brachiopod Terebratulina was investigated immunologically, using both antisera prepared against intraskeletal macromolecules from three species of this genus, as well as antisera similarly prepared against three terebratellacean genera (Dallina, Waltonia, Laqueus). The results of the sero-taxonomy experiments were compared with the known fossil record of Terebratulina and morphometric analyses of 9 shell parameters to generate a preliminary phylogenetic interpretation for this genus based on both morphological and molecular lines of evidence. The results indicate that two sympatric Japanese species, T.japonica and T. peculiaris, are morphometrically indistinguishable and very closely related at the molecular level and hence are best considered as synonymous. The immunological data also reveal two main clusters of Terebratulina species which may reflect a significant evolutionary event within the genus.

1 INTRODUCTION

Immunological techniques offer a rapid and reliable method of assessing the degree of molecular 'relatedness' between taxa. The technique exploits the specific reaction of antibodies against antigenic determinants to which they were elicited. The degree of molecular similarity is measured by the extent of reactivity between antibodies raised against one taxon with homologous antigens from other taxa. Antibodies raised against intraskeletal macromolecules from brachiopods have already proved to be of considerable taxonomic significance, and among Recent representatives can even distinguish between congeneric species (Collins et al, 1988).

Immunological taxonomy using intraskeletal macromolecules is unique among techniques of molecular systematics in that it has been applied to fossil materials. Since the macromolecules are protected by a biomineral, they may resist fossilisation for a considerable time period, while antibodies are elicited by small parts of a macromolecule that they might recognise an antigenic determinant even after the target molecule is partially degraded. Original antigenic materials were detected in Cretaceous belemnites (de Jong et al, 1974; Westbroek et al, 1979). Species specific reactivity has been reported from Pleistocene bivalve shells (Muyzer et al, 1988; see Lowenstein, 1986; Curry, 1987; Muyzer, 1988 for review).

Terebratulina (Cancellothyridacea, Cooper, 1973) is one of the commonest brachiopods living today (Cooper, 1973). It is represented in almost all modern seas, and a total of some 23 living species and 2 subspecies are known. The genus dates back to the Jurassic of Europe and India, and flourished in the Tertiary to become the most prolific genus in the Cenozoic era (Hatai, 1940). More than 480 articles have been published on Terebratulina since the middle of last century (Doescher, pers. comm.). This accumulated knowledge of morphology, distribution in time and space, ecology and palaeoecology provides

an indispensable background to the morphometric and molecular study on this genus.

2 MATERIALS AND METHODS

2.1 Materials

Table 1 indicates the species available for this study. Apart from 12 Terebratulina species and one subspecies, three terebratellacean species (Dallina septigera, Waltonia inconspicula, and Laqueus californicus) were selected as an outgroup, and a bivalve species (Codakia orbicularis) was used as a negative control in the immunological assays. Most samples were dried shells without softparts, but in some cases specimens preserved in 70% ethanol were utilised [see Collins et al (1988) on the effect of shell preservation on specific reactivity]. Antisera against four Terebratulina species and three terebratellacean species were available for immunological assays, while eight Terebratulina populations were available for the morphometric analysis (Table 1).

2.2 Morphometrics

Nine morphological characters were measured on complete specimens of each taxon (Fig.1). Length (L), width (W), and height (H) were measured to an accuracy of 0.1 mm using a dial caliper, while foramen length (fL), foramen width (fW1), and distance between deltidial plates (fW2) were measured to an accuracy of 0.1 mm using a graticule in a binocular microscope. The number of capilla (Ribs) was counted in a width of 4 mm on the dorsal valve at the point 4mm from the dorsal umbo. Shell weight of the brachial valve (bvWt) and the pedicle valve (pvWt) of dried specimens were measured separately to an accuracy of 0.1 gram. Every variable was standardized by subtracting its mean and dividing by its standard deviation prior to multivariate analyses to eliminate scaling problems between variables. Principal component analyses were carried out on an Apple computer using Odesta Corporation software, 'Datadesk Professional'.

2.3 Immunological techniques

The immunological technique utilised is described in Collins et al (1988) with slight modifications. Fibrous materials of shell secondary layer were isolated from thoroughly bleached and washed shells on the basis of differential precipitation rates. Organic material was extracted from the fibres by decalcification in 20% (w/v) ethylene diamine tetraacetate (EDTA) solution (pH 8), using either 20 g of shell fibre in 500 ml of 20% EDTA solution when extracting materials for immunization, or 46 mg of shell in 1 ml of 20% EDTA solution when preparing antigen solution for immunological assays. For immunization, the decalcified material in solution was concentrated and EDTA-calcium complex removed through ultra-filtration (MW cutoff: 10,000), followed by lyophilization. New Zealand white rabbits were immunized using conventional techniques (see Harlow and Lane [1988]).

The reactivity patterns between the resulting antisera and the antigens (crude extracts from various taxa) were measured by a sort of Enzyme linked immunosorbent assay (ELISA) using a fluorescent substrate. A volume of 5 µl of antigen solution in 95 µl of 10 mM Tris buffered saline, pH 7.4 (TBS) was incubated in flat bottom microtitre plates (Dynatech Labs) for 1.5 hours at 37°C using a Dynatech Shaker Incubator. The plates were then rinsed three times with 0.02% Tween 20 in TBS using a Dynatech Microplate Washer. The plates were incubated with 2% gelatin in TBS for 1 hour at 37°C. Antiserum (against brachiopod shell macromolecules) diluted appropriately in 0.2% gelatin in TBS was added and incubated for 1.5 hours at 37°C. The plates were washed five times with TBS/Tween and a goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma, A-8025) was added and incubated for 1.5 hours. The plates were washed five times with TBS/Tween, then substrate (4-methylumbellifenyl phosphate, dilithium salt, Boehringer Mannheim) in buffer was added. The fluorescence was measured automatically using a Dynatech MicroFLUOR plate reader. The assays were conducted in triplicate and the averaged read-

ings of cross-reactivity were expressed as a percentage of the cross-reaction strength between an antiserum and the antigen against which the antiserum was raised. Cluster analyses were carried out on these data to provide a graphical representation of the hierarchical relationships among investigated taxa.

3 RESULTS

3.1 Multivariate morphometrics

Table 2 shows the first four latent vectors for the principal component analysis on eight populations of Terebratulina. The first vector, which accounts for 78% of the total variation, is interpreted as a 'size component' because all elements of the vector, with the exception of the number of ribs, has similar and negative value. As the measured samples do not consist of specimens of a same stage of growth, this size component is not considered taxonomically significant, even though it does distinguish between some populations. The second vector (11% of the total variation) predominantly reflects the variation in the number of ribs. The number of ribs does have importance in discriminating species (for example distinguishing between T.septentrionalis and T. retusa). In general, however, the intra-specific variation is larger than the inter-specific variation, and this vector is not considered further in this paper.

The third and fourth vectors have values of mixed signs, and are therefore probably best considered as 'shape components' (Reyment et al, 1984). These components account for 4% and 3% of the total variations respectively. A scatter diagram using these components demonstrates a good separation of populations (Fig.2). The major contributing character to these two vectors is the height of the shell, and the signs of the components are both positive. The next most significant characters are the foramen size and shell weight, and these two characters have opposite signs in both vectors, although the signs are reversed in each vector (Table 2). Joint consideration of the third and fourth vectors suggests that a diagonal axis from the bottom left to top right in Figure 2 could be regarded as a 'shell height axis', with T.retusa and T.unguicula rotundata as end members (the former is the most convex species investigated, the latter the least convex). A second diagonal axis from the bottom right to top left separates the species into what could be considered as a 'life habit axis'. At one extreme of this axis, T.crossei has a smaller foramen and relatively heavy shell (usually indicative of a free-lying life habit) whereas, T. unguicula at the other end of the axis has a larger foramen and a lighter shell (suggesting a more pedicle-dependant mode of life).

As is clear from Figure 2, T.japonica and T.peculiaris could not be distinguished morphometrically. This was the case in the first, second and other components, a fact which indicates that very little morphological difference exist between these two 'species'.

3.2 Immunological assays

The pattern of immunological reactivity demonstrates overall consistency (Fig.3). Anti-T.retusa and anti-T.septentrionalis sera show a similar pattern, reacting most strongly with T.retusa, T.septentrionalis, T.japonica, and T.peculiaris, reacting to a less extent with T.unguicula and T.rotundata, and being least reactive with non-Terebratulina species. Anti-T.unguicula serum is highly reactive with every brachiopod species examined in this survey, but does not react with the bivalve species. Anti-T.crossei serum reacts most strongly with T.crossei, T.septentrionalis, T.japonica, T.peculiaris, and T.kiiensis, and least with the bivalve. The three sera against terebratellacean species (anti-Dallina, anti-Waltonia, and anti-Laqueus) all display a similar pattern. All react most strongly with themselves, followed in order of decreasing reactivity with (a) T. unguicula and T. unguicula rotundata, (b) T.retusa, T.septentrionalis, T.japonica, T.peculiaris, and T.cailleti. The remaining Terebratulina species show no significant reaction with these anti-terebratellaceans sera. Figure

Table 1. Terebratulina samples used in this investigation.

Species	Locality
T.retusa (Linnaeus) **	Firth of Lorn, Scotland, NE Atlantic
T.septentrionalis (Couthouy)**	Bay of Fundy, Canada, NW Atlantic
T.cailleti Crosse	Venezuela, Caribbean Sea
T.latifrons Dall	Pelican Island, Caribbean Sea
T.kiiensis Dall & Pilsbry	Valparaiso, Chile, SW Pacific
T.unguicula (Carpenter)**	Friday Harbor, USA, NE Pacific
T.unguicula rotundata Cooper*	Otuchi, Japan, NW Pacific
T.japonica (Sowerby)*	Izu Islands, Japan, NW Pacific
T.peculiaris (Hatai)*	Izu Islands, Japan, NW Pacific
T.crossei Davidson**	Otuchi Bay, Japan, NW Pacific
T.pacifica Hatai*	Izu Islands, Japan, NW Pacific
T.reevei Dall	Phillippines, Western Pacific
T.abyssicola Adams & Reeve	South Africa, Indian Ocean

* Samples used for morphometrics
**Samples used both for morphometics and to prepare antibodies

4 shows the hierachical clustering of the reactivity data set. Terebratulina species form a single cluster divided into two major subgroups. Half the number of species investigated in this study are from Western Pacific. The cluster analyses indicate that they are not an homogeneous group, but contain distinct taxa, some of which have obviously been genetically isolated for long periods of time. T.japonica and T.peculiaris are the most closely related pair of species, and indeed even more closely related than T.unguicula and its subspecies.

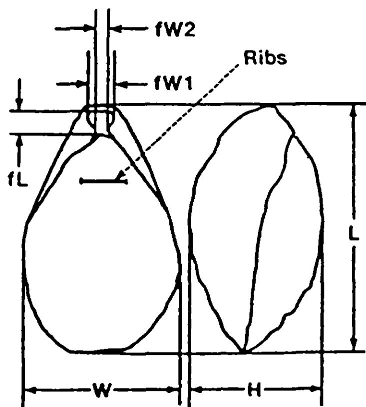

Figure 1. Diagram showing measurements used in principal component analyses. See text for explanation.

4 DISCUSSION

The pattern of immunological reactivity demonstrates a clear distinction between the long-looped brachiopods and the Terebratulina species, confirming the biological validity of this genus. However the combined morphometric-molecular data does throws considerable doubt on the taxonomic validity of one Japanese species. Whereas the majority of Terebratulina species which can be separated by principal component analysis on the basis of subtle but consistent morphological differences, two Japanese species, T.japonica and T. peculiaris, are mophometrically indistinguishable. Although the morphometric study seems to provide more information on ecology rather than taxonomy, it this case the immunological data provide strong supporting evidence for the suggestion that the morphological similarity between T.japonica and T. peculiaris is a phylogenetic rather than a ecophenotypic phenomenon. T.japonica and T. peculiaris are also the most closely related in the sero-taxonomy experiments, being more closely related than is the case between T. unguicula and its subspecies (Fig. 4). It is clear from such results that T.japonica and T. peculiaris are best considered as synonymous species. This conclusion is supported by the fact that they often occur together in Japanese waters (Hatai, 1940). By definition T.

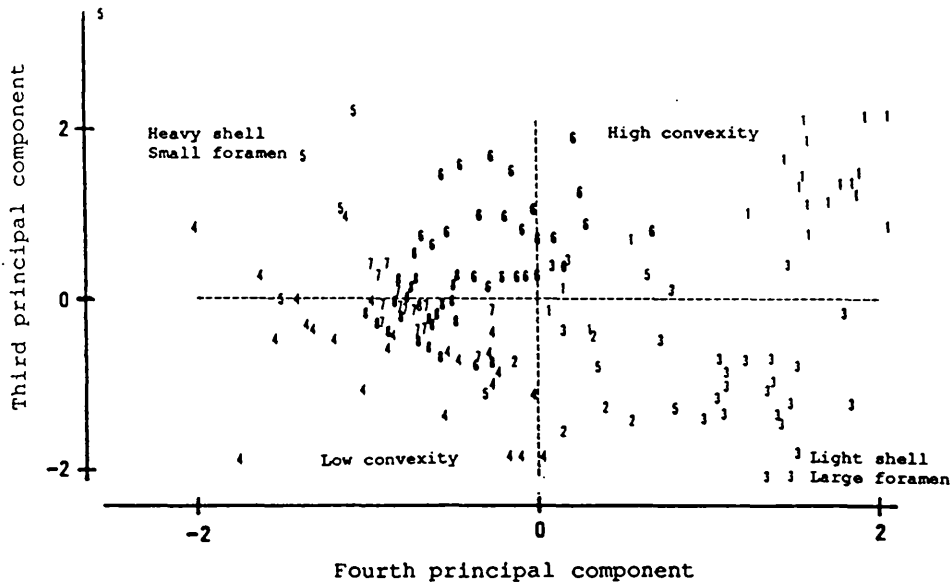

Figure 2. Principal component scores for the third and fourth principal components for the analysis of eight Terebratulina populations. 1, T.retusa; 2, T.septentrionalis; 3, T.unguicula; 4, T.unguicula rotundata; 5, T.crossei; 6, T.pacifica; 7, T.japonica; 8, T.peculiaris.

japonica is distinguished from T. peculiaris by stronger costellae, a pentagonal shell outline, and obsolete concentric growth lines (Hatai, 1940). Close examination of representatives of each species has revealed that an intermediate form exists, and in the light of the immunological and morphometric results such difference fall with the range of intraspecific variation.

The other interesting result of the immunology experiments is the clustering of the Terebratulina species into two well-defined subgroups (Fig 4). The significance of these subgroups is not immediately apparent from this preliminary investigation, but it is clear that it does not reflect geographic separation. Both groups are represented in the N. Pacific region and in the Caribbean area. The species from Japanese and nearby waters appear to be an particularly heterogeneous group in this respect.

The immunological data (Fig. 3) would suggest that one group of Terebratulina species (T. retusa, T. septentrionalis, T. unguicula, T. unguicula rotundata, T. japonica & T. peculiaris, and T. cailleti = RSUJC group) is more closely related

Table 2. First four latent vectors for component analyses of morphologic characters of the shell.

	V1	V2	V3	V4
L	-0.366	-0.008	0.097	-0.231
W	-0.366	-0.061	-0.035	-0.234
H	-0.322	-0.016	0.639	0.523
Ribs	0.112	0.979	0.088	-0.070
fL	-0.352	0.131	-0.229	0.373
fW1	-0.353	0.114	-0.234	0.429
fW2	-0.330	0.063	-0.617	-0.046
bvWt	-0.360	0.047	0.227	-0.388
pvWt	-0.362	0.043	0.182	-0.373

at the molecular level to the terebratellacean brachiopods than the other group (which shows no reaction with terebratellacean antisera).

Immunological techniques represent a relatively crude method of assess the overall degree of molecular similarity between species, and in this case the antibodies have been prepared against unknown shell macromolecules. Nevertheless immunology has proved to be a very successful method of phylogenetic reconstruction, and is interesting to consider the implications of our results.

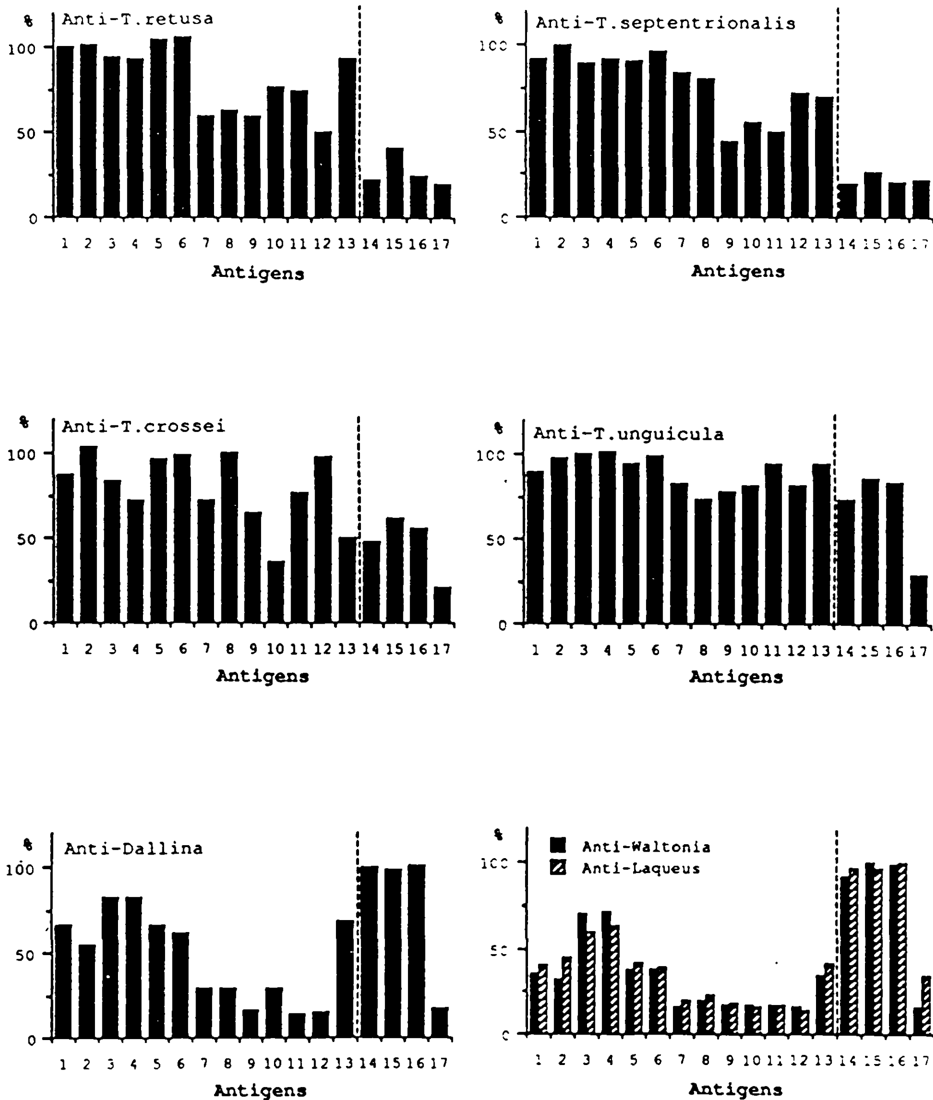

Figure 3. Cross-reactivity levels of seven antisera to seventeen antigens. Average of triplicate experiments. A, anti-T.retusa serum (dilution, 1:40000); B, anti-T.septentrionalis serum (dilution, 1:2000); C, anti-T.unguicula serum (dilution, 1:1000); D, anti-T.crossei serum (dilution, 1:1000); E, anti-Dallina serum (dilution, 1:40000); F, anti-Waltonia serum (dilution, 1:20000) and anti-Laqueus serum (dilution, 1:5000). Vertical axes: percentage of cross-reactivity (strength of cross-reactivity with homologous antigen = 100). Horizontal axes: antigens. 1, T.retusa; 2, T.septentrionalis; 3, T.unguicula; 4, T.unguicula rotundata; 5, T.japonica; 6, T.peculiaris; 7, T.pacifica; 8, T.crossei; 9, T.reevei; 10, T.abyssycola; 11, T.latifrons; 12, T.kiiensis; 13, T.cailleti; 14, Dallina septigera; 15, Waltonia inconspicula; 16, Laqueus californianus; 17, Codakia orbicularis (Bivalvia).

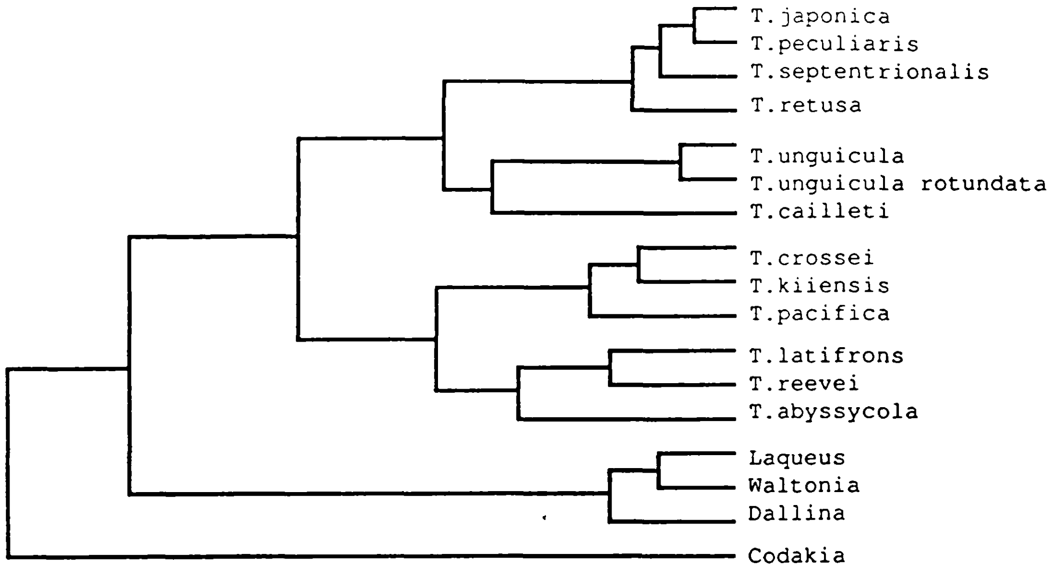

Figure 4. Cluster analysis based on the results shown in Figure 3.

One possible explanation is that the RSUJC species are representatives of an ancestral lineage which retains molecular evidence of the divergence from the terebratellacean lineage. By this interpretation the other group of Terebratulina species could be a more recent offshoot from the main Terebratulina lineage in which the rate of molecular evolution has increased, with the result that the level of molecular similarity with the terebratellaceans falls below that which can be detected immunologically.

An alternative explanation is that the species which do not react with the anti-terebratellacean sera represent the ancestral lineage, while the RSUJC group is a more recent offshoot which retains a higher level of gross molecular similarity with the terebratellaceans. This interpretation would involve a polyphyletic origin for the genus Terebratulina, and convergent molecular and morphological evolution.

Such suggestions are highly speculative, and there are a number of other complications in interpreting patterns of molecular evolution which prompt caution in trying to draw evolutionary conclusions from the preliminary data available. Another approach is to examine the other lines of evidence available.

The morphometric data do not reflect the subdivision of the Terebratulina species into two groups, but there are other qualitative morphological data which do appear to be pertinent. Examination of shell ribbing indicates that in general terms Terebratulina species are either strongly or weakly costellate. Without exception all the RSUJC group have strong ribbing. The other group have weak costellation, with the exception of T. latifrons and T. reevi. These two taxa are, however, unique among Terebratulina species in having a carinate shell and Cooper (1973) has even suggested that they should be classified as Rhyhchonellopsis species, because of their extreme deviation from the 'standard' Terebratulina form. This is a suggestion which immunology could readily investigate if suitable samples became available.

If the pattern of ribbing is indeed significant in terms of the subdivision of Terebratulina species, then the fossil record does tend to support the idea that the RSUJC lineage is ancestral and hence Terebratulina is monophyletic. Three of the earliest species from the Jurassic of Germany [T. silicea

(Quenstedt), T. substriata Schloth and T. quenstedti Suess] have strong ribs, while the weakly-ribbed species first appear in the late Cretaceous [T. floridana (Morton) from N.America, and T. striata (Whalenborg) from Sweden]. The first carinate Terebratulina (T.? palmeri) is known from the Miocene of Cuba (Cooper, 1979).

ACKNOWLEDGEMENTS

We gratefully acknowledge funding from the Systematics Association and NERC (KE), and the Royal Society of London (GBC). We thank R.Quinn, University of Glasgow, Drs G.Muyzer and M.J.Collins, Leiden University, for the preparation of antibodies. We also thank Drs M.J.Collins and G.Muyzer, Leiden University, for valuable discussion and reading the manuscript. Many thanks are due to Drs R.E.Grant and R.A.Doescher, Smithsonian Institution, for providing us with samples and information on literatures.

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CHAPTER 1.6
Intracrystalline Molecules from Brachiopod Shells

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Key words: Brachiopod, intracrystalline, protein primary sequence, amino acid analysis.

INTRODUCTION

Shells are composed of both organic and inorganic constituents. It is believed that the organic compounds have important functions at several stages during the formation of biominerals. In brachiopod shells the disposition of inorganic biominerals and their enclosing organic sheaths have been thoroughly investigated using both scanning and transmission electron microscopy but little is known about the biochemistry of the intracrystalline molecules i.e. those enclosed within the inorganic portion. Such information is crucial for an understanding of biominerals if, as has been suggested, these compounds (i) induce crystal nucleation by providing a surface for precipitation, (ii) form compartments that determine the shape and volume of the biocrystal and (iii) determine the pattern of growth in the mineral phase in what is termed 'matrix mediated mineralisation' [1].

This study presents the first details of the organic intracrystalline components from the shell of the articulate brachiopod, *Neothyris lenticularis* (Deshayes). The shell of *N. lenticularis* is composed of numerous long calcite fibres from which the proteins studied here have been extracted. Although this protocol has disadvantages in that it is very time-consuming and the great proportion of the shell is discarded, the major advantage of such a strategy is that it avoids the possibility of including extraneous molecules both from contaminating organisms, such as bacteria, which may infest the organic sheaths of shell calcite fibres and from human finger tips during collection and preparation.

Partial N-terminal sequence and amino acid analyses of two shell proteins are presented here alongside SDS PAGE and hplc analyses of the intracrystalline molecules of *N. lenticularis*.

MATERIALS AND METHODS

Extraction of Shell Proteins

Shells of living *N. lenticularis* were collected from Stewart Island, New Zealand and killed by dehydration. The shells were cleaned thoroughly and incubated for 2 h at 22°C in an aqueous solution of bleach (5% v/v) to destroy the organic sheath and any possible bacterial contamination. The shells were then powdered in a ceramic pestle and mortar before incubating overnight at 4°C in an aqueous solution of bleach (1% v/v). The bleach was removed by repeated washes with Milli Q™ water followed by centrifugation (8 g.h). The precipitate was washed until no bleach could be detected (typically ten 2 l washes) and then lyophilised and EDTA (20% w/v), pH 11 added in the ratio of 23 ml to 1 g shell. The entire mixture was agitated at 4°C for 72 h or until the inorganic phase had dissolved. Following centrifugation (20 g.h) the supernatant was concentrated and the EDTA removed using the Millipore Minitan™ tangential flow system. The preparation was further concentrated in a minicon static concentrator (Amicon) with a 10 kDa cut-off membrane.

Separation of Proteins by hplc

An aliquot of concentrated shell extract was applied to a reverse-phase Aquapore™ RP-300 narrow bore (2.1 mm diameter x 30 mm length) column in trifluroacetic acid (0.1% v/v) at a flow rate of 0.1 ml / min. After 5 min, a 40 min linear gradient of 0 to 70 % (v/v) acetonitrile, in 0.1% (v/v) trifluroacetic acid, was applied to fractionate the shell proteins. The eluate was monitored at 280 and 214 nm.

Separation of Proteins by SDS PAGE

Small gels (9 cm x 7 cm) of 0.75 mm thickness containing 15% polyacrylamide were prepared according to the method of Schagger and Van Jagow [2]. Glycine, which is used in most SDS PAGE systems is here replaced by tricine. Samples for electrophoresis were heated at 100°C for 4 min in an equal volume of sample buffer containing final concentrations of 0.15 M Tris / HCl, pH 6.8, 0.2 M 2-mercaptoethanol, 0.1% (w/v) SDS, 30% (v/v) glycerol and 0.0002% (w/v) of the tracking dye, bromophenol blue. Molecular weight standards; bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) were included on every gel. Electrophoresis of samples in the small gel system required a constant voltage of 100 V for 2 h. Following electrophoresis, proteins were either fixed in the gel and visualised using Coomassie Brilliant Blue-R or electroblotted onto ProBlott™ membrane (Applied Biosystems).

Electroblotting of Proteins

Following SDS PAGE, the proteins in the gel were transferred to ProBlott™ membrane. The transfer was performed in transfer buffer (10 mM CAPS buffer, pH 11, 10% (v/v) methanol) in a Bio-Rad Trans Blot cell. A constant voltage of 50 V for 0.5 h moved the proteins from the gel towards the membrane. Coomassie Blue staining was used to reveal the protein bands on the ProBlott™ membrane.

Amino-Terminal Sequence Determination and Amino Acid Analysis

Automatic Edman degradation was carried out on the stained bands using a pulsed liquid protein sequencer (Applied Biosystems 477A). Bands were also loaded onto the 420-H amino acid analyser with automatic hydrolysis (Applied Biosystems) to determine the overall amino acid composition. Amino acid analysis of the hplc fractions was employed to identify the major proteins by comparing with the analyses from the homogeneous electroblotted proteins. Stained ProBlott™ membrane with no protein attached and all buffer solutions employed were analysed to determine the background level of amino acids present.

RESULTS AND DISCUSSION

The shell of *N. lenticularis* contains a mixture of proteins of different molecular weight, as determined by SDS PAGE (Figure 1). The major proteins are of molecular weight 10.5, 13 and 47 kDa. An identical copy of the the protein pattern in an SDS gel is obtained on a membrane, such as ProBlott™, using electroblotting. This process, first described by Towbin *et al* in 1979 [3], is ideal for the isolation of single proteins from crude mixtures for N-terminal sequence analysis.

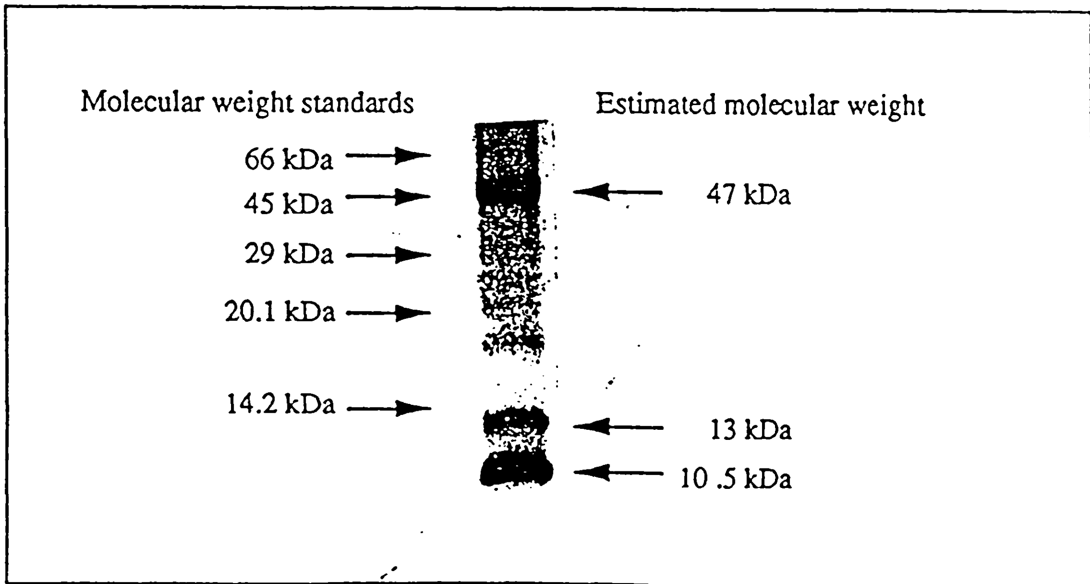

Figure 1 Intracrystalline Proteins of N. lenticularis shell

The proteins were separated by SDS PAGE (see Materials and Methods for details) and then revealed using Coomassie Brilliant Blue staining.

The elution profile of the shell proteins from the hplc system is presented in Figure 2. The light pink colour of Recent *N. lenticularis* becomes deep red as the shell extract is concentrated. The red pigment elutes from the hplc system in the elution volume 42 to 50 ml.(Figure 2). This corresponds with the elution of the 10.5 kDa protein. One possible explanation for the elution of the 10.5 kDa protein as several different peaks is that the molecule may in fact be a glycoprotein and the various peaks represent various degrees of glycosylation. The harsh conditions employed in SDS PAGE causes the protein(s) and pigment to dissociate.

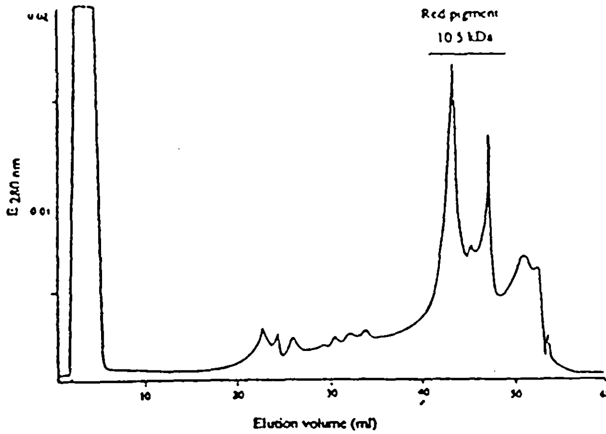

Figure 2 Elution profile of the intracrystalline molecules of N. lenticularis from reverse-phase hplc

An aliquot of the intracrystalline extract was applied to a reverse-phase Aquapore™ RP-300 narrow bore column and a linear gradient of 0 to 70 % (v/v) acetonitrile was applied to fractionate the shell proteins (See Materials and Methods for details).

The amino acid composition of the 10.5 and 47 kDa proteins are listed in Figure 3.

	47 kDa	10.5 kDa
Residue	mole %	
D/N	8.53	10.33
E/Q	8.18	9.06
S	5.57	5.65
G	8.89	11.50
H	1.10	0.76
R	0.10	3.21
T	6.12	6.21
A	13.02	9.21
P	6.47	16.23
Y	4.27	1.58
V	3.97	7.02
C	1.10	0.66
M	1.81	1.58
I	8.13	2.39
L	10.49	10.28
F	3.21	2.29
K	9.04	2.03

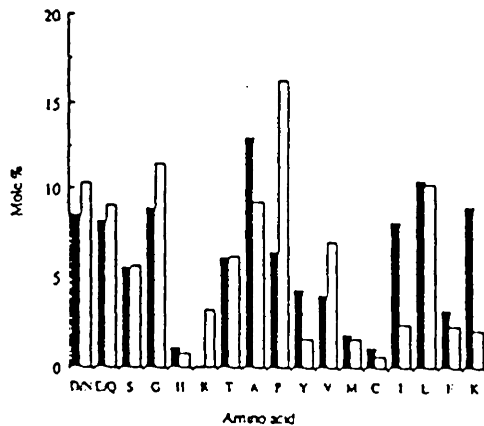

Figure 3 Amino acid composition of two of the major intracrystalline proteins of N. lenticularis shell.

Proteins were extracted and purified to homogeneity using SDS PAGE and electroblotted onto ProBlott™ and the amino acid composition determined using the 420-H amino acid analyser (Applied Biosystems). Values are presented for both the 10.5 (■) and 47 kDa (□) proteins.

Amino acid concentrations are presented as mole percentages to enable direct comparison of the two proteins. Actual yields of intracrystalline amino acids from the bulk mixture extracted from *N. lenticularis* indicate that intracrystalline amino acids occur at about 80 nmole/g shell material.

The conditions employed for the hydrolysis of the peptide bonds also destroys 100 and up to 80% of tryptophan and cysteine residues respectively. However,the hydrolysis reaction is automated and highly reproducible and thus each protein should lose the same proportion of these vulnerable residues allowing direct comparison of the compositions.

The N-terminal amino acid sequence of the 10.5 and 47 kDa proteins are presented in Figure 4.

	1	6	11	16
10.5 kDa	GPEQL	PYATM	ISKTS	NATKP
	1	6		
47 kDa	ANLVL	AGRGD		

***Figure 4** Amino-terminal sequence of 10.5 and 47 kDa protein from Neothyris lenticularis shell.*

Proteins were extracted from N lenticularis and then fractionated by SDS PAGE before being transferred to ProBlott™ for sequence determination (see Materials and Methods).

These protein sequences show no significant similarity to any protein sequence listed in the NBFR or EMBL data base or to any implied peptide sequence in the EMBL or GenBank DNA sequence data base on the basis of searches conducted by the 'FastA' and 'TFastA' programs in the GCG sequence analysis package (Version 6) [4]. Elucidation of the entire primary sequence of these intracrystalline proteins should make sequence comparisons more meaningful and may enhance our understanding of the possible role these proteins play in the process of biomineralisation.

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Biogeochemistry of brachiopod intracrystalline molecules

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SUMMARY

Brachiopods contain both proteins and lipids within the biocrystals of their shell. One intracrystalline chromoprotein causes red shell coloration, whereas the other molecules may be involved with biomineralization, may strengthen the biocrystal, or may simply have been inadvertently engulfed by calcite during shell growth. Evidence is presented which suggests that at least some of the breakdown products of indigeneous intracrystalline molecules can be recovered from the biocrystals of fossil brachiopod shells. Investigations of the remains of these intracrystalline molecules in fossils have geological application in fields such as environmental reconstruction, isotopic determinations, taxonomy and the interpretation of colour in extinct organisms.

1. INTRODUCTION

This paper deals with molecules that occur within the calcite of the brachiopod shell. These intracrystalline molecules are quite distinct from the intercrystalline organic membranes that surround the calcite biocrystals, and also from the 'metabolizing' evaginations of mantle tissue which penetrate many brachiopod shells (the caeca). This is not to say that the molecules recovered from within brachiopod calcite are uniquely distributed within the shell; indeed it is certain that they are synthesized within the secretory outer epithelium, and it is also possible that they have a function within the soft tissues of the organism.

The geological interest in intracrystalline molecules stems from the possibility that *in vivo* incorporation within resistant biocrystals may provide long-term protection from the various physical, chemical and biological factors that would otherwise bring about the destruction of these molecules. The implication is that the inevitable decay of these molecules over geological timespans may not only proceed at much slower rates within the enclosing biocrystal, but that it may occur essentially *in situ*. Intracrystalline molecules may therefore provide unique access to fossilized remains of the original organic constituents of an organism which have not been subjected to significant post-depositional ingress by the abundant organic debris of various sources and ages which occurs within, and migrates through, enclosing sediments. Brachiopod shells are composed of robust low magnesium calcite that remains stable and in its original configuration for hundreds of millions of years (Williams 1984). Intercrystalline molecules situated between, rather than within, brachiopod biocrystals do, by contrast, decay rapidly and are prone to bacterial infection (Collins 1986).

Intracrystalline molecular fossils will undoubtedly be greatly altered over geological time irrespective of the stability of their enclosing biocrystal. Factors that will affect fossilization potential include the range of molecules present inside biocrystals, their location within the shell, their interactions with the inorganic phase, their chemical stability, the environmental conditions (temperature, pressure, etc.), and the extent to which they can react with other intracrystalline molecules. As some of these factors are likely to have changed over geological time, there is no doubt that the breakdown of intracrystalline molecules will be extremely complicated. In this paper evidence for the *in situ* degradation of intracrystalline proteins and lipids in brachiopods is presented from the investigation of living and closely related fossil shells. The ultimate aim of this research is to determine the biological function of these molecules during biomineralization, and the geological applications of their preserved remains in fossils.

2. METHODS

Surface contaminant molecules and remnants of body tissue were removed by thoroughly cleaning the shells and incubating them for 2 h at 22 °C in an aqueous solution of bleach (5% by volume). The shells were then powdered in a ceramic pestle and mortar before incubating overnight at 4 °C in an aqueous

solution of bleach (1% by volume). The bleach was removed by repeated washes with MilliQ water followed by centrifugation (8 g.h). The precipitate was washed until no bleach could be detected (typically ten 2-litre washes) and the precipitate was then lyophilized.

The $CaCO_3$ shell was dissolved using EDTA (200 g l^{-1}) at a ratio of 23 ml to 1 g shell. The entire mixture was agitated at 4 °C for 72 h or until the inorganic phase had dissolved. Following centrifugation (20 kg.h) the supernatant was concentrated, washed, and the EDTA removed using the Minitan tangential flow system for Millipore. The preparation was further concentrated in a Minicon concentrator (Amicon) to obtain sufficient quantities of intracrystalline proteins for biochemical analysis.

EDTA is very difficult to remove, and various investigators have shown spurious spectrophometer readings, non-reproducible gel electrophoresis patterns and other distortions which have been attributed to the incomplete removal of EDTA (Weiner 1984; Benson *et al.* 1986). During our study the success in removing EDTA from preparations was monitored using an amino acid analyser, as EDTA produces a characteristic pattern of peaks on the reverse phase chromatography in this system. The results confirmed that EDTA cannot be entirely removed by dialysis or simple ultrafiltration. However, the Minitan system, which has a much greater filtration area and tangential flow preventing the blocking of filters, allowed the removal of EDTA down to a level undetectable on the amino acid analyser (i.e. less than 10^{-12} M). Typically the final solution represented a 10000-fold concentration. Samples intended for bulk amino acid analysis were prepared by dissolving the shells in 2 M HCl.

Samples for electrophoresis in 15% acrylamide gels were heated at 100 °C for 4 min in an equal volume of sample buffer containing final concentrations of 0.15 M Tris HCl, pH 6.8, 0.2 M 2-mercaptoethanol, 1 g l^{-1} SDS, 30% (by volume) glycerol and 0.002 g l^{-1} of the tracking dye, bromophenol blue. Electrophoresis of samples in the small gel system (9 × 7 cm) required a constant voltage of 100 V for 2 h. Following electrophoresis, a constant voltage of 50 V was applied to transfer the proteins onto ProBlott membrane in CAPS buffer (10 mM, pH 11) with methanol (10% by volume). The membrane was then washed briefly with water and then methanol before staining with Coomassie Brilliant Blue-R in destain (1 g l^{-1}) for 1 min. Background staining was reduced by using an aqueous dilution of methanol (50% by volume). High performance liquid chromatography (HPLC) analysis of intracrystalline molecules was carried out using a WATERS 650 HPLC fitted with a multiwavelength detector. Amino acid analysis was done using an ABI 420H amino acid analyser fitted with an automatic hydrolysis head. Amino acid sequencing was done on the stained protein bands immobilized on ProBlott membrane using a pulsed liquid protein sequencer (Applied Biosystems 477A).

For lipid analysis, the shells were cleaned as above, and then etched in cold dilute HCl until 20% of their mass was removed. The etched shells were further cleaned by repeated washes in dichloromethane, and then powdered in a ceramic pestle and mortar. Initially the quantities of lipids extracted from Recent and fossil brachiopod shells using a 93/7 (by volume) mixture of dichloromethane/methanol were insignificant. Similarly, demineralization of the shell powders did not yield significant levels of lipids, even with repeated extractions with dichloromethane. Successful extraction of intracrystalline lipids was, however, achieved by a modified method in which 2 M methanolic HCl was added once the shell had been dissolved in cold 6 M HCl, and the resulting aqueous mixture refluxed for 24 h. The solution was extracted with dichloromethane and the dichloromethane layer evaporated to dryness. The lipid fraction thus obtained was separated into two fractions using a short column of silica gel, eluted with 50/50 petroleum ether/dichloromethane (2 ml) and methanol (2 ml).

The individual fractions thus collected were evaporated to dryness and analysed via combined gas chromatography–mass spectrometry (GC–MS). Gas chromatography was done using a Carlo Erba Mega series 5360 gas chromatograph fitted with a DB-5 coated, 30 m fused silica capillary column (0.25 μm film thickness and 0.32 mm internal diameter (i.d.)). The column was temperature programmed from 50 to 300 °C at 4 °C per minute with an isothermal temperature of 300 °C for 20 min. Cold on column injection of the sample was used and a detector temperature of 310 °C was maintained. GC–MS analyses were done on a VG TS250 mass spectrometer (electron energy 70 eV; filament current 4000 mA; acceleration voltage 4 kV; source temperature 200 °C) interfaced with a Hewlett Packard 4890 GC fitted with a DB-5 coated, 30 m fused silica capillary column (0.25 μm film thickness and 0.32 mm i.d.).

The possibility of contamination was monitored at all stages of preparation by analysing reagents. In the text and figures the standard three-letter amino acid abbreviations have been used.

3. INTRACRYSTALLINE PROTEINS IN RECENT BRACHIOPODS

Polyacrylamide gel electrophoresis of intracrystalline molecules reveals a variable number of Coomassie-stained bands in different brachiopod groups. For example, *Neothyris* has three main bands with estimated molecular weights of 47 kDa, 16 kDa and 6.5 kDa (figure 1). In contrast, *Terebratulina* contains only a single band of molecular mass 30 kDa. These bands are sharp and readily stained by Coomassie Brilliant Blue, suggesting that these are proteins rather than glycoproteins. This interpretation has been supported by amino acid analysis which revealed no sign of galactosamine or glucosamine.

Brachiopod intracrystalline proteins appear to be very different from the sparse data available from other phyla. Weiner (1983) isolated two proteins of similar molecular mass to the two smaller proteins in *Neothyris* from the calcite component of the bivalve *Mytilus*, but the amino acid analyses are completely different (the *Mytilus* proteins have over 50% Asp and Glu,

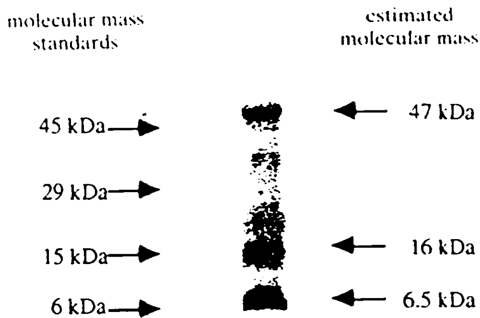

Figure 1. SDS-PAGE of intracrystalline proteins in the Recent brachiopod *Neothyris* from New Zealand.

compared with 20% for the brachiopod). Similarly phosphophryns, the major non-collagenous proteins of dentine, have a distinctive amino acid composition (Veis 1989) unlike that of any brachiopod intracrystalline proteins. In terms of electrophoretic behaviour and amino acid analysis, brachiopod intracrystalline proteins are again different from the glycoproteins extracted from sea urchins, which could not be stained by Coomassie (Benson *et al.* 1986).

The sequence of a 50 kDa sea urchin intracrystalline glycoprotein has been determined by nucleotide sequencing of the coding gene (Benson *et al.* 1987), but the development of a protocol which allowed purification of brachiopod intracrystalline proteins to homogeneity has permitted direct amino acid sequencing (Curry *et al.* 1991). As yet no similarity has been detected between the amino acid sequence of brachiopod intracrystalline proteins and published amino acid or nucleotide sequences (Curry *et al.* 1991).

The function of intracrystalline proteins is poorly understood. It has recently been suggested that they strengthen the shell (Berman *et al.* 1990), although it is also possible that some have been inadvertently incorporated into the shell during growth. As discussed below, the investigation of brachiopod shell proteins has revealed that one is responsible for shell colour. The indications are that there is considerable variation in the nature of intracrystalline proteins both within different groups of brachiopods and between different phyla.

4. INTRACRYSTALLINE AMINO ACIDS IN FOSSIL BRACHIOPODS

There are several lines of evidence that suggest that the remains of indigenous intracrystalline molecules can be recovered from fossil brachiopods. Firstly, amino acid analyses of progressively older fossil representatives of a single genus reveal a pattern of decay that is predictable from the chemical characteristics of individual amino acids. Within the shell of *Waltonia*, for example, relatively unstable amino acids (such as Asp, Glu, Pro and Ser) show a steady decline in relative abundance with increasing age (figure 2*a,c*). Alanine, one of the breakdown products of serine (Hoering 1980), shows a corresponding increase (figure 2*b*). This suggests that the parent proteins have progressively decayed *in situ* and have not been significantly contaminated by the abundant amino acids that occur in the surrounding sediments (Walton & Curry 1991).

Immunological techniques have also shown that original antigenic determinants, including peptides, have survived in these brachiopods, and such techniques offer an important method of extracting original molecular information from Pleistocene shells (see Lowenstein & Scheuenstuhl, this symposium). The immunological approach has proved less useful with older fossils, possibly due to interference from other intracrystalline compounds, and possibly EDTA, as well as the decay of determinants on the target molecules. However, amino acid analysis has suggested that intracrystalline molecules remain uncontaminated for much greater periods of geological time. For example, 27-million-year-old (Ma) specimens of *Terebratulina* have a similar amino acid composition to that of the single protein found in Recent shells of this genus. The major differences, the increase in Gly and Ala (both products of the decay of Ser), and decrease of Asp, Ser, Thr and Pro, are again predictable consequences of the known chemical properties of these amino acids (figure 3). The absolute abundance of soluble amino acids in 27 Ma shells of *Terebratulina* has dropped to about 50% of that in Recent shells. Some amino acids decay completely, but the most significant reduction is probably caused by incorporation of other amino acids into insoluble molecular agglutinations (melanoidins of Hoering 1980) which increase in abundance in fossils and are excluded from amino acid analysis of the soluble fraction.

The trends apparent from the investigation of *Waltonia* shells (figure 2) indicate that an amino acid such as Asp has decayed from 4% of total amino acids in Recent shells to about 2% in 2 Ma shells and to 1% in 2.5 Ma shells, and by extrapolation would be expected to disappear entirely in shells older than about 5 or 6 Ma. However, only a small decrease in Asp (and other amino acids) is apparent when comparing Recent and 27 Ma *Terebratulina* (in which Asp declines from 6% of the total to 1% – figure 3). This inconsistency may in part be explained by the different number and composition of the intracrystalline proteins present in these two genera, but may also reflect variations in the extent to which these proteins are intimately associated with the calcite matrix. Adsorption of amino acids such as Asp to calcium carbonate may increase their survival potential and retard their incorporation into the insoluble high molecular mass fraction. As more data become available it may be possible to reconcile these two results by investigating the stability of amino acids in a single taxon over much longer periods of time.

A third line of evidence for the *in situ* decay comes from principal component analyses (PCA) of amino acid analyses of intracrystalline proteins. In Recent shells, differences in homologous amino acid sequences, and the variation in the number and possibly also the proportions of proteins present, have produced differences in amino acid composition which can be revealed

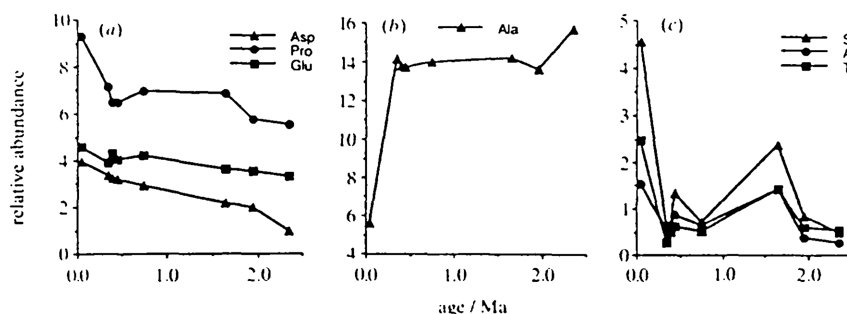

Figure 2. Relative abundance of intracrystalline amino acids in Recent and fossil shells of *Waltonia* from New Zealand.

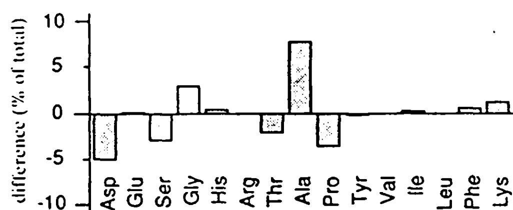

Figure 3. Comparison of the intracrystalline amino acid compositions of Recent *Terebratulina* and 27 Ma fossil shells of the same genus from New Zealand. The data for each analysis have been expressed in mole percentages, and the vertical axis indicates the difference detected in relative abundance for each amino acid between the fossil and Recent shells.

by PCA (figure 4). For example, four New Zealand brachiopod genera are readily distinguished on the basis of amino acid composition of their shells (figure 4*a*). The raw data from amino acid analyses are difficult to compare, and PCA not only has the advantage of presenting a clear graphical summary of the dispersion of the data but the eigenvector values provide a clear indication of which amino acids are important in distinguishing between different taxa (Walton & Curry 1991). The same four genera were collected as fossils from within a single horizon of the Shakespeare Group in New Zealand (approximately 600000 years before present (BP)), and were again distinguished by PCA analysis of their intracrystalline amino acid composition (figure 4*b*). The shells of the four genera have not homogenized in the fossil record, as would be the case if they had absorbed amino acids from the surrounding sediments. Amino acids are abundant in sediments, but in all cases investigated their compositions are readily distinguished from brachiopod shell amino acids when subject to PCA (Walton & Curry 1991). The differences detected by PCA most probably reflect back to the original compositional variability detected in their living representatives. Although shell amino acid composition may be affected by environmental conditions during the life of the organism, in this case these effects have been identical because the specimens lived together and certainly experienced identical diagenetic histories.

5. LIPIDS IN RECENT BRACHIOPODS

GC and GC–MS analysis of intracrystalline molecules from Recent brachiopod shell extracts reveals the presence of an homologous series of saturated acyclic

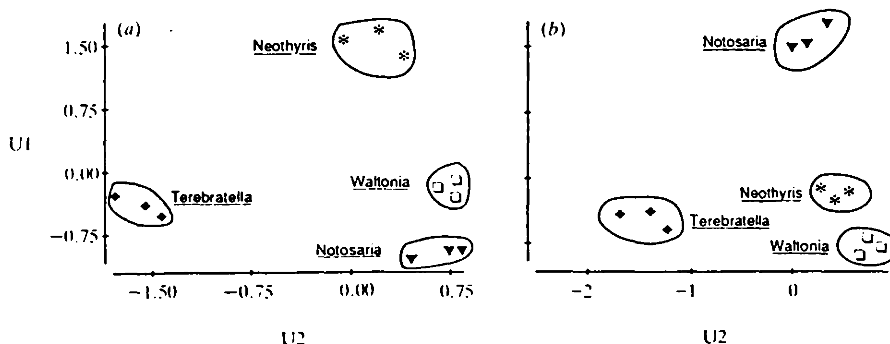

Figure 4. Plot of first (U1) and second (U2) principal component axes of amino acids in the shells showing the differentiation (*a*) between 4 Recent brachiopod genera and (*b*) between fossil representatives of the same taxa, collected from the Shakespeare Group, near Wanganui, N. Island, New Zealand. In (*a*) Gly has a high positive scoring on the first principal component axis, and Ser, Ala, Ile, Leu and Lys have high negative scores, whereas for the second principal component axis, Asp and Glu have high positive scores and His, Arg, Thr and Pro have high negative scores. In (*b*) Thr has a high positive scoring on the first principal component axis, and Gly, Arg, Valine and Leucine have high negative scores, whereas for the second principal component axis, Asp and Glu have high positive scores and Pro, Ile and Ala have high negative scores.

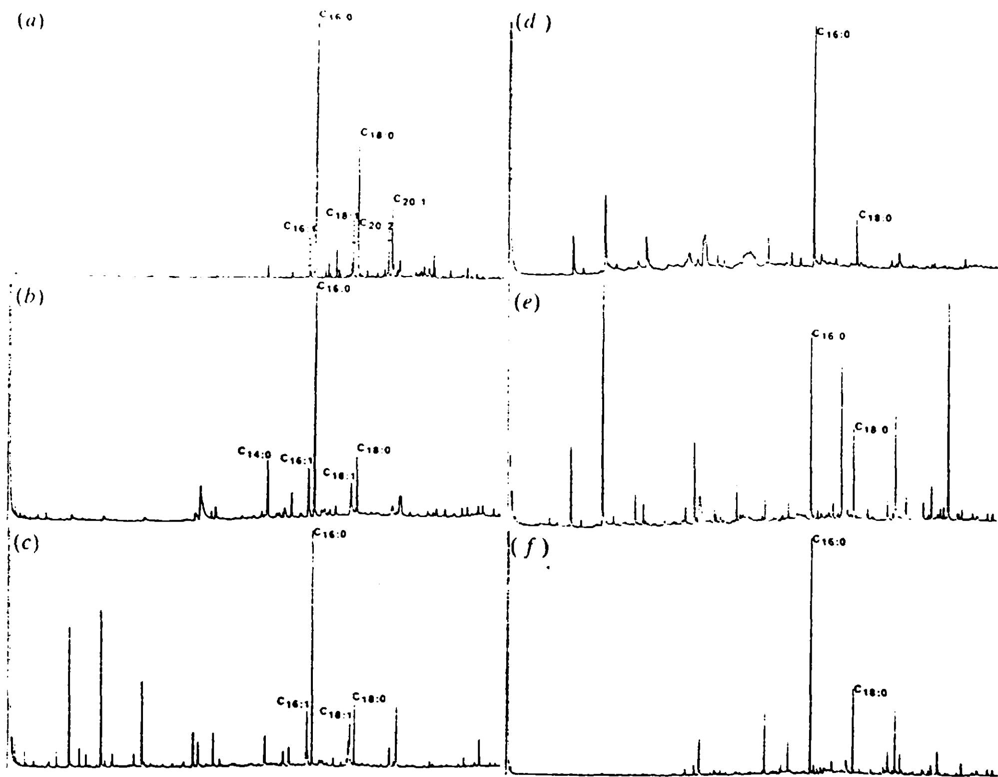

Figure 5. Gas chromatograms of petroleum ether:dichloromethane (50:50 by volume). Carbon numbers of the *n* fatty acids are indicated. (*a*), Recent *Neothyris*; (*b*), Shakespeare Group *Neothyris*; (*c*), Tewkesbury Fm *Neothyris*; (*d*) Castlepoint Fm *Neothyris*; (*e*), Gee Greensand Fm *Pachymagas*, (*f*), McDonald Limestone Fm *Liothyrella*. In (*e*) the major peak on right of chromatogram (retention time = 51 min) has been assigned to contamination.

carboxylic acids (derivatized as the methyl ester) as well as a number of unsaturated components. The dominant lipids detected in *Neothyris* in order of decreasing abundance are: n-$C_{16:0}$, n-$C_{18:0}$, n-$C_{20:1}$, n-$C_{17:0}$, n-$C_{18:1}$, n-$C_{20:2}$, fatty acids (figure 5*a*).

6. LIPIDS IN FOSSIL BRACHIOPODS

A range of *Neothyris* fossil extracts as analysed using the method described above. *Neothyris* shells from the Shakespeare Group (600000 years BP) contained a range of saturated and unsaturated carboxylic acids, namely n-$C_{16:0}$, n-$C_{18:0}$, n-$C_{14:0}$, n-$C_{16:1}$, n-$C_{18:1}$ in order of decreasing abundance (figure 5*b*). With increasing age, low molecular mass lipids begin to increase in relative abundance to the above acids, but as yet their assignments are equivocal. *Neothyris* from the Tewkesbury Formation (Fm) (1.6 Ma) contained n-$C_{18:0}$, n-$C_{18:0}$, n-$C_{16:1}$, n-$C_{18:1}$ fatty acids (figure 5*c*), whereas the same genus from the older Castlepoint Fm (1.85 Ma) contained n-$C_{16:0}$ and n-$C_{18:0}$, although there is a notable absence of unsaturated fatty acids (figure 5*d*).

Analyses of older fossil brachiopods of a different genus (*Pachymagas*) from the Gee Greensand Fm (South Island, New Zealand; 23 Ma) revealed n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids (figure 5*e*), and these same compounds were also recovered from the shells of *Liothyrella* from the McDonald Limestone Fm (30 Ma). These fossil extracts did not yield unsaturated fatty acids (figure 5*f*).

The presence of lipids within brachiopod shells is well known (Jope 1965; Stoyanova 1984) but until now there has been no attempt to distinguish between intercrystalline and intracrystalline lipids. The distinction is important as the intercrystalline lipids are more prone to post-depositional ingress. In this study intercrystalline lipids have been detected in extractions of the shell powders, and these are quite distinct from the intracrystalline lipids released from the subsequent demineralization of the residues. These results have therefore shown for the first time the presence of lipids within brachiopod biocrystals.

All Recent and fossil brachiopod shells examined contained normal saturated carboxylic acids, with $C_{16:0}$ (palmitic) and $C_{18:0}$ (stearic) fatty acids the dominant compounds present. With increasing age there is a decrease in the levels of lipids extracted from the shells, and a decrease in the levels of unsaturated fatty acids. In the samples studied, the unsaturated carboxylic acids disappear in shells older than approximately 2 Ma. Low molecular mass compounds increased in relative abundance with increasing age, with the exception of *Liothyrella* from the McDonald Limestone Fm (figure 5*b*–*f*): these compounds are not present in the shells of Recent brachiopods.

The function of these intracrystalline lipids is poorly understood, although these compounds may be responsible for establishing some of the essential conditions for calcification, as has been suggested for the lipid fraction of ostrich egg shells (Kriesten *et al.* 1979). The distribution and abundance of intracrystalline lipids may well be amenable to the kind of multivariate analysis applied to amino acids, and could augment the molecular discrimination between Recent and fossil taxa.

7. APPLICATIONS OF INTRACRYSTALLINE MOLECULES

The demonstration that brachiopod intracrystalline molecules do not appear to be contaminated by extraneous amino acids or lipids over time suggests several geological applications: their taxonomic uses have been discussed above and in immunological papers (Lowenstein & Schenenstahl, this symposium) and will not be expanded here. The short amino acid sequences recoverable from fossils have little geological application, but extensive research has already highlighted important applications for amino acids in absolute dating, stratigraphic correlation (including correlation from land to sea (Bowen *et al.* 1989)), environmental reconstruction, and nitrogen and carbon stable isotope determinations to investigate the diets of ancient organisms. It has been suggested that racemization studies and stable isotope ratios from individual amino acid enantiomers will also allow the indigenity of the amino acid to be demonstrated (Bada, this symposium; Engel & Macko 1986), especially from biominerals (such as the apatite in bone) which has a more porous structure than brachiopod calcite.

Previous isotopic determination from shells must inevitably have included carbon and nitrogen from molecules as well as from calcite, but as the absolute quantities of molecules involved is probably of the order of 1% of shell mass (amino acids alone account for about 0.04% of shell mass in *Terebratulina*, not including the substantial quantities of lipids, carbohydrates and other molecules present) the difference is likely to be comparatively insignificant and within the range of experimental error. However, if there was any preferential release of carbon and nitrogen from intracrystalline molecules during the preparation of shells for isotopic investigation then the significance of these molecules would increase.

There are indications that the decay rates of proteins in brachiopod shells provide useful environmental information. For example, several relatively unstable amino acids appear to be much better preserved in the *Waltonia* specimens from the Tewkesbury Fm than in most other Plio-Pleistocene horizons investigated (i.e. the peak at approximately 1.6 Ma in figure 2*c*). The Tewkesbury Fm represents a shallow marine environment, as compared with the fully marine faunas of the other formations investigated (Fleming 1953). The improved preservation of serine and other usually fragile amino acids in the Tewkesbury Fm may reflect superior preservation conditions or a relation between environmental conditions and the types of amino acids incorporated. These possibilities are being investigated given the widespread distribution of Recent brachiopods and their fossil ancestors in New Zealand ecosystems and Plio-Pleistocene successions. Other applications may appear once the function of intracrystalline molecules is revealed; for example, if any were toxic they would discourage predation.

8. SHELL COLOUR

During the biochemical investigation of Recent brachiopod intracrystalline molecules, it became clear that red shells retained their colour throughout the preparation protocol which stripped off all intercrystalline molecules. The solution produced by dissolving the calcium carbonate of the shell was also red, suggesting that shell coloration is caused by a soluble intracrystalline molecule. Reverse-phase HPLC of these samples allowed the isolation of the coloured fractions, which were then re-analysed by SDS-PAGE (polyacrylamide gel electrophoresis). In all six red-shelled brachiopod genera analysed, the molecule responsible for shell colour is an intracrystalline protein (ICP1) with an apparent molecular mass of 6.5 kDa. All other intracrystalline molecules are colourless, and non-coloured shells do not have ICP1.

Detailed analyses of ICP1 have confirmed that this chromoprotein has a very similar N-terminal amino acid sequence in the three red brachiopod genera *Waltonia*, *Neothyris* and *Terebratella*. The proteins are clearly homologous (there is no need to insert gaps to align them) but searching of recent versions of the protein and DNA sequence databases (EMBL, NBRF, GenBank & SwissProt using the GCG package) has so far failed to reveal any significant match with the primary structure of any known protein.

UV spectrophotometry and standard colour tests (Dunning 1963) have suggested that the prosthetic compound may be a carotenoid, which as a similar role in crustacean chromoproteins (Zagalsky 1976). ICP1, like other chromoproteins, is soluble in water, but the prosthetic chromophore becomes insoluble when separated, which is characteristic of a lipid–protein association. This emphasizes the importance of preparation technique because the chromophore could not be isolated for analysis in organic solvents until it had been stripped from the protein segment of the molecule.

The importance of showing that the red colour of brachiopods is caused by an intracrystalline protein, results from the fact that fossil brachiopods with preserved colour patterns have been documented from many geological horizons. During the investigation of New Zealand Pleistocene brachiopods a number of specimens with red coloured shell corresponding to that of Recent con-generic species have been recovered from sediments up to 400000 years old. Records of colour pattern do extend much further back in time, indeed as far back as the Palaeozoic. Surprisingly perhaps, some of the best recorded examples of patterned brachiopod shells occur in Devonian and Carboniferous rocks, with ages ranging from 350 to over 400 Ma (Blodgett *et al.* 1988).

At these ages the colour has usually faded or altered, although there are some reports of original colours being preserved that fade rapidly on exposure to light and oxygen. Most fossil 'coloured' brachiopods simply show colour patterns that are reddish brown or black, suggesting that the original prosthetic molecule has altered over geological time. The majority of fossil brachiopods do not show preserved colour patterns, although shells which were originally uniformly coloured, rather than patterned, are easily overlooked. If some molecules do indeed survive within the shell for long periods of geological time, then it may be possible to recover the alteration or breakdown products of the chromoprotein from shells that have lost their colour as a result of the light or oxygen sensitivity of the prosthetic group. This may open up the possibility of reconstructing the colour of fossil shells from an intracrystalline biomarker, an important new ability given that colour may be a crucial factor in evolutionary history (radiations into shallow water and onto land) because it provides protection from predators and radiation. Blumer (1965) has shown that this is possible by determining the breakdown pathway of a chromophore found in the skeleton of fossil crinoids more than 160 million years old.

9. DISCUSSION

Apart from lipids and proteins, it is thought that other types of molecules (e.g. carbohydrates) are present inside biocrystals. The full exploitation of this phenomenon will only be realized when these compounds have been localized, characterized and their function determined. It is possible to recover amino acids, lipids and probably other types of molecules from early Mesozoic or even Palaeozoic brachiopods (i.e. up to 550 Ma), but undoubtedly it becomes progressively more difficult to utilize these molecules in older specimens. Potential problems which must be addressed include the possibility of regeneration (e.g. compounds decay and then are produced *de novo* from simple building blocks within the biocrystal), coelution and hence misidentification of decay products due to identical chromatographic behaviour, and the generation of entirely new molecular species. The precise location of molecules within the shell, and their relation with the calcite matrix, are also crucial factors that have considerable bearing on the extent to which intracrystalline molecules survive in the fossil record, are free to interact with other components, and can be utilized in palaeobiological studies.

We thank the Royal Society, NERC (GR3/7218) and BP for research funding and studentships.

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Discussion

J. L. Bada (*Scripps Institution of Oceanography, University of California at San Diego, U.S.A.*). The pigments Dr Curry finds in the shell layers are likely photosynthetic in origin. Is it thus possible that the protein components that are present are derived from the surrounding seawater and are not directly synthesized by the brachiopods?

G. B. Curry. We certainly consider it likely that the pigment portion of the chromoprotein is derived from the brachiopod diet, possibly from some planktonic organism present in the surrounding seawater. We have not considered the possibility that the entire carotenoprotein had been directly assimilated, rather than synthesized by the brachiopod. We may get some information on this from a current survey of the pigments present in other parts of the brachiopod (for example the gonads) and of the pigmentation present in pelagic microorganisms that occur in the vicinity of brachiopod populations.

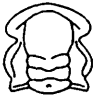
M. H. Engel (*School of Geology and Geophysics, University of Oklahoma, Norman, U.S.A.*). I was curious about the potential preservation of unstable amino acids (e.g. serine) in some of the higher molecular mass fractions of proteins from fossil brachiopods. Wherever the presence of serine is commonly attributed to a modern overprint (contamination), several investigators (e.g. Weiner, Lowenstein) have reported the presence of relatively high concentrations of serine in proteins isolated from fossil ammonoids that are Cretaceous in age. Perhaps Dr Curry could comment on the relative abundance of serine in his various molecular mass fractions going back in time. Also, I would be interested in any thoughts he might have concerning the racemization of amino acids in the various molecular mass fractions of the brachiopods!

G. B. Curry. Serine and various other 'unstable' amino acids are indeed recovered by hydrolysing the insoluble high molecular mass fraction extracted from brachiopod shells. We have not measured the racemization state of amino acids in fossil brachiopods, but intend to do so in the near future.

Immunological responses from brachiopod skeletal macromolecules; a new technique for assessing taxonomic relationships using shells

GORDON B. CURRY, ROSALIND QUINN, MATTHEW J. COLLINS, KAZUYOSHI ENDO, STEPHEN EWING, GERARD MUYZER AND PETER WESTBROEK

LETHAIA



Curry, G. B., Quinn, R., Collins, M. J., Endo, K., Ewing, S., Muyzer, G. & Westbroek, P. 1991 10 15: Immunological responses from brachiopod skeletal macromolecules; a new technique for assessing taxonomic relationships using shells. *Lethaia*, Vol. 24, pp. 399–407. Oslo. ISSN 0024-1164.

The phylogenetic relationships of living, calcareous-shelled brachiopods have been assessed on the basis of immunological responses from intracrystalline macromolecules. Calculations of immunological distances between representatives of the order Terebratulida have revealed a primary threefold division which correlates precisely with a proposed subdivision of the order into three superfamilies but refutes attempts to establish a fourth superfamily. This conclusion was confirmed by carrying out immunological investigations of small shell fragments from other brachiopod genera which were so rare that no antisera could be prepared. The immunological results also indicate a fundamental subdivision of the long-looped brachiopods, with one group perhaps being derived from short-looped terebratuloids rather than long-looped terebratelloids. Sero-taxonomy of skeletal macromolecules provides an ideal method of acquiring molecular phylogenetic data in many groups because a large number of taxa can be surveyed in a short period of time, and microscopic pieces of shell contain sufficient antigenic determinants for many reactions. The technique can also be applied to specimens which have been stored without special treatment in museum collections, making the technique particularly applicable to rare taxa for which no other form of molecular data is available. □ *Immunology, Brachiopoda, taxonomy, intracrystalline molecules.*

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There is no doubt that molecular techniques have revolutionized evolutionary studies, but it is equally clear that such techniques are, for various reasons, neither widely available nor universally applicable. An important factor is the extent to which a particular organism has already been investigated biochemically, as prior knowledge and proven purification protocols are essential both to identify and recover molecules which are likely to provide information on the problem under investigation. Another even more obvious limitation is that sufficient quantities of soft tissues must be available to allow purification of the molecule or molecules of interest. Virtually no biochemical work has been conducted on living brachiopods, and some taxa are so rare, and live in such inaccessible environments, that there is no realistic prospect of being able to obtain intact proteins or DNA. In effect, therefore, precise molecular phylogenetic methods (such as DNA or protein sequencing) would be restricted to a subset of brachiopods and the necessary data needed for sequence comparison from a representative range of modern taxa will take many years to accumulate; this will be determined primarily by the sporadic availability of specimens which have been suitably treated or maintained to yield DNA or proteins.

An alternative method of acquiring such highly desirable molecular data is to utilize indirect methods of assessing the extent of molecular similarity between taxa, which have the advantage of being more widely applicable but the disadvantage of being less precise than direct gene or protein sequencing. The novel aspect of our use of immunological techniques to assess the gross molecular similarity between representatives of modern terebratuloid brachiopods is that the tar-

get macromolecules are situated within the shell and not from the body tissue. This has the major advantage, for brachiopods and for many other taxa, of allowing molecular phylogenetic assessments from tiny pieces of shells, even those which have been stored in museum collections. In effect this is a compromise, trading off a certain degree of precision in the molecular technique against the major advantage of being able to investigate a wide range of taxa relatively rapidly and accurately. For a group such as the brachiopods, for which no molecular data are available, this is undoubtedly an acceptable compromise and a meaningful step along the road to fully integrating molecular and morphological methods of phylogenetic interpretation.

This is not to say that immunology is not a powerful method of assessing phylogenetic relationships. The intensity of immunological reactions, between target molecules and antisera prepared from different taxa, has been widely used as a taxonomic indicator (Nei 1987) and, indeed, is thought to be the longest established branch of 'biochemical systematics' (Ferguson 1980). This technique, known as sero-taxonomy, provides a reliable and relatively rapid method of assessing gross molecular relatedness between taxa; and, with modern high sensitivity detection systems, it has been demonstrated that antibodies can even detect single amino acid changes in proteins (Maxon & Maxon 1979). Purified molecules such as albumins have been successfully used as target molecules in sero-taxonomy, but complex mixtures of uncharacterized macromolecules such as whole body homogenates can also be used (Olsen-Stojkovitch *et al.* 1986; Price *et al.* 1987), and hence immunology is an ideal method of generating molecular phylogenetic data for organisms which are poorly characterized at the molecular level.

This is certainly the case for brachiopods, sessile marine lophophorate invertebrates, which have an extensive fossil record but which are represented by relatively few genera in present-day seas. Many living brachiopods have a patchy distribution in deep water or geographically isolated localities, and can only be collected by dredging. As a result fresh or frozen tissue samples are only available sporadically for a small proportion of living genera making it impossible to obtain DNA or protein samples from a representative range of taxa. Yet molecular data are urgently required because there are controversial inconsistencies in the classification of brachiopods which show no signs of being resolved solely by morphological comparisons.

The number of brachiopod genera which could be included in this study was further increased by preparing antibodies against that component of brachiopod organic mass which is incorporated within their shells. Brachiopod shells are particularly rich in organic compounds, with between between 40 and 50% of their total organic mass being situated within the shell (Curry *et al.* 1989). A proportion of these skeletal organics is known to be situated within the constituent biocrystals, and it is these compounds, utilized as antibody targets, which have proved to be useful taxonomic indicators both in a preliminary study (Collins *et al.* 1988) and in subsequent, more specific, immunological distancing investigation (Collins *et al.* 1991). For the study presented here we have been able, (a) to increase the number of taxa for which antibodies (and hence immunological distances) are available; (b) to demonstrate that a higher sensitivity fluorescent detection system yields the same results as a more traditional enzyme detection system of lower sensitivity; and (c) to further extend the scope of the study (and hence prove its significance as a new taxonomic tool of acceptable resolution and widespread application) by incorporating a number of additional and rare brachiopod taxa to confirm the major conclusions reached. Intracrystalline macromolecules are poorly known in most phyla, but at least some of these compounds are thought to be ubiquitous due to their presumed central role in the processes of biomineralization, while others may have simply been incorporated within shell during growth (for example, fragments of the secretory epithelium which may or may not have any function within the shell).

By adopting an immunological approach and using soluble skeletal macromolecules as targets, it has been possible to include a high proportion of all known living brachiopod genera in this sero-taxonomy study. Because of the sensitivity of immunology it was possible to carry out reactions with microscopic fragments of shells and hence to include some rare taxa. A further advantage of this approach is that entombed macromolecules seem to be protected from short-term decay within the shell. In fact it was possible to obtain reproducible results from specimens which had been subjected to long-term storage in museum collections. Prior to this investigation brachiopod

molecular taxonomy had only been investigated sporadically within a single genus (Hammond & Poiner 1984; Cohen *et al.* 1986) and no major systematic study of brachiopod genera at the molecular level had been attempted.

Methods

Macromolecules were mostly extracted from the shells of living brachiopods using a technique which allows the isolation and NaOCl-cleaning of the secondary shell fibres prior to etching in 20% v/w EDTA. Subsequent ultrafiltration or dialysis was used to removed EDTA prior to sample concentration and immunization. Variations in brachiopod shell ultrastructure made this preparation procedure impractical in some taxa, and in these genera the shell was powdered and cleaned before EDTA treatment. A detailed description of preparation techniques is given in Collins *et al.* (1988, 1991). Antibodies were produced by New Zealand white rabbits which were immunized four times with extracts of purified skeletal macromolecules. Measurements of immunological reactivity were determined using Enzyme-Linked Immuno-Sorbent Assay using a fluorescent substrate (FELISA) on a DYNATECH Plate Reader.

Immunological distancing in brachiopods

Antisera were prepared against all taxa for which sufficient shell material was available, and the reactivity levels between each antiserum and all other taxa were then measured. Immunological distances were determined from the formula of Sarich and Wilson (1966), in which ID = $100 \times \log_{10}$ of the factor to which the antiserum concentration must be raised to produce the same level of reactivity with a heterologous antigen as with the homologous antigen. These distances were obtained from the linear regions of semi-logarithmic binding curves plotted against a dilution series of antibody concentrations.

A total of 11 brachiopod genera were available from world-wide locations (see key to Fig. 1 for details), representing approximately 10% of all

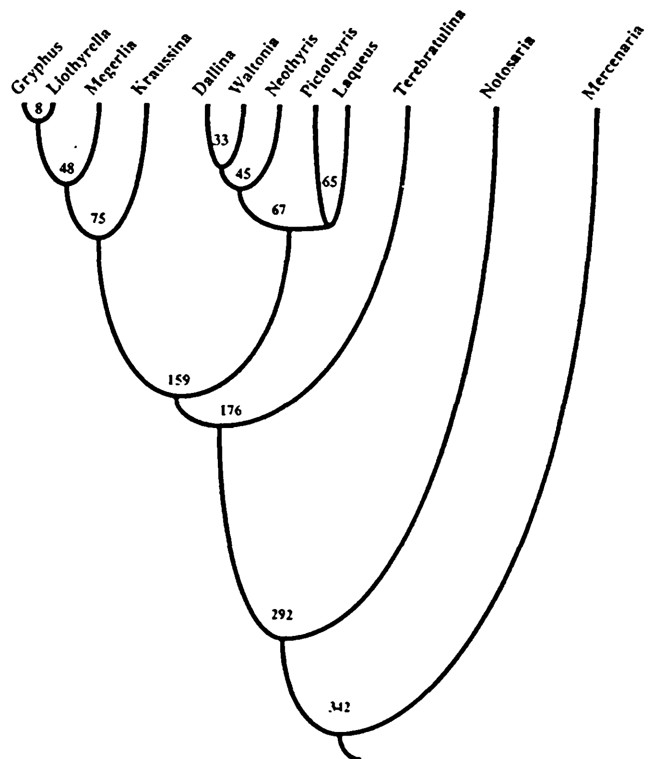

Fig. 1. UPGMA dendrogram based on immunological distance data (Table 1) for the following brachiopod species: *Gryphus vitreus* (Born). Mediterranean. *Liothyrella neozelandica* (Thomson). New Zealand. *Megerlia truncata* (Gmelin). Mediterranean. *Kraussina rubra* (Pallas). S. Africa. *Dallina septigera* (Loven). Scotland. *Waltonia inconspicua* (Sowerby). New Zealand. *Neothyris lenticularis* (Deshayes). New Zealand. *Pictothyris picta* (Dillwyn). Japan. *Laqueus rubellus* (Sowerby). Japan. *Terebratulina retusa* (Linnaeus). Scotland. *Notosaria nigricans* (Sowerby). New Zealand.

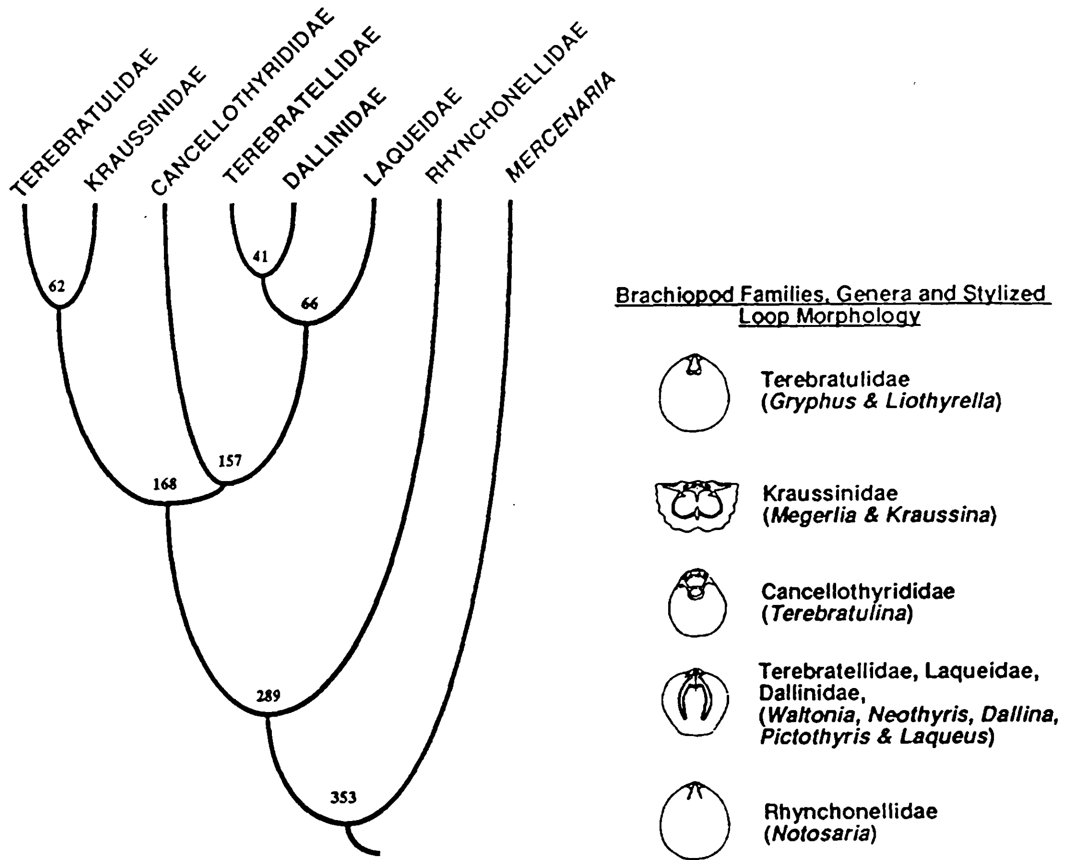

Fig. 2. UPGMA dendrogram of immunological distance data (Table 1) for living brachiopods grouped by family. Family assignment illustrated in key, along with stylized representation of loop morphology within each grouping.

living brachiopod genera. These taxa are predominantly terebratuloid brachiopods, which are distinguished from other brachiopods by the presence of a calcareous loop, developed in the interior of the dorsal valve for support of the lophophore (see sketches in Fig. 2). It was also possible to include a rhynchonelloid brachiopod, *Notosaria*, a representative of a separate brachio-

Table 1. Immunological distances among brachiopods and the bivalve *Mercenaria*.

	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	242	386	280	392	109	282	322	287	x	
Mercenaria	249	327	352	362	447	470	151	362	313	408	324	x

pod order which does not have a brachial loop. Antibodies were also available against skeletal macromolecules from the bivalve mollusc *Mercenaria*, which was used as an outgroup. The FELISA measurements of the reactivity of these antisera against the other taxa included in the immunological distances are summarized in Table 1; the figures cited represent the mean of at least two repetitions of each reaction. The pattern of antibody cross-reactivity is best summarized graphically, and the brachiopod data have been processed using both the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA – see Sneath & Sokal 1973), and the least squares method of Cavalli-Sforza and Edwards (1967). The former calculations were carried out by hand in the manner outlined by Sneath and Sokal (1973), while the latter analysis were carried out on an Apple Macintosh II computer using the appropriate options in the FITCH program (part of the PHYLIP package – Felsenstein 1988).

Comparison with existing classification of terebratuloid brachiopods and the fossil record

At the higher taxonomic levels the pattern of antibody reactivity is entirely consistent with the current classification. The bivalve *Mercenaria* consistently shows the least immunologically detectable molecular similarity, and hence the greatest immunological distance, to the brachiopod genera (Fig. 1). It seems probable that the skeletal organic components, which are common to such distantly related groups, have some fundamental role in biomineralization processes in calcareous shelled organisms. The major Precambrian radiation of metazoan phyla cannot be dated by direct palaeontological evidence, but this event, which must have provided the last common ancestors of brachiopods and bivalves, has been variously dated between 700 and 900 million years ago.

Within the brachiopod stocks, the rhynchonelloid *Notosaria*, lacking a loop, is strongly resolved from all the remaining loop-bearing terebratuloid taxa (Figs. 1, 2). Rhynchonelloids 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 million years to early Ordovician times (Williams & Rowell *in* Williams *et al.* 1965). The rhynchonelloids are thought to be the oldest and least specialized of a third major phase of anatomical development which characterized the Palaeozoic history of brachiopods (Williams & Rowell *in* Williams *et al.* 1965). The terebratuloids and the spiriferoids are the other components of this third evolutionary phase and various lines of evidence suggest that these three orders were originally closely related, with the rhynchonelloids possibly giving rise to the spiriferoids in mid-Ordovician times (~460 million years ago), and the spiriferoids in turn acting as the parent group of the terebratuloids in late Silurian times (~410 million years ago) (Williams & Rowell *in* Williams *et al.* 1965).

Terebratuloid brachiopods dominate present-day brachiopod faunas, and the ability to prepare antibodies against skeletal macromolecules from 10 genera from this order allows a comprehensive investigation of the degree of molecular similarity within this major grouping. The immunological data points to a threefold subdivision of the terebratuloids irrespective of whether the data are plotted for genera (Fig. 1) or for families by using mean data for all available constituent genera in each family (Fig. 2). In contrast the last major summary of brachiopod classification (in the Treatise on Invertebrate Paleontology) adopted a twofold 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 Fig. 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 Cancellothyrididae form a third major grouping virtually equi-distant from the Terebratulidae–Kraussinidae cluster and the long-looped Terebratellidae, Laqueidae and Dallinidae (Fig. 2). The oldest geological representatives of these three groups are of very similar age (Upper Triassic–Lower Jurassic; approximately 210 million years ago) suggesting that there may well be a rough correlation between immunological distance and the timing of divergence of common ancesters, as has been reported in other serotaxonomic studies (Sarich & Wilson 1967).

Figs 1 and 2 have both been produced using UPGMA, a method which makes the fundamental assumption that the rates of evol-

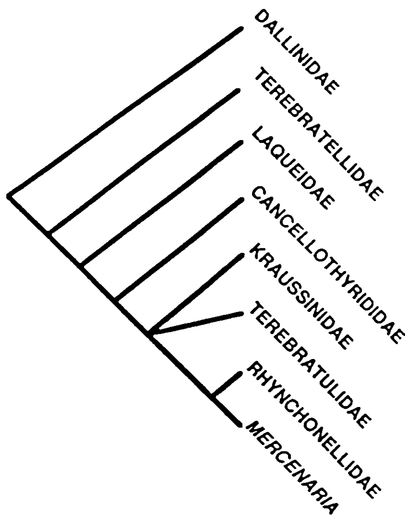

Fig. 3. Least-squared method of analysis applied to the same family immunological distance data as used in Fig. 2, but using the FITCH program of the PHYLIP package (Felsenstein 1988).

utionary change are equal in all lineages (in effect assuming that the 'molecular clock' is a reality). The brachiopod family data were also analysed using the FITCH least-squares method, which does not depend upon this assumption (Fig. 3), and the separation of the cancellothyrids is again evident. The rate of evolutionary change within brachiopod skeletal macromolecules is currently unknown, but the absence of such information clearly does not affect the major conclusion of this study.

Some elements of the immunological clustering can readily be reconciled with developments in morphology-based taxonomy since the publication of the brachiopod Treatise. For example, Cooper (1973a) suggested that the morphology of the short-looped Family Cancellothyrididae was sufficiently distinct to warrant its elevation to superfamily status. This is consistent with the immunological data, in which the cancellothyrid representative plots well away from other short-looped stocks with which it was previously classified (Figs. 2, 3). The combination of independent molecular data, along with distinctive morphological characters and a separate geological history stretching back 160 million years to late Jurassic times, provides multidisciplinary justification for the establishment of the Cancellothyridacea Superfamily (Cooper 1973a).

In contrast, a new superfamily has also been established for the Dallinidae (Cooper 1981), but this group plots closely with other long-looped taxa in the immunological clustering, and hence the molecular data provide no support for the proposed superfamily Dallinacea, and indeed constitute evidence against such a grouping, at least among living stocks. The dallinid pattern of loop development is distinctive and is known from some of the earliest terebratelloids to appear 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 the related Megathyrididae) are thought to have arisen by neoteny from terebratelloid ancesters; 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* (Fig. 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 realized. 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 assumed. Certainly Elliott (1950) has recorded finding both long and short-looped adult forms within a single terebratelloid species from one Jurassic locality, and such discoveries may well be indicative of an underlying plasticity in loop morphology in some stocks.

The incorporation of additional brachiopod genera

In this study it was desirable to incorporate additional taxa to see if the patterns described above were consistent in other representatives of the groups investigated. For this purpose small fragments of a wide range of other taxa have been obtained from private and museum collections.

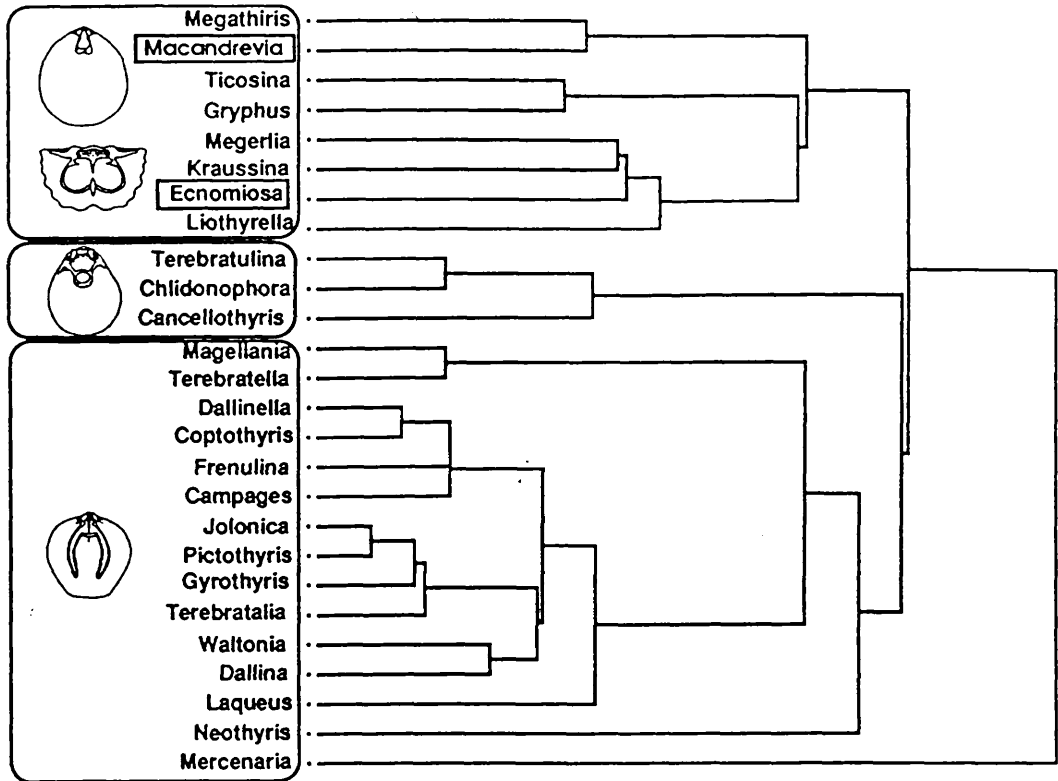

Fig. 4. Single-linkage cluster analysis of 25 terebratuloid brachiopods with the three major groups outlined and represented by stylized loop sketches. The anomalous positions of *Macandrevia* and *Ecnomiosa* are indicated by boxes.

Although immunological distances cannot be calculated for such taxa, it is possible to assess the similarity between molecular extracts of these new taxa and the existing panel of brachiopod antibodies and then to carry out a simple clustering exercise to investigate their relationships (Fig. 4).

The results of a single-linkage clustering of such data (equivalent to the nearest-neighbour method–Sneath and Sokal 1973) re-inforce the major conclusions of the immunological distancing experiments. Two additional cancellothyrid genera were available (*Cancellothyris* and *Chlidonophora*). They plotted, along with *Terebratulina*, in a tight cluster well separated from other short-looped and long-looped brachiopods. Additional dallinid, laqueid and terebratelloid genera again clustered together as a coherent group, while the mixed long- and short-looped terebratuloid–kraussinoid–megathyroid grouping remained intact with the incorporation of a larger number of genera. The only inconsistencies are the position 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 indeed 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.

Discussion

The major conclusion of this study is that skeletal macromolecules in brachiopods vary taxo-

nomically to an extent which can be detected immunologically. In particular, the suggested threefold division of the terebratuloids is reinforced by the incorporation of additional cancellothyrid genera into the immunological study, the results of which confirm their separation from the terebratelloid and terebratuloid stocks.

Although the molecular basis for such discrimination is as yet unknown, the sero-taxonomy data have provided an invaluable molecular perspective in a group in which evolutionary interrelationships have primarily been determined from morphological characteristics of the shell. Immunological data do not represent a general panacea for the problems of interpreting brachiopod evolutionary history, and considerable arguments exist about the methods used to draw trees from this type of data, because they inevitably involve a blurring of the original data. UPGMA has mostly been used here because it has a long, proven track record, but the main conclusion concerning the separation of the cancellothyrids can readily be seen in the raw data for the cancellothyrid *Terebratulina* against all other brachiopods, as compared to the immunological distances among genera within the other two main groups (Table 1). The fact that there are reciprocity differences between some taxa in the raw data set is a consequence of the inherent experimental error of such techniques, but this would only seriously disrupt attempts to draw much more specific taxonomic conclusions than has been attempted here.

In general, there is no particular reason for favouring the molecular over the morphological approach to phylogenetic reconstruction. There is even the risk of invoking a circular argument in first testing one against the other and then refuting one or the other. But in the case of the brachiopods there is a very encouraging correlation between the two approaches; and a combined molecular–morphological synthesis, using information from the fossil record on the stratigraphic and geographic distribution of the ancestors of living brachiopods, does seem to offer the best possible opportunity of accurately tracing evolutionary histories within the phylum. The indication that intracrystalline macromolecules have taxonomic significance suggests that determination of the amino acid (and underlying nucleotide) sequence of these macromolecules would provide further precise information both on evolution and biomineralization processes, and with recent technological developments this may shortly become a viable prospect.

Acknowledgements. – We thank Dr. C. H. C. Brunton, Department of Palaeontology, Natural History Museum, London and Dr. R. E. Grant, Smithsonian National Museum of Natural History, Smithsonian Institution, Washington DC, for providing shell samples of living brachiopods. Dr. A. Ansell, Oban, Dr. C. Emig, Marseille, Dr. M. James, Dunedin, and Dr. L. Peck, Cambridge, also kindly provided us with material for this study. We thank Sir Alwyn Williams for reading and commenting upon the manuscript. This work was supported by NERC grants GR3/6213 and GR3/7218 and several grants from the Royal Society of London (all to GBC) and a NERC research fellowship (to MJC), all of which are gratefully acknowledged.

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Migration of brachiopod species in the North Atlantic in response to Holocene climatic change

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ABSTRACT

The brachiopod *Terebratulina retusa* has migrated 3000–4000 km from the Iberian Peninsula as far north as Spitsbergen during the past 10 ka. This migration was caused by the progressive northward reestablishment of the North Atlantic Current, which had been deflected south during the last ice age and which represents the primary method of dispersal for short-lived pelagic brachiopod larvae. This migration has resulted in two similar but morphometrically distinct species, *Terebratulina retusa* and *Terebratulina septentrionalis*, occasionally occurring together. This allows the identification of periods of rapid climatic change in the geologic record because of the effect such changes have on currents that control the distribution of sessile marine organisms.

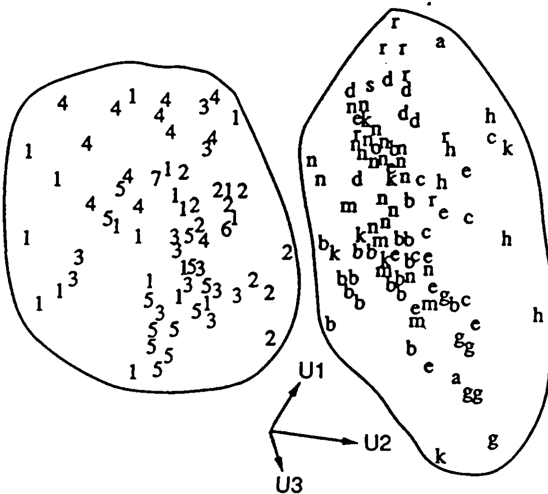

Figure 1. Principal component analysis (PCA) showing clear morphometric distinction between *T. retusa* and *T. septentrionalis*; the two species occupy nonoverlapping areas of multidimensional morphospace. Measurements taken from shell of each specimen were length, width, and height of joined valves, rib width, foramen length, and foramen width. *T. septentrionalis* populations are represented by numbers and *T. retusa* populations by letters. Diagram shows rotating three-dimensional plot of first three principal component axes (which together account for 96% of total detected variability) arrested at position that shows clearest separation between clouds of points representing the two species. Arrows labeled U1, U2, and U3 show relative positions of three axes. Separation is primarily achieved along second principal component axis, eigenvector values for which indicate that this mainly reflects differences in rib width. All analyses were carried out by Datadesk Professional (or its 1989 upgrade Datadesk 3.0) running on Macintosh II microcomputer fitted with 68881 math coprocessor. Populations included are, for *T. septentrionalis*, 1—Finnmark, northern Norway; 2—Cape Farewell, Greenland; 3—Maine; 4—Massachusetts; 5—Cape Cod; 6—Trondheimfjord, Norway; 7—Bukken, near Stavanger, Norway. For *T. retusa*, populations are, a—Spitsbergen; b—Iceland; c—Trondheimfjord, Norway; d—Hardangerfjord, Norway; e—Bukken, near Stavanger, Norway; g—Sound of Jura, Scotland; h—Roscoff, northern France; k—Sardinia, Mediterranean; m—Corsica, Mediterranean; n—Rhodes, Mediterranean (Pleistocene); o—Canary Islands; r—Messina, Italy (Pliocene). Specimens measured were collected for this study or are housed in Natural History Museum, London; Smithsonian Institution, Washington, D.C.; Zoological Museum and Institute of Palaeontology and Historical Geology, Copenhagen; Institut Royal des Sciences Naturelles, Brussels; and Musée National d'Histoire Naturelle, Paris. All five museums have specimens collected from Finnmark that have characteristic morphology of *T. septentrionalis*.

INTRODUCTION

The most abundant brachiopod in the North Atlantic is *Terebratulina*, and although it has long been accepted that two species are probably present (Davidson, 1886), it has been impossible to determine their individual distributions because they are so similar. Recently the morphology of the *Terebratulina* shell has been investigated extensively by using principal component analyses (PCA). This study has revealed that there is a clear and unambiguous distinction between the two species (Fig. 1), primarily reflecting the fact that *T. septentrionalis* has many more ribs than *T. retusa* (Fig. 2). Having established reliable morphometric criteria for distinguishing between these species, it was then possible to map out their separate distributions in the North Atlantic by measuring precisely located specimens that were either collected for this purpose or available in museum collections.

DISTRIBUTION OF TWO SPECIES IN THE NORTH ATLANTIC

The results of the investigations of several thousand specimens have demonstrated that *T. septentrionalis* is found predominantly in the western

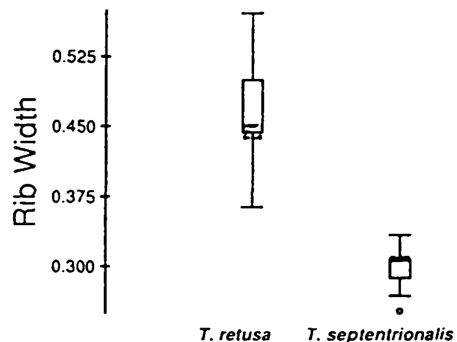

Figure 2. Boxplots showing width of ribs on two species in 4-mm transverse sector situated 4 mm posteriorly of umbo on brachial valve. Boxes depict data between 25th and 75th percentiles; vertical bars illustrate extent of main body of data (circle is extreme datum point); patterned areas represent 5% confidence levels around medians for each species. Measurements are from living and fossil specimens included in Figure 1. Means for each sample (*T. retusa*—0.47 mm, standard deviation = 0.043, 98 samples; *T. septentrionalis*—0.30 mm, standard deviation = 0.025, 67 samples) are significantly different at 0.01 level.

North Atlantic from Baffin Bay and Davis Strait, west of Greenland, and down the east coast of North America (Fig. 3). Specimens with the characteristic morphology of *T. septentrionalis* are also found along the coast of the Finnmark region in northern Norway. *T. retusa* is present in the eastern Atlantic from the Canary Islands and the Mediterranean in the south, around the coasts of Spain and northern France, off Iceland, all along the east coast of Norway, to as far north as Spitzbergen (Fig. 3).

A CLINE?

The confusion over the distribution of these two species has led to the suggestion that a morphological cline exists between the two species in the northern North Atlantic (Wesenberg-Lund, 1938, 1940a, 1940b, 1940c). However, this morphometric investigation demonstrates that the two species are just as clearly distinguishable from one another in localities separated by a few hundred kilometres along northern Norway as in localities separated by the several thousand kilometres between Canada and Scotland (Figs. 1-3). Throughout its range, each species displays a highly characteristic morphology, and examination of more than 2000 specimens has revealed no sign of the intermediate morphotypes that would be expected if a cline existed between the two species (Fig. 1). As discussed below, genetic and immunological data have also confirmed that the two taxa are valid species and that there is no indication of genetic exchange between the populations studied (Cohen et al., 1991b).

BRACHIOPOD DISPERSAL AND OCEAN CURRENTS

The present distribution of *T. retusa* closely follows the path of the North Atlantic Current, which brings relatively warm water across the Atlantic from the Caribbean and flows northeastward across Europe as far north as Spitsbergen, the most northerly location of *T. retusa*. The disperal of *T. retusa* is achieved during a short pelagic larval stage, probably lasting only a few days in this species (Curry, 1982). These larvae, being weak swimmers, positively phototactic, and nonplanktotrophic, depend predominantly on ocean currents for dispersal (Brunton and Curry, 1979; Curry, 1982). In their preferred habitats at depths of 200–500 m along the continental margins of Europe, the primary agent of dispersal must, therefore, be the North Atlantic Current.

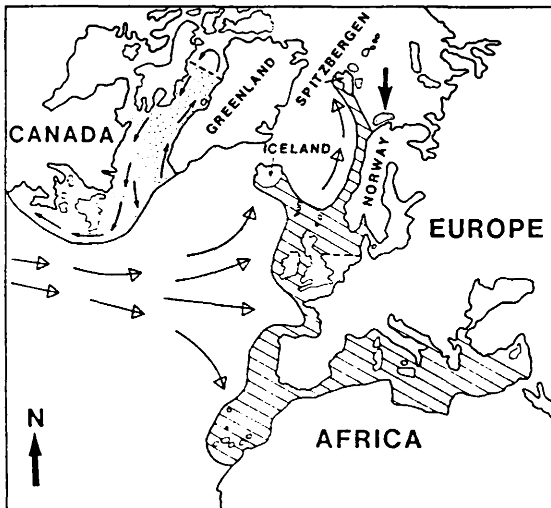

Figure 3. Main distribution of *T. retusa* (diagonal pattern) in eastern North Atlantic and *T. septentrionalis* (dot pattern) in western North Atlantic; large solid arrow indicates small relict population of *T. septentrionalis* off northern Norway. Distributions have been determined from specimens with accurate location data in museum collections. Direction of North Atlantic Current represented by medium open arrows, Baffin and Labrador Currents by small solid arrows. Two western Norwegian localities where species occur together cannot be illustrated on map of this scale; other instances of overlapping distribution are likely in areas where replacement of one species by another is still going on.

The last Ice Age caused severe disruption of ocean currents. It has been estimated that the North Atlantic Current was deflected south of the Iberian Peninsula at the peak of the most recent glaciation, ca. 18 ka (Golikov and Scarlat, 1989). As the ice melted, the current is thought to have pushed progressively northward along the coast of northern Europe—producing considerable changes in marine faunas (Thomsen and Vorren, 1986)—and extended (or reestablished) its influence over 3000–4000 km of continental margin that had recently been in arctic or subarctic water conditions. *T. retusa* appears to be well adapted for temperate marine environments, and on the basis of its present distribution, there is a likelihood that it was excluded from much of northern Europe during the last glaciation. This implies that the present range of *T. retusa*, as far north as Spitzbergen, reflects a recent, and probably ongoing, colonization as its larvae were carried northward by the North Atlantic Current.

T. septentrionalis, by contrast, appears to be adapted for colder water. Its present distribution follows the path of the cold-water Baffin Current, with a southern extension into the North Atlantic coincident with the Labrador Current. Throughout much of its range, *T. septentrionalis* lives in temperatures of 0.5–5 °C (e.g., shelf temperatures west of Greenland; Rudels, 1986). The comparable and presumably optimal figures for *T. retusa* in Scotland are 6.5–13 °C for open-marine (Brunton and Curry, 1979; Curry, 1982) and 6.8–11 °C for sea-loch (James et al., 1991) populations, although it can clearly tolerate lower temperatures. *T. septentrionalis* is therefore likely to have been more widely distributed in the North Atlantic during the last ice age. Indeed, the Finnmark animals may be relicts isolated by the northward advance of warmer currents for which they are poorly adapted. Isolated communities of arctic benthic invertebrates have been recorded from many temperate-latitude fjords or sea lochs (Blacknell and Ansell, 1975; Vahl, 1980), and the deep, cold waters of these fjords afford ideal refuges for arctic or subarctic organisms. Certainly, such habitats could well accommodate breeding populations of *T. septentrionalis* along continental margins now dominated by *T. retusa*. *T. septentrionalis* is known to brood larvae until shortly before they are released from the shell to attach to the seabed (Webb et al., 1976), but *T. retusa* releases fertilized ova much earlier (Brunton and Curry, 1979; Curry, 1982). *T. septentrionalis* will therefore have a much shorter pelagic larval stage (and indeed may settle immediately on release from the shell) and will have more likelihood of being cut off by rapidly changing environments than *T. retusa*.

DIRECT EVIDENCE OF SPECIES MIGRATION

The suggestion that *T. septentrionalis* has gradually been replaced by *T. retusa* in relatively recent times is supported by several independent lines of evidence. First, there is direct evidence of the two species occurring together in two Norwegian fjords. The Natural History Museum in London contains several brachiopods collected from Bukken, near Stavanger, Norway. All living specimens from this locality display the characteristic morphology of *T. retusa* (labeled "c" in Fig. 1), but a single dead, sediment-filled shell (ZB3299) in the same collection was definitely *T. septentrionalis* on morphometric criteria (labeled "6" in Fig. 1). A similar association was found in samples from Trondheimfjord that are stored in the Zoological Museum in Copenhagen, although in this case one living specimen of *T. septentrionalis* (7 in Fig. 1) had been collected from a fjord where the remaining specimens are *T. retusa* (c in Fig. 1).

MOLECULAR EVIDENCE

A second, entirely independent, line of evidence supporting the above hypothesis comes from the results of recent biochemical investigations of *Terebratulina* in the North Atlantic. Analyses of mitochondrial and nuclear DNA and immunology of intracrystalline shell proteins have shown that a large genetic distance exists between *T. retusa* and *T. septentrionalis*, indicating that they are two distinct, noninterbreeding species that, on the basis of known DNA mutation rates, probably diverged about 11-18 m.y. ago (Cohen et al., 1986, 1991a, 1991b; Collins et al., 1988; Endo and Curry, 1991). Throughout the genetic work, complete congruence was demonstrated between the molecular and morphological analyses. These molecular data also reveal no detectable genetic distance between populations of *T. retusa* from localities as widely separated as the Mediterranean and Norway, consistent with the interpretation that they have been established relatively recently. The alternative possibility, that the populations are much more ancient and that homogenization of the gene pool occurs by long-distance larval transport, is much less likely. As already noted, the larval stages of *T. retusa* are of only a few days duration, and complete homogenization over such distances is unrealistic given the vagaries of larval dispersal, settlement, and survival.

DISCUSSION

A striking aspect of the distribution of *T. retusa* is the speed at which this sessile marine invertebrate has been able to extend its range in response to changing climates. With generation times of the order of 3-5 yr in this species (Curry, 1982), more than 3000 km of continental margin appear to have been colonized in about 3000 generations. This dispersal rate is not unrealistic, as a planktonic larva of this species could be transported for 10 or more km northward along the European continental shelf by the North Atlantic Current. The implication is that the biogeographical distribution of sessile benthic organisms such as brachiopods is a sensitive marker of climatic change.

There are also significant paleontological, paleobiogeographical, and evolutionary consequences of the rapid, climate-induced movements of *Terebratulina*. During the past 10 ka, both of these species have migrated over large distances of the European continental shelf, occasionally occurring together as one replaced the other. *T. retusa* has migrated an average of 300 km/ka, and during this period a sediment layer perhaps only 10-50 mm thick will have accumulated on the European continental shelf (Anders et al., 1987). The rapid mixture and replacement of two species with very similar morphologies will be difficult to recognize in the geologic record, but would be revealed by using a rigorous identification scheme capable of distinguishing subtle but reliable specific morphological features (such as PCA). Indeed, this phenomenon may have already been detected in benthic fossils, but may have been attributed erroneously to sexual dimorphism.

It is clear that *T. retusa* has been present in the North Atlantic for at least 3 m.y., because fossil specimens morphometrically identical to living ones are present in Pliocene (ca. 3 Ma) rocks in Italy (*r* in Fig. 1) and Pleistocene (ca. 2 Ma) rocks in Rhodes (*n* in Fig. 1). During this time there have been many major climatic perturbations, presumably reflected in the migration of *Terebratulina* species up and down the North Atlantic continental margin, which will produce a distinctive pattern of mixed species in the fossil record, patterns that could be detected morphometrically. Although there are dangers in extrapolating far back in time, *Terebratulina* has great potential for this form of analysis because it has a worldwide distribution in present seas and a fossil record extending back to the Late Jurassic (ca. 160 Ma).

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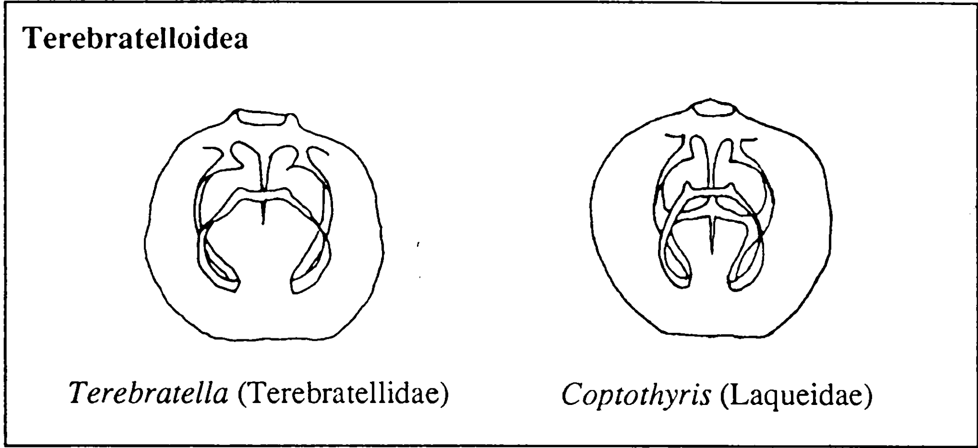
ACKNOWLEDGMENTS

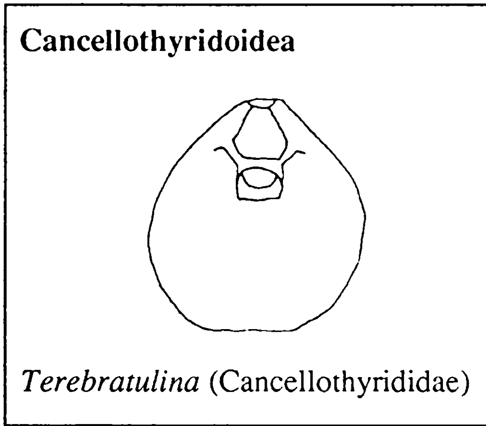
Supported by the Royal Society of London, Natural Environment Research Council (grant GR3/6760), and the University of Glasgow. We thank Ulla Asgaard, Alan Ansell, Richard Bromley, Howard Brunton, Bernard Cohen, Jean-Loup Dhondt, Dick Grant, Annie d'Hondt, Claus Nielson, and Elsebeth Thomsen for providing or helping to locate specimens.

Manuscript received April 29, 1991
Revised manuscript received July 26, 1991
Manuscript accepted August 5, 1991

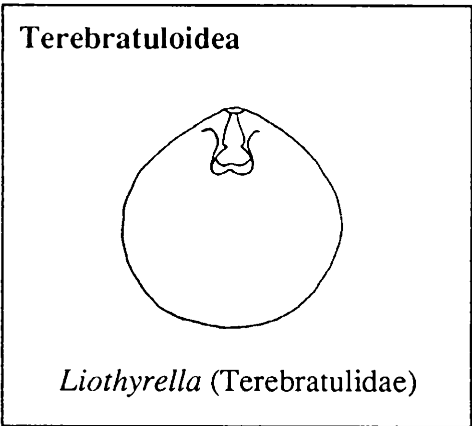
Appendix III Stylised loop morphology of some terebratulide genera



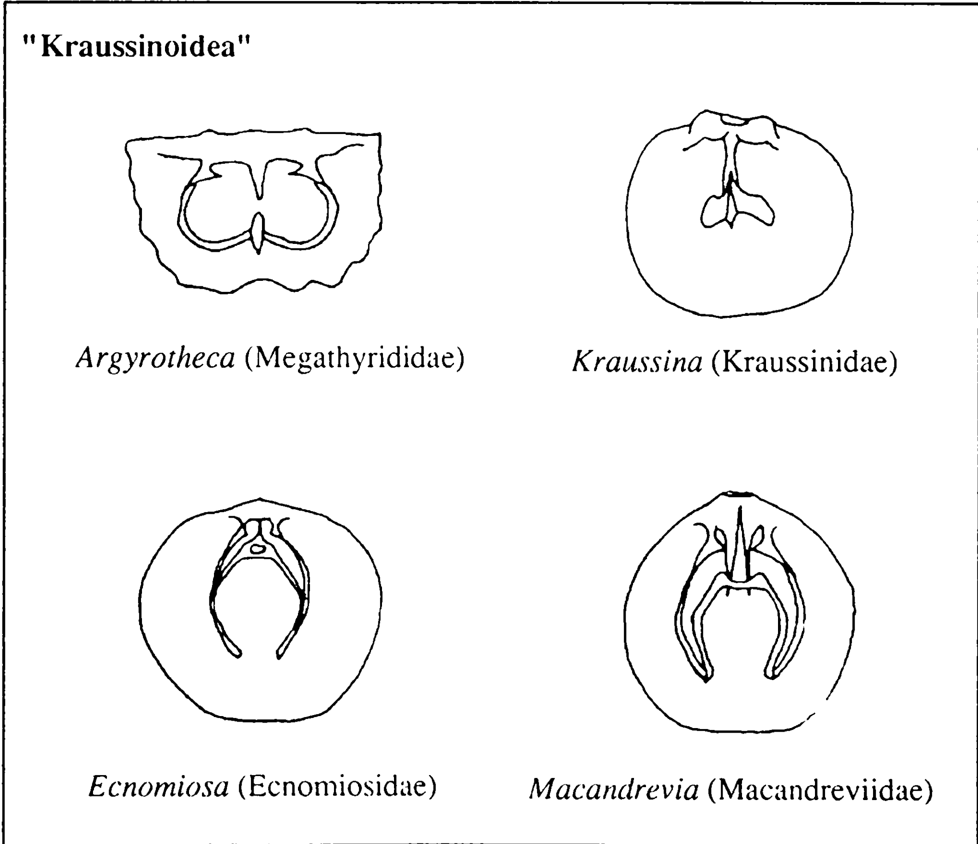
Terebratella (Terebratellidae) *Coptothyris* (Laqueidae)



Terebratulina (Cancellothyrididae)



Liothyrella (Terebratulidae)



Argyrotheca (Megathyrididae) *Kraussina* (Kraussinidae)

Ecnomiosa (Ecnomiosidae) *Macandrevia* (Macandreviidae)