THE UNIVERSITY OF CALGARY

Comparative Morphology and Evolutionary Trends in the Class Gastropoda through Three-Dimensional Tomography and DNA sequence analysis

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

December, 1999

ABSTRACT

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This study evaluated the validity of the recently developed multidisciplinary scheme for gastropod subclass phylogeny. The multidisciplinary scheme appeared to be more suitable than the longstanding, firmly entrenched classical scheme, which is seen currently in textbooks and many journals. In order to ascertain this, I described and analyzed novel molecular and morphological characters as cladograms, which I then compared to the two phylogenetic schemes. I accumulated these characters using two techniques: 18S ribosomal DNA sequencing and three-dimensional magnetic resonance microscopy (MRM). For this study, I sequenced DNA encoding the 18S rRNA subunit of seventeen previously uncharacterized gastropods in order to develop a more complete molecular survey of the Gastropoda. The result was a robust computer analyzed consensus tree. The MRM portion of my thesis dealt with the acquisition of the first 3D non-destructive models of the musculo-skeletal arrangements within the gastropod foot. The eight models generated represent a pan-class selection and were analyzed for and resulted in several morphological and functional trends regarding the tarsos and columellar musculature and their integration with each other. In short, the phylogenetic analysis of these two datasets supported the new multidisciplinary scheme but with modifications. It also shows that the smoothly graded classical prosobranch-opisthobranch-pulmonate scheme is probably artificial. Additionally, both these techniques have been adapted for addition to the toolbox of malacologists and other biologists for uses that extend beyond phylogenetic studies.

ACKNOWLEDGMENTS

I am a lucky fellow... Although this field of science is, in my opinion, of true value and fills most of its workers with a miraculous feeling of discovery and self worth, rising through the educational column is often a gaunt prospect with respect to personal finances and time. These obstructions, in my case, have been amply overcome with the help of my family both genetically related and adopted. These people, to whom I am in debt, I list below:

My wife Angela for all her support through and help with this thesis.
My Mom and Dad for help in reading through my thesis and growing me up right.
My supervisor Dr. G. Bourne, who is my first mentor and good friend.
My brother Keith for being a good brother and helping with the software.
My best buddy Mike Newel for being a great lab mate and an invaluable third hand in many of the protocols.

Professionally, I am in debt to many, many people. I look at the M.Sc. program, and life in general, as an opportunity to learn as much as you possibly can and there are people that have gone out of their way to grant me the chance to learn the skills I hold so dear:

My committee, who I've noticed is much more interactive and helpful than most: Dr. Bourne, Dr. Walker & Dr. Syed. I would also like to thank Dr. Syme for serving as my external examiner and Dr. Phillips, who was originally on my committee and who came to my aid countless times whenever I had a computer visualization problem or needed critical reading.
Those who taught me about sequencing: Bob Winkfein (who went out of his way to do most of the sequencing), Dr. Honda and the Bamfield Marine Station.
The guys at the Center for *In Vivo* Microscopy at the Duke University Medical Center, especially Dr. Brad Smith (now at U of Michigan at Ann Arbor), Mr. Gary Collier and Dr. Al Johnson for technical guidance, microscope time and critical readings.
My 3D rendering guru, and one author of the great computer program Sterecon, Mr. Mike Marko of the Wadsworth Institute in Albany, New York.
I'd also like to thank my department and Dr. Tim Buell of the Learning Commons for indispensable funding received for my research.
Those people who helped me out here and there with administrative stuff and a plethora of small jobs: Karen & Wilma, Dr. Barclay, Shanna, and many others.
Lastly, although my time with them was brief, I would like to thank Dr. Bill Kier of the University of North Carolina at Chapel Hill, Dr. John Gosline of the University of British Columbia and Drs. Vogel and Wainwright of Duke University for giving me much perspective and hope for the future.

DEDICATION

I dedicate this thesis to my family and
especially to my late grandparents,
all of whom believed that
"Education is the Great Separator of Men and Gentlemen".

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Function without structure is a ghost.
Structure without function is a corpse.

Dr. Wainwright & Dr. Vogel,
Autograph in my copy of "Mechanical Design in Organisms"

There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.

Charles Darwin,
Last paragraph in "The Origin of Species"

Orange and speckled and fluted nudibranchs slide gracefully over the rocks, their skirts waving like the dresses of Spanish dancers.

John Steinbeck,
First paragraph of the sixth chapter in "Cannery Row"

CHAPTER ONE
INTRODUCTION

The class Gastropoda is the largest and most diverse class within the phylum Mollusca (Ponder, 1998). Although it is one of the classes with the most research literature devoted to it (the other one being the class Cephalopoda), there is confusion with respect to its subclass phylogeny due to recent changes in analytical techniques and the introduction of new data. The role of phylogenetics is to arrange organisms into patterns that show their evolutionary relationships so that they form assemblages of monophylys. Their relationships are usually displayed as phylogenetic trees or cladograms. To build such a phylogenetic tree, one must develop a set of characters for a wide number of organisms within a group. During the mid to late nineteenth century with the first publications of such studies (e.g., Milne-Edwards, 1848 and Spengel, 1881), the characters consisted mostly of shell morphology and some single organ systems. Then, much later, other considerations such as numbers and morphology of many different organs and ultrastructure were incorporated (e.g., Golikov & Starobogatov, 1975 [gill morphology]; Healy, 1988 [sperm ultrastructure]; Haszprunar, 1985 [osphradia fine structure]) along with the descriptions of newly-found deepsea organisms (e.g., Fretter, 1990). With the advent of technology such as high resolution electron and optical microscopes, molecular techniques and better analytical and collection procedures, scientists are uncovering many more characters and testing them to develop more robust and holistic schemes. Even with the use of all this novel technology, there are still basic questions in the field of gastropod phylogeny. The higher subclass taxa are underrepresented in terms of research and as a result are unstable at best and unresolved at worst (Harasewych, 1994). I believe that with the latest flourish of research data collected in the last half of this decade, the state of the higher taxa is becoming much more stable.

As we come to the close of the twentieth century and the second millennium there are two general groupings of schemes in use. The first group is the one derived from

Milne-Edwards (1848). These schemes arrange the gastropods into three subclasses, the Prosobranchia, the Opisthobranchia and the Pulmonata (referred to as classical schemes) and their use is currently reinforced as this arrangement is used by the International Commission on Zoological Nomenclature and the taxonomical journal Zoological Record. One often sees these schemes referenced in journal titles. The other group of schemes is supported by many of the active molecular and morphological experts because of their multidisciplinary approach to character collection. Thus, these are hereafter referred to as the multidisciplinary schemes. These schemes (as put forward by authors such as Ponder and Lindberg, 1997; Haszprunar, 1988; Harasewych *et al.*, 1997a) generally include the Vetigastropoda, Patellogastropoda, Caenogastropoda, Neritopsina, Cocculiniformes and Heterobranchia as their subclass taxa.

In reviewing these two sets of schemes, classical and multidisciplinary (Chapter 2), because of their increased data set and acceptance among specialists in the field, I concluded that the multidisciplinary schemes have more validity, although they currently do not enjoy the wide use that the classical schemes do within the scientific community. I set out to test the validity of the multidisciplinary schemes. However, it was not my purpose to re-evaluate the characters used to define these two sets of schemes but rather to generate my own set of characters, particularly morphological/biomechanical and genetic, in order to support or refute the current design of the multidisciplinary schemes.

From a technical point of view, there are many methods of data collection that have rarely or never been used with gastropods as specimens. Therefore, it is also within the purpose of this thesis to broaden the technique toolbox as well as the character database available to the gastropod systematist, molecular biologist and anatomist/morphologist by developing techniques that work on these interesting organisms.

CHAPTER TWO
LITERATURE REVIEW

2.1 Topics covered

Initially, I review the literature that describes the spectrum of techniques that may be used to recruit both molecular and morphological characters for analysis. I state the criteria that I used to select magnetic resonance microscopy (MRM) and DNA sequencing, used to develop morphological and genetic characteristics, respectively. The second part of this chapter is dedicated to three important topics that fall within the subject of gastropod systematics. Firstly, I describe the current state of gastropod systematics with emphasis on subclass taxa. Secondly, I present the historical data and character types that lead up to the current systems of classifying the snails. This area also encompasses the idea of how the morphological and genetic data have been handled in the past. Finally, I present an assortment of recently published information to aid in better resolving the question of how gastropods should be grouped.

2.2 Overview of the spectrum of techniques

2.2.1 Spectrum and selection of imaging techniques

Biological imaging is an area of data acquisition to which a lot of attention has been focused recently, probably because of its effectiveness in aiding medical research (e.g., the Computer Axial Tomography [CAT] Scan and the Positron Emission Tomography [PET] Scan). These areas have been receiving attention because they are non-destructive and in many cases allow *in vivo* imaging. The direct benefits are the reduction of artifacts (many of which are described in the survey below) and the

representation of three-dimensional spatial information with a decreased amount of subjectivity in output and analysis.

Tomography is a technique that defocuses activity from surrounding planes by means of relative motions at the point of interest (Photonics Spectra, 1999). This is a more general definition than has been used in histological research because it does not imply that the object being sectioned must be physically altered nor does it have to be investigated in a linear or planar method.

Tomographic techniques include gross dissection and clearing and staining of whole organism, destructive sectioning, motorized stage optical microscopy and the emerging techniques of MRI or magnetic resonance imaging and its offshoot, MRM. Finally, I would like to describe forms of data output and their use in this project.

2.2.1.1 Gross dissection and whole organism imaging

Gross dissection is an intuitive and useful technique by which one can learn the anatomy of organisms. However, for the purposes of imaging, recording and careful re-examining the morphology of the structures, one must rely on rather subjective methods such as the *camera lucida* or simplified drawings. Otherwise, one must rely on techniques such as photography that do not have the ability to remove unavailing information. Regardless, the presentation of such dissections is two-dimensional and if internal structures are to be recorded one must expose them and thereby disturbing their important three-dimensional relationships.

2.2.1.2 Destructive sectioning

Histological or mechanical microtomy is a method of sectioning that is well developed. As documented by Humasen and others (Presnell & Schreibman, 1997), there are already techniques devised specifically for molluscan tissues. However, in a practical sense this system only works for a range of organisms that fall into volume classes between 5 mm^3 and 10 cm^3. Generally, data are recorded by photographic means. The well-documented problems with this technique are many. Firstly, one has to fix tissues without any damage. Cell rupture and ion-flux related problems often occur here

(Presnell & Schreibman, 1997). Then one must correct for compression as the blade goes through the tissue as well as artifacts pertaining to reorientation of the sections in the x and y axis. This problem, often called fiducial, registration or realignment error, although addressed vigorously, has never really been overcome effectively (Jones *et al.*, 1994, Lyroudia *et al.*, 1997, Carlbom *et al.*, 1994). Histological sectioning is not a very useful imaging technique because of these problems, but it is very useful in determining cell types and fine structure because of the well-developed toolbox of stains, dyes and organic labels (e.g. Presnell & Schreibman, 1997).

2.2.1.3 Optical sectioning

Since its introduction in the 1950s, the use of confocal technology for the purpose of tomography has grown into the most popular form of optical sectioning (Wallén *et al.*, 1992). I have used the Confocal Laser Scanning Microscope (CLSM) at the Geological Survey of Canada in Calgary, Alberta and have found that this technique allowed me to focus into specimens while omitting all out-of-focus information. Another benefit is that the CLSM scanning can be performed on live narcotized specimens. Unfortunately, I have found crippling limitations, the most severe of these being photobleaching or tissue damage due to high intensity light and the limited depth through which the light source can penetrate (currently UV spectrum light is used, the depth of which is only about 0.5 to 1 mm). I included a description of this technique to acknowledge my attempt to use it and to suggest that it would be useful for extremely high resolution imaging of larvae, which, unfortunately is not within the scope of this thesis.

2.2.1.4 Magnetic resonance sectioning

Another method of tomography that is rapidly gaining favour is nuclear magnetic resonance imaging (MRI). The technique was invented in 1973 by Lauterbur (1973) and has gained ready acceptance in medicine and mammalian biology (Wehrli *et al.*, 1988). A recent innovation in this field is the extension of the MRI technique to much higher spatial resolution, i.e., to magnetic resonance microscopy (MRM). MRM was first developed in 1986 by Johnson *et al.* (1986) and Eccles (Eccles & Callaghan, 1986) and is

accomplished using specialized magnets, gradient coils, radio-frequency coils and pulse sequences (Zhou & Johnson, 1995, Hurlston *et al.* 1997). This technique is described in more detail below (Section 5.1.2). My work marks the initial use on a mollusc, however this machine has been used once before on an invertebrate in a crustacean physiological study (Brouwer *et al.*, 1992). In the end, this technique was chosen as the main form of data acquisition for its adaptability to the size of specimen, fairly good resolution, and absence of artifacts induced during preparation.

2.2.2 Spectrum of genetic techniques

The basic premise of any study of which the goal is to reconstruct phylogeny from DNA sequence information, is that different organisms or groups of organisms have different underlying gene sequences. Another premise that I embraced is Ockham's idea of parsimony (Sober, 1993). Together, these two ideas suggest that phylogenetic development from basal to derived species is caused by mutations in gene sequences (fewer in the basal and more derived species) and that the most probable phylogenetic tree is the one that necessitates the fewest genetic mutations (Swofford *et al.*, 1996). The practical implication is that a project is best served by a technique that resolves the differences between the DNA sequences of each organism. There are several modern techniques that accomplish this, as follows: Restriction Fragment Length Polymorphisms, Amplified Fragment Length Polymorphisms, Random Amplified Polymorphic DNA, Variable Number of Tandem Repeat analysis and DNA nucleotide sequencing (gene sequencing). I also explain the reasons for selecting gene sequencing. Lastly, I survey research that has been completed in molluscan molecular phylogeny.

2.2.2.1 Random Amplified Polymorphic DNA

Random Amplified Polymorphic DNA (RAPDs, pronounced "rapids") is a technique that brings to light randomly selected differences in sequences (Grosberg *et al.*, 1996). This technique is useful for tracking various alleles of genes and if one knows enough about the gene sequence, one can pre-amplify a gene of interest before testing it

with RAPDs (Griffiths *et al.*, 1993). Even if one has no *a priori* knowledge of the genomic DNA, it is possible to group together animals with the same DNA segment sizes. These segments are generated by performing a Polymerase Chain Reaction (PCR), which is an integral part of the methodology of RAPDs. Another added benefit of this technique is that one can test a very large number of individuals because of the small amount of work involved in the generation of Southern blots (agarose gel electrophoretic separation of DNA segments), a method of imaging DNA segments of differing size. Finally, RAPDs can be applied to very minute amounts of DNA. This technique is therefore efficient and inexpensive, but Rabouam *et al.*, (1999) have pointed out many, potentially crippling problems with this method. Firstly, one only runs into mutations by means of random primer selection, therefore there is a lot of potential work involved in optimizing the DNA products. Secondly, there is very little control in producing the RAPD products, which may contain confusing secondary structures when separating the products by gel electrophoresis. Research by Davin-Regli *et al.* (1995) showed that one could expect difficulties in reproducibility in RAPD fingerprinting (characterization) attributable to variations in DNA concentrations.

2.2.2.2 Restriction Fragment Length Polymorphisms

Restriction Fragment Length Polymorphisms or RFLPs (pronounced "ar-flips") is a technique roughly similar to RAPDs but its strategy tackles the problem of mutation visualization from a different direction. RFLPs have traditionally been used to resolve the coexistence of polymorphic alleles within populations (Griffiths *et al.*, 1993) and therefore have been used extensively in population ecology. RFLP protocol makes use of the existence of a large assortment of DNA cutters known as restriction enzymes. One selects a series of cutters and restricts the Polymerase Chain Reaction amplified DNA to reveal differences in size of cut pieces, which in turn reveals mutational differences (Dowling *et al.*, 1996). Fleischer's work (1996) suggested that the technique would have been especially useful for resolving systems such as those encountered in this study, in which gene variation (mutational rate) is low. However, one may encounter similar

problems to RAPDs because the imaging step at the end of both consists of a Southern blot and would therefore experience the same drawbacks.

2.2.2.3 Amplified Fragment Length Polymorphisms

AFLP (pronounced "ay-flip") is the acronym for Amplified Fragment Length Polymorphism and is quite similar to RFLPs in its manner of visualizing mutational differences between related specimens. Vos *et al.* (1995) described the technique which involved the following steps: the genomic DNA is restricted and the fragments are processed with a set of random primers. Next, a set of sequences of nucleotides (called adapters) is ligated to the ends of these fragments. Secondly, instead of the fragments being separated by a Southern blot (as in RFLPs), they are thermocycled in a PCR protocol and the products are run out on an acrylamide gel. This style of imaging displays the presence or absence of restriction fragments rather than length differences and so sidesteps many of the artifacts of the RAPD technique. Problems with this technique include the increased cost due to the PCR step and insufficient use to date. Although it is as technically difficult and costly as complete gene sequencing, Jones *et al.* (1997) and Blears *et al.* (1998) pointed out that it does have the added benefits of not relying on *a priori* knowledge of the sequence, as well as being highly reproducible.

2.2.2.4 Variable Number of Tandem Repeat markers

Variable Number of Tandem Repeat (VNTR) analyses are described by Fleisher (1996) as coming in two different types, minisatellites and microsatellites (or Short Tandem Repeats [STRs] or Simple Sequence Repeats [SSRs]). Both are useful because STRs and SSr are inheritable and have different mutational rates and so can be used as a molecular clock.

Minisatellites are nucleotide sequences (15-100 bases) that are present in tandem copies of between 20-50, usually totaling 100 to 5000 base pairs and are located mostly at the telomeres (Fleisher, 1996, Dowling *et al.*, 1996). They are thought to be caused by sequential and unequal crossing over during meiotic division. Microsatellites are

repetitions of only 2-10 base pairs and are present in tandem arrays of thousands of sets of copies in random order (Strassmann, 1996). The problem with this approach is that it is expensive to search and locate VNTRs within an uncharted genome. Assessement of their worth as a mutational clock is also very resource consuming. Generally, however, it is quite a robust system after this initial assessment (Strassmann, 1996).

2.2.2.5 DNA sequencing

DNA sequencing is a more complete method of analysis. The act of sequencing refers to the characterization of every single base pair position within a given set of primers via PCR, and then subsequent reading of the basepair products via gel electrophoresis. Kary Mullis (the Nobel Prize-winning inventor of PCR; 1991) summarized that PCR sequencing requires selection of a region to sequence and then building a set of bounding primers to amplify only that one sequence from the entire genome. This requires a large amount of *a priori* knowledge of the area of interest and therefore necessitates a selection of a specific gene. Often, primers are built by reverse modeling proteins of varying importance so as to predict the DNA sequence and then search around the gene for areas of appropriate mutational rate (Winnepenninckx & Backeljau, 1996). If resources are available to perform complete sequencing, I believe this is the preferable method because within a given area delimited by primers, one knows the entire relationship between each of the basepairs of every organism in the study. I chose a sequence coding for a portion of the 18S ribosomal RNA gene for this study because of its previous use and appropriate mutational rate (*see* section 2.2.2.6 below for further clarification).

2.2.2.6 Gene selection

Since the most appropriate technique is the complete sequencing of a specific universal piece of DNA and its subsequent analysis for differences (Honda, pers. comm., Swofford, *et al.*, 1996), the next step is the selection of this piece of DNA. This DNA should be a gene or gene segment of considerable functional importance since one needs

a fairly low rate of mutation to resolve at the subclass level (Kenchington, *et al.*, 1994). The appropriate mutational rate is the most important character of a prospective gene candidate, but there are many other factors that have to be considered. Several authors have described many of the necessary characteristics of a specific gene for this type of research. These are as follows:

1. The gene must be found in all the organisms in the survey i.e., the gene must be "universal" within the group. (Winnepenninckx *et al.*, 1994).
2. It must be "unambiguously homologous" or without having complicating multiple forms among different gene copies within a species (Dover, 1986).
3. The gene must be complex enough to make convergence of nucleotides or back mutations highly unlikely (Boore and Brown, 1994).
4. The product for which the sequence is coded must have the same function in all organisms in the survey (Winnepenninckx *et al.*, 1994).
5. It must have an alternate in conserved and variable regions to allow phylogenetic studies at a broad range of taxonomical levels (Winnepenninckx *et al.*, 1994).
6. All the organisms in the study must have sites that are strongly conserved to be able to make primers (Hillis & Dixon, 1991).
7. There should be a conserved secondary structure that facilitates the identification of homologous positions in regions with little sequence similarity (useful in alignment) (Winnepenninckx *et al.*, 1994)
8. In practical terms, the DNA sequence should be short enough for successful sequencing. The technique of multiple primer "gene walking" to characterize sections larger than ~600 base pairs is costly, especially if one uses the more accurate autoradiograms as opposed to fluorescence based auto sequencers (Uyeno, unpublished findings).

These criteria must be met for one to choose a DNA sequence and these are present in this study's sequence, a partial sequence encoding the 18S ribosomal RNA subunit.

The ribosome is present in any organism that produces proteins through translation, or the process by which messenger RNA copies of genes encoded in the DNA are decoded and serve as a blueprint for the assembly of amino acids into proteins (Frank,

1998). This important duty is so critical to an organism's survival that the portions of DNA that codes for the functional proteins remain relatively unmutated (De Rijk, *et al.*, 1992). Furthermore, this gene is safeguarded by being present in many separate copies in the genome (Wolfe, 1993, Griffiths, *et al.*, 1993).

Eukaryotic ribosomes consist of two subunits (Figure 1), which are termed 60S (or large subunit) and 40S (or small subunit), where S, the sedimentation coefficient, is measured in *Svedberg* units. These denote size by the relative rate at which molecules descend in a centrifugal gradient under standard conditions (Wolfe, 1993). The entire ribosome, composed of the 60S and the 40S components, sediment as an 80S particle (Griffiths *et al.*, 1993). The two subunits that fit together to form a ribosome themselves contain subunits that are composed of both RNA molecules and protein molecules. The 60S subunit contains three pieces of RNA which sediment at 28S (composed of about 4800 bases), 5.8S (160 bases) and 5S (120 bases), as well as 50 proteins labeled L1, L2, etc. The smaller 40S subunit contains only one piece of RNA, the 18S rRNA subunit, which is about 1900 nucleotide bases long. The protein moiety of the 40S subunit contains 33 proteins and these are labeled S1, S2, etc. (Griffiths *et al.*, 1993).

The many copies that encode for the rRNA subunits are arranged in clusters at one or more locations in the chromosomes of each species (Griffiths *et al.* 1993). All of the rRNA subunits, except for the 5S, are encoded in the genomic DNA in the following sequence from the 5' end to the 3' end: 18S, 5.8S and 28S. This DNA is transcribed into a very large pre-rRNA strand, which is then processed to form the three subunits.

The DNA coding sequences for these subunits are separated by intragenic spacers, which in some cases may be removed as late as the pre-rRNA processing stage. These intragenic spacers are areas where the mutational rate is extremely high (i.e., highly polymorphic) and sequencing may reveal very low-level phylogenetic details (Potts, 1996).

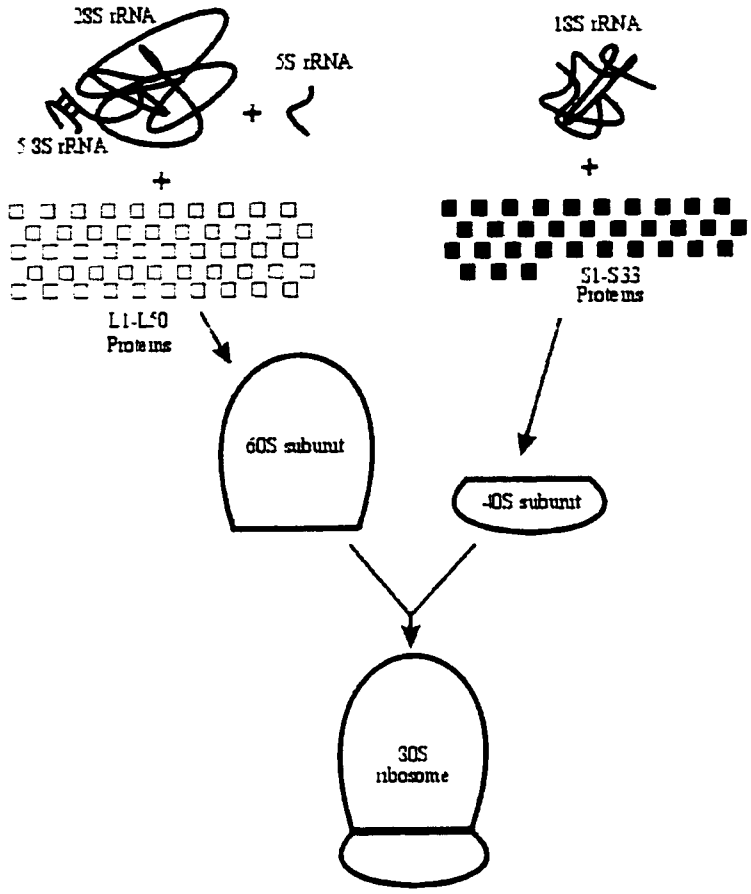

Figure 1. The eukaryotic ribosome (after Griffiths *et al.*, 1993).

The secondary structure of many invertebrate rRNA subunits has been resolved since the size of the RNA molecule is within the resolving power of some electron microscopes (Frank, 1996). De Rijk *et al.* (1992) and Winnepenninckx and Backeljau (1996) (Figure 2) described the folding rRNA structure that is the 18S subunit and identified various secondary structures of which the more common are hairpins, stems, bulge loops, interior loops, multibranched loops and pseudoknots (Wolfe, 1993).

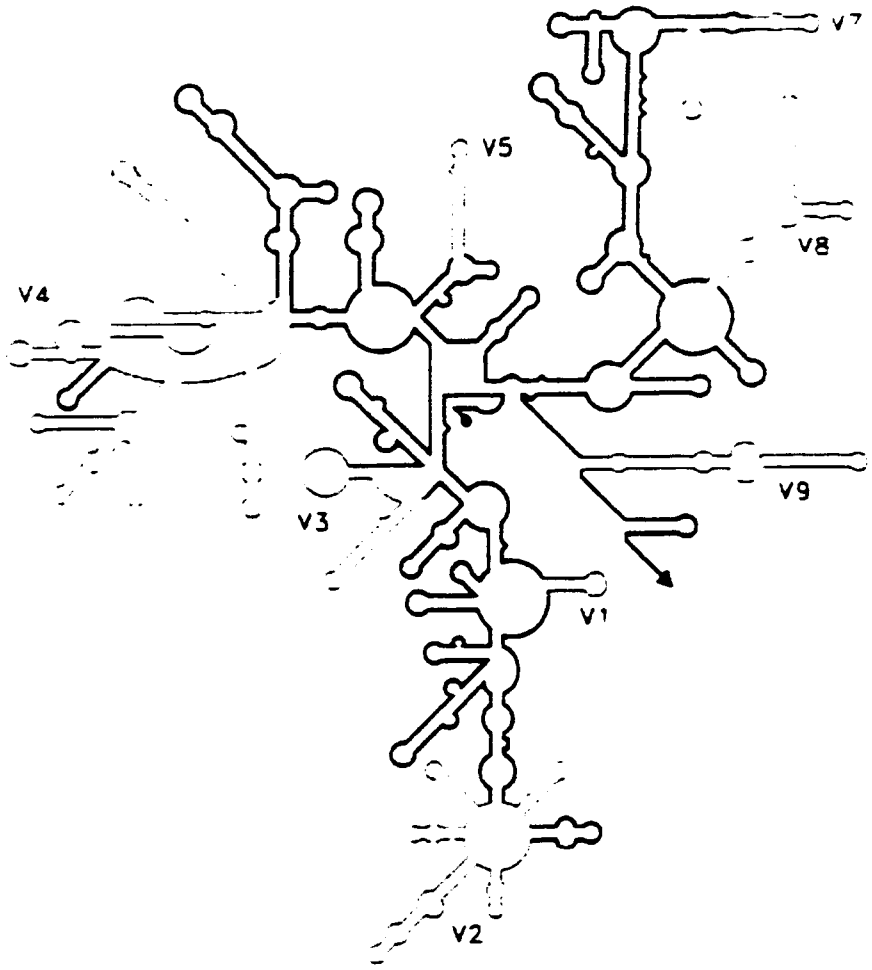

Figure 2. A composite18S rRNA secondary structure (after De Rijk *et al.*, 1992 and Winnepenninckx & Backeljau, 1996). Note: The solid heavy lines are conserved regions, the solid light lines are moderately conserved regions and the broken lines represent regions that do not occur in all species. The dotted end represents the 5' end and the arrow represents the 3' end.

Because of the flexibility of RNA double helices (formed in the paired regions of RNA), little is known about the higher-level tertiary structures (i.e. its three-dimensional folding) (Wolfe, 1993). As mentioned above, the secondary structure of the 18S rRNA has been studied in a multitude of organisms, both prokaryote and eukaryote, for the general areas of hairpins and loops.

This analysis (De Rijk *et al.*, 1992) has led to the definition of areas of variable primary and secondary structure, V1 to V9 (the variable region V6 is very conserved amongst eukaryotes and therefore that term is skipped). The variable regions are interspersed between helices that are composed of paired nucleotides. The helices are very important to the tertiary structure, which in turn is very important to functionality. Consequently these helices are highly conserved (De Rijk *et al.*, 1992). This means that there are sequences of variable regions and conserved regions. It is this quality that allows one to tune the "molecular clock" by selecting an appropriate section with the right amount of conserved and variable sequences in order to resolve at the desired taxonomic level. Harasewych *et al.* (1997a) found that the subclass level could be resolved adequately by characterizing a piece of the gene that codes for about 450 bases at the 5' end of the 18S gene and so I have used the primers that target this region in my thesis. This area codes for several highly conserved helices, and two variable regions (V1 and V2 in Figure 2).

2.3 The development of phylogenies of the class Gastropoda

2.3.1 The state and history of current gastropod schemes

Gastropods are and have been a very important group of animals to humans since prehistory (e.g. Varley, 1984). Many species in this class variously affect us by affording us food and conversely, vectoring parasites and feeding on crops (Pechenik, 1996). Thus, we have been studying them for generations. In order to study any group of organisms, one must first classify them in some meaningful way. Classifying organisms based on phylogeny is the most intuitive and reliable in that the groups are formed using strict evolutionary principles to find genealogic relationships (Wiley, *et al.*, 1991). However, phylogenetic information comes in many forms. The earliest gastropod studies were the domain of amateur conchologists (Kay *et al.*, 1998), who collected and classified

aesthetically pleasing shells according to colour, shape and geographical location. The second form of information arrived with the development and understanding of paleontological studies. The study of gastropod hard structures along with some gross dissection and in rare cases, histological sectioning, produced much of the information used by Milne-Edwards in 1848 (Bieler, 1992). The former proposed a class with three subclasses, Prosobranchia, Opisthobranchia and Pulmonata (Graham, 1985). Thiele (1929) subdivided the prosobranchs into three orders, the Archaeogastropoda, Mesogastropoda and Neogastropoda (Ponder & Lindberg, 1997) (Figure 3).
This highly-used scheme is referred to as the "classical" scheme within this thesis. It is the one supported by Zoological Record and it places 54% of the North American species within the Prosobranchia (although there is ample evidence of polyphyletic origins [Ponder & Lindberg, 1997]), 19% within Opisthobranchia and 26% within the Pulmonata (Turgeon *et al.*, 1988) (*see* Appendix IV sections 1 and 2 for numbers of species and familial lists). Tables 4 and 5 are a systematic classification of the organisms used in this study based on this classical scheme.

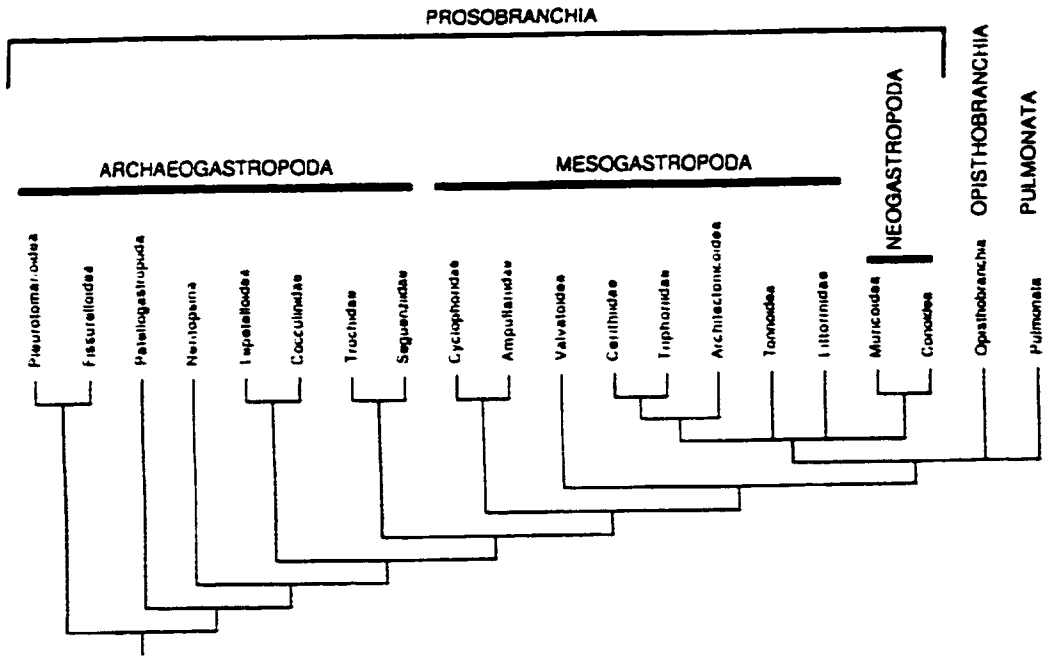

Figure 3. Gastropod phylogeny of Thiele, 1929 (after Ponder & Lindberg, 1995).

Table 1. Systematic structure of sampled polyplacophorans. (after Turgeon *et al.* 1988 and Zoological Records, 1998)

Class Polyplacophora		
Order	Family	Genus/species
Neoloricata	Ischnochitonidae	*Lepidozona mertensii*
	Mopaliidae	*Mopalia muscosa*

Table 2. Classical systematic structure of sampled organisms in the class Gastropoda. (after Turgeon *et al.* 1988 and Zoological Records, 1998)

Class Gastropoda			
Subclass	Order	Family	Genus/species
Prosobranchia	Archaeogastropoda	Haliotididae	*Haliotis rufescens*
		Fissurellidae	*Diodora aspera*
		Trochidae	*Calliostoma canaliculatum*
			Tegula pulligo
			Tegula funebralis
		Turbinidae	*Turbo castanea*
	Mesogastropoda	Pilidae	*Marisa cornuarietis*
			Pomacea bridgesi
		Naticidae	*Polinices lewisii*
	Neogastropoda	Muricidae	*Nucella lamellosa*
			Nucella ostrina
			Ceratostoma foliatum
		Buccinidae	*Searlesia dira*
Opisthobranchia	Nudibranchia	Discodoridídae	*Anisodoris nobilis*
			Diaulula sandiegensis
Pulmonata	Basommatophora	Lymnaeidae	*Lymnaea stagnalis*
		Planorbidae	*Helisoma trivolvis*

Between Thiele's work in 1929 and the late 1980s one interesting scheme was put forward by Golikov and Starobogatov (1975). Figure 4 is an adaptation of this tree which was based mostly on gill morphology.

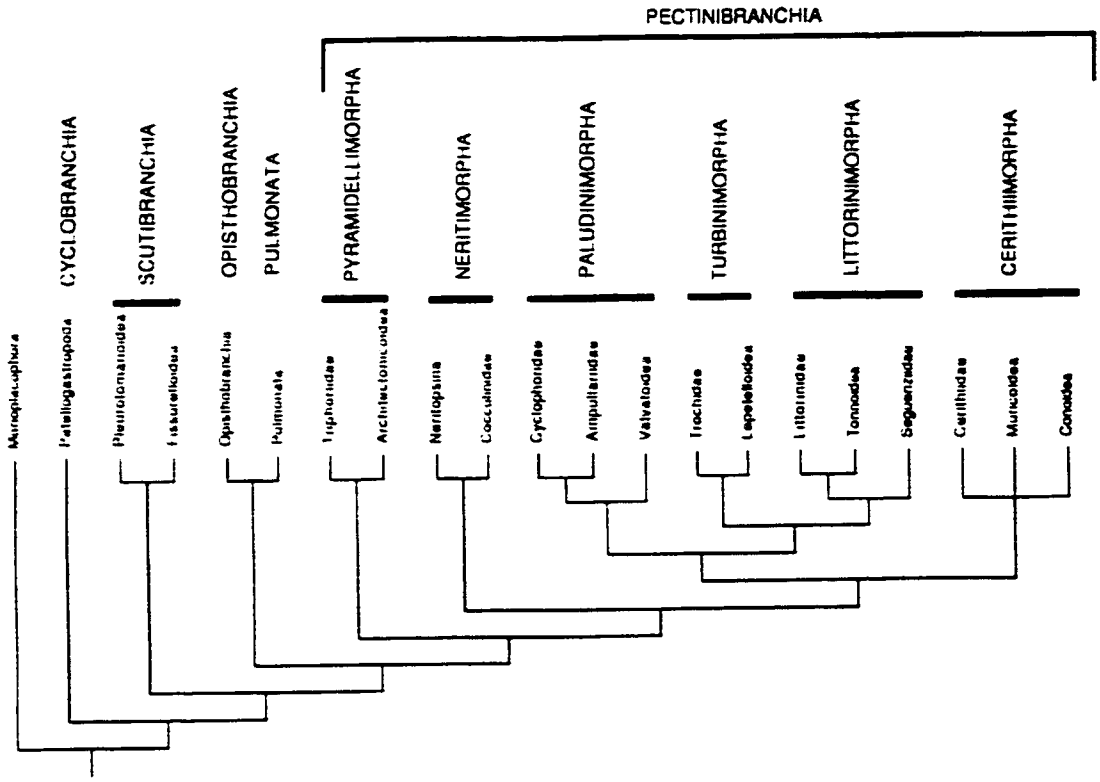

Figure 4. Gastropod phylogeny according to Golikov and Starobogatov, 1975 (after Ponder & Lindberg, 1995).

In the closing years of the 1980s, Hasprunar (1988) developed a very important model that attempted to concatenate morphological data for the expressed purpose of characterizing the higher taxa. His tree (Figure 5) was a breakthrough because it was designed using a large, multi-character matrix. A significant number of characters put forward in this tree have been incorporated into the current, multidisciplinary phylogeny of the Gastropoda and has helped direct gastropod phylogeny to its current state.

Ponder and Lindberg (1995) revolutionized Haszprunar's approach by codifying the large number of morphological characters into an analyzable matrix (Figure 6). Since its publication, the findings of many current malacologists have seemingly converged on this scheme, such as the recent, elegant embryological work done by Van Den Biggelaar and Haszprunar (1996) and work performed by Harasewych *et al.* (1998).

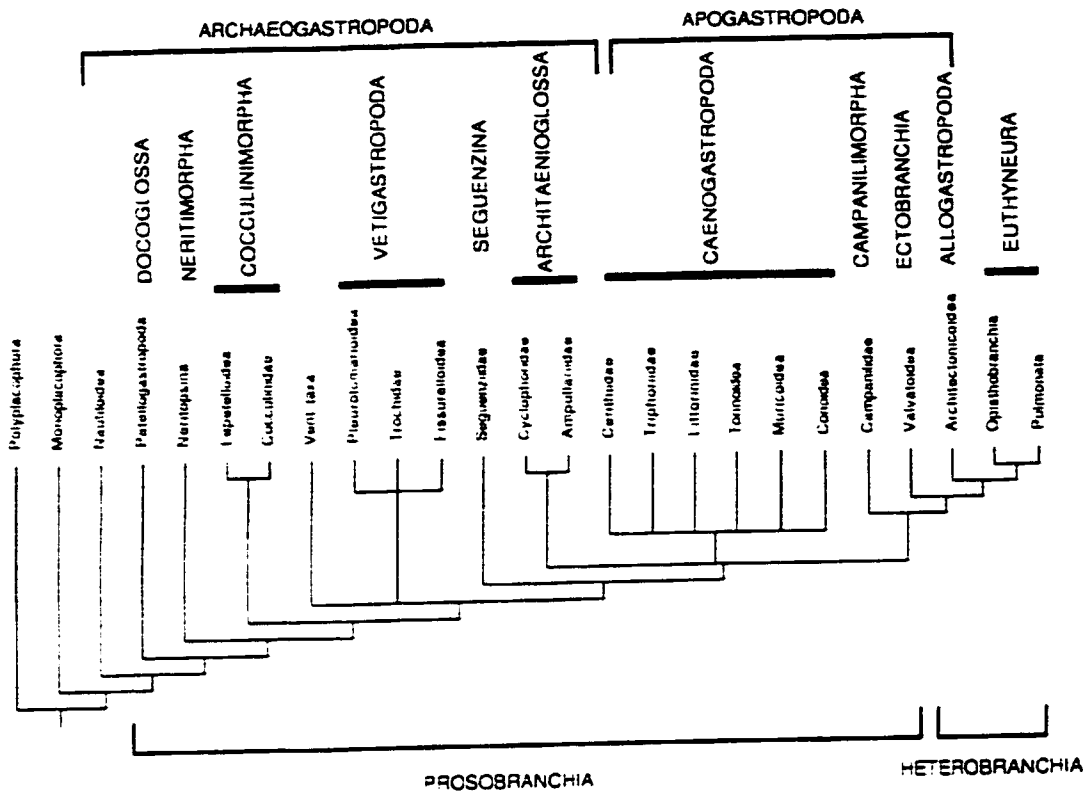

Figure 5. Gastropod phylogeny according to Haszprunar, 1988 (after Ponder & Lindberg, 1995).

Ponder and Lindberg (1997) continued to add to their codified character matrix, as they and many others realized the value in it as an important framework on which one could build and computer analyze new characters.

Winston Ponder of the Division of Invertebrate Zoology at the Australian Museum (1999) echoed a relieved sentiment shared by many gastropod phylogeneticists: "There is some general agreement now about what the higher groups in the gastropods are". Thus most gastropod workers (and unfortunately it seems to be restricted to gastropod workers) agree on a recently expanded core set of characters and groupings that is embodied in the latest and most complete morphological character phylogenetic scheme (Ponder & Lindberg, 1997) (Figure 7). This is the scheme referred to as the multidisciplinary scheme.

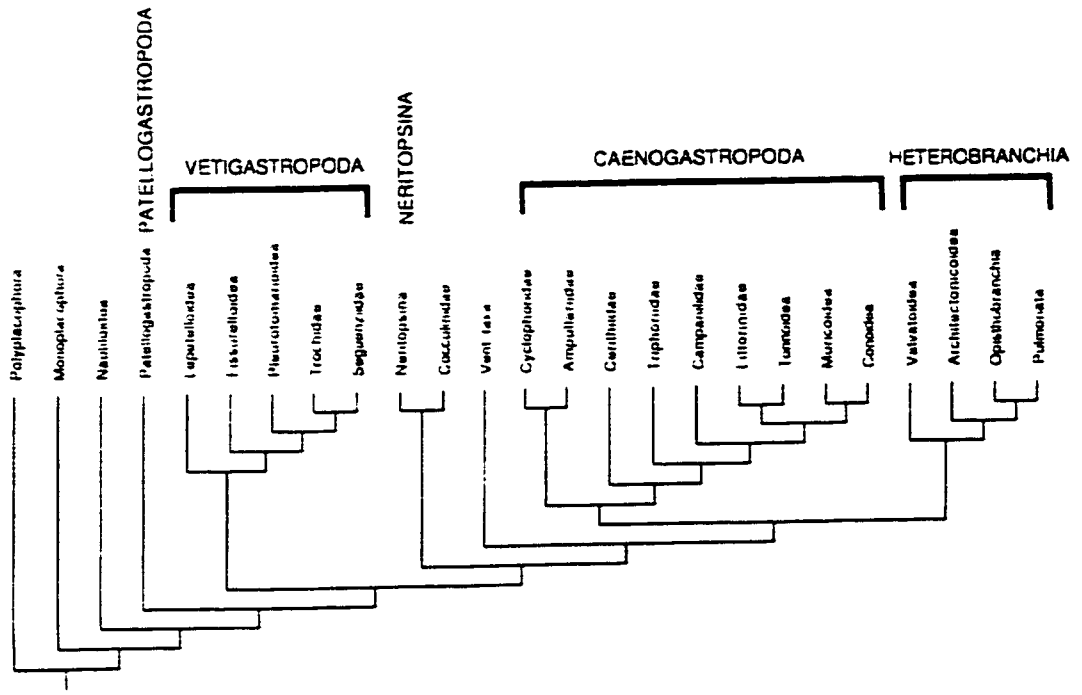

Figure 6. Ponder and Lindberg's preliminary phylogeny, 1995 (after Ponder & Lindberg, 1995).

The Vetigastropoda comprise the majority of the old Archaeogastropoda, the remnants of which are placed in the Patellogastropoda and some other small groups. The Patellogastropoda are now considered to be the most primitive of all the Gastropoda. This is true to such an extent that Ponder and Lindberg (1995) suggested that they should be placed as a sister group to the rest of the gastropods.

The notion of the Caenogastropoda is an important theme put forward by Haszprunar (1988) and Ponder and Lindberg (1995), although it is now generally thought that the caenogastropods are comprised of the previous Mesogastropoda and Neogastropoda. Finally there is the Heterobranchia, which include what some consider higher caenogastropods and the Euthyneura (opisthobranchs and pulmonates). However, the relationships of the major clades to one another are still somewhat fluid as well as the placement of certain enigmatic groups such as the Cocculinidae (Ponder, pers. comm., 1999). Table 3 shows the organisms used in this study and their new taxonomic designations.

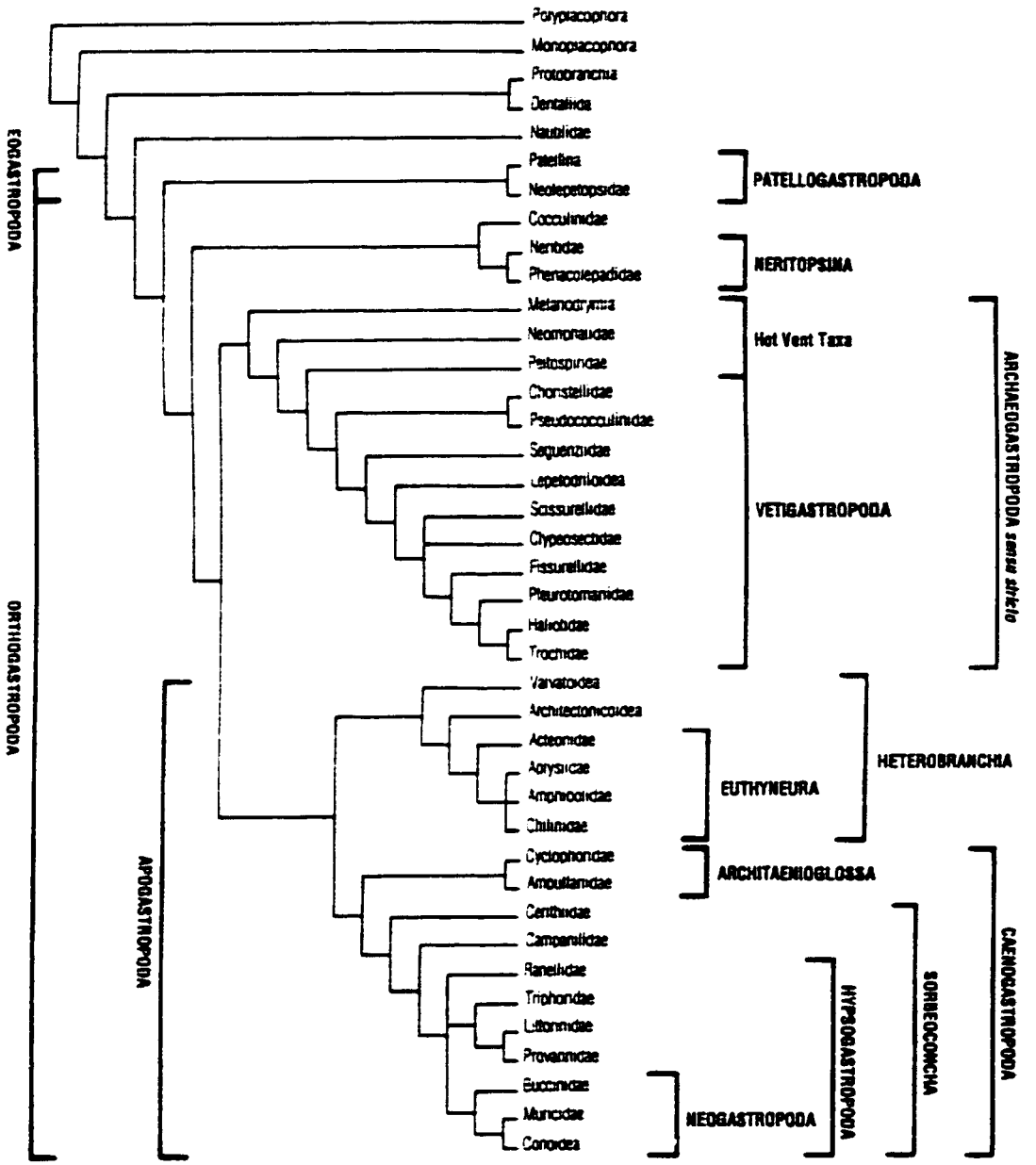

Figure 7. Ponder and Lindberg's phylogenetic synthesis, 1997 (after Fretter *et al.*, 1998).

Table 3. Multidisciplinary systematic structure of my study organisms.

Class Gastropoda				
Subclass	Superorder	Order	Family	Species
Vetigastropoda			Haliotididae	*Haliotis rufescens*
			Fissurellidae	*Diodora aspera*
			Trochidae	*Calliostoma canaliculatum*
				Tegula brunnea
				Tegula funebralis
			Turbinidae	*Turbo castanea*
Caenogastropoda	(Eucaenogastropoda)		Naticidae	*Polinices lewisii*
			Muricidae	*Nucella lamellosa*
				Nucella ostrina
				Ceratostoma foliatum
			Buccinidae	*Searlesia dira*
	(Architaenioglossa)		Pilidae	*Marisa cornuarietis*
				Pomacea bridgesi
Heterobranchia	Euthyneura	Opisthobranchia	Discodoridídae	*Anisodoris nobilis*
				Diaulula sandiegensis
		Pulmonata	Lymnaeidae	*Lymnaea stagnalis*
			Planorbidae	*Helisoma trivolvis*

It should be noted that these phylogenetic trees are, for the most part, based on morphological and not molecular characteristics. This is because the molecular data are only now starting to be accumulated. Harasewych *et al.* (1997) (*see* Figure 8) and Tillier, *et al.*, 1992 (*see* Figure 9) are among the first to publish molecular-based trees of higher

gastropod taxa. It is comforting that these new techniques deliver cladograms that roughly agree with the morphological consensus because they validate and build upon each other.

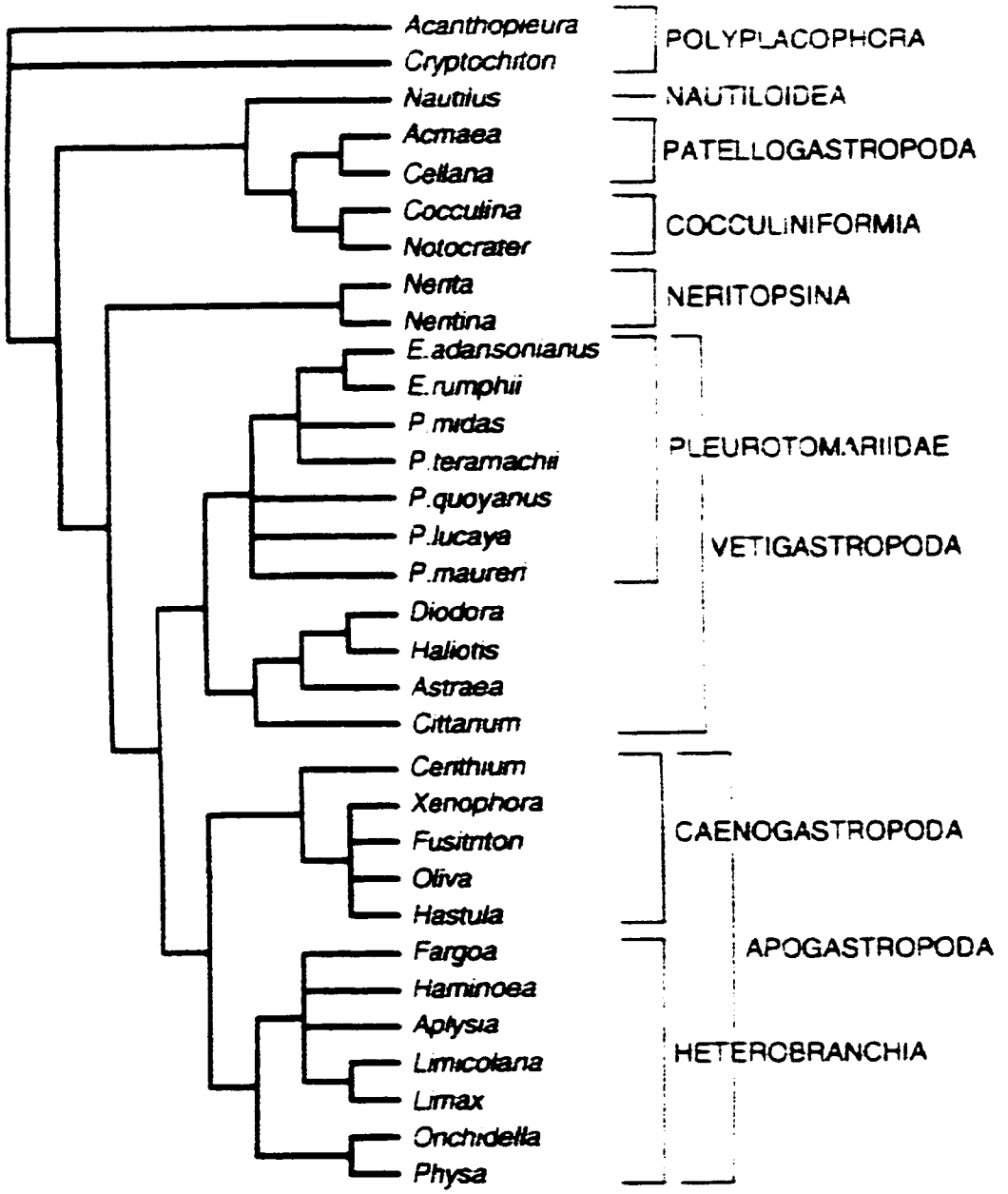

Figure 8. Molecular (18S rRNA) based phylogeny according to Harasewych *et al.*, 1997a.

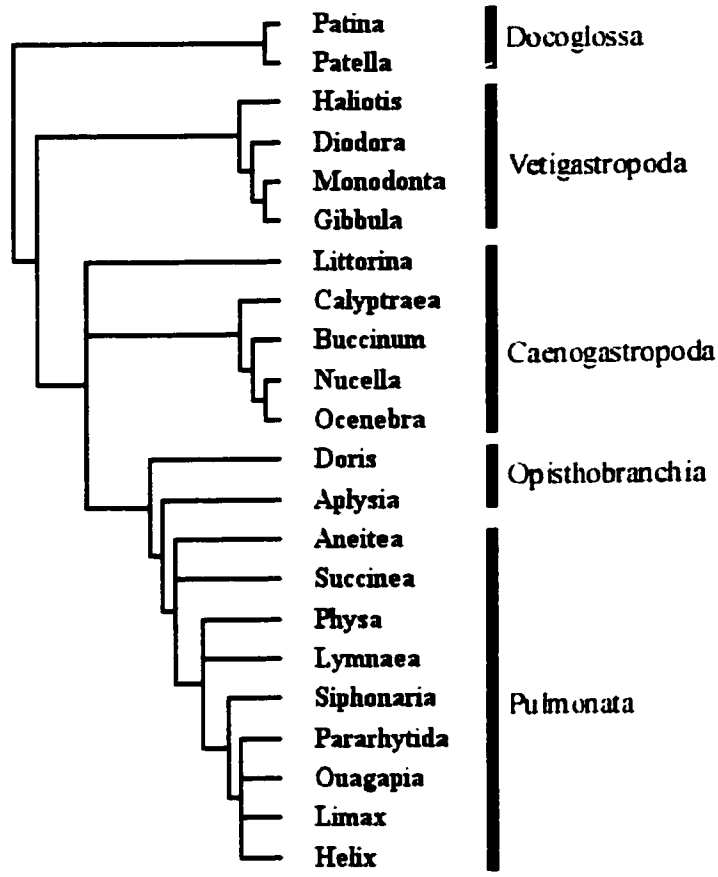

Figure 9. Molecular (28S rRNA) based phylogeny according to Tillier *et al.*, 1992.

2.3.2 Past research in gastropod phylogeny

Past research in published literature pertaining to gastropod phylogeny falls into one of four categories. The first category includes those papers that deal with molecular phylogeny which are often about species identification and occasionally phylogeography (e.g. Kyle & Boulding, 1998). However, there are some papers dealing with the relationships of families and orders that in turn have bearing on subclass structure (e.g. Harasewych, *et al.*, 1997a). The second category represents evidence from the geological record, which lends support to more conservative systematic schemes (e.g. Runnegar & Pojeta, 1985). A third category is represented by morphological data where locomotory musculature and other organ systems have greater relevance (e.g. Croft, 1955 or Miller,

1974). Fourthly are papers dealing with developmental and embryological information (e.g. Van Den Biggellaar & Haszprunar, 1996). In terms of where knowledge is lacking, many gastropod phylogeneticists (Haszprunar, 1988, Bieler, 1992, Ponder & Lindberg, 1997 and Harasewych, 1994) point out the many gaps, and more specifically in the areas of development, ultrastructure and especially molecular biology.

2.3.2.1 On the geological evidence pertaining to gastropod phylogeny

Other than conchological initiatives, research interests pertaining to paleontological endeavours are the first useful studies in gastropod systematics. Please refer to the geological timescale in reference to the following review (Figure 10).

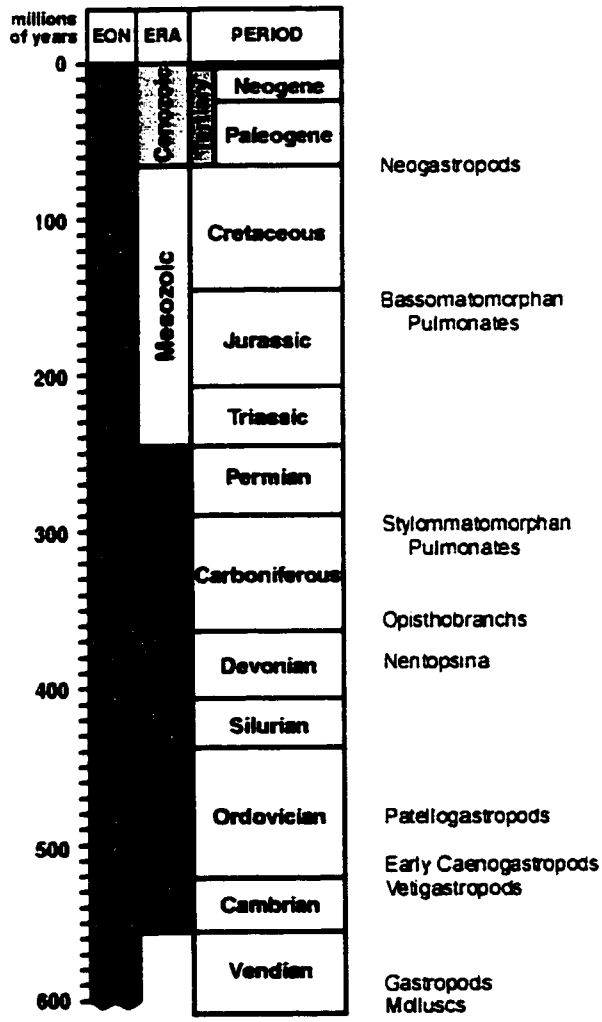

Figure 10. Geological timescale (adapted from MacRae, 1996).

According to Runnegar and Pojeta (1985) and Vostokova (1962) the oldest known molluscan fossils are about six hundred million years old, which places the origin of the phylum before the Precambrian/Cambrian boundary (Runnegar & Pojeta, 1985). These early Cambrian molluscs included monoplacophorans, rostroconchs, bivalves and gastropods. In fact, all the major groups from the class Gastropoda, which possess biomineralized structures, are found with relatively complete representation in the fossil record (Graham, 1985, Schmekel, 1985, Solem, 1985). These early gastropods were very small and the generic and specific diversity remained low for quite a while (Runnegar, 1983). Some belong to the primitive subclass taxon of Paragastropoda. The Paragastropoda are thought to be extinct sinistrally-coiled precursors (Linsley & Kier, 1984) to the Patellogastropoda, which appear in the Middle Ordovician (Yochelson, 1988). Yochelson (1998) thought that the class may be diphyletic, which is an idea supported by Ponder and Lindberg (1997). Runnegar (1981) proposed that torsion developed twice in the Gastropoda. The first instance was in the dextral pelagiellids and the second was in conjunction with the development of torted ultradextral forms from untorted dextrally coiled forms. Runnegar's findings (1981) lend further support to the polyphyletic nature of this class.

The Vetigastropoda are among the first extant subclasses to appear, present in the Upper Cambrian (Knight *et al.*, 1960). Yochelson (1963) noticed a second evolutionary event in the Late Cambrian/Early Ordovician stages that saw the development of polyplacophorans and cephalopods as well as observing larger gastropods that were previously small to microscopic. This development is hypothesized to have allowed the further development of other prosobranchs (including the early Caenogastropoda or mesogastropods).

The mesogastropod grade of caenogastropod was first to appear during the Late Cambrian to Early Ordovician, as mentioned earlier. Organisms that undoubtedly could be considered neogastropod (Cyclophoroidea and Ampullarioidea) do not appear in the fossil record until the Cretaceous and early Cenozoic, however (Taylor & Morris, 1988, Ponder, 1973).

The other major group, the Euthyneura, is comprised of the opisthobranchs and pulmonates. The opisthobranchs descended from the Cambrian ultradextral gastropods and appeared for the first time in the Lower Carboniferous (Kollmann and Yochelson, 1976, Yoo, 1994). The Pulmonata, represented by stylommatomorphan snails, begin in the Upper Carboniferous and are followed by basomatomorphan land snails in the Upper Jurassic (Solem & Yochelsom, 1979).

Finally, there are the rather systematically fluid taxa of the Neritopsina and the Cocculiniformia. The Neritopsina appear first in the Middle Devonian Epoch (Knight *et al.*, 1960). The taxon Cocculiniformia was designated by Salvini-Plawen and Haszprunar (1987) to describe the Cocculinoidea and the Lepetelloidea. However, Ponder and Lindberg (1995) challenged this designation and sorted the Cocculinoidea with the Neritopsina and the Lepetelloidea with the Vetigastropoda.

2.3.2.2 On molecular phylogeny research

Recently there has been a flourish of activity in the field of phylogenetic character development based on molecular techniques. Molecular techniques have been used for some time as a tool to discriminate mostly among lower taxa. Davis (1994) noticed that microbiologists, mammalogists and herpetologists have been using these techniques, in some cases, for four decades now (e.g. Hunter & Markert, 1957 and Harris, 1966) and malacologists are just now beginning to take full advantage of this powerful tool. The first studies that incorporated molecular techniques involved allozymes comparisons for resolving relationships (Buth, 1984). It has only been in the last ten or so years that the vast majority of molluscan DNA and RNA sequences have been published (e.g., Tillier *et al.*, 1992, 1994, Harasewych *et al.*, 1994, 1997a,1997b, 1998, Winnepenninckx *et al.*,1993, 1994, 1996, 1998). In fact, at the time of writing, Davis (1994) was quoted as saying that "Allozyme electrophoresis is an ideal tool for population genetics as applied to delineating species", and that "DNA-RNA sequencing was in its infancy", and "literally exploding in dimensions of use, problems and surprises".

Since the incorporation of DNA analysis in gastropod research, many workers have begun testing gene suitabilities and characterizing novel gene sequences. Testing

gene suitabilities, especially for mutational rate is very important to this research (*see* Section 5.1) and many people are credited (as noted below) with much of this testing on specimens belonging to several different subclasses. The results of this testing seems to favor several genes as being useful in resolving the hierarchy of higher taxa. Tillier *et al.* (1992, 1994) sequenced many gastropods (especially the Hot-Vent limpets) using primers that focus on 28S rRNA gene. Winnepenninckx *et al.* (1994, 1998) focused on several sites included in the number of genes that code for the smaller RNA portions of the ribosome (generally referred to as the SSU rRNA complex), including the 18S rRNA subunit. Harasewych, McArthur and Adamkewicz have routinely used 18S rRNA genes to analyze relationships within pleurotomariid gastropods (Harasewych *et al.* 1997a), resolve neogastropod phylogeny (Harasewych *et al.* 1997b) mesogastropod phylogeny (the lower Caenogastropoda, Harasewych *et al.* 1998) and more recently limpets and deep-sea cocculinids (McArthur & Harasewych, pers. comm., 1999).

2.3.2.3 On research pertaining to morphological phylogeny

Ponder and Lindberg (1997) presented a description of the research completed on morphological characters in their excellent and comprehensive work that I discuss below. In searching through the literature in preparation of this review, I was only able to find papers dating back twenty years, or else papers that were published in the late 1800s or early 1900s. Ponder and Lindberg (1997) noticed that this initial flourish of systematic research was based on single organ systems (Ponder and Lindberg [1997] reference Troschel's [1956] work on radulae and Spengel's [1881] and Bouvier's [1887] work on nervous systems). To this I would add that much embryological work was completed at this time (*see* Section 2.4.2.4 below). Surveying these works, including that of Milne-Edwards (1848), Thiele (1929-1931) wrote his *Handbuch der Systematischen Weichtierkunde* with the tripartite classification scheme (Prosobranchia, Opisthobranchia, Pulmonata). There have been many recent papers dealing with phylogeny using morphological characteristics such as osphradia (Haszprunar, 1985), sperm ultrastructure (Healy, 1988), excretory systems (Andrews, 1988), neurobiology, (Dorsett, 1986), larvae (Fioroni, 1982) and respiratory structures (Lindberg, 1989). Many more papers were

descriptive in nature and published on specific organisms (e.g. Crofts, 1929 (*Haliotis*) or Bekius, 1972 (*Lymnaea*)). A synthesis of many of these ideas was brought together in Wilbur's (1988) "The Mollusca" series. A current and celebrated publication on this topic are volumes 5a and 5b (entitled "Mollusca: The Southern Synthesis") in the series "Fauna of Australia" (Beesley *et al.*, 1998). Haszprunar suggests that this is probably the most detailed and best recent review on the subject and will be the standard for the next few years (pers. comm., 1999). Unfortunately, all the information in these publications is descriptive and in a format that does not lend itself to being rigorously tested using phylogenetic analysis programs.

As sufficient data was compiled to make a robust analysis of gastropod phylogeny, the next logical step was to begin the immense work of sifting, comparing, and grading all this published information into a system whereby one could compare the data. Ponder and Lindberg (1995) published such a data set in which 25 taxa and 95 characters were analyzed. This data set was extremely interesting in that the results suggested that the gastropods were possibly not monophyletic and so the authors coined the terms Eogastropoda (the very primitive Patellogastropoda and their coiled ancestors) and the Orthogastropoda (all the other gastropods). In order to test this distinction and to build a more rigorous phylogeny, Ponder and Lindberg (1997) gathered more characters and published the work described below.

The largest and most complete work put forward in the field of morphological phylogeny is the recent monograph by Ponder and Lindberg (1997). Published in the Zoological Journal of the Linnean Society, it is recognized as being the most comprehensive framework of morphological characters. It contains a survey of 117 characters and includes 40 taxa and five outgroup taxa. The characters include aspects of the shell, operculum, muscle, ctenidium, renopericardium, reproduction, digestion, nervous system, development, hypobranchial gland and foot morphology.

2.3.2.4 On the research based on ontogeny and embryology

There are many descriptive works on gastropod development, most of which are on organisms that are of economic importance. Among the gastropods examined are *Haliotis* (Crofts, 1929,1937), *Aplysia* (Carazzi, 1905), *Crepidula* (Conklin, 1897), *Littorina* (Delsman, 1914), and *Limax* (Kofoid, 1894). More recent papers dealing with gastropod ontogeny tend to be parts of more complete morphological description of lesser known species or because the organisms are important as models in other fields of science. Some examples are *Sinotaia* (a pond snail) (Tanaka *et al.*, 1987), *Patella* (Damen & Dictus, 1994) and *Lymnaea* (Van Den Biggelaar, 1976, Martindale *et al.*, 1985).

Van Den Biggelaar and Haszprunar (1996) surveyed the literature on fate mapping and mapped several new species and analyzed the data for ontogenetic clues to the phylogeny of the class. They (*ibid.*, p. 1520) concluded that "the comparison of the early cleavage patterns appeared to be a powerful method for investigating the evolutionary relations between major gastropod taxa", and that " the larger gastropod taxa are characterized by distinctive cleavage patterns"(Van Den Biggelaar & Haszprunar, 1996). The results of this survey showed that there were modifications to how the 3D cell (an early stem cell) divided and gave rise to the mesentoblast. In the gastropods, the mesentoblast is formed between the 24-cell stage and the 63-cell stage (*see* Figure 11).

Polyplacophorans, the outgroup for this thesis, develop their mesentoblasts late, at around the 70-cell stage. The next group, the Docoglossa (=Patellogastropoda), and the Vetigastropoda form their mesentoblasts at the 63-cell stage. The difference between these groups is that the Docoglossa 63-cell stage lasts relatively long whereas in the Vetigastropoda the stage terminates quite quickly. The next slower group are the Architaenioglossa (or lower Caenogastropoda, which includes *Pomacea*) which form their mesentoblasts at the 44- to 48- cell stage. The rest of the Caenogastropoda seems to develop their mesentoblast more quickly, before the 40-cell stage. In fact, some of the more advanced caenogastropods develop their mesentoblasts at the 24-cell stage, which is the stage that the pulmonates and opisthobranchs (the Euthyneura) develop theirs. An

interesting problem is that the Valvatoidea (belonging to the Ectobranchia or Heterobranchia) is often considered a primitive euthyneuran or an advanced apogastropod. The Valvatoidea develop their mesentoblast relatively late, which seems to be contrary to much of the other phylogenetic data.

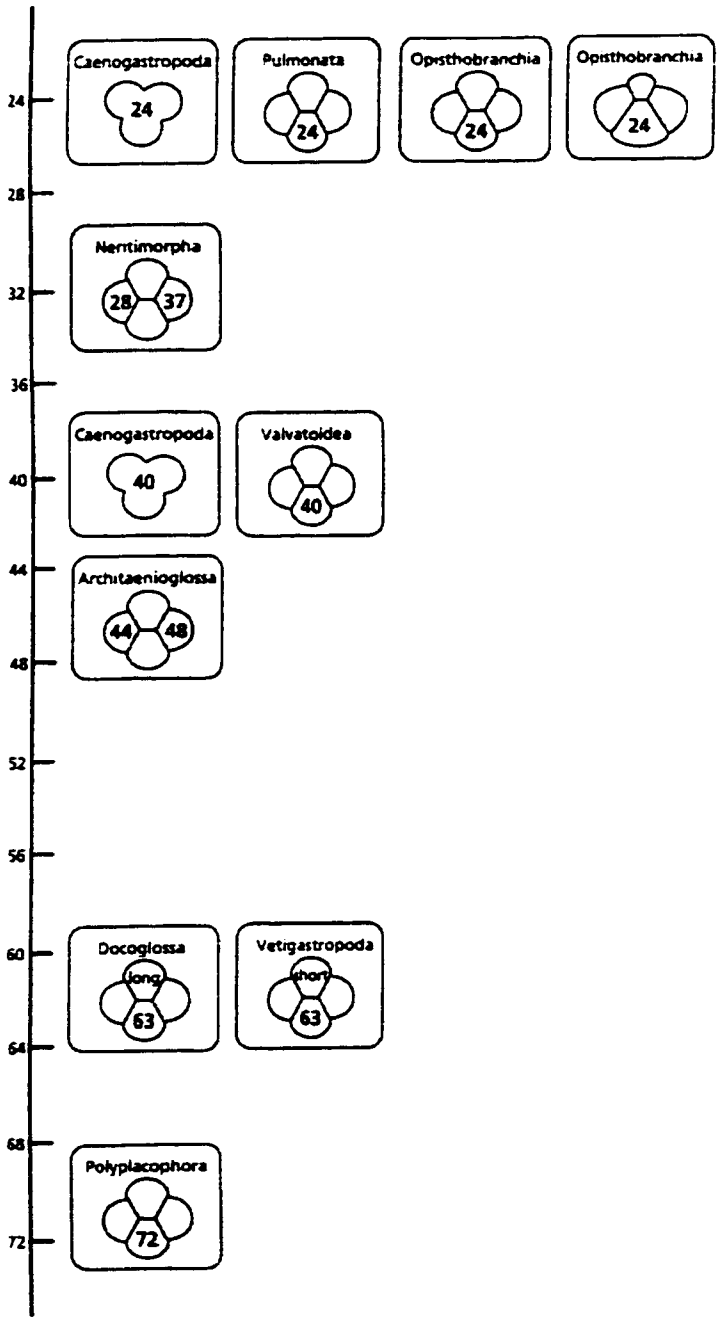

Figure 11. The classification of Polyplacophora and higher gastropod taxa with respect to mesentoblast formation. (after Van den Biggelaar and Haszprunar, 1996)

CHAPTER THREE
THE GASTROPODS OF THESE STUDIES

This chapter introduces the animals that were directly and indirectly used in both the molecular and morphological studies. It briefly introduces the organisms, their localities and their biology. Over and above this general overview, I will give specific specimen information within the studies themselves in the appropriate places. All the organisms used in this study were given care and were handled in procedures that followed the guidelines approved by the Canadian Council of Animal Care.

I have selected gastropods and polyplacophorans present in North America. The majority of the marine species were collected in the vicinity of the Bamfield Marine Station on the southwestern edge of Vancouver Island in British Columbia, Canada. All of the animals that were imaged (*Calliostoma canaliculatum*, *Diaulula sandiegensis*, *Haliotis rufescens*, *Lymnaea stagnalis*, *Marisa cornuarietis*, *Nucella lamellosa*, *Pomacea bridgesi* and *Searlesia dira*) were also sequenced and subjected to histological treatments. Tables 4 and 5 list the full binomen, the author/year of description and the common name.

Table 4. Scientific and common names of polyplacophorans used in this research.

Polyplacophorans (Chitons)	
Binomen, author, year	Common name
Lepidozona mertensii, Middendorff, 1847	Red chiton
Mopalia muscosa, Gould, 1846	Mossy chiton

The polyplacophorans listed in Table 4 were selected as an outgroup to define the extent of differences found within the gastropods. In this thesis, there were two possible outgroups to which the gastropod cladogram could have been rooted. Bieler (1992) quoted various authors as saying that the two closest classes were the Polyplacophora and the Cephalopoda. To this I would add the monoplacophorans, however, their scarcity precluded my investigation of them. The polyplacophorans were selected over the

cephalopods as they were much easier to collect and maintain until extraction. The second set of reasons for their selection was that they inhabit similar niches, have similar diet, and externally resemble many of the gastropods studied herein. In this study, two northern Pacific chitons were sequenced and their DNA analyzed.

Table 5. Scientific and common names of gastropods used in this research.

Gastropods (Snails/Slugs)	
Binomen, author, year	Common name
Anisodoris nobilis, MacFarland, 1905	Pacific Sea-lemon
Calliostoma canaliculatum, Lightfoot, 1786	Channeled topshell
Ceratostoma foliatum, Gmelin, 1791	Foliate thornmouth
Diaulula sandiegensis, J.G. Cooper, 1863	Ringed doris seaslug
Diodora aspera, Rathke, 1833	Arcuate keyhole limpet
Haliotis rufescens, Swainson, 1822	Red abalone
Helisoma trivolvis, Say, 1817	Small ramshorn
Lymnaea stagnalis, Linnaeus, 1758	Common swamp pond snail
Marisa cornuarietis, Linnaeus, 1758	Giant ramshorn
Nucella lamellosa, Gmelin, 1791	Frilled dogwhelk
Nucella ostrina, Gould, 1852	Northern dogwhelk
Polinices lewisii, Gould, 1847	Pacific moon snail
Pomacea bridgesi, Reeve, 1856	Spiketop Applesnail
Searlesia dira, Reeve, 1846	Dire whelk
Tegula pulligo, Gmelin, 1791	Dusky tegula
Tegula funebralis, A. Adams, 1855	Black tegula
Turbo castanea, Gmelin, 1791	Chestnut turban

The common names in Tables 5 and 6 were agreed upon by the Committee on Scientific and Vernacular Names of Mollusks of the Council of Systematic Malacologists and the American Malacological Society (formerly the American Malacological Union) and were published by Turgeon *et al.* (1988).

I find that more intuitive systematic decisions can be made if one is familiar with other aspects of the biology of the sample organisms. Consequently, I prepared Table 6 based on some other biological information, which will allow the reader to paint a picture of the organism in its surroundings and aspects of its life style. I cite Kozloff (1996), Thorpe (1962) and Strathmann (1987) as well as my own observations as sources on polyplacophoran information. Strathmann (1987), Kozloff (1996) are two major sources

on general localities and development types for the marine gastropods. Abbott & Haderlie (1980) is the source on *Diodora*. Palmer (1980) and Newel (pers. comm., 1999) are the sources on *Nucella* species. Bloom (1976) is a source on sea slugs. Perera and Walls (1996) is the source for *Marisa* and *Pomacea*, the large freshwater species. Fretter & Graham (1962) and Abbott (1974) wrote on the *Turbo* species, and they are also useful general morphological and conchological sources, respectively.

Table 6. Some background information on each species in this thesis

Species		Character				
		Zonation	Size	Development	Nutrition	Appearance
Polyplacophora	*Lepidozona mertensii*	Low intertidal/ subtidal	11 mm long	Free spawning, hatch as trochophore	Unknown, probably biofilms	Brownish red and plates with white lines
	Mopalia muscosa	Mid intertidal	50 mm long	Free spawning, hatch as early trochophore	Algae and other biofilms	Brownish green with one central white stripe. Rimmed w/ bristles
Gastropoda	*Anisodoris nobilis*	Low intertidal/ subtidal to 85m	60 mm long	Whorled egg masses contain capsules, hatch as veligers	Sponges only, especially *Halichondria*	Slug like body, white with black/brown spots
	Calliostoma canaliculatum	Low intertidal/ subtidal	15 mm tall	Hatch as veligers with protoconch	Herbivores (kelp beds)	Squat top-like shell
	Ceratostoma foliatum	Mid to high intertidal	25 mm long	Hatch as crawl away juveniles from stalked capsules	Carnivores, bivalves and barnacles	Cream coloured trilobed shell with obvious tooth near opening
	Diaulula sandiegensis	Low intertidal /subtidal to 37m	70 mm long	Hatch from capsules within whorled egg masses as veligers	*Halichondria* and *Haliclona* only (sponges)	Large fleshy yellow sluglike body with black spots
	Diodora aspera	Low intertidal	25 mm wide	Eggs are shed in soft gelatinous spawn. Little else known	Encrusting Sponges and Bryozoans	Caplike shell with hole in top. Circular foot
	Haliotis rufescens	Subtidal	55 mm long	Eggs are shed in gelatinous spawn, hatch as veligers	Herbivores, Kelp beds	Flat dishlike shell, broad strong oval foot

Helisoma trivolvis	Fresh water	15 mm dia.	Large soft clumps of eggs hatch crawlaway juveniles	Herbivores/ detritivores feed on lettuce in lab	Planispiral shell that is slightly red
Lymnaea stagnalis	Fresh water	28 mm long	Small soft eggs in gelatinous strip, hatch as crawl away juveniles	Herbivores, detritivores, feed on lettuce or kale in lab	Long transparent spire. Soft yellowish foot
Marisa cornuarietis	Fresh water	35 mm dia.	Eggs in bright orange gelatinous matrix, hatch as crawl away juveniles	Herbivores, feed on lettuce and carrots in lab	Large planispiral shell, yellow with brown stripes
Nucella (=*Thais*) *lamellosa*	Low intertidal/ subtidal	30 mm long	Eggs hatch from stalked capsules as crawl away juveniles	Carnivores, mussels and barnacles	Very hard spired shell
Nucella (=*Thais*) *ostrina*	High intertidal	23 mm long	Eggs hatch from stalked capsules as crawl away juveniles	Carnivores, mussels and barnacles	Thinner shell, high spire
Polinices lewisii	Low intertidal/ subtidal	70 mm long	Eggs packaged in mucous semented sand, crawl away juveniles	Carnivores, Clams and other bivalves	Tan spherical shell with a highly extensible foot
Pomacea bridgesi	Fresh water	47 mm long	Above water surface hard green egg masses hatch as crawl away juveniles	Herbivores, feeds on lettuce in the lab	Brownish green spherical shell with dark stripes
Searlesia dira	Low intertidal	33 mm long	Blister-like capsules hatch carnivorous crawl away juveniles	Carnivores, specializes on scavenging hurt prey	Long spindle like shell similar to Nucella
Tegula pulligo	Mid to high intertidal	30 mm high	Little known	Herbivores, biofilms	Squat black spiralling shell
Tegula funebralis	High intertidal	25 mm high	Little known	Herbivores, biofilms	Squat brown/red spiralling shell
Turbo castanea	Low intertidal	40 mm long	Little known	Calcareous encrusting algae	Chalky knobbed squat spiralling shell.

Table 7 summarizes the physical localities from where these organisms were collected and in which studies they appear. I am indebted to The Abalone Farm, Inc. of Cayucos, California, U.S.A. for providing appropriately sized abalones from their grow-out facility. Although I used SCUBA to collect marine snails, the majority were collected for me by Shane Servant, Boat and Diving Officer at the Bamfield Marine Station. *Helisoma trivolvis* was acquired from Franko Wu and Bill Ho at the Department of Biological Sciences, University of Calgary. *Marisa cornuarietis* and *Pomacea bridgesi* were purchased at a local shop, which ships them in from Venezuela. Finally Dr. Syed donated many *Lymnaea stagnalis* from his stock, which are used in his neurobiological research at the Department of Physiology and Biophysics, University of Calgary.

Table 7. Types of study carried out and localities from which the organisms were obtained.

Species		Character			
		DNA	MRM	Histology	Locality
Chitons	*Lepidozona mertensii*	Yes			Bamfield Marine Station, BC
	Mopalia muscosa	Yes			Bamfield Marine Station, BC
Gastropoda	*Anisodoris nobilis*	Yes			Bamfield Marine Station, BC
	Calliostoma canaliculatum	Yes	Yes	Yes	Bamfield Marine Station, BC
	Ceratostoma foliatum	Yes			Bamfield Marine Station, BC
	Diaulula sandiegensis	Yes	Yes	Yes	Bamfield Marine Station, BC
	Diodora aspera	Yes			Bamfield Marine Station, BC
	Haliotis rufescens	Yes	Yes	Yes	The Abalone Farm, Inc. Cayucos, CA, USA
	Helisoma trivolvis	Yes			Calgary, AB (Department of Biological Sciences)
	Lymnaea stagnalis	Yes	Yes	Yes	Calgary, AB (Department of Physiology & Biophysics, Dr. Syed's lab)
	Marisa cornuarietis	Yes	Yes	Yes	Venezuela via Riverfront Aquariums, Calgary, AB
	Nucella lamellosa	Yes			Bamfield Marine Station, BC
	Nucella ostrina	Yes	Yes	Yes	Bamfield Marine Station, BC
	Polinices lewisii	Yes			Bamfield Marine Station, BC
	Pomacea bridgesi	Yes	Yes	Yes	Venezuela via Riverfront Aquariums, Calgary, AB
	Searlesia dira	Yes	Yes	Yes	Bamfield Marine Station, BC
	Tegula pulligo	Yes			Bamfield Marine Station, BC
	Tegula funebralis	Yes			Bamfield Marine Station, BC
	Turbo castanea	Yes			Florida via U of C teaching labs.

CHAPTER FOUR
PHYLOGENIES USING DNA SEQUENCE ANALYSIS

4.1 Introduction

In section 2.2.2, I described the techniques available and indicated some of the reasons for using DNA sequence analysis. As an introduction to the development and analysis of genetic sequence data, I will describe the history and the reasons behind the selection of the partial sequencing of the DNA coding for the 18S RNA ribosomal subunit. I also provide background information necessary for understanding the analysis of the collected data.

An important idea in genetic research is that of an "evolutionary clock" or a measure of mutational rate. Genes may be passed on from a parent to offspring in an imperfect manner. Throughout life, the genes possessed by an organism must be packed, copied, unfolded, repacked while being subjected to attack by viruses or exposed to damaging chemicals and radiation, which may induce mutations. Most mutations are repaired by safeguarding mechanisms within the cells (Wolfe, 1993). Occasionally a nucleotide insertion or deletion may occur and exist unrepaired in the reproductive cells of a parental organism and get passed on to its offspring. More often this mutation will have a deleterious effect in the offspring. However, in rare cases, the mutation is in an intron (which gets cut out of the gene before being translated into a protein) and so it has no effect and the gene produces a molecule that works. In even rarer cases, the mutation, although within the coding portion of the gene, results in no effect or even a positive effect in the final product (Griffiths *et al.*, 1993). Therefore, genes with many regions that can support sequence changes and still function (introns) are more likely to change at a faster rate. As a result, each gene has an inherent mutational rate. Genes coding for essential, life sustaining products mutate more slowly than less important ones and therefore can be used to measure changes over long periods. These "slow evolutionary

clock" genes tend to be very conserved and exist in large numbers of copies throughout evolution. One of the goals of this experimentation was to select a "slow evolutionary clock" gene sequence so I could resolve the subclass taxa.

Dr. Barry Honda, one of my instructors in the 1998 Bamfield Marine Station summer course "DNA manipulation techniques", suggested that I use sequencing as the preferred technique for my research. During the course, he offered my laboratory partner, Christian Jurha, and me commercially available universal 16S ribosomal subunit primers (designed from seastars, *Pisaster* sp.) to sequence a variety of molluscs. Since the gene for the 16S ribosomal subunit, an important molecule, is from the mitochondrial genome, we assumed that this would decrease the mutational rate and would be useful for higher taxa. Though these primers did amplify a product, when sequenced and analyzed, they proved to be too variable to resolve at the class level (Uyeno & Jurha, unpublished, 1998). One result of the analysis showed that among the animals sequenced (*Haliotis kamtschatkana*, *Tegula funebralis*, *Littorina sitkana*, *Littorina scutulata*, *Nucella emarginata*, *Haminoea vesicula Lymnaea stagnalis*, *Katharina tunicata* and *Cryptochiton stelleri*), that animals considered to be prosobranchs were shown to be polyphyletic. Thus my first analysis of gastropods suggested that the Prosobranch-Opisthobranch-Pulmonate scheme was probably artificial.

Tillier and his coworkers (1992, 1994), suggested that the major gastropod taxa could be resolved with the gene for the genomic 28S ribosomal subunit. Although this was not the gene that I finally selected, the background information suggested that it would have been just as good in terms of mutational rate and the ability to resolve at the subclass level.

To cover other possible options, I contacted Dr. Andrew McArthur of the Marine Biology Laboratory at Woods Hole and Dr. Jerry Harasewych, Curator of Mollusks at the Smithsonian Institution, and they both indicated that they were having great success with all types of gastropods using primers that were characterizing the 18S ribosomal subunit gene. Preliminary results demonstrated that it was indeed resolving at the appropriate

level for this study. With the choice between the 18S gene and the 28S gene, I chose the former since there was a larger body of information available using the 18S primers, and both Dr. McArthur and Dr. Harasewych were very helpful in giving hints regarding the actual protocol.

4.2 Materials and Methods

4.2.1 Protocols

Mr. Bob Winkfein (of the University of Calgary, Department of Physiology and Biophysics' MRC-Group Molecular Biology Laboratory) and I developed the following technique in which DNA was isolated, amplified using 18S primers and sequenced. It is based on a simple proteinase K-PC:IA-ethanol extraction, but effected with many added steps to overcome the problems inherent in working with snail DNA. The following is my protocol (greatly enhanced by Winkfein) that rendered my sequences:

Live specimens were flash frozen and maintained at –80°C. At the appropriate time, specimens were briefly and partially thawed, and small (2-3 mm^3) non-pigmented sections of the pedal muscles were removed and placed into a 2.0 ml Eppendorf tubes containing 300µl of lysis buffer (Proteinase K buffer), and then ground in an Eppendorf pestle. Samples were incubated for 4 h at 55°C and subjected to sequential phenol (buffer saturated), chloroform:isoamyl alcohol (24:1) extraction. One-tenth volume of 5M ammonium acetate and one volume of isopropyl alcohol were added to precipitate the DNA. Pellets were washed with 70% ethanol and resuspended in Tris-HCl buffered at pH 8.5. The resulting DNA was evaluated by electrophoresis on 0.8% (w/v) agarose gels.

Holland *et al.* (1991) developed the primers used in this study to amplify rDNA regions (forward: 5'-GCCAAGTAGCATATGCTTGTCTC-3' and reverse: 5'-AGACTTGCCTCCAATGGATCC-3'). PCR amplifications were performed using

Perkin-Elmer 2400 or 9700 thermal cyclers. PCR reactions were performed in a total volume of 50 µl, containing 250 ng of genomic DNA, 1.25 µl of Taq polymerase (Life Technologies), 200 µM of each dNTP, 0.25 µM of each primer, 1.5mM MgCl2 and 5µl of 10x PCR buffer. Amplification parameters used were as follows: an initial denaturation step of 5 min at 94°C followed by 30 cycles of: 45 seconds at 94°C (denaturation), 45 seconds at 62°C (annealing) and 1 min at 72°C (extension). An additional 10 min extension period was added after the last cycle and tubes containing the end product were held at –4°C until use.

PCR products were gel isolated prior to sequencing as follows: the entire PCR product was loaded onto a 1.3% preparative agarose gel, which was electrophoresed until sufficient band separation had occurred. Gel slices containing the correct sized PCR product were excised and the DNA purified using the Qiaquick Gel Extraction Kit according to the Qiagen's protocol.

Sequence reactions were performed according to the manufacturer's directions (Thermosequenase Radiolabelled Terminator Sequencing Kit, Amersham) using 10 µL of the purified PCR product. Both primers were used to amplify the product of interest, as well as an internal primer to generated overlapping sequence data. Sequence ladders were separated on 6% denaturing polyacrylamide gels, with two staggered loadings per sample. Gels were fixed for 30 min in 20% methanol/5% acetic acid and dried under vacuum. Autoradiography using Kodak BioMax MR film was employed to visualize ladders after overnight exposure at room temperature.

4.2.2 Analysis

There are three steps in the analysis of sequence data. The first is the alignment of the sequences of the various taxa. The second step is to produce a phylogenetic tree based on probability and the final step is to draw the most probable cladograms.

Data from the sequenced snails in the form of autoradiograms were manually input to Cabot's (1998) PC computer program, Eyeball SEquence Editor (ESEE version 3.2), the file was then saved to the Fast A format. My own sequenced species did not adequately cover the number of taxa represented by the class Gastropoda and so supplemental data were added to my findings. As the sequences for these supplemental taxa were developed using the same primers both results are directly comparable. The supplemental data were collected from the following sources: Harasewych *et al.*, 1997a, 1997b, 1998 and the Genbank *ex* National Center for Biological Information's BLAST server. In total, 68 taxa were added to my 19 sequences to total 87 taxa (*see* Table 8 for sources), which include all the subclass taxa including those that are still considered somewhat fluid.

The alignment of sequences may be a tricky endeavour. Essentially one is trying to identify the homology of every single nucleotide base between sequences originating from different taxa. Ideally, the result is the alignment of every single base with its counterpart or else, if that base happens to be an insertion or deletion, alignment of it with a gap or insertion of a gap, respectively. The most intuitive way to align a series of DNA nucleotides is to incorporate the rRNA secondary structure information. This secondary structure can suggest that a given base is actually, based on its natural function, evolutionarily homologous to a base in another organism's sequence. I elected not to follow this method for three reasons. Firstly, I did not have the means to collect secondary structure information for all the taxa. Secondly, there were a large number of taxa (86) to align. Thirdly, the results of Winnepenninckx and Backeljau (1996) indicated that DNA sequences can be aligned differently based on the secondary structure one uses. Of course the primary structure (the sequence of RNA bases) of the 18S ribosomal RNA is fixed for a given taxon, but the secondary structure models are often modified or optimized in the presence of the ever growing number of sequences available for comparative studies.

To standardize all the sequence information, I decided not to rest the alignment of all the sequences on one arbitrarily chosen organism's rRNA secondary structure but instead to rely on a uniform application of a probability algorithm to align all the

sequences. Such an algorithm must identify common sequences and then based on distances of neighboring nucleotides, calculate the probability of the position of the given base by leaving it aligned with all the other organisms' corresponding bases or inserting a gap in the sequence (suggesting that the other sequences have mutated by inserting a base or that this sequence has that base deleted). The algorithm that I chose for this purpose is implemented in ClustalX, a program designed originally by Dr. Des Higgins (Higgins & Sharp, 1988, 1989, Thompson *et al.*, 1997). This program calculates a separate distance score between every pair of entire sequences. The score is then used to construct a guide tree and which in turn, is used to go through the data again to calculate percent identity scores (the final multiple alignment). Please see appendix II.2 for the complete alignment and base pair sequences for all the specimens.

All the sequences, presented in the Fast A format, were opened together in ClustalX and subsequently aligned using the multiple alignment mode and once again without end gap penalization. The resultant file of aligned sequences (in ClustalX format) was then saved for further analysis in a phylogenetic inference software package. This ClustalX formatted output is a series of aligned sequences for each organism, where every base pair in the alignment is in a column and every column represents homologous positions in the sequence. This output is therefore ready for the second step of developing a phylogenetic tree.

The two programs most used for phylogenetic inference are Swofford's (1996) PAUP (Phylogenetic Analysis Using Parsimony, originally developed for Macintosh) and Felsenstein's (1993) PHYLIP (PHYLogeny Inference Programs, originally developed for PC systems). After evaluating both programs, I decided to feed my alignment information into the PHYLIP programs to develop phylogenetic trees based primarily on a bootstrapping and neighbor joining process.

PHYLIP Version 3.5c contains four programs used to perform the building of trees. The data processing was performed as follows:

1. A preparatory bootstrap analysis using the program SEQBOOT was performed on the aligned sequences using 1000 replicates. 1000 replicates were suggested by Felsenstein (1993) as being adequate to create a robust analysis.

2. The 1000 replicates were then fed into a phylogeny analysis program named DNADIST. This program calculated the Jukes/Cantor distances (Jukes and Cantor, 1969) between pairs of species from the nucleotide sequences. The Jukes/Cantor distance was the chosen method of distance calculation because it is accepted as a robust method. A large number of studies rely on this method (e.g. Winnepenninckx & Backeljau, 1996) and it assumes that within a short 640 base pair sequence that a nucleotide can mutate to any of the other three nucleotides.
3. I used the resultant distance matrix as input for the program NEIGHBOR to exhaustively construct trees using the neighbor-joining method. The program produced 1000 possible trees. The neighbor-joining method was used because it is not statistically susceptible to the order in which the species are listed in the bootstrap replication (Felsenstein, 1993).
4. All the neighbor-joining trees were then used as input for the program CONSENSE to develop a consensus tree from the data by weighing the branches in the order of appearance in the possible trees. CONSENSE delivers a type of confidence index in its evaluation of the analysis by combining the most robust trees and supporting each branch with bootstrap values. Only branches with bootstrap values than 50% were considered and only branches with robust values of 70% or better were used to build the final cladogram (Figure 14) unless there were extenuating circumstances.

Finally, although seemingly trivial, some consideration was put into method of graphically displaying the phylogenetic tree. The program must be able to organize branches in a meaningful way and it also must be able to define an outgroup and subsequently root the tree to that outgroup. The two most used programs to do this type of display are Roderic Page's (1996) TreeView (Version 1.5.2, developed for Windows 3.X/9X/NT systems) and Maddison & Maddison's (1992) MacClade (Version 3, for Macintosh MacOS 7.5.2 or higher). Both basically perform the same tasks and I have used both TreeView (for vertical trees) and MacClade (for horizontal trees) in preparing the figures in this thesis.

4.3 Results

In presenting the results from the genetic analysis, I include a description of the important elements that were present and an untouched phylogenetic tree resulting from the PHYLIP Version 3.5c CONSENSE program (Felsenstein, 1993). As there were 1000 possible trees, CONSENSE concatenated only those branches and nodes that occurred in all the trees 50% of the time or more (in fact the major branches occurred in over 70% of the trees). CONSENSE weighted the branches so that those branches occurring more often were more likely to appear in the consensus tree. The result is the tree depicted in Figure 12 (*see* Appendix II.1 for direct output of this file which was used to build the cladogram)).

Figure 13 is a modified phylogenetic tree that was developed from the output of the PHYLIP CONSENSE program and the tree in Figure 12. The branches are rotated to elucidate some of the interesting and problematic areas as well as to clearly illustrate the higher taxa relationships. Please note, rotation of branches does not constitute any modification to the phylogenetic tree, it is simply a different arrangement for the sake of clarity. Each major taxon is represented by its constituent species printed in a different typeface. The typefaces corresponding to the taxa are given in Table 8 in the order in which they appear in the figure.

The analysis of the sequence data resulted in the following subclass taxa. The polyplacophorans formed a robust monophyly in every tree analysed. The Gastropoda therefore necessarily form a monophyly with the polyplacophorans as an outgroup. In describing the basic divisions of the gastropod portion of the phylogenetic tree, I found that it could be broken down into the following subclasses: Neritopsina, a group of primitive gastropods, Caenogastropoda and Heterobranchia.

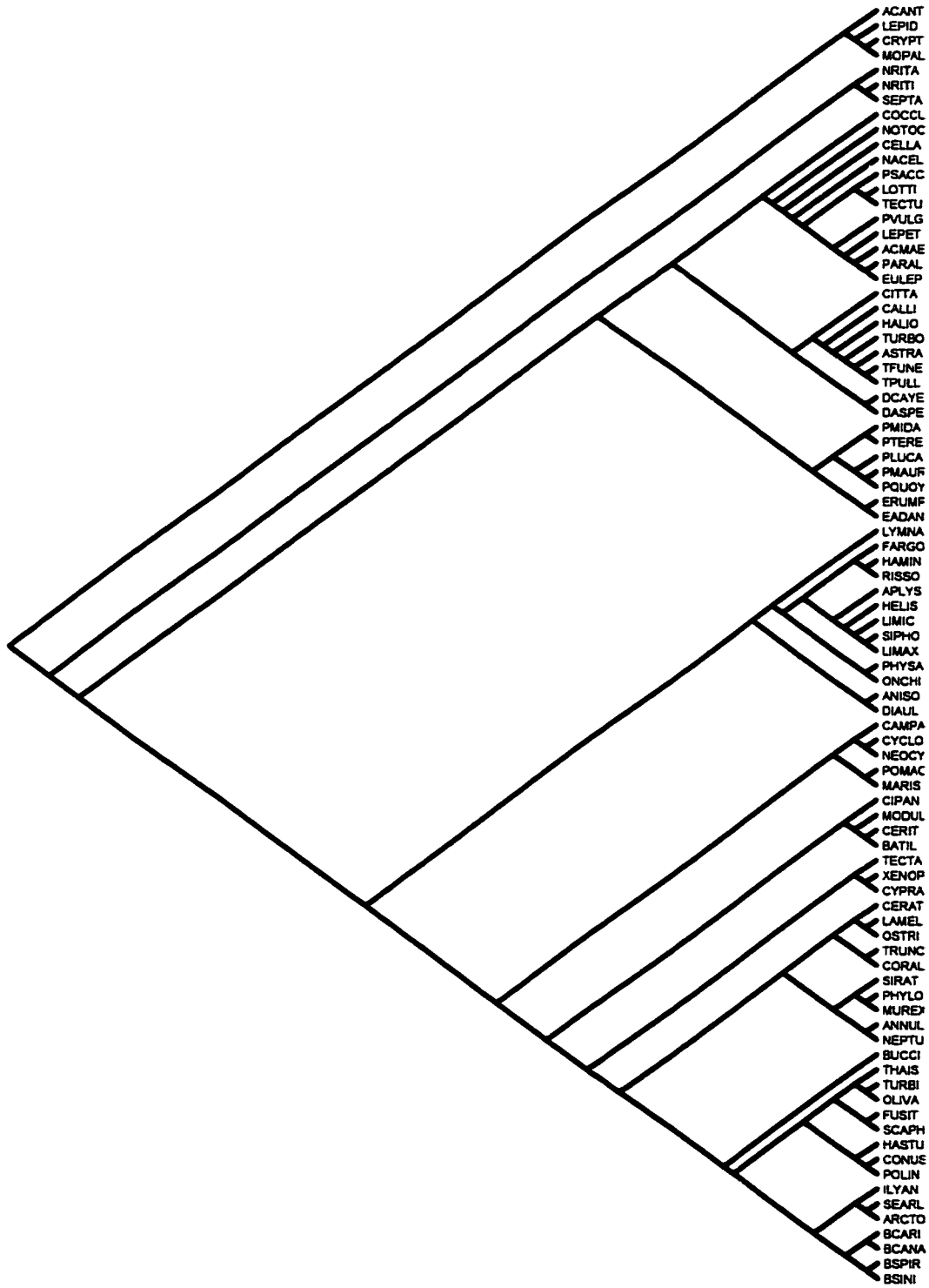

Figure 12. PHYLIP CONSENSE output, a phylogenetic tree of the Polyplacophora and Gastropoda (Note: Species code and Genus/species names are listed in Table 9).

Table 8. Key to typefaces and taxonomic names for Figure 13.

	Typeface	Taxon name
1	**TIMES NEW ROMAN BOLD**	Polyplacophora
2	*AVANT GARDE ITALICS*	Neritopsina
3	**CENTURY GOTHIC BOLD**	Vetigastropoda
4	DAUPHIN	Cocculiniformia
5	**FUTURA BLACK**	Patellogastropoda
6	**DOM CASUAL**	Lower Caenogastropoda/Mesogastropoda
7	KABEL	Upper Caenogastropoda/Neogastropoda
8	***TIMES NEW ROMAN BOLD ITALIC***	Heterobranchia

Neritopsina includes three species, *Nerita versicolor*, *Neritina reclivata* and the purported vetigastropod, *Septaria porcellana* (from the family Neritidae). This subclass represents a very primitive group of gastropods that are closest to the polyplacophorans.

The next group includes a relatively primitive set of gastropods, which appear to be monophyletic. Upon further inspection, this group may be subdivided into two groups, the Vetigastropoda and the Patellogastropoda/Cocculinidae. All vetigastropods are contained in this group except *Septaria porcellana*. The majority of vetigastropods are separated into two groups, the family Pleurotomariidae and the other vetigastropods. All of the patellogastropods are segregated into their own group, but a few vetigastropods are found to align with them (*Paralepetopsis floridensis*, *Euleptopsis vitrea*, *Lepeta caeca* and *Nacella magellanica*).

The Caenogastropoda are monophyletic with the exception of *Rissoella caribea*, a eucaenogastropod that unexpectedly sorts with the Heterobranchia. The mesogastropod grade is shown to bifurcate from the main caenogastropod line before the neogastropod grade, suggesting that they are a more primitive group. The neogastropod grade is shown to be monophyletic although four of the more developed mesogastropods sort with them (*Truncatella guerinii*, *Annularia fimbriatula*, *Fusitriton oregonense* and *Polinices lewisii*), which is probably responsible for their lower bootstrap confidence value.

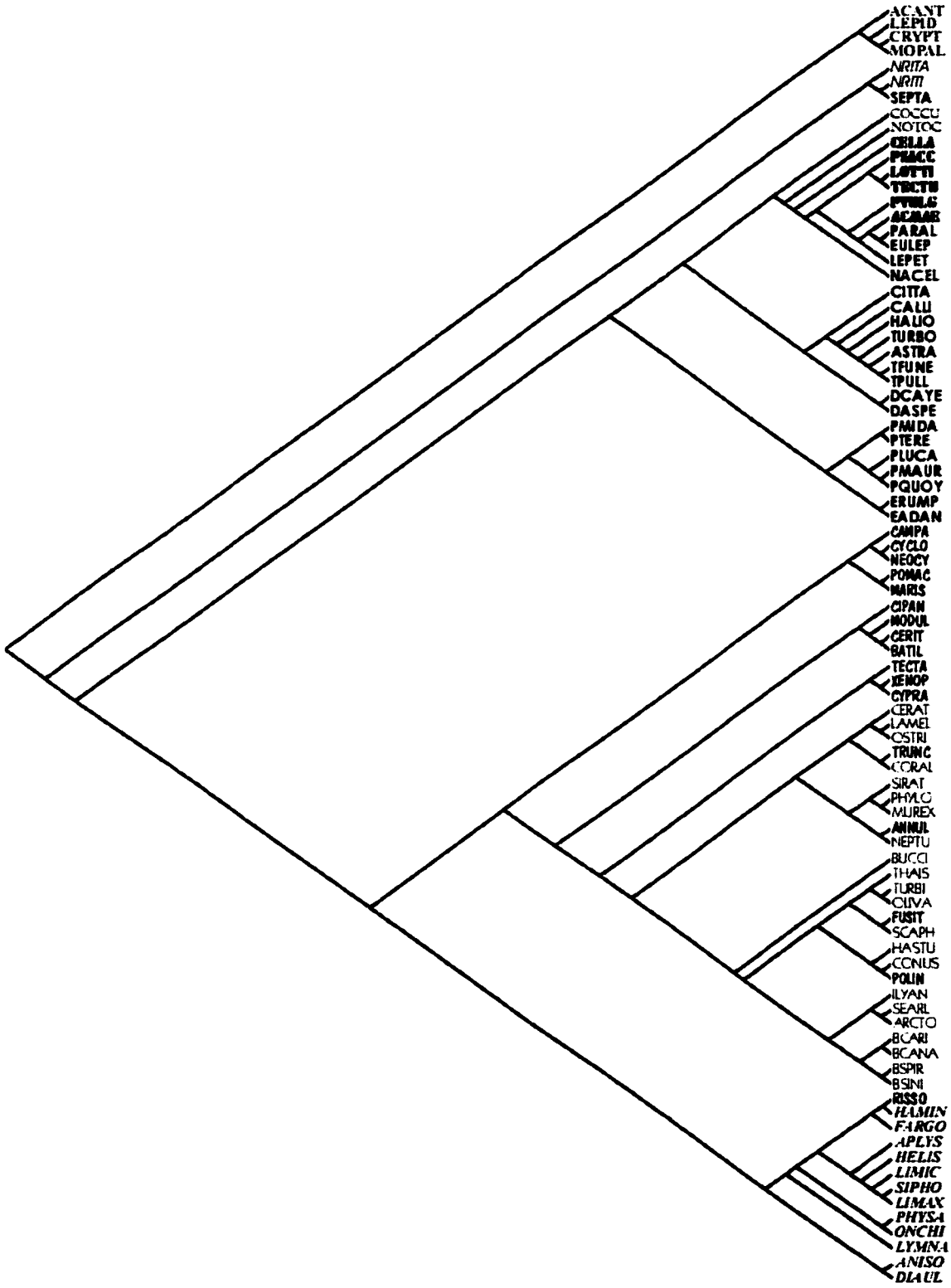

Figure 13. A modified phylogenetic tree to show relationships (Note: Species code and Genus/species names are listed in Table 9).

Finally, the last group that sorts as a monophyly is the Heterobranchia, the group that contains the Pyramidelloidea and the Euthyneura. This group is certainly a monophyly although it is interesting to note that this study did not appear to be successful in resolving between the opisthobranch and pulmonate members. *Fargoa bushiana* is a pyramidelliodean (the only heterobranch in this study that is not also a euthyneuran). In the traditional and multidisciplinary schemes, *Haminoea antillarum*, *Aplysia dactylomela*, *Anisodoris nobilis* and *Diaulula sandiegensis* are opisthobranchs and *Onchidella celtica*, *Physa heterostropha*, *Limicolaria kambeul*, *Limax maximus*, *Siphonaria pectinata*, *Helisoma trivolvis* and *Lymnaea stagnalis* are pulmonates. However, these organisms do not sort in this manner. There is a heterogeneous mix of Opisthobranchia-Pyramidellioidea-Pulmonata, although there is also an interesting close grouping of *Rissoella caribea* (a mesogastropod), *Haminoea antillarum* (a shelled opisthobranch) and *Fargoa bushiana* (a primitive heterobranch).

4.4 Discussion

An important matter of discussion is the number of unexpected elements that arose from the PHYLIP analysis, and how I can account for them and/or what implications they have with regards to gastropod phylogeny. Equally important, but more technically related, is the resolution of problematic issues that accompany the extraction and sequencing of molluscan tissue.

4.4.1 Interesting elements of the phylogenetic tree

Table 9 shows how the exhaustive list of species used in this study place in the systematic scheme of Ponder & Lindberg (1997) and Haszprunar (1988), or the "multidisciplinary" scheme as described in Table 5 in section 2.4.1. Please remember that

this scheme is based on a very large collection of morphological and developmental characteristics. There is considerable similarity between this phylogeny and the higher taxa represented within the phylogenetic tree developed by the molecular data.

Table 9. List of species represented in the DNA sequence analysis along with their superorders, orders, families, genera and species' names and species code used in figure 11 & 12. (Source: 1 = Sequence data generated for this study, 2 = Harasewych *et al.*, 1997b, 3 = Harasewych *et al.*, 1998, 4 = Harasewych *et al.*, 1997a, 5 = McArthur unpublished data (with GenBank accession numbers))

Superorder Order	Family	*Genus/species name*	Species CODE	Source
		Acanthopleura japonica	ACANT	4
		Cryptochiton stelleri	CRYPT	4
		Lepidozona mertensii	LEPID	1
		Mopalia muscosa	MOPAL	1
Patellogastropoda				
		Acmaea mitra	ACMAE	1
		Cellana nigrolineata	CELLA	4
		Lottia pelta	LOTTI	5, af046049
		Patelloida saccharina lanx	PSACC	5, af046051
		Patella vulgata	PVULG	5, af046046
		Tectura scutum	TECTU	5, af046050
Cocculiniformia				
	Cocculinidae			
		Cocculina messingi	COCCU	5, af046054
	Lepetelloidea			
		Notocrater houbricki	NOTOC	4
Neritopsina				
		Nerita versicolor	NRITA	3
		Neritina reclivata	NRITI	3
Vetigastropoda				
	Fissurellidae			
		Diodora aspera	DASPE	1
		Diodora cayenensis	DCAYE	3
	Haliotididae			
		Haliotis rufescens	HALIO	1,2
	Lepetidae			
		Lepeta caeca	LEPET	5, af046048
	Neritidae			
		Septaria porcellana	SEPTA	5, af046055
	Neolepetopsidae			
		Eulepetopsis vitrea	EULEP	5, af046052
		Paralepetopsis floridensis	PARAL	5, af046053
	Trochidae			

Superorder Order	Family	*Genus/species name*	Species CODE	Source
		Astraea caelata	ASTRA	3
		Cittarium pica	CITTA	4
		Calliostoma canaliculatum	CALLI	1
		Tegula pulligo	TPULL	1
		Tegula funebralis	TFUNE	1
	Turbinidae			
		Turbo castanea	TURBO	1
	Nacellidae			
		Nacella magellanica	NACEL	5, af046047
	Pleurotomariidae			
		Entemnotrochus adansonianus	EADAN	4
		Entemnotrochus rumphii	ERUMP	4
		Perotrochus lucaya	PLUCA	4
		Perotrochus maureri	PMAUR	4
		Perotrochus midas	PMIDA	4
		Perotrochus quoyamus	PQUOY	4
		Perotrochus teremachii	PTERE	4
Caenogastropoda				
Mesogastropoda				
Architaenioglossa				
	Cyclophoroidea			
		Cyclophorus hirasei	CYCLO	3
		Neocyclotus seminudus	NEOCY	3
	Ampullariidae			
		Pomacea bridgesi	POMAC	1,3
		Marisa cornuarietis	MARIS	1,3
Neotaenioglossa				
	Cerithiidae			
		Cerithium atratum	CERIT	3
		Batillaria minima	BATIL	3
		Modulus modulus	MODUL	3
	Campaniloidea			
		Campanile symbolicum	CAMPA	3
Eucaenogastropoda				
	Xenophoridae			
		Xenophora exutum	XENOP	3
	Tonnoidea			
		Fusitriton oregonense	FUSIT	3
	Naticidae			
		Polinices lewisii	POLIN	1
	Viviparidae			
		Cipangopaludina japonica	CIPAN	3
	Rissoellidae			
		Rissoella caribea	RISSO	2
	Littorinidae			
		Tectarius muricatus	TECTA	3
		Annularia fimbriatula	ANNUL	3
	Truncatellidae			

Superorder Order	Family	*Genus/species name*	Species CODE	Source
		Truncatella guerinii	TRUNC	3
	Cypraeidae			
		Cypraea tigris	CYPRA	3
Neogastropoda				
	Muricidae			
		Phylonotus pomum	PHYLO	2
		Thais haemastoma	THAIS	2
		Ceratostoma foliatum	CERAT	1
		Murex troscheli	MUREX	2
		Nucella lamellosa	LAMEL	1
		Nucella ostrina	OSTRI	1
		Siratus beauii	SIRAT	2
	Coralliophilidae			
		Coralliophila abbreviata	CORAL	2
	Olividae			
		Oliva sayana	OLIVA	3
	Volutidae			
		Arctomelon stearnsii	ARCTO	2
		Scaphella junonia	SCAPH	2
	Turbinellidae			
		Turbinella angulata	TURBI	2
	Terebridae			
		Hastula cinerea	HASTU	3
	Conidae			
		Conus floridanus	CONUS	2
	Melongenidae			
		Busycon carica	BCARI	2
		Busycon sinistrum	BSINI	2
		Busycotypus spiratus	BSPIR	3
		Busycotypus canaliculatus	BCANA	2
	Buccinidae			
		Buccinum oedematum	BUCCI	2
		Searlesia dira	SEARL	1
		Neptunea polycostata	NEPTU	2
	Nassariidae			
		Ilyanassa obsolete	ILYAN	2
Heterobranchia				
Pyramidelloidea				
		Fargoa bushiana	FARGO	4
(Euthyneura)				
Opisthobranchia				
		Haminoea antillarum	HAMIN	4
		Aplysia dactylomela	APLYS	3
		Anisodoris nobilis	ANISO	1
		Diaulula sandiegensis	DIAUL	1
Pulmonata				
		Onchidella celtica	ONCHI	4
		Physa heterostropha	PHYSA	4

Superorder Order	Family	*Genus/species name*	Species CODE	Source
		Limicolaria kambeul	LIMIC	4
		Limax maximus	LIMAX	4
		Siphonaria pectinata	SIPHO	3
		Helisoma trivolvis	HELIS	1
		Lymnaea stagnalis	LYMNA	1

Interesting comparisons can be made between the morphology-based scheme represented in the Table 9 and the molecular data. Firstly, the supposedly monophyletic Vetigastropoda seem to separate into two distinct groups (a polyphyly). This is not an accurate representation of the state of the Vetigastropoda and can be explained quite readily. Within the vetigastropods there appears to be a divided group consisting of five species of the genera *Entemnotrochus* and *Perotrochus*. These five sequences belong to two closely related genera in the family Pleurotomariidae for the purposes of investigating the pleurotomarid phylogeny (Harasewych *et al.*, 1997a) and therefore group together to give the appearance of a polyphyly. The distance matrices of the phylogenetic analysis program that I used have weighted the group as a whole more heavily because there were more constituent members. Nowhere else in this study is there a larger collection of more closely related species.

It is interesting to note that several of the vetigastropods sort closely to the Patellogastropoda. Ponder and Lindberg (1997) found that the patellogastropods are so very different from all other gastropods that they proposed a new taxon of Eogastropoda to encompass those snails and the Neritopsina. This study shows that the Patellogastropoda and the Vetigastropoda are possibly more closely connected. These findings are not supported by the findings of Harasewych *et al.* (1997a) who, using a smaller number of species (thirty-two), found that the Patellogastropoda were quite separate from the Vetigastropoda. I suggest two possibilities for this discrepancy. The first is the limited number of taxa in the Harasewych *et al.* (1997a) study and the second is the possible lower quality alignment in this study (at least for the primitive gastropods, with their generally larger genomes). I mentioned in the section dealing with data

analysis (4.1.2) that although the primers were developed with secondary structure in mind, no alignments were based on them because of the great number of taxa involved, the lack of knowledge of these secondary structures and low reproducibility with multiple structures. This indicates that the Patellogastropoda and Vetigastropoda are primitive gastropods. In my opinion, the Vetigastropoda and Neritopsina segregate well enough that Thiele's (1929) term "Archaeogastropoda" that incorporates all gastropods that are considered more primitive than caenogastropods is of little use. Furthermore, this study very strongly supports outdating the term Prosobranchia for its polyphyletic nature. As one can see from these results, the primitive gastropod groups neatly segregate from the Caenogastropoda and Heterogastropoda. I strongly urge the cessation of the use of the term Prosobranchia as it is not supported by these results.

The next issue is the position of the smaller, more fluid groups: the Neritopsina and Cocculiniformia. Harasewych *et al.* (1997) suggested, based on molecular research, that the Cocculiniformia align with the Patellogastropoda and that the Neritopsina align with the Vetigastropoda. This thesis further supports that claim by showing the tree length is shorter (more parsimonious) than the trees built by either Haszprunar (1988) or Ponder and Lindberg (1997). Ponder and Lindberg (1997) found that the Neritopsina are closely aligned with the cocculinids and these two groups are intermediate between the Caenogastropoda and Vetigastropoda. Haszprunar (1988) hypothesized that the Neritomorpha (=Neritopsina) were a sister group to the Vetigastropoda/Cocculinimorpha (=Cocculiniformia). This study agrees with Haszprunar's (*ibid.*) results in that the Neritopsina clearly branch off before the Cocculiniformia/Vetigastropoda group. Ponder (pers. comm., 1999) suggested that the positions of the Cocculinids and Neritopsinids will be quite fluid for some time to come.

The caenogastropod clade represents the largest collection of species that I analyzed. It is clear from my results that the Caenogastropoda represent a monophyly. It is also evident that the mesogastropods branch off before the neogastropods for the large part. This is supported by the fossil record, with the neogastropods first appearing in the Cretaceous (Taylor & Morris, 1988), almost 450 million years after the early mesogastropods appeared (*Ibid.*, Ponder, 1973). However, I found that several

"mesogastropods" share higher caenogastropod positions with the neogastropods, but the reverse is not true. These results suggest that the meso/neogastropod groups are probably not clades *per se*, but more representative of grades. It also suggests that the terms mesogastropod and neogastropod are still of value in the general description of the Caenogastropoda. This idea that the neogastropods are advanced caenogastropods and the mesogastropods are more primitive is also strongly supported by morphological studies such as that of Ponder and Lindberg (1997).

Haszprunar (1988) favoured the terms Architaenioglossa, Neotaenioglossa and Eucaenogastropoda (= Hypsogastropoda = Sorbeoconcha [Harasewych *et al.*, 1998]) to further divide the Caenogastropoda. This study has found support for these terms as grades within the Mesogastropoda (Architaenioglossa containing primitive characteristics, Neotaenioglossa occupying a middle position and Eucaenogastropoda being more advanced). Looking at the phylogenetic trees, the Architaenioglossans are *Cyclophorus hirasei*, *Neocyclotus seminudus*, *Pomacea bridgesi* and *Marisa cornuarietis*. These snails group together with *Campanile symbolicum*, a neotaenioglossan. The other neotaenioglossans branch off at the next node and contain the typical neotaenioglossans, *Cerithium atratum*, *Batillaria minima* and *Modulus modulus*. Once again, a more advanced snail is sorting with this group (*Cipangopaludina japonica*), which suggests that these groups could be considered grades as opposed to clades.

The heterobranchs in this study are monophyletic except for the possible single case of *Rissoella caribea* (traditionally a mesogastropod), which aligns with this group. This result provides some molecular support for the pattern of evolution of the heterobranchia. As mentioned in section 2.4.2.1 that until the late Cambrian/Early Ordovician epochs, gastropods were primitive and small. After this period, they increased in size, theoretically allowing caenogastropods to evolve (Yochelson, 1963). The Euthyneura descended from ultradextral gastropods (probably these larger caenogastropods) and appeared for the first time in the Carboniferous (Kollmann and Yochelson, 1976). Fretter and Graham (1962) made the popular suggestion that the euthyneurans evolved from eucaenogastropods such as the Rissoaceans and Cerithiaceans, although there have been other suggestions for ancestral taxa (e.g., the

Littorinoidea; Gosliner [1981]). These mesogastropods qualify for the position of being ancestral to at least the opisthobranchs by having several of the important shell, foot, reproductive and gut morphologies thought to be needed in the evolution of the Euthyneura. Several authors have different opinions on whether Rissoaceans sort with the Heterobranchia or with the Caenogastropoda. I elected to label *Rissoella caribea* as a mesogastropod to draw attention to this subject. Fretter and Graham, (1962) and Turgeon *et al.* (1988) listed *Rissoella* as a lower caenogastropod whereas Rudman and Willan (1998), Harasewych *et al.* (1997b) and Ponder and Lindberg (1997) placed them within the heterobranchs. Either way, this analysis provides molecular support for the alignment of the rissoaceans within the Heterobranchia and points to the lower caenogastropods for the origin of the Heterobranchia. Finally, it is interesting to note that *Rissoella caribea* sorts with *Haminoea antillarum* (a primitive shelled opisthobranch) and *Fargoa bushiana* (a primitive heterobranch (pyramidelloidean)), which also supports this theory of the origin of the Heterobranchia (although this last observation is relatively tenuous given this study's inability to resolve between the pulmonates and the opisthobranchs).

4.4.2 Technical matters

As an aim of this study was the evaluation or development of techniques suitable for use with molluscan tissue, I will discuss problems pertaining to molluscan DNA extraction.

4.4.2.1 High molecular weight DNA

An often discouraging characteristic of many gastropods, especially the more primitive ones, is that their genomic DNA is of relatively high molecular weight. Winnepenninckx *et al.* (1993) published a technical tip on how to counteract this problem. Such papers are rare but I found that most technical information is passed along by either word of mouth or by e-mail. The problem with extracting high molecular weight DNA is that there are greater chances in having the extraction complicated,

especially by two specific compounds: polyphenolic proteins and mucopolysaccharides. Both of these are present in snail secretions and both copurify with the DNA and interfere with the enzymatic processing of nucleic acids (Winnepenninckx *et al.*, 1993). Plant molecular biologists have developed methods for dealing with these polyphenolic proteins and mucopolysaccharides for both are often present in some plants (Jobes, *et al.*, 1995). In fact, commercial products are available, aimed at the molecular plant market (eg., the Nucleon™ PhytoPure system, which contains free boric acid groups which covalently bind polysaccharides).

While learning to manipulate DNA in Dr. Honda's preparatory course at the Bamfield Marine Station, I used several compounds that provided solutions for handling these secreted molecules. Many researchers (e.g. Harasewych (1997a, 1997b), Winnepenninckx, *et al.* (1993), McArthur (pers. comm.) and I) find that a cationic detergent, CTAB (hexadecyltrimethylammonium bromide or cetyltrimethylammonium bromide) helps by forming complexes with polysaccharides and other residual proteins. Another helpful product is Biorad's Chelex. It is a resin that may prevent the degradation of high molecular weight DNAs by chelating metal ions. Purification steps are also important to clean up slimy DNA. I found using GFX Columns from Amersham Pharmacia Biotech's GFX Genomic Blood DNA Purification Kit was effective. Bob Winkfein, found that brief sonication of the DNA was an inexpensive and rapid way of fragmenting the high molecular weight DNA for easier isolation.

4.4.2.2 Hyperactive gastropod DNAses

Within cells, damage to DNA can arise from various viruses, failed mitotic and meiotic events as well as natural degredation. Such damage often results in fragments that are cleaned up in the cell by catabolic enzymes known as DNAses (Wolfe, 1993). I have experienced, as have other workers (e.g., Boulding, pers. comm.,1998), that the DNAses are hyperactive in gastropods. Normally these enzymatic actions are controlled by the cell, however minutes after death DNAses begin to render the DNA unusable (Uyeno,

unpublished data). The best solution to this problem that I found was freezing of the animal (using liquid nitrogen) while living and then extracting the DNA. All of my other attempts not using this method failed to produce DNA. However, I would imagine that the excision of tissue from a live organism and direct placement into an extraction buffer could work as well.

4.5 Summary and concluding remarks

4.5.1 Molecular conclusions for the phylogeny of the subclass taxa

I now draw attention to the state of the subclass taxa based on the results of the analysis of the DNA sequence that codes for the 18S ribosomal RNA molecule. Generally, this study renders a phylogenetic tree (Figure 14) that is similar in many respects to the multidisciplinary tree (Haszprunar, 1988, Ponder & Lindberg, 1997). Of the two types of systematic schemes reviewed, it is least similar to the classic tree because of contradictions at many crucial points. Thus my work supports and validates the more modern scheme that is based upon multidisciplinary research as opposed to the classic scheme.

Figure 14 illustrates the simplified results of the molecular analysis, retaining only the subclass taxa. Note the bootstrap confidence values placed at each branch. The numbers refer to the number of times these branches appear in 1000 tree replicates. Please refer to it as I summarize the major findings of the molecular analysis and their impact on the phylogenetic tree.

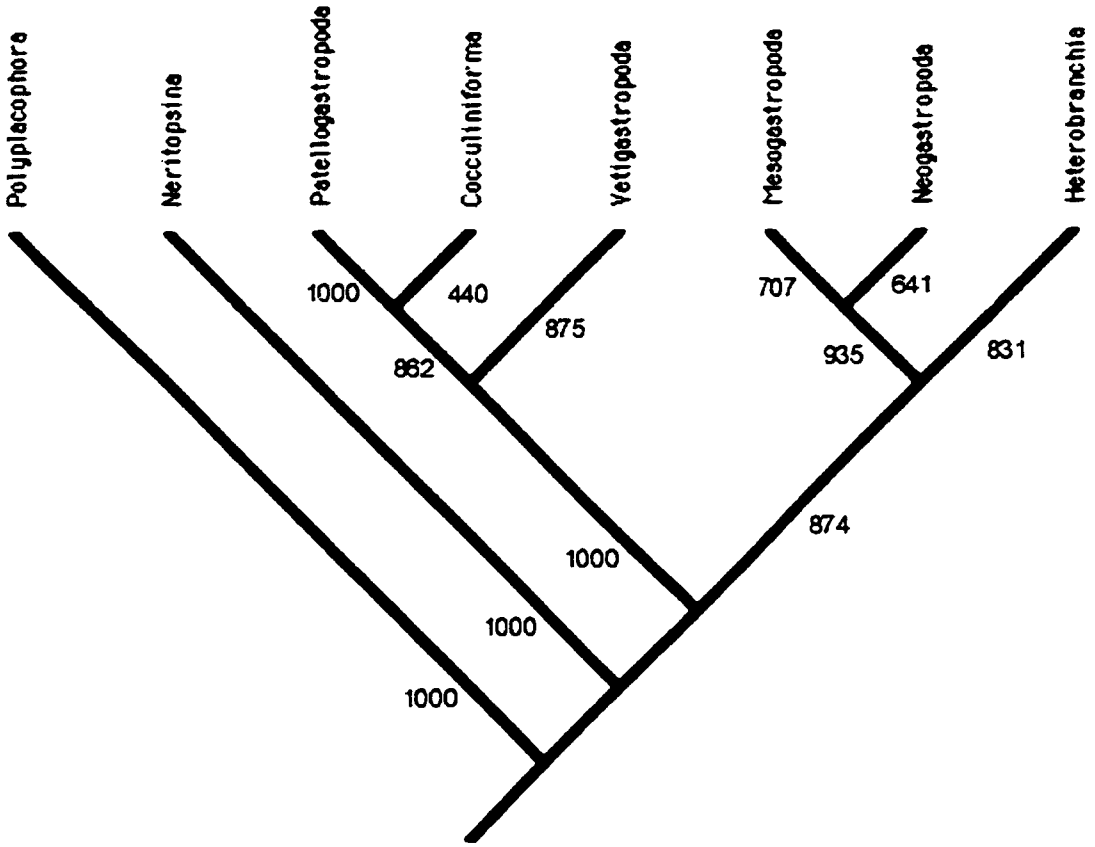

Figure 14. Cladogram of gastropod subclass taxa derived from molecular data (The numbers represent bootstrap confidence values out of 1000).

4.5.2 Summary

1. Contrary to Ponder and Lindberg's (1997) findings, this part of the study finds that the basal gastropod (gastropods other than caenogastropods and heterogastropods) subtaxa are quite close. This is more in agreement with Haszprunar's work (1988), which groups these organisms into the Archaeogastropoda. However, I suggest that the term Archaeogastropoda is at very best a grade in that there is a monophyly indicated by the Vetigastropoda.
2. The Neritopsina is monophyletic and can be considered a sister group to all other gastropods, which was also found by Haszprunar (1988), but not supported by Ponder and Lindberg (1997). Ponder and Lindberg (1997), however, do show the Neritopsina as being quite primitive and monophyletic. This contradicts Thiele's

(1929) scheme, which shows the Neritopsina within the Archaeogastropoda and more closely related to and deriving from the vetigastropod/patellogastropod group.

3. This study shows the Patellagastropoda/Cocculiniforma and the Vetigastropoda as closely related. This is the original position of these two group arising from Thiele (1929) and his concept of Prosobranchia. This situation is probably not correct as there is considerable evidence to the contrary, both morphological (Ponder & Lindberg, 1997) and molecular (Harasewych *et al.*, 1997a). I described possible reasons for this finding as being a low number of primitive gastropods analyzed or poor alignment within this evolutionary region of gastropods.
4. There is strong support for the idea that the Caenogastropoda form a monophyly or a clade. Both Haszprunar (1988) and Ponder & Lindberg (1997) supported this idea that the earlier use of the term Prosobranchia to include the primitive snails as well as the Caenogastropoda (Mesogastropoda & Neogastropoda) is probably inaccurate.
5. Within the Caenogastropoda, there is strong support that the terms Mesogastropoda and Neogastropoda represent grades of development (due to their low, but significant bootstrap value) within the strongly monophyletic Caenogastropoda.
6. I support Haszprunar's (1988) and other authors' use of the terms Architaenioglossa, Neotaenioglossa and Eucaenogastropoda (= Hypsogastropoda = Sorbeoconcha) to describe grades of development within the Mesogastropoda.
7. There is strong support for the monophyly of the Heterobranchia clade (the Opisthobranchs, Pulmonates and the Pyramidelloidea) although resolution beyond this level was not possible.
8. The inclusion of species in the family Rissoellidae within the Heterobranchia is supported by molecular evidence and gives credence to the concept that the Heterobranchia, which includes the euthyneuran Opisthobranchia and Pulmonata, were derived from lower caenogastropod stock. This idea was earlier advanced by Fretter and Graham (1962) and Kollmann and Yochelson (1976), based on proposed morphological precursors and paleontological evidence, respectively.

CHAPTER FIVE
TOMOGRAPHY AND SNAIL MORPHOLOGY

5.1 Introduction

5.1.1 Characters of structure and function

Scientists wishing to understand the use of a structure must first evaluate its form, because the latter is inextricably related to function. This relationship works on many levels, from molecules to cells to organs and organisms. An example is evident in the previous chapter in which the function of the 18S rRNA molecule was shown to be directly related to its sequence, secondary and tertiary structures. This form-function relationship is especially evident in the area of hydrodynamic biomechanisms (Vogel, 1994). Within the gastropod foot, there are no hard skeletal structures for providing leverage and allowing muscles mutually antagonistic forces. All such skeletal arrangements are provided by hydrostatic means (Voltzow, 1985). Since the gastropod foot is a highly synapomorphic (shared and derived) characteristic, differences of the form and function of this structure within and between various taxa of gastropods can give valuable phylogenetic information and trends.

Ponder and Lindberg (1997) warned that overemphasis on form and function may mask certain morphological patterns in data by subsuming them into large and multifarious character complexes; that is, one should look at these systems in a descriptive manner and guard against attaching outrageous functions. Ponder and Lindberg (1977) suggested breaking down structure complexes into character states to be able to reconstruct phylogeny.

When trying to characterize the full nature of the form and function of gastropod structures, it is necessary to view the animal internally. In the past, anatomists, such as Crofts' (1937) work on *Haliotis*, had to cut into the structure using gross dissection

techniques. However, since the gastropod's foot uses fluid-filled areas as its hydrostatic elements, dissection is particularily disruptive to the arrangement of these tissues and fluid spaces. One approach is to produce an entire histological data set of the organism, which is an exhausting undertaking. As a result, there are relatively few papers that look at this aspect of gastropod morphology. The earlier papers therefore tended to focus more on fine structure as opposed to general function (Rotarides, 1945, Gainey, 1976, Voltzow, 1985). Here, I use a non-invasive technique for viewing the intact foot as it appears in life.

Firstly, I describe the two techniques used in this chapter, magnetic resonance microscopy (MRM) and histological microtomy. Also, I outline the theory behind MRI and the mechanics of the MRM technique and explain why I created histological sections. The second area of background serves as a starting point by identifying the organisms in this study and mapping them to the molecular phylogenetic tree as seen in the previous chapter. Then, I relay a more indepth morphological background upon which I build a more holistic picture while describing the hydrostatic skeletal morphology. Finally, the last area is the description of the form and function of the hydrostatic elements that are characterized in this study.

5.1.2 Theory and mechanics of magnetic resonance microscopy

I learned much from the Duke University team on the basics of magnetic resonance theory. The background information presented here is adapted from Callaghan's (1993) MRI. The MRM technique began with the research of Felix Bloch and Edward Purcell (Stanford and Harvard Universities respectively), which led to the discovery of nuclear induction (a discovery which awarded them the Nobel Prize in 1952) (Hulthén, 1952).

In their natural state, the nucleons of an atom (neutrons and protons) are spinning. When the nucleons of an atom are unpaired, they wobbling as they spin or "precess". This wobbling results in a net nuclear moment. When this spinning magnetic moment is placed in a magnetic field, it will tend to align and precess about the applied field.

The simplest case in the description of magnetic resonance and nuclear induction is the description of hydrogen in water. This is the phenomenon of which most MRM takes advantage. The hydrogen atoms in water consist of one unpaired proton. Since the proton is a positively charged particle, which spins with an angular momentum, it has a magnetic moment that will cause it to precess once placed in a magnetic field. As living organisms contain much water, there is a huge abundance of these precessing protons all randomly wobbling. When these protons are put into a magnetic field that is strong enough, the hydrogen ceases to randomly precess and begin to align with this field. This means that as protons align with the field and they begin to precess at the same angle and frequency around this field.

This frequency of precession, known as the Larmor frequency (ω), is calculated by the following formula:

$$\omega = \gamma \cdot \mathbf{B_0}$$

Where γ is a constant and $\mathbf{B_0}$ is the external magnetic field. As everything is precessing together, the collective wobble causes a net magnetization ($\mathbf{M}$) precessing at the Larmor frequency. Nuclear induction is the addition of another external magnetic field $\mathbf{B_1}$ with a frequency of ω at an angle different from $\mathbf{B_0}$. This causes $\mathbf{M}$ to try to align with both $\mathbf{B_1}$ and $\mathbf{B_0}$. If an antenna (i.e., an Rf coil, see below) is set up around the protons, as $\mathbf{M}$ is being pushed out of its original alignment with $\mathbf{B_0}$, it will create an electromagnetic (EM) signal in the antenna with a frequency of ω (in the case of MRM, the frequency lies within the radio frequency range 85-400 MHz).

To make an image, the NMR signal is encoded by the use of magnetic field gradients that alter the local magnetic field and thus altering the local Larmor frequency. For example, applying a gradient along the specimen while the initial Rf excitation pulse is applied will cause the field to vary along this z-axis. If the applied Rf pulse is a narrow band of frequencies, only a narrow "slice " of the specimen will be at the correct field to satisfy the Larmor equation. Only the spins in that slice will be excited and signal will only be generated within this slice. Additional gradients and Rf pulses can be applied to produce a wealth of encoding and excitation strategies. The particular

encoding scheme used in this work is called three-dimensional spin warp encoding. First introduced by Edlestein *et al.* (1980), the technique has subsequently been modified to allow three-dimensional imaging (Johnson *et al.*, 1983) and extended to very large arrays for MRM by Suddarth (Suddarth & Johnson, 1991). Image arrays as large as 256 x 256 x 256 were acquired with isotropic resolution along all three axes (Johnson *et al.*, 1992).

With these varied "pulse sequences", it is possible to differentiate soft tissues on a wide range of biophysical parameters, such as proton density, spin lattice relaxation time (T1), and diffusion, all of which reflect some properties of the water in the tissue. The contrast in the images shown in this work is most probably due to differences in local proton density. Extensive descriptions of the contrast mechanisms for MRI in general and MRM at high fields are available (Wehrli *et al.*, 1985, Johnson *et al.*, 1985, Dockery *et al.*, 1989).

5.1.3 Identification of structures

Since MRM is new and has not been tested on molluscan tissue before, the first step was to scan with natural hydrogen densities. This initial imaging shows natural differences of tissues with varying hydrogen densities. If a given tissue appears darker in the resulting image, it must be denser and contain less fluid and conversely if a tissue has a higher percentage of water (i.e. less dense) it will appear as a lighter structure in the scan. Future studies may include doping procedures such as perfusion of proton-suppressing materials or hyper protonated materials, which would show up on the scan as very dark or very light structures. Much of these doping experiments have been conducted on live mice (e.g., Möller *et al.*, 1999, Viallon *et al.*, 1999, Benveniste *et al.*, 1998, Chen *et al.*, 1998).

Doping procedures, however, were not performed in this study because of the restricted access to the microscope. Instead, I identified the tissue types by comparing the natural hydrogen density MRM scans to histological sections of the very same specimens. This was possible because the MRM scans were not destructive. The

organisms were saved and later underwent paraffin wax imbedding, rotory or sled microtome sectioning and histological staining for muscles, connective tissue, etc.

5.1.4 Organisms of Study

The number of organisms tested (eight) was based on number of hours of "magnet time" that the Center for *In Vivo* Microscopy afforded me. The organisms were selected based on availability and their taxonomic placement. The selected animals were as follows: *Calliostoma canaliculatum*, a small marine snail, is variously considered as a prosobranch archaeogastropod or a vetigastropod. *Diaulula sandiegensis* is a shell-less marine opisthobranch known as a sea slug. *Haliotis rufescens*, the red abalone, is considered either a primitive archaeogastropod or vetigastropod. *Lymnaea stagnalis* is a common freshwater pulmonate. *Marisa cornuarietis* is a freshwater ramshorn that is considered either as a mesogastropod or a primitive caenogastropod. It represented the only planar coiling shell in this study. *Nucella ostrina* is a member of the very large family Muricidae and is considered as either a neogastropod or an advanced caenogastropod. *Pomacea bridgesi* is one of the largest freshwater snails, and is of the same family as *Marisa cornuarietis*. *Pomacea bridgesi* was chosen for comparative reasons as it has a more conventional spiral shell. *Searlesia dira* is a common marine snail from the very large family Buccinidae and is considered a neogastropod or an advanced Caenogastropod.

5.1.5 Survey of the large subclass taxa

A morphological characterization of the three major subclass taxa is in order to understand the organisms of this study as well as the analysis that is to follow. This review is written with a focus on the organisms in this morphological study and with the systematic point of view as resolved in the previous chapter.

The first group under consideration is the Opisthobranchia since it is a taxon that is conserved within all the schemes under review. (*see* Rudman & Willan, 1998)

Opisthobranchs are among the most colourful and structurally diversified of the gastropods. Their evolution shows a trend in shell reduction, to the extent of loss in many cases, and are generally classified into grades based on this characteristic (Beeman & Williams, 1980). The primitive opisthobranchs have a general body shape similar to that of *Lymnaea stagnalis* (personal observation). Many opisthobranchs move about by creeping using cilia and mucous, although the larger ones crawl by muscular waves or even swim using lateral extensions of the foot or by dorso-ventral flexion of the whole body (initially observed by Jordan, 1901). These animals are almost always marine and are all known to be hermaphroditic (Rudman & Willan, 1998). Like most molluscs, their main body cavity is haemocoelic and not coelomic (the coelom is usually confined to the kidneys, the pericardial sac and the reproductive system [Kay *et al.*, 1998]). The haemocoel, which is a part of the circulatory system, is also used as a hydrostatic skeleton and extends as small sinuses and lacunae within the musculature (Rudman & Willan, 1998).

The Pulmonata are similar to the opisthobranchs in that their monophyly has not been questioned until recently. Currently, they are grouped with the Opisthobranchia as the Euthyneura whereas they were considered separate subclasses before the 1970s. Haszprunar *et al.* (1997a) supported the move to have them grouped with the Pyramidelloidea as the Heterobranchia, a move further supported by the results of the molecular data within this thesis. Morphologically, pulmonates are molluscs that use a mantle lung as their respiratory surface. The large majority of pulmonates live in terrestrial or freshwater habitats, although some primitive pulmonates inhabit intertidal areas. Physically, most have a spirally-coiled shell and have lost the operculum and most are usually simultaneous hermaphrodites (Smith & Stanisic, 1998). Plesche *et al.* (1975) found that the musculature of the terrestrial pulmonate body wall is more developed than in aquatic forms. However, in either case it seems that the arrangement surrounds a hydrostatic cavity to various extents. In many pulmonates relative to other gastropods, Kier (1988) noted that the muscle density is low and hydrostatic lumena are extremely important in these animals. It is clear that the haemocoel, with its various thin septa, is

the hydrostatically active cavity in providing support for the shell and organs (Smith & Stanisic, 1998).

The Caenogastropoda is a large group of freshwater and marine gastropods that include the Mesogastropoda and Neogastropoda as defined in the molecular study (Chapter 4). Paleontogical research shows that the Mesogastropoda first appeared in the early Ordovician Epoch and from them, the Neogastropoda arose in the Cretaceous Period (*see* section 2.3.2.1). This evolutionary path is supported by the molecular study herein, as well as by morphological studies (Haszprunar, 1988, Ponder& Lindberg, 1997). The Caenogastropoda as a group have several advancements over the patellogastropods and vetigastropods. The caenogastropods have compact nervous system (Fretter *et al.*, 1998), which may have evolved as the caenogastropod moved from the herbivory and detritivory of the lower mesogastropods to carnivory in some mesogastropods and the neogastropods (Ponder, 1998). With carnivory, these organisms developed new radular complexes, proboscises (particularily the introvert or an inward turning snout) and even hypobranchial secretions containing poison (Graham, 1985).

The final group investigated in this study is the primitive Vetigastropoda which, in the classical scheme, were considered part of the prosobranch Archaeogastropoda. The animals selected here are representative of a large number of vetigastropod grazers with the rhipidoglossate radular condition for scraping up biofilms. These animals have broad feet suitable for maintaining a good hold on firm substrate. The sole of the foot contains a complex and dense array of dorso-ventral and transverse or longitudinal muscles that extend and fill the foot (Voltzow, 1988, 1994, Kier, 1988). The vetigastropod foot is a solid and dense structure that is very complex. Fretter *et al.* (1998) outlined this complexity by noting that there are three important locomotory features of the foot, the epithelium of the sole (which has to be elastic), the haemocoelic vesicles above the epithelium (at least some of these vesicles are now thought to be glands [Kier, pers. comm., 1999]) and the muscles (extrinsic dorso-ventral columellar muscles and transverse intrinsic muscles).

5.1.6 The evolution of form and function

The snail's foot is a dynamic structure that is intriguing because of its range of use. Some of the more spectacular uses that I have observed are the limpet's ability to clamp onto the substrate with more force than it is attached to its shell, the ability of an abalone to rapidly swim away by undulating its broad foot, the careful effort of a whelk as it uses its foot to mould its egg cases, or the ability of the moon snail to cover its entire shell with its inflatable foot and even more miraculously pull its entire foot back into its shell.

The foot, although providing myriad functions, is based on a singular plan that is indirectly the basis of the most synapomorphic character that defines the Gastropoda (Kay *et al.*, 1998). The gastropod foot is synapomorphic by virtue of its asymmetry, which is the result of torsion. Indeed, torsion, which occurs in all gastropods (although many secondarily "detort") causes much more than the foot to become asymmetrical, for it introduces asymmetries in most body structures including shifting the anal and kidney openings. Regardless of why torsion occurs, the more important question to this thesis is how this occurs. The answers may provide some clues as to how to analyse my data for evolutionary trends.

Authors generally agree (Voltzow, 1985, Brusca & Brusca, 1990, Kay *et al.* 1998) that torsion results from differentially developed muscles that arise from left and right mesodermal bands (Smith, 1935, Crofts, 1937), the result of which is a counter-clockwise rotation of the visceral mass and shell over the head and foot to as much as 180 degrees. The reason it is always counter-clockwise is the precise method by which the musculature interacts to create the torsion. Kay *et al.* (1998) suggested there is evidence that torsion occurs in some animals, before muscles are developed, however the right mesodermal band differentiates into the larval retractor muscles (Voltzow, 1985) and the left eventually develops into the major columellar muscle (the right one in the adult since torsion reverses the positions of the muscles) (Crofts, 1937). The larval retractor muscles

gives the veliger the ability to pull its velum into the protoconch. Although during torsion, these muscles contract and remain contracted to rotate the shell 90 degrees (the rest of torsion occurs by differential tissue growth [Brusca & Brusca, 1990]). Crofts, (1937, 1955) noted that the contracted retractor muscles become a very small left columellar muscle in primitive organisms and usually incorporates into the head musculature in most others. In all adult gastropods, the columellar muscle is actually the hypertrophied right columellar muscle that was derived from the left mesodermal band.

Janice Voltzow's (1985) doctoral dissertation was novel in describing the functional and internal morphology of the pedal musculature. She noted that there were two muscular elements within the foot, the columellar muscle, the ontogeny of which is described above, and the less well understood tarsos musculature. The term "tarsos" (greek = flat bottom) was coined by Voltzow (1985) and described ventral and peripheral muscles of the foot that are not directly attributable to the columellar muscle. The tough columellar muscle is attached along the columella and extends ventrally towards the sole and, in species with opercula, angled to the posterior to insert on the operculum. The spongy tarsos take on a ventral position and form a loose three-dimensional network of interwoven muscles. The tarsic muscles extend from the ventral side of the columellar muscle and continuously subdivide into finer and finer bundles until they insert into the foot wall. It was Voltzow's (1985) finding that about 70% of the bundles remain within the columellar muscle and the rest branch off into the tarsos so that one cannot easily segregate one muscle type from the other. Tarsos and columellar musculature interweave in the central third of the foot.

Muscles only provide tension or shortening in contraction and thus require an external agent for restoration, which may be provided in several ways. In vertebrates and arthropods, muscles are arranged in pairs through the intervention of hard skeletal framework. Gastropods generally do not use hard structures in this way and thus rely on other forms of antagonism. One form is the well-described fluid-filled hydrostatic skeleton seen in many soft-bodied animals (Brusca & Brusca, 1990). In 1983, Kier described another mode of antagonism and he coined the term muscular hydrostat to describe it. Kier & Smith (1989) used the term to define the operation of the human

tongue or elephant trunk that showed no hydrostatic cavity around which muscles could interact. Instead, they found the muscles were directly inserting and originating on each other and in effect using the turgidity of the fluid encapsulated within each cell as the hydrostatic element. These two types of hydrostatic skeletons both occur in various forms in the gastropod foot.

Almost all gastropods use the foot in some way to effect locomotion. There are however, different methods of using the foot to move around. Considering only the movement over a substrate using the sole of the foot, one finds that there are two strategies: gastropods can move by either ciliary movement or by muscular waves. Generally, ciliary movement is reserved for smaller organisms and slower speeds and is characterized by the entire sole of the foot being evenly in contact with the substrate and a uniform movement over a layer of mucous (Kay *et al.*, 1998). Audesirk and Audesirk (1985) found that the fastest of the snails using this type of locomotion is *Tritonia* at a top speed of 1.9 mm/second. The other form of locomotion, using muscular waves, has been reviewed by Miller (1974). Forward movement by gastropods can be effected by moving a series of waves either forward or backward, along the axis of the foot, usually in conjunction with secretion of mucopolysaccharides (Denny, 1981). This scheme of moving the wave forward by passing the wave from the posterior to the anterior in the direction of travel is termed direct locomotion. The opposite condition is referred to as retrograde. Miller (1974) noticed that the speed of movement is a function of the muscular contraction speed and the amplitude of the ripples. Increasing the amplitude increases the speed. Extreme speed can be achieved by either functionally or physically splitting the foot longitudinally into two equal halves. In the most extreme cases, this technique seems to take on the characteristic of walking where one side stays attached while the other moves forward, and it constitutes the greatest form of amplitude aggrandizement. Such a splitting of the foot is termed ditaxic, whereas if the wave extends over the entire width of the foot, the term used is monotaxic.

5.2 Materials and Methods

5.2.1 Protocols

5.2.1.1 MRM

The snails represented here are a subset of the organisms discussed in Chapter 3 and hence animals are from the same sources. The protocol used here was designed by Dr. Bradley Smith, Duke University Medical School's Center for *In Vivo* Microscopy. His technique was designed for the imaging of mouse embryos in order to develop an embryological atlas but with his help I modified it to produce high-resolution images of snails. The following is the modified technique.

All the animals were anesthetized by either slowly adding a 0.7% MgCl w/v solution to their seawater holding tank or slowly adding a saturated propylene phenoxetol solution to their water (the latter was brought to the Bourne laboratory's attention by Dr. Bob Shadwick of the Scripps Institution of Oceanography). The animals were then fixed for imaging (2% (v/v) gluteraldehyde/ 1% formalin in phosphate buffer at 300 milliosmoles/litre). All specimens were packed under vacuum in fixative within a custom-built imaging container. Plastic syringes with the ink volume markings removed, were used for small animals or, for the larger snails, heavy gauge plastic bags that were hermetically sealed (using a commercial "Seal-a-meal" vacuum device).

All data, except for *Haliotis* and *Diaulula*, were acquired at magnetic strength of 7 Tesla (T) by using a GE NMR Instruments Omega system modified for MR microscopy (*Haliotis* and *Diaulula*, the two large specimens were acquired at 2T). The 7 T magnet (Oxford Instruments, England) has a 15 cm bore (Bruker Instruments, Fremont, CA) with a 90 Gauss/cm gradient. The 2T magnet (Oxford Instruments, England) has a 30 cm bore (General Electrics NMR Instruments, Fremont, CA) with a gradient of 20 Gauss/cm. Two appropriate "bird-cage" resonators (Rf coils) had been custom designed

and fabricated from a single sheet of dielectric microwave substrate to suit the two size classes. All studies were GRASS (Gradient Refocused Acquisition in the Steady State) Scans with a Rf flip angle of 30° for the 7T and 60° for the 2T scans. For all data acquisition, the echo time was 2 ms (TE = 2 ms) and the repetition time was 200 ms (TR = 200 ms). Scanning data were reconstructed by Fourier transform on a Sparc 1 workstation (Sun Microsystems, Mountain View, CA). The resulting 256, 16-bit image slices were archived and then scaled to 8 bits for volume rendering on a Silicon Graphics workstation (Iris 4D/320VGX, Silicon Graphics, Mountain View CA) using VOXELVIEW-ULTRA 2.0 (Vital Images, Fairfield, IA). Residual matrix noise was manually removed from the resultant bitmap sequences using a PenPartner 4 x 5 tablet (Wacom) and Photoshop 5.5 (Adobe) running on a 500MHz Intel PC (Win 98, dual head, 128MB RAM, 9GB HD, 4x CD/WR).

Dr. Smith reloaded the data for each snail into VOXELVIEW-ULTRA 2.0 and we reoriented them such that the snail images were situated into the orthogonal planes. The data were then resectioned into transverse, sagittal and frontal sections (*see* Figure 15).

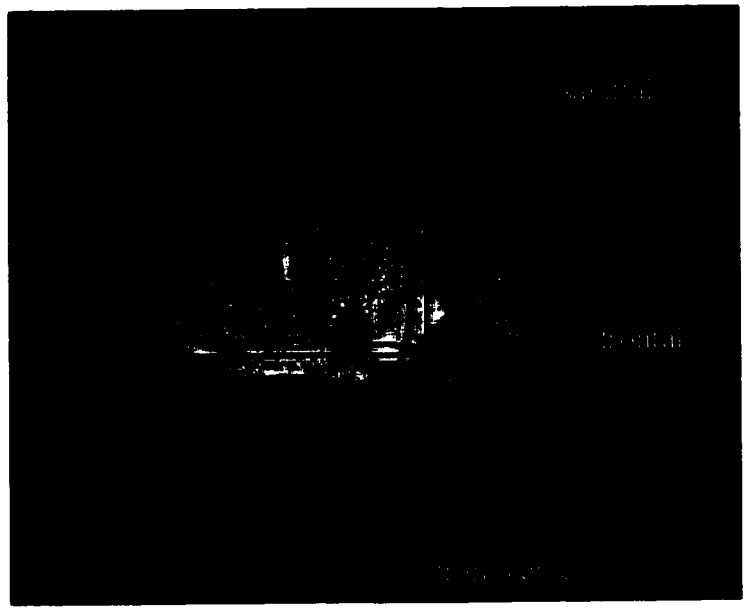

Figure 15. The three orthogonal planes in which all eight snails have been sectioned via MRM. (Example snail is *Lymnaea stagnalis* oriented with its anterior to the left, dorsal surface up, and left side out of the plane toward the reader).

5.2.1.2 Histology

The intact specimens were saved, transported back to the University of Calgary in the same buffered fixative used for MRM and were subsequently sectioned via conventional microtomy. The animals underwent the following imbedding protocol using fresh solutions at each step:

1. 70% Ethanol dehydration bath for 3 h
2. 95% Ethanol dehydration bath for 2 h
3. 100% Ethanol dehydration bath for 2 h
4. 100% Ethanol dehydration bath for 2 h
5. Clear for 2 h (Stephens Scientific Clearing solution, CAS 5989-27-5)
6. Clear for 2 h
7. Infiltrate using Paraplast X-tra tissue embedding medium (Oxford Labware) for 24 h.
8. Infiltrate in Paraplast for 1 h
9. Infiltrate in Paraplast for 1 h
10. Infiltrate in Paraplast for 1 h
11. Block out in appropriate mould.

An American Optical rotory microtome (model 820) was used to section the smaller animals. The larger animals were sectioned using a Lipshaw sled microtome (model 80A). The resultant sections were then all stained using a modified Milligan's trichrome stain (based on the protocol in Presnell & Schreibman, 1997). The staining procedure is as follows:

1. Clear and deparaffinize (Stephens Scientific Clearing solution, CAS 5989-27-5) for 3 minutes.
2. Clear and deparaffinize for 2.5 min
3. 100% Ethanol hydration bath for 3 min
4. 95% Ethanol hydration bath for 1 min
5. Mordant for 7 min

   Mordant bath composition:

Solution A:	Potasium dichromate ($K_2Cr_2O_7$)	3.0 g
	Deionized H_2O	100.0 ml
Solution B:	100% HCl	10.0 ml
	95% Ethanol	100.0 ml

   Mix Solution A and Solution B together and use within 4 h
6. Deionized H_2O rinse for 30 s

7. Acid Fuchin stain for 8 min
   Acid Fuchin bath composition:
   Fuchin (C.I. 42685) 0.2 g
   Deionized H_2O 200.0 ml
8. Deionized H_2O rinse for 30 seconds
9. Phosphomolybdic acid preparation for Orange G for 5 min
   Phosphomolybdic acid bath composition:
   Phosphomolybdic acid ($20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) 4.0 g
   Deionized H_2O 400.0 ml
   (use half in Orange G stain below)
10. Orange G stain for 6 min
    Orange G bath composition:
    Orange G (C.I. 16230) 4.0 g
    1% phosphomolybdic acid (above) 200.0 ml
11. Deionized H_2O rinse for 30 s
12. 1% Acetic acid for 2 min
    Acetic acid bath composition:
    Glacial acetic acid 20.0 ml
    Deionized H_2O 180.0 ml
13. Fast Green stain for 10 min
    Fast Green bath composition:
    Fast Green FCF (C.I. 42053) 2.0 g
    2% acetic acid 20.0 ml
    (19.6 mL Deionized H_2O & 0.4 ml Acetic acid)
    Deionized H_2O 180 ml
14. 1% Acetic acid fix for 3 min
15. 95% Ethanol dehydration bath for 5 min
16. 95% Ethanol dehydration bath for 5 min
17. 100% Ethanol dehydration bath for 3 min
18. 100% Ethanol dehydration bath for 3 min
19. Final Clearing in clearing solution for x min (such that 5 min < x < 4 h)
20. Mount slides with coverslips using Permount (Fisher-Scientific, SP 15-500)

The resultant thin sections picked up stain as follows:

Nuclei and muscle tissue	Magenta (a deep purplish red)
Collagen and fibrinoid connective tissue	Green/blue

The sections were photographed using a Nikon Eclipse TE 300 inverted microscope with a Nikon F-601 Automatic SLR camera attached and loaded with Kodak EliteCHROME,

I.S.O. 100, slide film. The developed slides were digitized using a Kodak SprintScan 35 slide scanner with a resolution of 600 dpi.

5.2.2 Analysis

After describing the hydrostatic elements of the gastropod feet, I analyzed the results for phylogenetic trends. The morphological characteristics were not analyzed by computer, but were instead mapped manually to the molecular tree of Chapter 4. This was done because the morphological characters were not conducive to being inputted to a character weight matrix. The morphological description of MRM data was completed by the use of a slice selection program developed by Uyeno & Uyeno (unpublished, 1999 see Appendix V). A comparative description of the histological sections was completed to aid tissue identification of the MRM data. Each organism was described based generally on the characteristics discussed in Section 5.1.6, that is, the interplay of reliance on the two types of hydrostatic skeletons (muscular hydrostat *versus* fluid-filled cavity), the morphology and reliance on each of the tarsos and columellar musculatures and the morphological basis of locomotory type.

5.3 Results

Following are descriptions of the magnetic resonance microscope images and histological slides for the eight snails in alphabetical order (See Table 9 for summary):

Calliostoma canaliculatum shows a very thick columellar muscle that is attached to the spiraling columella for at least one whorl (Figures 16-18).

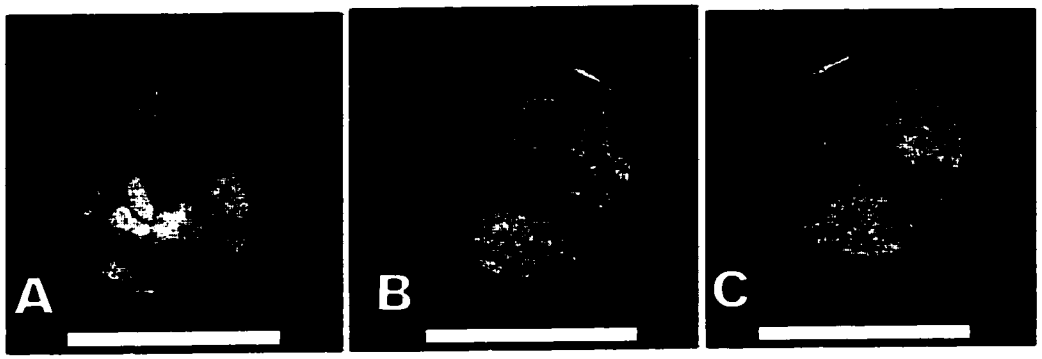

Figure 16. Series of frontal sections through the spiral region of *Calliostoma canaliculatum* obtained by MRM. The columellar muscle attachment to the columella is marked in red (A to C are ventral to dorsal sections). Scale bar = 1 cm.

It extends from the columella down into the foot and splits into two halves, left and right which is indicative of ditaxic locomotion (Figure 17).

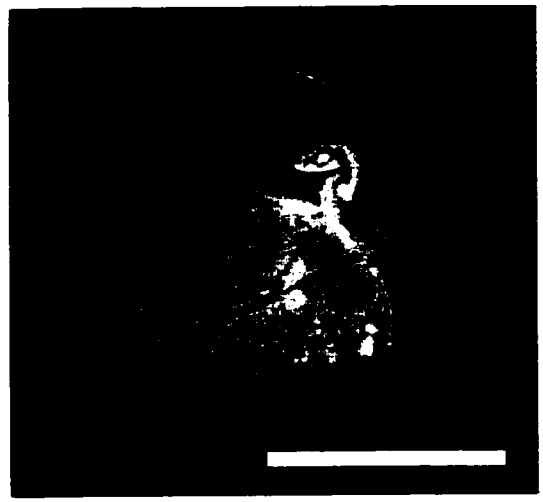

Figure 17. A frontal section through the lower foot region of *Calliostoma canaliculatum* obtained by MRM. Red arrows point out the division plane of the foot. Scale bar = 1 cm.

These two halves curve posteriorly and insert on the operculum (Figure 18). The differentiation of the collumellar and the tarsic muscles is quite difficult to distinguish as the densities of both muscles are similar. The muscular density is high and there seems to be no sizable hydrostatic cavity within the foot.

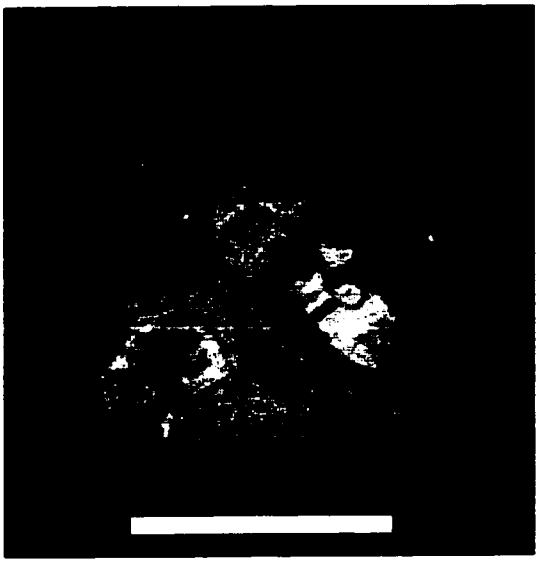

Figure 18. A sagittal section through the midline of *Calliostoma canaliculatum* obtained by MRM. The red arrows show the thickness of the columellar muscle. Scale bar = 1 cm.

There is evidence in the histological sections (Figure 19, left photo) that spaces open up within the haemocoel in extended portions of the foot. This indicates that in the foot the haemocoel exists as a lacunar network.

Figure 19. Histological sections through the foot of *Calliostoma canaliculatum.* (Left photograph [black bar = 100 μm] shows the edge of the foot with its spaces. Right photograph [black bar = 100 μm] shows the dense foot matrix).

With respect to the composition of the foot matrix, there is a very solid and dense arrangement of connective tissue with muscle fibres running through them at even intervals (Figure 19, right photo).

Diaulula sandiegensis is the only non-shelled organism in this study and so appears quite different externally.

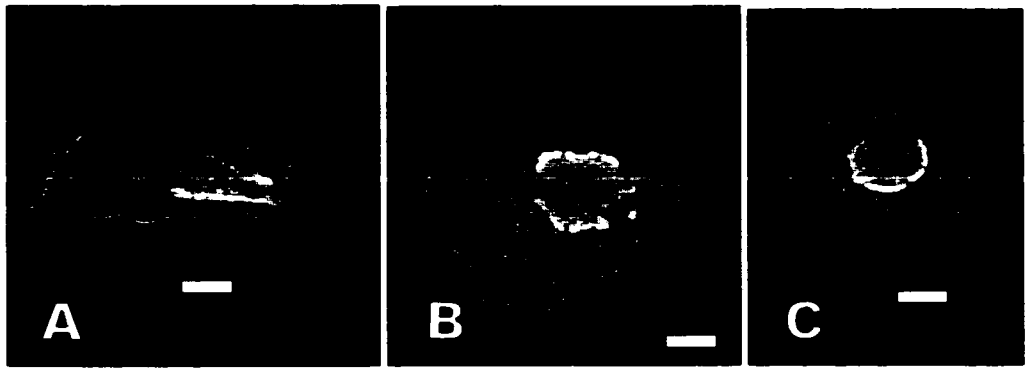

Figure 20. Sagittal (A), frontal (B) and transverse (C) sections through the midline region of *Diaulula sandiegensis* obtained by MRM. (A shows the connection of the anal papillae to the haemocoel, B shows the bilateral nature of this organism, and C shows the central viscera as well as the thick sole [marked in red]). Scale bar = 1 cm.

Since this group of gastropods detort, they take on a bilateral form (Figure 20 B & C). The visceral mass is now within an extensive body cavity (Figure 20 A & B). This main body cavity appears to be haemocoelic because it is separate from and encloses the pericardial cavity (Figure 20 A,B & C). The surface of this organism is dotted with many hard calcareous spicules inbedded within an extremely thick notum. The bulk of the haemocoelic volume is composed of a central cigar shaped space that contains the internal organs (Figure 21).

Figure 21. Whole animal image (dorsal view) of *Diaulula sandiegensis* obtained by MRM. The area shaded red denotes the bulk of the haemocoelic volume and red arrows point out examples of imbedded spicules. Scale bar = 1 cm.

External to this central core is a mantle skirt and the flat long foot. There are dense muscles running from the foot dorsolaterally to the dorsal surface (Figure 22), which are described as the pedal retractor muscles and are thought to be of similar ontogenetic origins as the columellar muscle (One can also see these denser areas of tissue in an oblique manner in Figure 20 C).

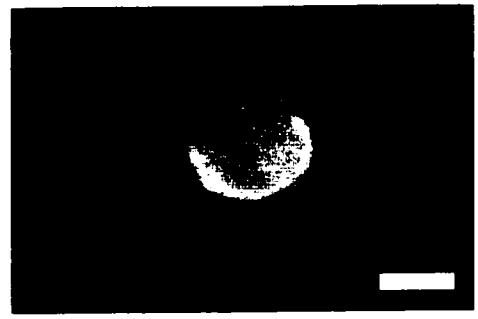

Figure 22. Whole animal image (anterior view) obtained by MRM, of the dorsal-ventral musculature in *Diaulula sandiegensis*, which appear as dense bands between the red arrows. Scale bar = 1 cm.

The histological sections (Figure 23) show thick-wall musculature and a dense matrix of connective tissue with bundles of dorso-ventral muscles running through out.

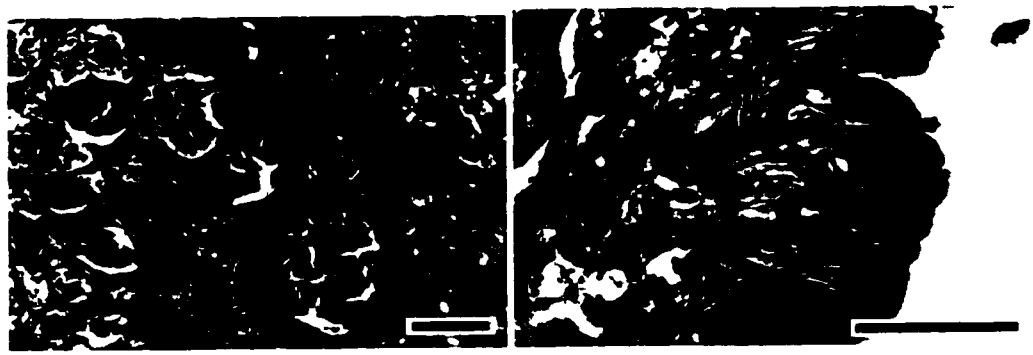

Figure 23. Frontal histological sections through the foot of *Diaulula sandiegensis*. (Left photograph [black bar = 100 μm] shows dorsal-ventral muscular bundles running through the foot matrix. Right photograph [black bar = 100 μm] shows the edge of the foot.)

The matrix contains many small haemocoelic spaces and dorso-ventral muscles (Figures 23 & 24). Tarsos musculature appears as thin strands within the matrix and appears to be running at angles perpendicular to the pedal retractor muscles and inserting on the foot and mantle skirt wall (Figure 24).

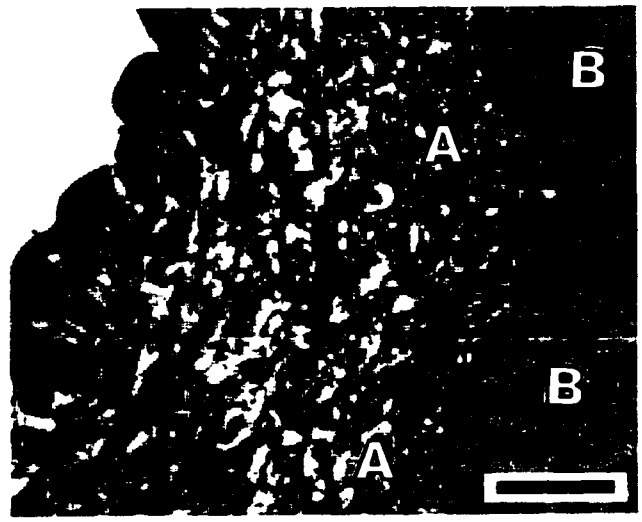

Figure 24. Frontal histological section through the foot of *Diaulula sandiegensis* showing small muscular bands (labeled A) oriented in a perpendicular fashion to the dorsal-ventral muscles (area shaded red and labeled B on right) (black bar = 100 μm).

Haliotis rufescens has a shallow dishlike shell, which cannot be closed by an operculum. A result of this is that the columellar muscle becomes barrel-shaped and oriented in a strict dorso-ventral orientation (Figure 25).

Figure 25. Sagittal section through the midline of *Haliotis rufescens* obtained by MRM showing massive dorsal-ventral columellar muscle (shaded red). Scale bar = 1 cm.

The insertion of the columellar muscle on the shell is massive and takes up a large amount of the surface area under the shell. The muscle appears to be strictly dorso-ventral until it reaches the mid-sagittal line where the bundles seem to spread out laterally into the tarsos. Only the very peripheral areas such as the extreme edges of the foot and the epipodial skirt seem to contain less dense tissue (Figure 26).

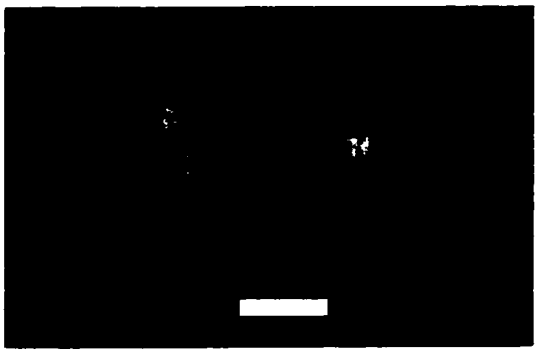

Figure 26. Transverse section through the midline of *Haliotis rufescens* obtained by MRM showing areas of tarsos musculature in the epipodial skirt and foot edges as pointed out by the red arrows. Scale bar = 1 cm.

This being the case, it is difficult to see any regional differences between tarsos and columellar areas. The foot is composed of dense tissue with no significant hydrostatic cavity present (Figure 26). The histological section confirms this and shows mostly a solid matrix of connective tissue and muscle with only a sparse number of haemocoelic vessels interrupting (Figure 27).

Figure 27. Histological sections through the foot of *Haliotis rufescens*. (Left photograph [black bar = 100 µm] shows edge of foot. Right photograph [black bar = 100 µm] shows dense foot matrix and haemal spaces).

Lymnaea stagnalis shows a very thick foot epithelium. The haemocoel is extremely pervasive and the tarsos is very thin (Figure 28).

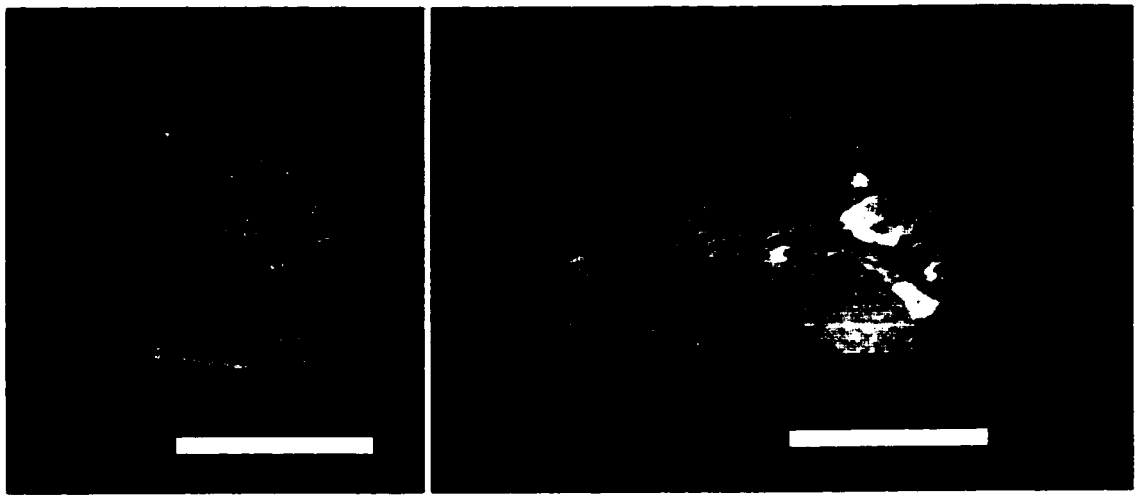

Figure 28. Transverse (left) section near the anterior and sagittal (right) section through the midline of *Lymnaea stagnalis* obtained by MRM. Left scan shows red arrows pointing out the columellar muscle secondarily bifurcating and red lines outlining the thickness of foot wall. Right scan shows columellar muscle extending and bifurcating across haemocoel in foot. Scale bar = 1 cm.

The columellar muscle originates along the columella within the first three quarters of the body whorl. As the columellar muscle descends ventrally into the foot it bifurcates and then further subdivides as it gets closer to the edge of the foot (Figure 28). The foot appears in the MRM scans as a large haemocoelic space with muscular elements running through them. Generally, the musculature in this region takes the form of dense strands spanning across the haemocoelic cavity. There appears to be very little that can be described as tarsos musculature as the columellar muscle can be followed from the columella directly to the foot wall. This is reasonable since *Lymnaea stagnalis* has no operculum.

In a living organism, the locomotory type is strictly ciliary and I can see no muscular contraction wave types (direct/retrograde, mono/ditaxic). The histological sections (Figure 29) show that the muscles traverse the foot matrix as discrete bundles and fragment relatively infrequently as they insert on the highly muscled foot wall. The matrix has many haemocoelic spaces and takes on a spongy quality.

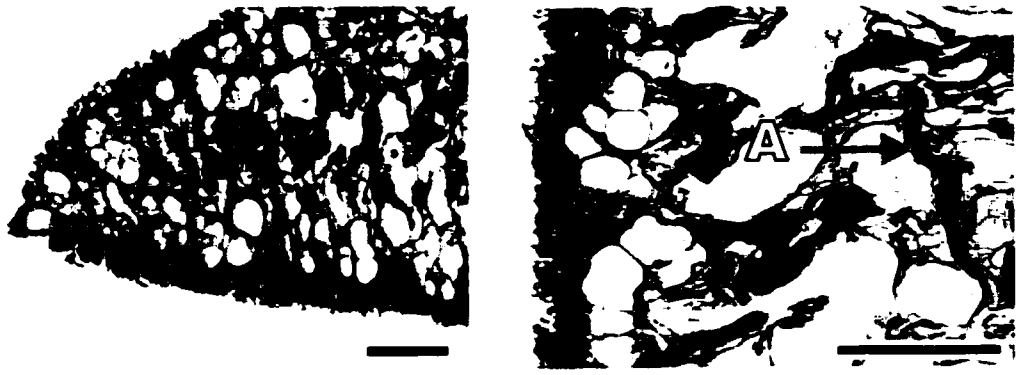

Figure 29. Histological sections of the musculature and haemocoel in the foot of *Lymnaea stagnalis*. (Left photograph [black bar = 100 µm] shows edge of foot. Right photo [black bar = 100 µm] shows large spaces within the foot matrix [a thin muscular bundle is labeled A in the right photograph]).

Marisa cornuarietis is the only snail in this study with a flat coiling or planispiral shell and therefore has a vanishingly small columella. The columellar muscle seems to insert on the inside edge of the first half of the body whorl.

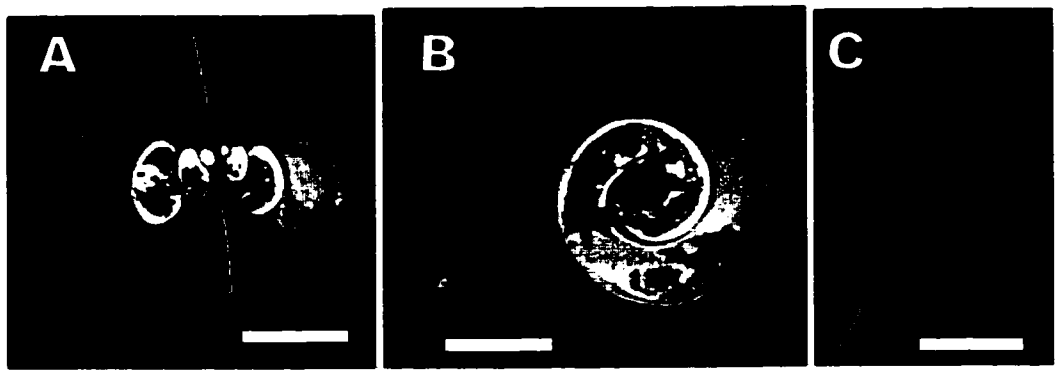

Figure 30. Frontal (A), sagittal (B) and transverse (C) section through *Marisa cornuarietis*. (A shows the condensed planispiral columella. B has the columellar muscle shaded red. C shows red arrow pointing to typical low density area of foot). Scale bar = 1 cm.

The most obvious point is that the tissue is not very dense (i.e., darker) and it is difficult to distinguish the columellar and tarsic musculature. The opacity of the MRM scans show that the tissue itself is of low density and has a large water content (Figure 30). The scans do suggest that there is a loose band of slightly denser muscle (i.e., lighter) running from the operculum to the muscle origin on the inside of the shell (Figure 30 B). The foot

seems to have a relatively dense foot wall with respect to the internal composition. The histological slides (Figure 31) match the MRM scans in that there seems to be a sheath of muscle at the foot wall, and the internal matrix is shown to be connective tissue with large haemocoelic spaces.

Figure 31. Histological sections through the foot of *Marisa cornuarietis*. (Left photograph [black bar = 100 μm] shows edge of foot and haemocoelic spaces. Right photograph [black bar = 100 μm] shows loose foot matrix with large haemal sinuses).

Nucella ostrina shows a columellar muscle originating on the first whorl of the columella and descending towards the sole of the foot and abruptly turning caudad and inserting on the operculum (Figure 32). It is interesting to note that the columellar muscle inserts on only about one-quarter of the surface area of the operculum. The columellar muscle appears to divide into two connected sides as it bends towards and inserts on the operculum. The tarsos musculature originates from within the two halves of the columellar muscle and as such, effectively splits the foot into left and right halves (Figure 33). This arrangement implies ditaxic locomotion.

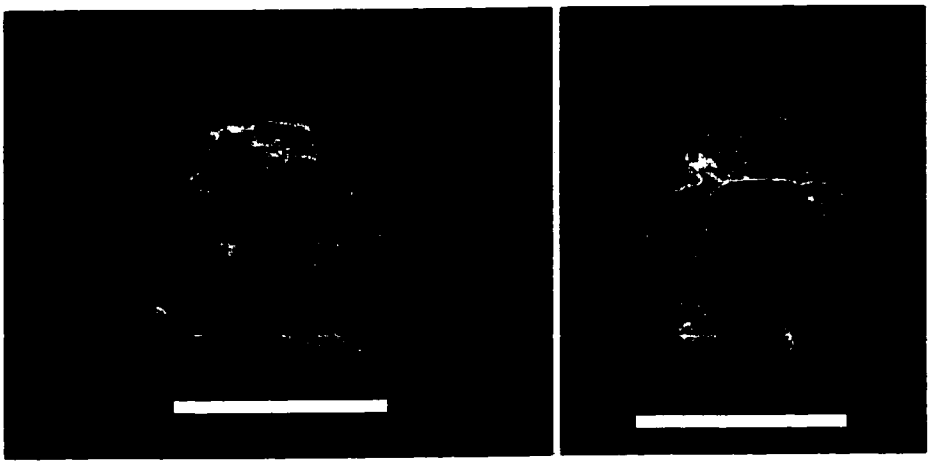

Figure 32. Sagittal (left) and transverse (right) sections through the midsection of *Nucella ostrina* obtained by MRM showing the columellar muscle (shaded red). (Scan to the right also shows thickness of the foot wall delineated by red lines). Scale bar = 1 cm.

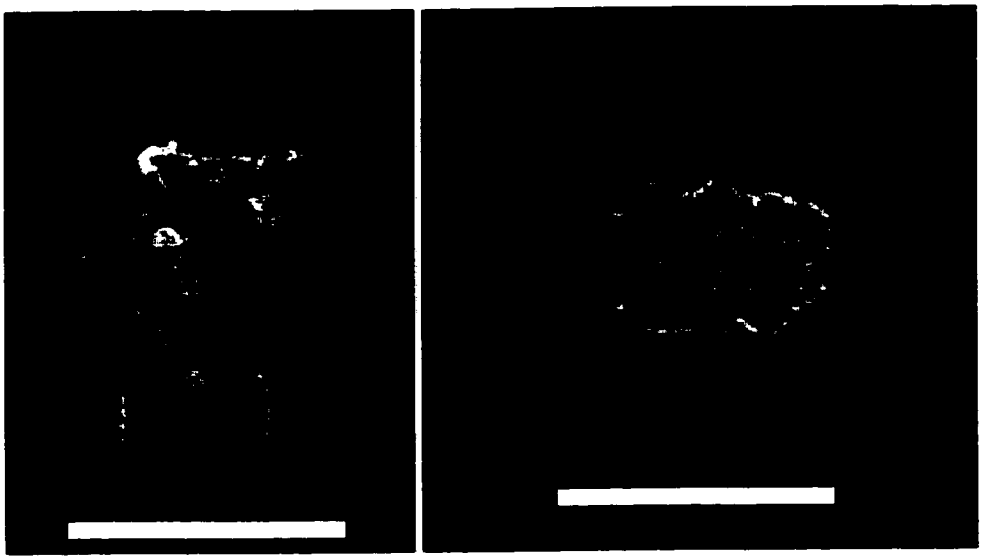

Figure 33. An anterior section in the transverse plane (left) and ventral section in the frontal plane (right) through the foot of *Nucella ostrina* obtained by MRM, showing the divided halves of the foot using red arrows. Scale bar = 1 cm.

There seems to be no central haemocoelic cavity, although the tarsic area seems to be less dense and quite distinguishable from the columellar muscle (Figure 32). The foot wall is very thick and within the musculature, and connective tissue matrix is of medium density. The histological sections (Figure 34) show a very dense tarsos matrix with many muscle

fibres running through and inserting on a thick foot wall. The system has a small number of haemocoelic spaces arranged throughout it.

Figure 34. Histological sections through the foot of *Nucella ostrina*. (Left photograph [black bar = 100 μm] shows dense oriented connective tissue within the foot matrix. Right [black bar = 100 μm] shows the thick foot wall, haemal spaces and tarsic musculature where it separates from the columellar muscle.

Pomacea bridgesi shares the same lower relative density characteristic with *Marisa cornuarietis*. The internal tissue density is low and shows a signal that is similar to water in some parts. There is a very strong columellar muscle inserting directly onto approximately half of the opercular inside surface area (Figure 35).

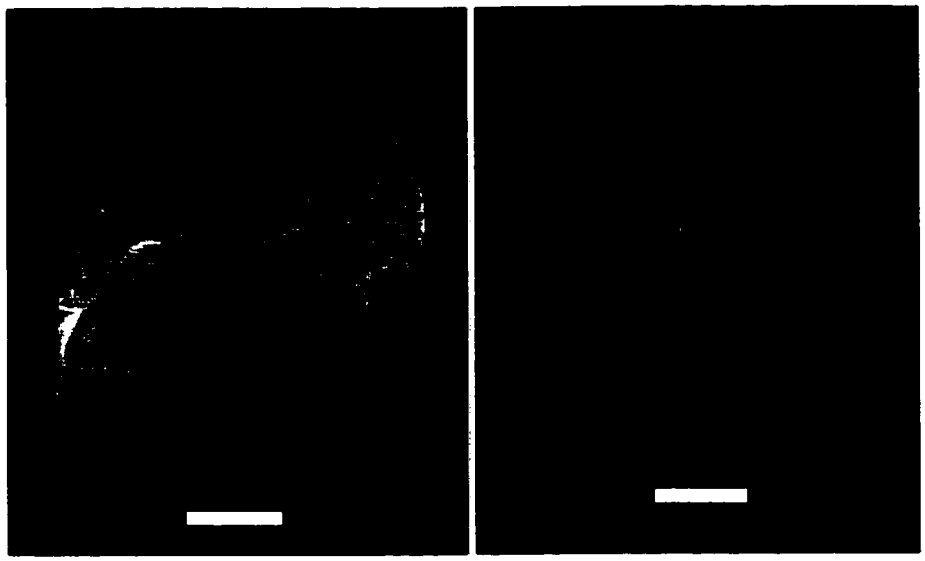

Figure 35. Sagittal (left) and transverse (right) sections through the midline of *Pomacea bridgesi* obtained by MRM. The left scan shows a strong columellar muscle shaded red. The right scan shows the columellar muscle shaded red as well as red lines delineating the thickness of foot wall. Scale bar = 1 cm.

The columellar muscle is similar to *Marisa cornuarietis*, in that it originates on the columella within one half whorl of the opening. In *Pomacea bridgesi* there seems to be a relatively larger attachment surface area due to lengthened columella. *Pomacea bridgesi* also differs from *Marisa cornuarietis* in that the columellar muscle is clearly differentiated from the tarsic musculature. In fact the tarsic muscle bands originating from the columella can be observed (Figures 35 & 36).

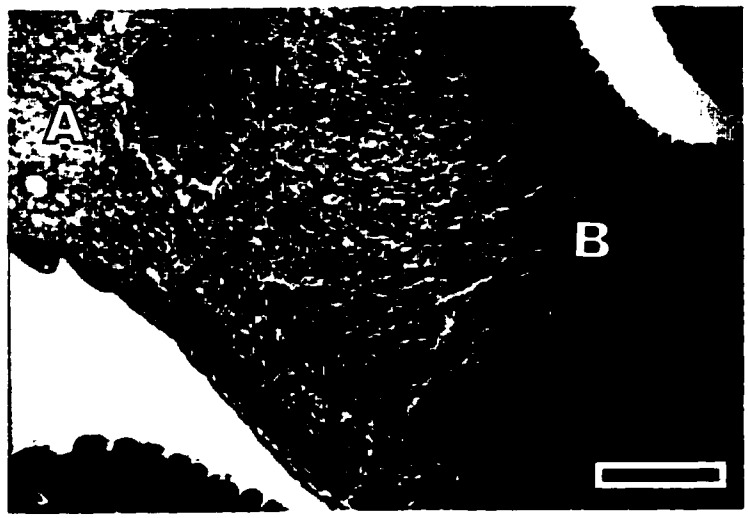

Figure 36. A thick histological sagittal section through the columellar/tarsos musculature of *Pomacea bridgesi.* (The red line shows the boundary between differently staining tarsos (A) and columellar (B) muscles. The columellar stains darker because of denser musculature.) (black bar = 1mm).

Pomacea bridgesi shows an increase in density at the border of the foot (Figure 35, left scan). The sole appears to be heartshaped and not physically divided in two. The histological sections (Figure 37) show the columellar muscle as dense bundles of muscle and connective tissue. The less dense tarsos is composed of lesser amounts of muscle relative to connective tissue and the muscle appears as thin bundles radiating from the columella and extending and imbedding into the foot wall. The tarsos is also pervaded by many haemocoelic vessels and is bounded by a thick and heavily muscled foot wall.

Figure 37. Sections through the foot of *Pomacea bridgesi.* (Left photograph [black bar = 100 μm] shows a low magnification photo of large haemocoelic vessels. Right photograph [black bar = 100 μm] shows close up of the foot wall and small haemocoelic spaces and muscles within a dense connective tissue matrix).

Searlesia dira has similar characteristics to *Nucella ostrina* in terms of muscle densities. The foot wall, however, seems to be less thick. The columellar muscle inserts on more surface area of the operculum and originates higher up the columella within the spire than in *Nucella ostrina.*

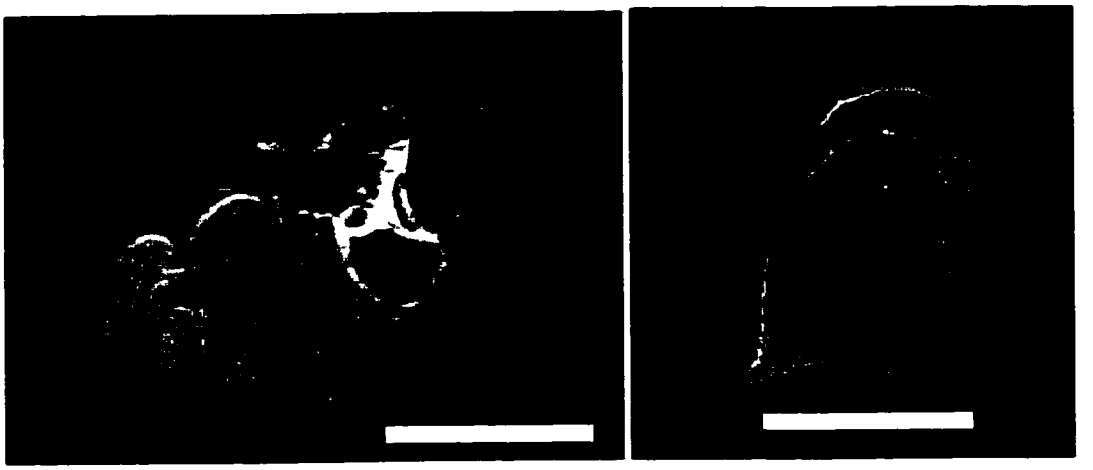

Figure 38. Sagittal (left) and transverse (right) sections through the midline of *Searlesia dira* obtained by MRM. The columellar muscle is shaded red in both scans. Scale bar = 1 cm.

The columellar muscle seems to be quite thick (thicker than that of *Nucella ostrina*) and very integrated with the tarsic musculature (Figure 38). There appears to be very little in terms of hydrostatic spaces and even vessels throughout the foot are difficult to discern.

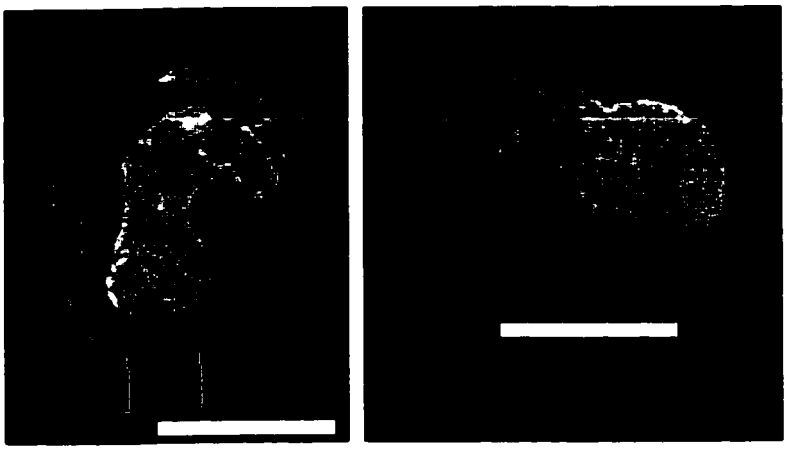

Figure 39. Posterior transverse (left) and ventral frontal (right) sections through the foot of *Searlesia dira* obtained by MRM. The red arrows show the divided nature of the foot. Scale bar = 1 cm.

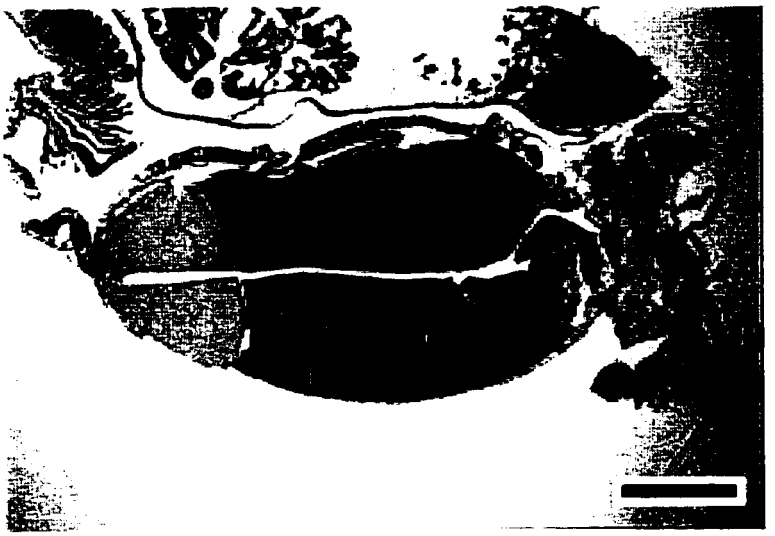

Figure 40. A histological frontal thick section through the foot of *Searlesia dira* showing the heavily muscled and divided mid-region of the foot (stained red) and dense, though lightly muscled, anterior and posterior portions of the foot (stained blue/green) (black bar = 1mm).

The tarsos musculature appears to be very dense (eg., Figure 38) and the histological sections agree in revealing extremely dense and highly muscled matrix (Figures 39-40). *Searlesia dira* exhibits a columellar muscle splitting as it descends ventrally and then bends posteriorly to the operculum (eg., Figure 40). This would suggest a ditaxic locomotory type.

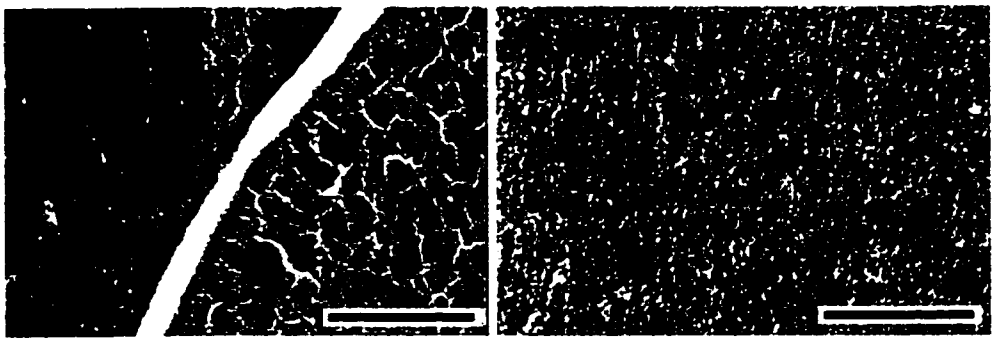

Figure 41. Left (black bar = 100 µm), section through the foot of *Searlesia dira* showing the dense, though lightly muscled, anterior portion of the foot and right (black bar = 100 µm) dense, heavily muscled matrix of the median portion.

Table 10. Characters and character states for the species in this study. (MH = Muscular Hydrostat, HC = Hydrostatic Cavity, CO = Columellar muscle, T = Tarsos musculature)

Specimen	**Hydrostat composition**	**Foot boundary**	**muscle composition**
Haliotis rufescens	heavily MH	Thin	massive CO little T
Calliostoma canaliculatum	heavily MH	Thin	heavy CO little T
Marisa cornuarietis	ligher MH	Medium	lighter CO expansive T
Pomacea bridgesi	ligher MH	Medium	heavy CO expansive T
Nucella ostrina	medium MH	Thick	heavy CO heavy T
Searlesia dira	medium MH	Thick	heavy CO heavy T
Diaulula sandiegensis	HC	Thick	pedal retractors heavy tarsos
Lymnaea stagnalis	HC	Thick	heavy CO integrated into T

5.4 Discussion

There are three evolutionary trends that are supported by the results. The first is a decreasing reliance on muscular hydrostats with a concomitant increase in use of haemocoelic hydrostats. Although, both methods allow muscle antagonism, the difference between the two is the number and volume of the encapsulated hydrostatic fluid compartments (Kier, 1983). In muscular hydrostats, because the muscle fibre itself is the encapsulated space, there are increased numbers of smaller amounts of encapsulated fluid (Kier & Smith, 1989) (e.g., within the columellar muscle and the tarsos muscles of *Haliotis rufescens*). Whereas at the other end of the spectrum, a hydrostatic cavity can be a large volume of fluid encapsulated in a single space (Kier & Smith, 1989)(e.g., the foot of *Lymnaea stagnalis*). I suggest that larger volumes of encapsulated fluid allow the organism to make rapid overall changes in the body shape (albeit with a loss of regional control [Vogel, 1994]). In the foot of basal species, muscular hydrostats occur across its entirety and support slower, strong and fine movement, while in the more advanced feet, there is only a reliance on muscular hydrostats along the edge, which allows integration of the benefits of hydrostatic cavities. As the edges of a foot are most often used for fine movements, such as waves of muscular compressions for locomotion and moulding egg capsules (Voltzow, 1985), it stands to reason that such areas would make use of muscular hydrostats. The greater number of smaller encapsulated space allows finer control over localized movements (Kier & Smith, 1989).

In terms of the organisms described, *Haliotis rufescens* and *Calliostoma canaliculatum* were grouped as vetigastropods in the first study (Chapter 4). Here they are grouped together by virtue of their dense and almost homogeneous foot matrix made of connective and muscle tissue. The next group, *Marisa cornuarietis* and *Pomacea bridgesi*, were both classified as mesogastropods (Chapter 4) and possess a different foot morphology. These mesogastropods have quite spongy feet, which is beginning to resemble a hydrostatic cavity and show a moderate amount of muscularization of the foot

wall. *Searlesia dira* and *Nucella ostrina*, although classified as neogastropods and therefore more derived than the mesogastropods, show a combination of the well developed haemocoelic vessels of the mesogastropod and the heavy and dense muscled foot matrix of the vetigastropod. This mixture of characteristics supports the theory (Chapter 2) that the neogastropods did not evolve from advanced mesogastropods, but rather broke away from the mesogastropod lineage at an early point. The differences could be related to the habitats of the animals, however, for the mesogastropods of this study are freshwater types. Finally, in the previous chapter, I showed that the most derived group is the Heterobranchia or the Euthyneura (opisthobranchs and pulmonates). Both *Diaulula sandiegensis* (opisthobranch) and *Lymnaea stagnalis* (pulmonate) heavily rely on a hydrostatic haemocoel. In *Diaulula sandiegensis*, the animal seems to be using the haemocoelic space as the main hydrostatic cavity, with many subsequent rami penetrating into the mantle skirt and the foot. *Lymnaea stagnalis* uses the foot as a functional haemocoelic, hydrostatic cavity. One final observation is that with an increased reliance on a larger hydrostatic cavity, there seems to be an increase in thickness of the foot wall. This is reasonable since a thicker wall is needed to prevent rupturing when pressurizing a larger volume of fluid (The law of Laplace states that the circumferential tension in the wall of a cylinder is equal to the product of the radius and the internal pressure (Wainwright *et al.*, 1982)).

The second evolutionary trend seems to be increased integration of the tarsic musculature with the columellar musculature. Voltzow (1985) noticed two trends of the evolution in the pedal musculature in "prosobranch" snails. The first was that as progression is made from primitive vetigastropods to the more advanced neogastropods, there is a transition from round simple feet to more complex narrower feet capable of more plastic behaviours. The other trend she noted was the tarsic musculature becomes more complex and seems to integrate more with the columellar muscle, thus combining the flexibility of the tarsos with the strength of the columellar muscle. My study extends this observation beyond the "Prosobranchia" to the entire class. Concurrent with the trend to increase reliance on hydrostatic cavities with advancement, there is a trend towards direct linkage between the tarsos and the columellar muscles. The extreme case is

Lymnaea stagnalis where it is nearly impossible to tell where the columellar muscle ends and the tarsos begins. An idea supported by these data is that although the columellar muscle is already well developed in the more primitive gastropods, such as the Vetigastropoda, it is the Caenogastropoda that have enhanced the tarsos and integrated it into the columellar muscles. This is apparent in the more complex functions the caenogastropods can perform with their feet (Miller, 1974, Voltzow, 1985, Uyeno pers. obs.) and is observable in the MRM scans and histological sections as being a complex interplay of discrete tarsos muscles and haemocoel vessels (e.g. *Nucella*, figure 33).

Haliotis rufescens and *Calliostoma canaliculata* show massive columellar muscles but very little in terms of tarsos (the fringe musculature, such as the epipodium is herein described as being tarsic in nature). This finding supports Voltzow's (1985) conclusion in that there is a general evolutionary reduction in columellar muscle and an increase in tarsic musculature. *Marisa cornuarietis* and *Pomacea bridgesi* as well as *Nucella ostrina* and *Searlesia dira* all show a strong columellar muscle. In all these caenogastropods, however there is an increase in tarsic musculature so that the majority of the foot is supported by tarsos, and the columellar muscle is strictly originating on the columella and inserting on the operculum.

Marisa cornuarietis and *Pomacea bridgesi*, although from the same family and with very similar feet are different in their shell forms. *Marisa cornuarietis* has a planispiral shell and it is interesting to note that this shell form seems to allow for lesser surface area for the columellar muscle origin and forces the columellar muscle to follow the shell along its inside circumference rather than the direct path it takes in a spiral shell where the columella projects out of the plane of rotation. This arrangement causes a longer curving distance for the muscle to span and contract over and may cause a weaker connection. I suspect this may explain in part why planispiral shells are less common in nature. Neither the opisthobranch nor the pulmonate has opercula, and show the extreme condition of columellar muscle and tarsos integration. The columella or pedal retractor muscles descend directly from a dorsal position and then begin to bifurcate until they have split up into small discrete bundles and have inserted into the foot wall. Finally, there is the observation of a trend to developing thicker foot walls as larger hydrostatic

cavities are developed. I suspect this trend is has two causes. Firstly, Laplace's law states the wall must become thicker to handle increase pressure due to larger volumes. Also, as there is an increased reliance on tarsic musculature (which inserts on the foot wall), there may be a necessity for a stronger attachment area.

Based on time sequence photographs of gastropods walking over a platen glass, Miller (1974) noted that there was the evolutionary trend from monotaxic waves to ditaxic waves. The morphological evidence supports Miller's findings (1974) that ditaxy was developed quite early on in the archaeogastropods (patellogastropods/vetigastropods) as *Calliostoma canaliculatum* shows a physical predisposition to ditaxy. *Haliotis rufescens* does not show this characteristic of division and is therefore functionally ditaxic rather than structurally ditaxic. In listing the locomotory modes used by caenogastropods, Miller (1974) notes an extremely wide variety, including monotaxy, ditaxy, leaping and ciliary movement. This finding is also supported by the morphological data because the neogastropods seem to by physically predisposed to ditaxy whereas the mesogastropods have musculature with no splitting of the columellar muscle and may even rely in part on ciliary gliding. Miller (1974) found only monotaxy and ciliary gliding in the opisthobranchs. My results show that either may be possible in *Diaulula sandiegensis*, because of its dorso-ventral muscle array. Finally, Miller (1974) suggested that pulmonates inherited monotaxic locomotion from the opisthobranchs and secondarily reverted to ciliary movement. If this is the case, then this study shows a greater integration and reduction of both the columellar and tarsos muscles to specialize in ciliary movement.

5.5 Summary and concluding remarks

In summary, this study has found characteristics pertaining to three general biomechanical categories: hydrostatic skeleton composition, muscular type composition and physical basis for locomotory style. Based on the characterization of these categories

within the specimens found in this study, the concluding general evolutionary trends and resultant cladogram implication are summarized as follows:

1. There is an evolutionary trend to increase the volume contained within a hydrostatically active cavity and to reduce the number of these cavities. Primitive gastropods tend to rely more heavily on muscular hydrostats whereas more derived gastropods seem to rely more on haemocoelic cavities and vessels. The cladogram inferred is: (Vetigastropoda, (Neogastropoda, (Mesogastropoda, (Opisthobranchia, (Pulmonata))))). However, the mesogastropod specimens in this case are quite derived.
2. As gastropods rely more on hydrostatic cavities and develop the tarsos muscle bundles that insert into the wall musculature, the width of the foot wall become thicker. This suggests a cladogram as follows (Vetigastropoda, (Caenogastropoda, (Euthyneura))).
3. Neogastropod and the vetigastropod feet posess a similar muscle/connective tissue foot matrix and a physical basis for ditaxy. As well, neogastropods and mesogastropods share similar tarsic musculature and foot wall characteristics. This supports the idea of neogastropods arising from early mesogastropods and not derived mesogastropods, such as the ones in this study. This suggests the following cladogram for the Caenogastropoda: ((early Mesogastropoda, (Neogastropoda)), derived Mesogastropoda).
4. This study supports the idea that there is an evolutionary trend from large columellar (pedal retractor) muscles with little or no integrated tarsos musculature to a more derived state of a more focused columellar muscle with a larger number of integrated tarsic muscle bundles. The integration and development of the tarsos increases the functional plasticity of the foot. This suggest the following cladogram: (Vetigastropoda, (Caenogastropoda, (Opisthobranchia, (Pulmonata)))).

5. A possible reason for the small number of planispiral shells found in nature could be the smaller origin area of the columellar muscle on the columella, which in turn causes the columellar muscle to be longer and forced to contract around the circumference of the shell.
6. This study supports many of Miller's ideas (1974) with morphological data as follows: *Calliostoma canaliculatum* has developed a physical basis for ditaxy, which supports Miller's idea that ditaxy developed quite early in the Gastropoda. Miller (1974) classified *Haliotis rufescens* as ditaxic, however it does not have a split foot, thus it should be considered a functional ditaxic gastropod. The caenogastropods in this study have different muscle morphologies in that the mesogastropods do not possess a split foot whereas this is the case with the study's two neogastropods. This finding suggests, as does Miller (1974) that within the caenogastropods there has been much experimentation with locomotory type. This suggests a parsimonious cladogram as follows: (Vetigastropoda, (Mesogastropoda, (Opisthobranchia, (Pulmonata))), Neogastropoda).

When one concatenates and takes into consideration all the phylogenetic details in each of the summary points' cladograms (Figure 42), the most reasonable conclusion supports the molecular study, with the vetigastropods being considered the most primitive, the caenogastropods arising next and the Euthyneura the most derived. The conclusion further supports the idea that neogastropods arose from early mesogastropods as did the euthyneuran line. A point that this study clarified from the molecular study (Chapter 4) is that the opisthobranchs and pulmonates belong to different groups where the Pulmonata are more derived.

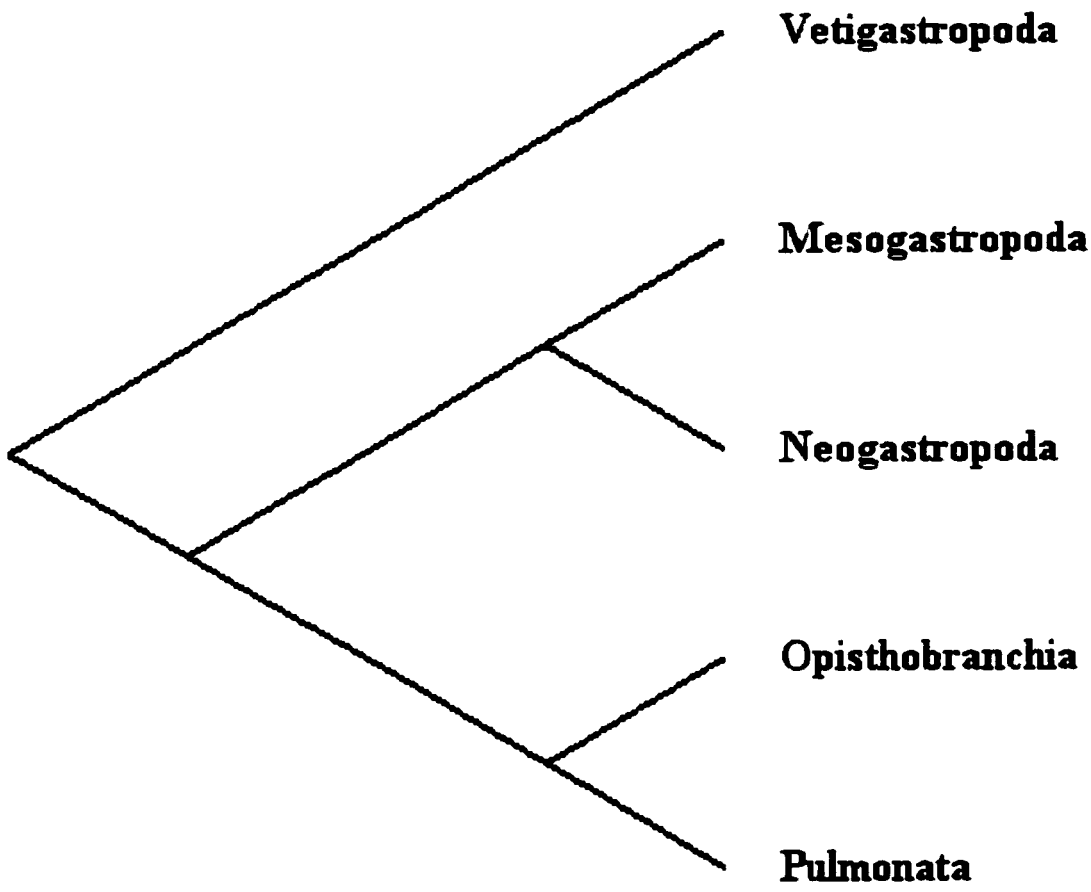

Figure 42. A general consensus tree based on morphological characters.

CHAPTER SIX
CONCLUSION

6.1 Conclusions based on the concatenation of morphological and molecular phylogenetic trees

When compared and combined, the data matrix within the morphological study, although small, mapped well to a fairly robust molecular phylogenetic tree.

The molecular data, when analyzed showed the phylogenetic tree seen in Figure 43 A and shows, among its main points effective grouping of primitive gastropods, resolution within the Caenogastropoda and a lack of resolution of the Heterobranchia. In Figure 16 B, one sees the positioning of the Vetigastropoda as primitive, resolution of the Caenogastropoda as the Mesogastropoda and Neogastropoda as well as the resolution of the Heterobranchia (in this case equal to the Euthyneura) as the Opisthobranchia and the Pulmonata. It is gratifying to note this lack of resolution within the Heterobranchia of the molecular study is effectively filled in by morphological data.

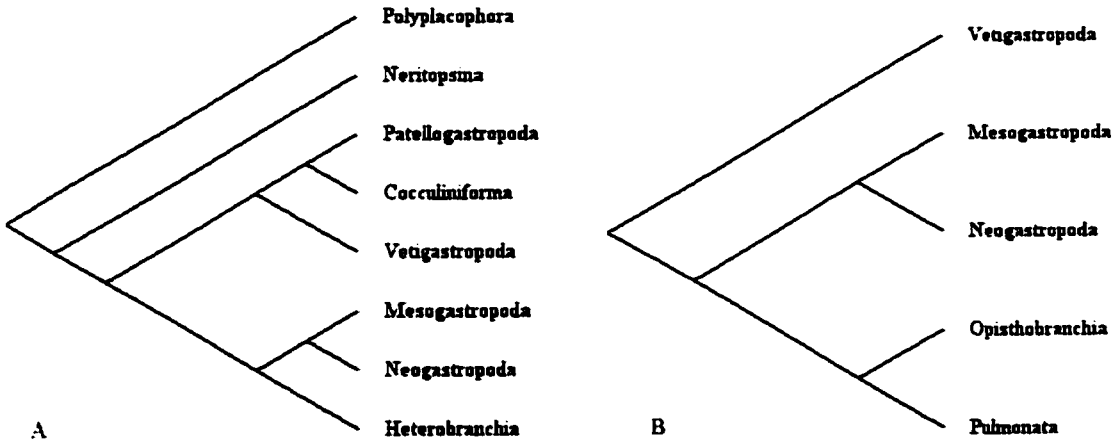

Figure 43. The final molecular (A) and morphological (B) trees.

In short, I offer a final concatenated tree that reflects evolutionary trends found in both the molecular study as well as the morphological study (Figure 44).

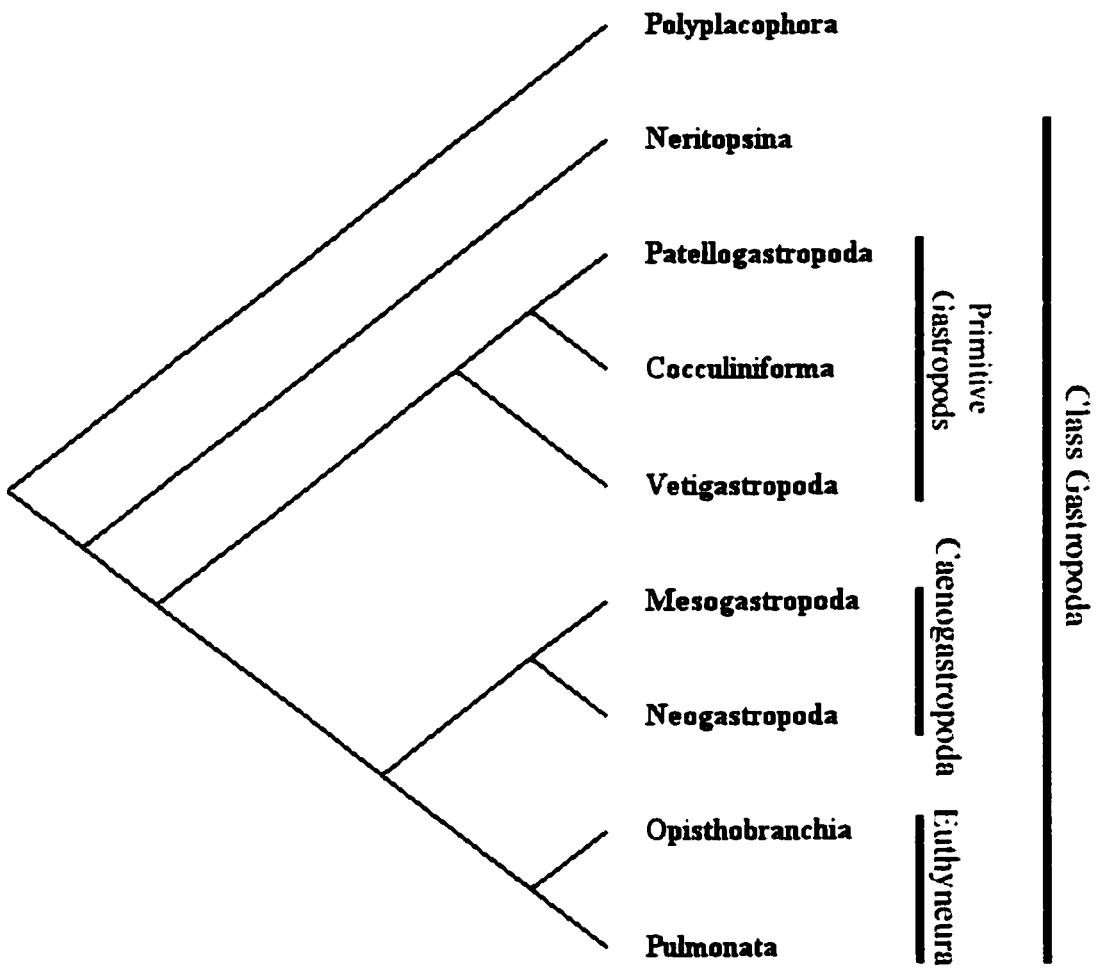

Figure 44. A phylogenetic tree based on combining the findings within the morphological and molecular studies.

6.2 Comparison of the summary tree found by this research to other trees.

In this thesis I described two schemes, the classical scheme, which was the product of the first scientific studies of gastropod phylogeny and the interdisciplinary scheme. The latter scheme is supported by evidence accumulated by relatively modern techniques. Of these two schemes the classical scheme is still being used as a basis of teaching and learning about gastropods, even though the interdisciplinary scheme

(spearheaded by Ponder and Lindberg, 1997) has incorporated robust analytical techniques and many more characteristics. It was the objective of this thesis to validate the interdisciplinary scheme and hopefully support it with novel data.

Figures 45 and 46 are depictions of the classic and multidisciplinary schemes in order to highlight points of comparison.

My data leads to the rejection of the classical scheme (Figure 45) mainly because it does not support the basic premise under which the classical scheme was developed, a smooth gradation of organisms from most primitive to the most derived.

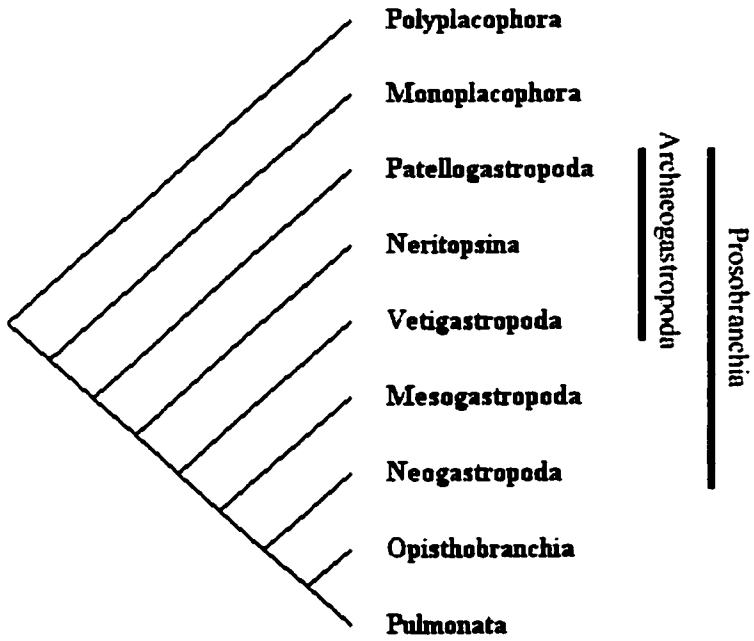

Figure 45. Classic scheme based on Thiele (1929)

My results in Figure 44 show a number of points where many of the groups within the classical model are artificial and polyphyletic in nature. Figure 46 shows the multidisciplinary scheme, which attempts to correct these failings in the following manner. The classic grouping of the archaeogastropods have now been divided into the major subclass taxa of the Patellogastropoda and the Vetigastropoda. The caenogastropods are noted for being a monophyly and the neogastropods are derived from early mesogastropods (architaenoglossans are primitive caenogastropods).

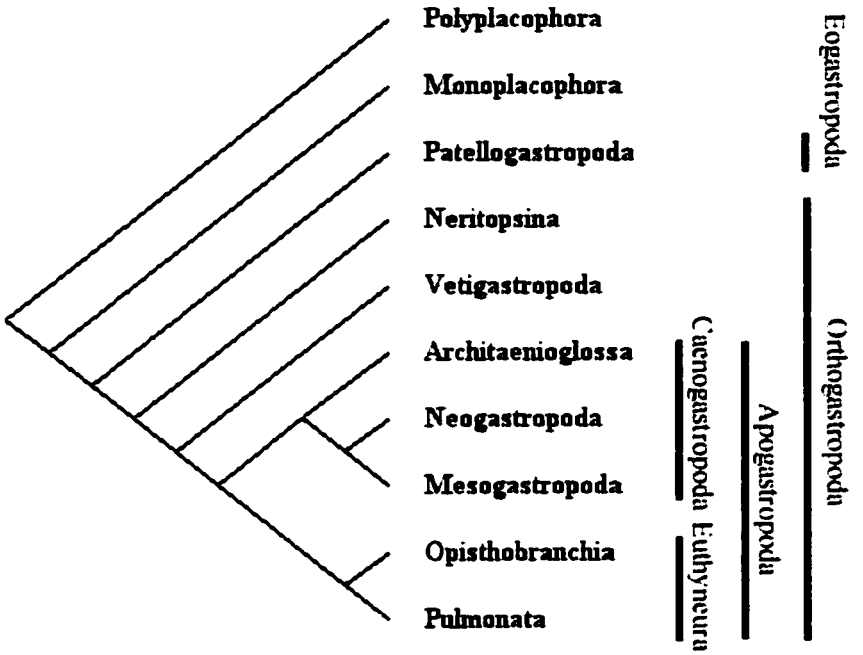

Figure 46. Multidisciplinary scheme based on Ponder & Lindberg (1997)

The Heterobranchia is now a subclass taxon and include the classical subclasses of the Opisthobranchia and Pulmonata as well as some former primitive caenogastropods. Generally, the results of this thesis (Figure 44) agree with the multidisciplinary scheme, in fact, the only revelation of the multidisciplinary scheme (Figure 46) that is not present in my data is the resolution of the primitive subclasses of the Patellogastropoda and the Vetigastropoda.

6.3 Future directions

As character and character type collection is a neverending job, there is no end to the research that could be done to improve our view of the systematics within the Gastropoda. In terms of the scope of this thesis however, there are a few areas in which further study would be of interest.

Firstly, it would help the phylogenetic picture if more organisms could be sequenced for the 18S ribosomal DNA. This is especially true with the Vetigastropoda, and the other more primitive groups. Another area in which there should be further resolution via data collection is from the non-Euthyneuran heterobranchs, this would allow one to develop molecular support for their ancestors. Resolution is important within the Euthyneurans as well because of because the molecular study of this thesis could not separate the Pulmonata from the Opisthobranchia.

With respect to the morphological study, I believe that many more evolutionary trends can result from studies, which try to fit structure to function. Studies of molluscan biomechanics are relatively new and so there is a lot to investigate. With respect to the morphological study, I believe that the digitization of more specimens would obviously help with technological advances leading to increased resolution. I believe that digital preservation may be a unique way of viewing especially rare organisms or organisms that face extinction and will serve as both a research tool as well as a teaching tool. This study has helped to expand the number of species in which we know something about the columellar and tarsic musculature to the class level, however there are many holes in the snail family tree. I therefore mimic Voltzow (1985) in suggesting that observation of further snail pedal musculatures is in order.

Magnetic resonance microscopy has the potential to revolutionize several areas of study. Among these is the use of labelling or marking techniques, which could be used to perfuse the haemocoelic cavity or to imbed markers in muscle for biomechanical vector analysis. Finally, the data output of the MRM lends itself to isosurfacing routines, which may then be usable in finite element and biomechanical vector analyses. I believe that the future will bring many revelations with this amazingly adaptable technology.

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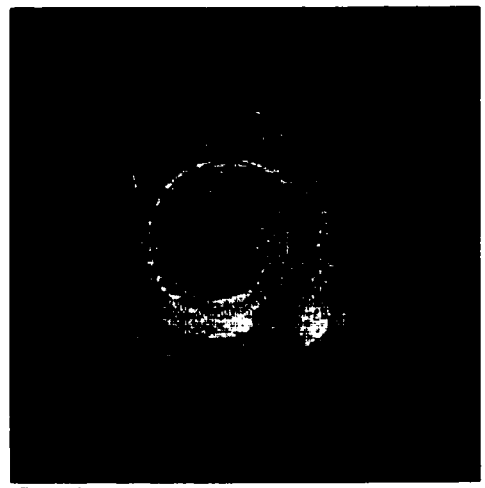
APPENDIX I: Whole snail MRM Images of the front, top and left side

Below is a series of opaque and transparent views from different angles of the snails in the MRI study to give the viewer a 3D perspective of the organism and their internal and external structures. Sizes for these organisms are given in Table 6.

I.1.1 *Calliostoma canaliculatum* (Opaque view)

Left side view



Top view

Front view

I.1.2 *Calliostoma canaliculatum* (Transparent view)

Left side view

Top view

Front view

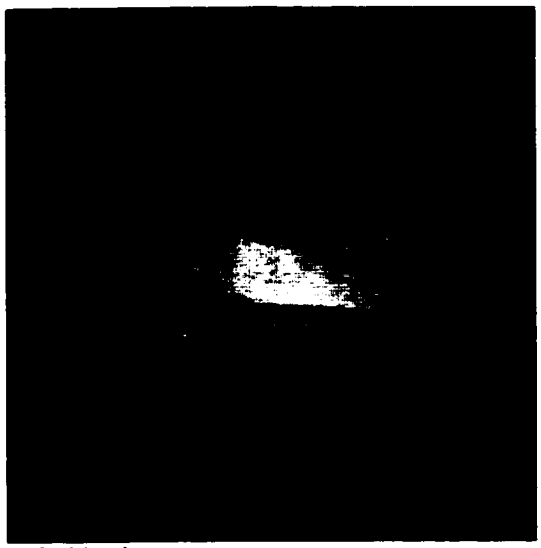
I.2.1 *Diaulula sandiegensis* (Opaque view)

Left side view

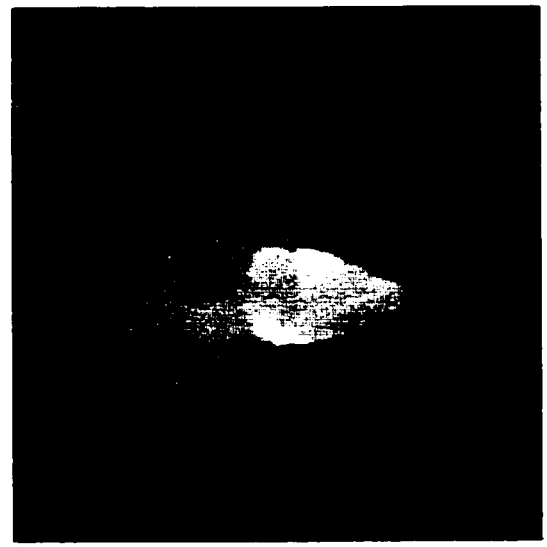
Top view

Front view

I.2.2 *Diaulula sandiegensis* (Transparent view)



Left side view

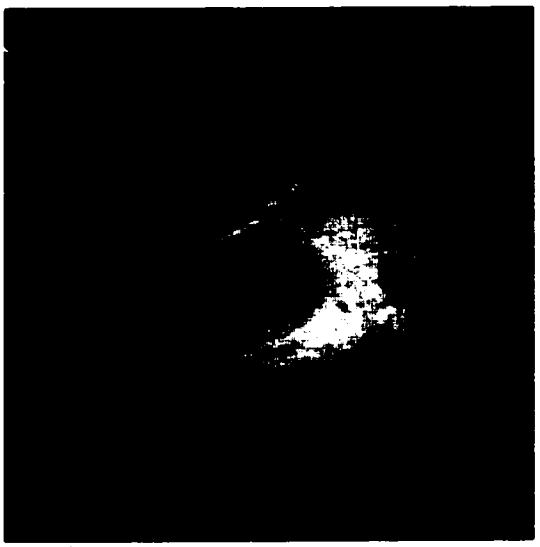


Top view

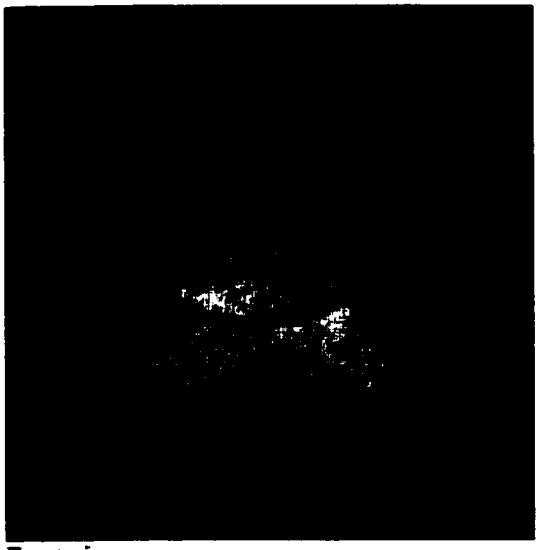
Front view

I.3.1 *Haliotis rufescens* (Opaque view)

Left side view



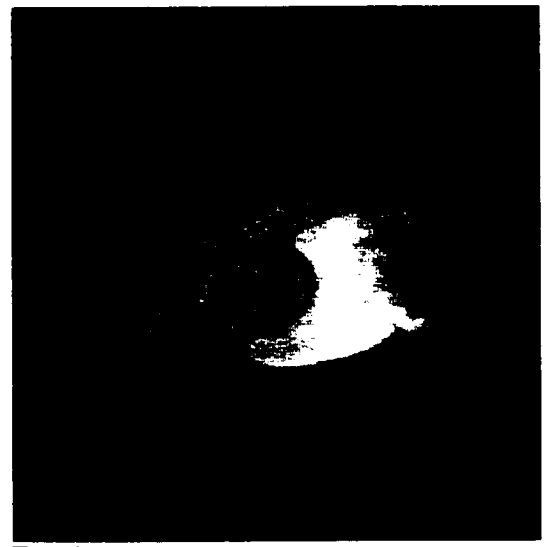
Top view



Front view

I.3.2 *Haliotis rufescens* (Transparent view)

Left side view

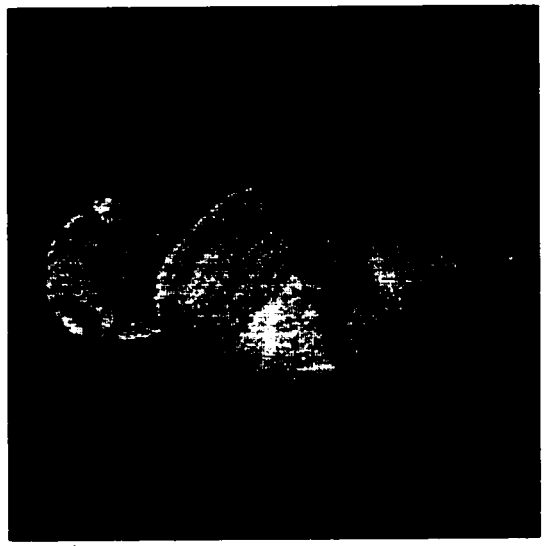


Top view

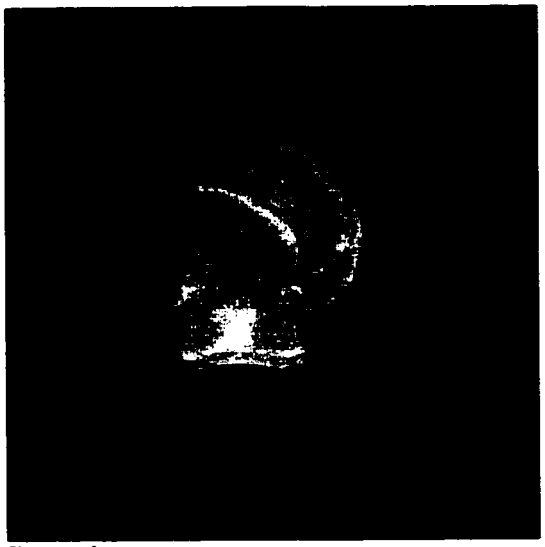
Front view

I.4.1 *Lymnaea stagnalis* (Opaque view)

Left side view

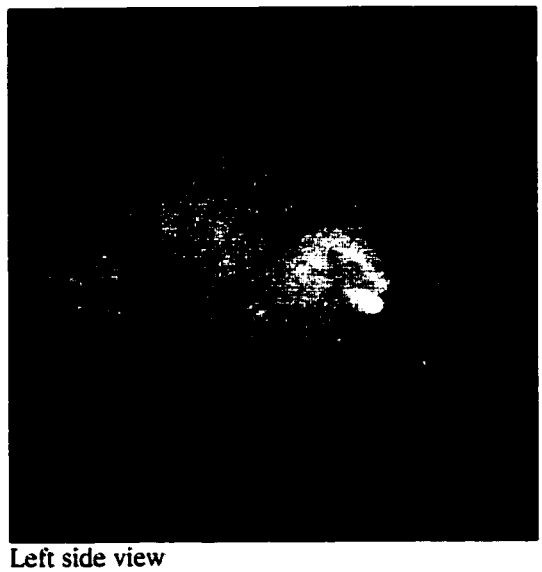


Top view

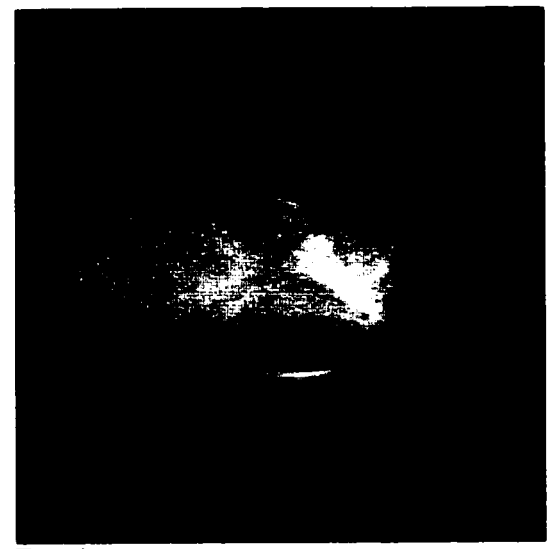


Front view

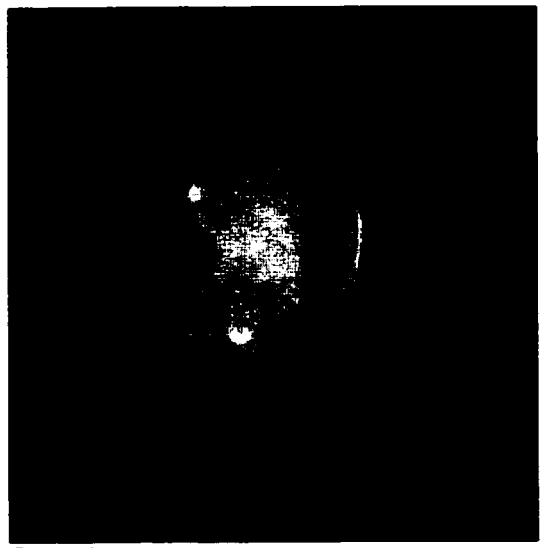
I.4.2 *Lymnaea stagnalis* (Transparent view)



Left side view



Top view

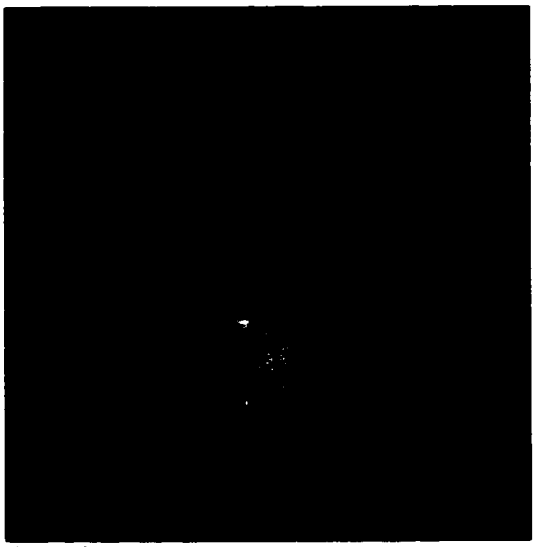


Front view

I.5.1 *Marisa cornuarietis* (Opaque view)

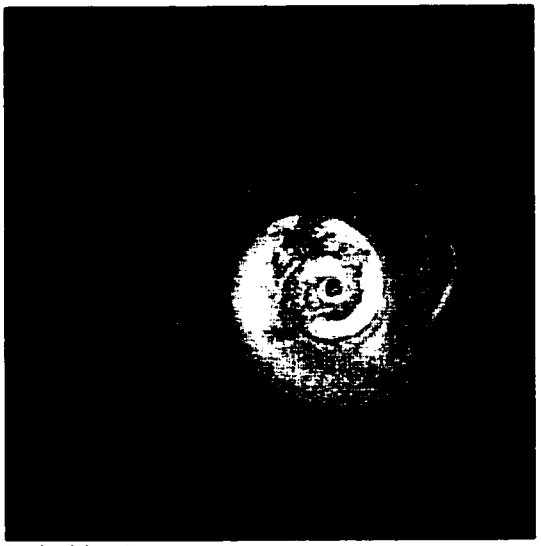
Left side view

Top view



Front view

I.5.2 *Marisa cornuarietis* (Transparent view)



Left side view

Top view

Front view

I.6.1 *Nucella ostrina* (Opaque view)

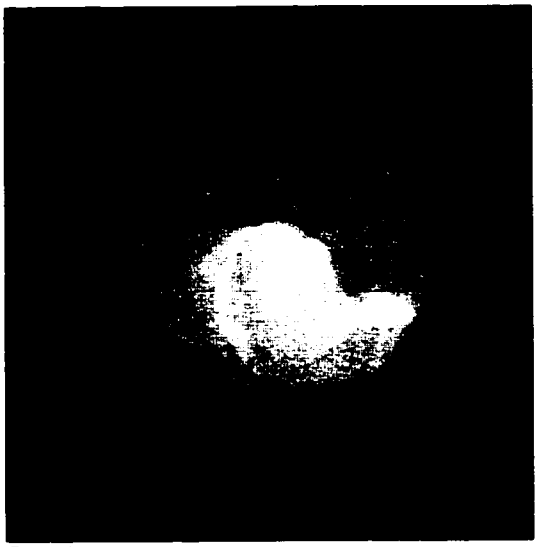
Left side view

Top view

Front view

I.6.2 *Nucella ostrina* (Transparent view)

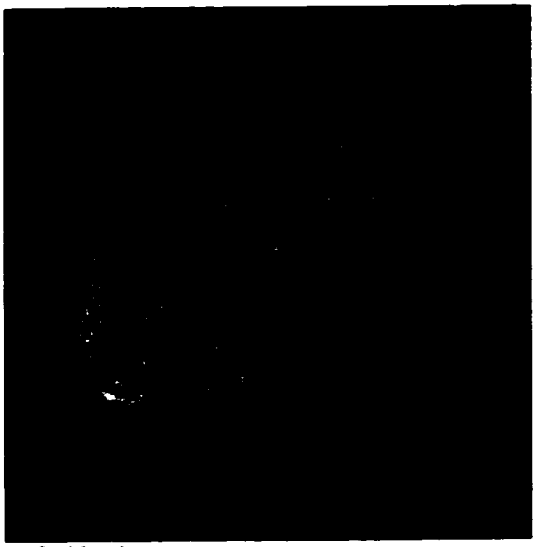
Left side view



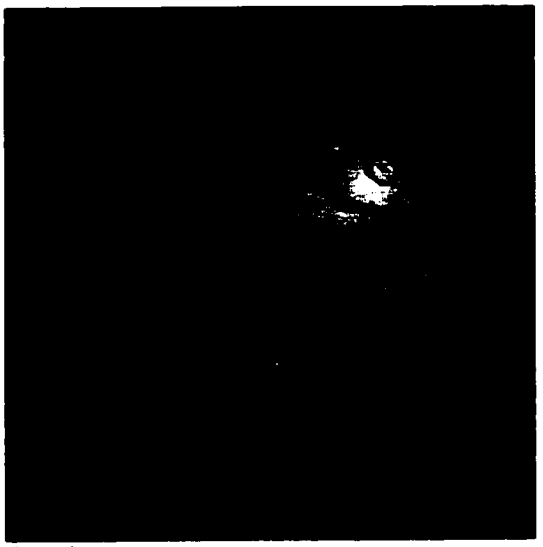
Top view

Front view

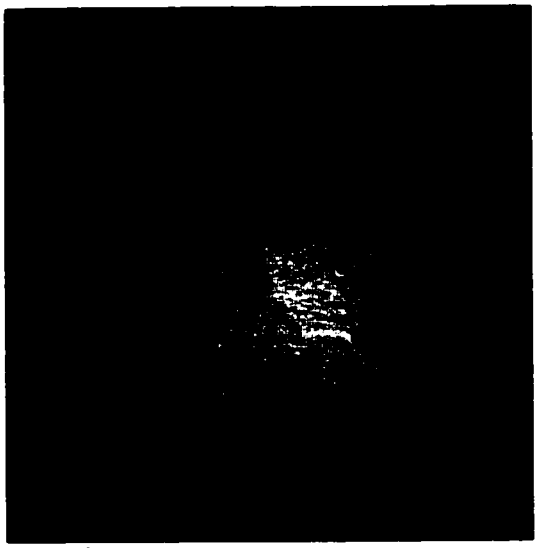
I.7.1 *Pomacea bridgesi* (Opaque view)



Left side view

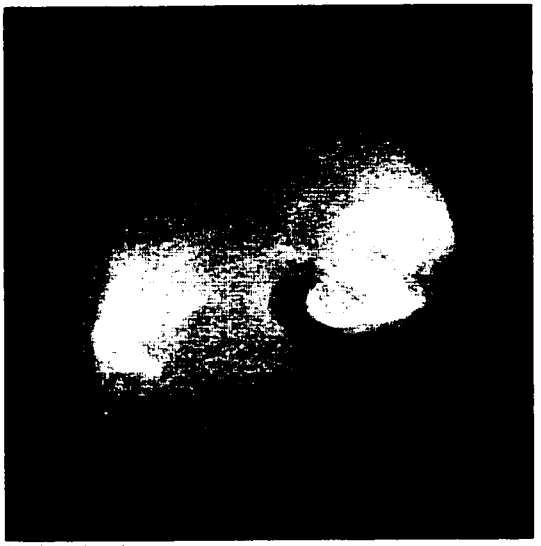


Top view



Front view

I.7.2 *Pomacea bridgesi* (Transparent view)



Left side view

Top view

Front view

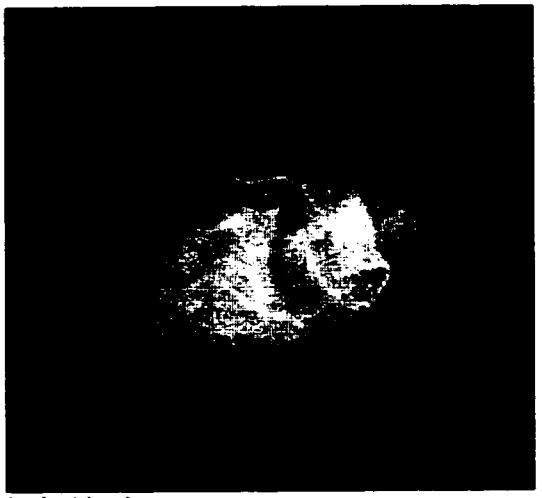
I.8.1 *Searlesia dira* (Opaque view)

Left side view

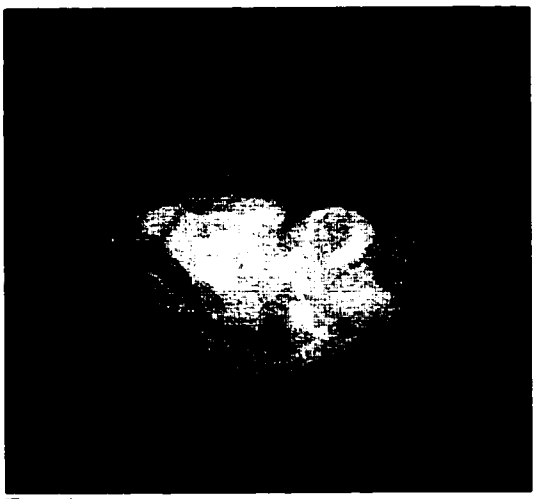
Top view

Front view

I.8.2 *Searlesia dira* (Transparent view)



Left side view



Top view

Front view

APPENDIX II: Molecular data

Below is the raw output (II.1) and input (II.2) molecular data should anyone wish to view my output on one's own or wish to re-analyse the input data. Sequence sources are credited to those who characterized them as noted in Table 9 (pp. 47-50).

II.1 PHYLIP CONSENSE tree results in the New Hampshire standard form

((ACANT,(LEPID,(CRYPT,MOPAL))),((NRITA,(NRITI,SEPTA)),(((((COCCU,(NOTOC,(CELLA,(NACEL,((PSACC,(LOTTI,TECTU)),(PVULG,(LEPET,(ACMAE,(PARAL,EULEP)))))))),((CITTA,(CALLI,(HALIO,(TURBO,(ASTRA,(TFUNE,TPULL)))))),(DCAYE,DASPE))),(((PMIDA,PTERE),(PLUCA,(PMAUR,PQUOY))),(ERUMP,EADAN))),(((LYMNA,(((FARGO,(HAMIN,RISSO)),(APLYS,(HELIS,(LIMIC,(SIPHO,LIMAX)))))),(PHYSA,ONCHI))),(ANISO,DIAUL)),(((CAMPA,(CYCLO,NEOCY)),(POMAC,MARIS)),((CIPAN,(MODUL,(CERIT,BATIL))),((TECTA,(XENOP,CYPRA)),((((CERAT,(LAMEL,OSTRI)),(TRUNC,CORAL)),((SIRAT,(PHYLO,MUREX)),(ANNUL,NEPTU))),(BUCCI,((((THAIS,(TURBI,OLIVA)),(FUSIT,SCAPH)),(HASTU,(CONUS,POLIN))),((ILYAN,(SEARL,ARCTO)),((BCARI,BCANA),(BSPIR,BSINI))))))))))));

II.2 18S ribosome DNA sequences (Aligned and interleaved) in Clustal X format. The abbreviated names of species are given in full in Table 9.

```
CLUSTAL X (1.8) multiple sequence alignment


NAUTI           TAAGTTCA--GCCGATTGAAT--GGG---CG-----AAA-CCGCGAA-CGGCTCA--GGA
NOTOC           TAAGTACG--CCGGTTCCTATTTGGG---CG-----AAA-TG--GAA-CGGCTC---GTA
TFUNE           TAAGTA----CTTACTCTAGC-ACAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
TPULL           TAAGTA----CTTACTCTAGC-ACAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
ASTRA           TAAGYA----CA-AMTCTAGC-ACAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
TURBO           TAAGTA----CAAACTCTAGC-ACAG---TG-----AAA-CTGCGAA-TGGCTCTATTAG
HALIO           TAAGTA----CAAACTCTAGC-ACAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
CALLI           TAAGTA----CAAACTCTCGC-CCAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
CITTA           ------------------------------------------------------------
DASPE           TAAGTC----CAAACTCTCGC-CCAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAC
DCAYE           TAAGTA----CAAACTCTCGC-CCAG---TG-----AAA-CTGCGAA-TGGCTC-ATTAC
LEPID           TAAGTA----CAGACTTTTAC-ACGG---TG-----AAA-CCGCAGA-TGGCTC-ATTAA
ACANT           TAAGTA----CAGACTTTCAC-ATAG---TG-----AAA-CCGCAAA-TGGCTC-ATTAA
MOPAL           TAAGTA----CAGACTTTCAC-ACAG---TG-----AAA-CCGCAAA-TGGCTC-ATTAA
CRYPT           TAAGTA----CCGACTTTCAC-ATAG---TG-----AAA-CCGCAAA-TGGCTC-ATTAA
ANISO           TAAGTT----CACCCCCTCGA-ACGG----GT----AAA-CCGCGAA-TGGCTC-ATTAA
DIAUL           TAAGTT----CACCCCCTCGA-ACGG----GT----AAA-CCGCGAA-TGGCTC-ATTAA
HELIS           TAAGTT----CACACTGTCTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
LYMNA           TAAGTT----CACACTGTTTG-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
HAMIN           TAAGTT----CACACTGTGTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
RISSO           TATGT-----CACACTTTGGT-ACAG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
LIMIC           TAAGTT----CACACTGTCTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
SIPHO           TAAGTT----CACACTGTCTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
LIMAX           TAAGTT----CACACTGTCCC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
APLYS           TAAGTT----CA-ACTGTCTC-ACGG---TGT----AAA-CCGCGAA-TGG-TC-ATTAA
FARGO           TAAGTT----CACACTGTCTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
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ONCHI    TAAGTT----CACACTGTCTC-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
PHYSA    TAAGTT----CACACTGTCCC-ATGG---TG------AA-ACGCGAA-TGGCTC-ATTAA
OLIVA    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
THAIS    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
TURBI    TAAGTT----CACACCCTCGT--CGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
LAMEL    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CERAT    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
OSTRI    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BUCCI    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
FUSIT    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
SCAPH    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BSPIR    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BSINI    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
SEARL    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BCARI    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BCANA    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
ILYAN    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
NEPTU    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
POLIN    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CONUS    TAAGTT----CACACCCTCGT-ATGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
HASTU    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
PHYLO    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAAATGGCTC-ATTAA
MUREX    TAAGTT----CACACCCTCGT-ATGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
SIRAT    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
ANNUL    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
TRUNC    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CORAL    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAAATGGCTC-ATTAA
ARCTO    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
XENOP    TAAGTT----CTC--CCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC--TTAA
CYPRA    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CERIT    TAAGTTA---CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
BATIL    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
POMAC    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
MARIS    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CYCLO    TAAGTT----CCAACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
NEOCY    TAAGTT----CCAACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CAMPA    TAAGTT----CACACCCTTGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
CIPAN    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
MODUL    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
TECTA    TAAGTT----CACACCCTCGT-ACGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAA
EADAN    TAAGTA----CACGCCCTCGGYACGG---CG-----AAA-CTGYGAA-TGGYTC-ATTAG
ERUMP    TAAGTA----CACGCCCTCGGCACGG---CG-----AAA-CTGCGAA-TGGCTC-ATTAG
PQUOY    TAAGTA----CACGCCCTCAGTACGG---TGT----AAA-TGG--AA-TGGYTC-ATTAG
PMAUR    TAAGTA----CACGCC-TCAGTACGG---TGT----AAA-CTGYGAA-TGGYTC-ATTAG
PLUCA    TAAGTA----CACGCCCTCAG-ACGG---TG-----AAA-CTGCGAA-TGGCTC-ATTAG
PMIDA    TAAGTA----CACGCCCTCAGCACGG---CG-----AAA-CTGCGAA-TGGCTC-ATTAG
PTERE    TAAGTA----CAC-CCCTCAGCACGG---CG-----AAA-CTGCGAA-TGGCTC-ATTAG
NRITI    --AGTA----CAAACCTTCAC-ATGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAG
SEPTA    TAAGTA----CAAACCTTCAC-ATGG---TG-----AAA-CCGCGAA-TGGCTC-ATTAG
NRITA    TAAGTT----CAAACTTTCAC-ATAG---TG-----AAA-CCGCGAA-TGGCTC-ATTAG
COCCU    CAAGT-----ACGATCGGTACAATGA----G-----AGA-CTGCGAA-TGGCTC-ATTAG
EULEP    TAAGTTCAGGCTTGTTCCTTTCGGGG-AGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
PARAL    TAAGTTCAGGCTTGTTCCTTTCGGGG-AGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
PVULG    TAAGTTCAGGCTTGTTCCTTTCGGGG-AGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
ACMAE    TAAGTTCAGGCTTGTTCCTTTCGGGGCAGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
LEPET    TAAGTTCAGGCTTGTTCCTTCGGGAG---CGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
LOTTI    TAAGTTCAGGCTTGTTCCTTTCGGGG-AGCRAGCCGAAAACTGCRAA-CGGCTC-ATTAG
TECTU    TAAGTTCAGGCTTKYTCCTTTCGGGG-AGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
PSACC    TAAGTTCAGGCTTGTTTCTTTCGGGG-AGCGAGCCGAAA-CTGCGAA-CGGCTC-ATTAG
NACEL    TAAGTTCAGGCTCGTTCCTTTCGGGG-AGCGAGTCGAAA-CTGCGAA-CGGCTC-ATTAG
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CELLA       TATGTTCAGACTCGTCCTTTCGGGGAGCGCGA-TCGACA--TGTGAA-CGGCTC-ATTAG


NAUTI       ACCGGACGTAATCCATTAGATCGTA-CCGACCCT-AC--TTGGATAACTGTGGCAATTCT
NOTOC       CACGGTCGTAATTTAGCGGGCGATT-CGTTCCCTTAC--TTGGATAACTGTGGAAAATCT
TFUNE       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
TPULL       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
ASTRA       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
TURBO       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
HALIO       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
CALLI       ATCAGTTATGGTTCCTTAGATGATA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
CITTA       ATCAGTTATGGTTCCTTAGATGATA-CTAT--CCTAC--TTGGATAACTGTGGTAATTCT
DASPE       ATCAGTTATGGTTCCTTGGACGATA-CCAT--CCTAC--TTGGATAACTGTGGTAATTCT
DCAYE       ATCAGTTATGGTTCCTTGGACGATA-CCAT--CCTAC--TTGGATAACTGTGGTAATTCT
LEPID       ATCAGTTATGATTTCTCAGATCGTA-CACT--CCTAC--TTGGATAACTGTGGTAATTCT
ACANT       ATCAGTTATGATTTCTTAGATCGTA-CACT--CCTAC--TTGGATAACTGTGGTAATTCT
MOPAL       ATCAGTTATGATTTCTTAGATCGTA-CAAT--CCTAC--TTGGATAACTGTGGTAATTCT
CRYPT       ATCAGTTATGATTTCTTAGATCGTA-CMAT--CCTAC--TTGGATAACTGTGGTAATTCT
ANISO       ATCAGTCGAGGTTCCTTAGATGACA-CGAAA-CTGAC--TCGGATAACTGTGGCAATTCC
DIAUL       ATCAGTCGAGGTTCCTTAGATGACA-CGAAA-CTGAC--TCGGATAACTGTGGCAATTCC
HELIS       ATCAGTCGATGTTTATTAGATGAAAACTAT--CCTAC--TTGGATAACTGTGGCAATTCT
LYMNA       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
HAMIN       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
RISSO       ATCAGTCGAGGTTCCTTAGATGACA-CGTTTTCCTAC--TTGGATAACTGTGGCAATTCT
LIMIC       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
SIPHO       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
LIMAX       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
APLYS       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
FARGO       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTAGCAATTCT
ONCHI       ATCAGTCGAGGTTCCTTAGATGACA-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
PHYSA       ATCAGTCGAGGTTCCTTAGATGACW-CGAT--CCTAC--TTGGATAACTGTGGCAATTCT
OLIVA       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
THAIS       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
TURBI       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
LAMEL       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
CERAT       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
OSTRI       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
BUCCI       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
FUSIT       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TW-TAC--TTGGATAACTGTGGTAATTCT
SCAPH       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
BSPIR       ATCAGTCGAGGTTCCTTAGATGATC-CCAA-TT-TAC--TTGGATAACTGTGGTAATTCT
BSINI       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
SEARL       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
BCARI       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-CT-TAC--TTGGATAACTGTGGTAATTCT
BCANA       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-CT-TAC--TTGGATAACTGTGGTAATTCT
ILYAN       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
NEPTU       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
POLIN       ATCAGTCGAGGTTCCTTAGATGGTC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
CONUS       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
HASTU       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
PHYLO       ATCAGTCGAGGTTCCTTAGATGATC-CCAA-TT-TAC--TTGGATAACTGTGGTAATTCT
MUREX       ATCAGTCGAGGTTCCTTAGATGATC-CCAA-TT-TAC--TTGGATAACTGTGGTAATTCT
SIRAT       ATCAGTCGAGGTTCCTTAGATGATC-CCTA-TT-TAC--TTGGATAACTGTGGTAATTCT
ANNUL       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TC-TAC--TTGGATAACTGTGGTAATTCT
TRUNC       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TC-TAC--TTGGATAACTGTGGTAATTCT
CORAL       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
ARCTO       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
XENOP       ATC-GTCGAGGTTCCTTAGATGATC-CAA--TT-TAC--TTGGATAACTGTGGTAATTCT
CYPRA       ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TT-TAC--TTGGATAACTGTGGTAATTCT
CERIT       ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGTAATTCT
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BATIL    ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGTAATTCT
POMAC    ATCAGTCGAGGTTCCTTAGATGATC-CAAA-TC-TAC--TTGGATAACTGTGGCAATTCT
MARIS    ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGCAATTCT
CYCLO    ATCAGTCGAGGTTCCTTAGATGATC-CTTT-ACCTAC--TTGGATAACTGTGGTAATTCT
NEOCY    ATCAGTCGAGGTTCCTTAGATGATC-CTTT-TCCTAC--TTGGATAACTGTGGTAATTCT
CAMPA    ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGTAATTCT
CIPAN    ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGTAATTCT
MODUL    ATCAGTCGAGGTTCCTTAGATGATC-CATT-TC-TAC--TTGGATAACTGTGGTAATTCT
TECTA    ATCAGTCGAGGTTCCTTAGATGATC-CCAA-TC-TAC--TTGGATAACTGTGGTAATTCT
EADAN    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
ERUMP    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
PQUOY    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
PMAUR    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
PLUCA    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
PMIDA    ATCAGTTATGGTTCCTTAGACGGTA-CGAT-TCCTAC--TTGGATAACTGTGGCAATTCT
PTERE    ATCAGTTATGGTTCCTTAGACGGTATCGAT-TCCTAC--TTGGATAACTGTGTCAATTCT
NRITI    ATCAGTTATGGTTCCTTAGATCGTA-CAAC--TCCAC--TCGGATAACTGTGGCAATTCT
SEPTA    ATCAGTTATGGTTCCTTAGATCGTA-CAAC--TCCAC--TCGGATAACTGTGGCAATTCT
NRITA    ATCAGTTATGGTTCCTTAGATCGTA-CAAC--TCCAC--TCGGATAACTGTGGCAATTCT
COCCU    ATCAGTTATAATTTTCTGGATCGTA-CGAT-CCTTAC--TTGGATAACTGTGGCAATTCT
EULEP    T-CAGATAAGGTTCCTTGGCGAAAAGCGGGTCGGTTT-AATGGATAACTGTGGTAATTCT
PARAL    T-CAGATAAGGTTCCTTGGCGAAAAGCGGGTCGGTTT-AATGGATAACTGTGGTAATTCT
PVULG    T-CAGATAAGGTTCCTTGGCGAAAAGCGGGTCGGTTT-AATGGATAACTGTGGTAATTCT
ACMAE    T-CAGATAAGGTTCCTTGGCGAAAAGCGGGTCGGTTT-AATGGATAACTGTGGTAATTCT
LEPET    T-CAGATAAGGTTCCTTGGCGAAAAGCGGGTCGGTTT-AATGGATAACTGTGGTAATTCT
LOTTI    T-CAGATAAGGTTCCTTGGCTAAAAGCGGGTCGGTTTTAATGGATAACTGTGGTAATTCT
TECTU    T-CAGATAAGGTTCCTTGGCTAAAAGCGGGTCGGTTTTAATGGATAACTGTGGTAATTCT
PSACC    T-CAGATAAGGTTCCTTGGCTAAAAGCGGATCGGTTTTAATGGATAACTGTGGTAATTCT
NACEL    T-CAGATAACGTTCCTTGGCAAATAGCGGGTCGATTTGAATGGATAACTGTGGTAATTCT
CELLA    --CAGACGAGGTTCCTTGGTTCCTATCGGGTCCGTAA-AATGGWTAACTGTGGTAATTCT
           * *      *      *       *                ** *******   ** **

NAUTI    AGAGCTAATACATGCAAC-GATGCTCCGACTTCTTT-------CGTGGAAGGAAGAGCGC
NOTOC    AGAGCTAATACATGCACA-CATGCCCGTCCGCCGTG-------CTTTTCGCCGGGTGCGT
TFUNE    AGAGCTAATACATGCACT-ATAGCTCCG-ACCCT---------TTCGCG--AGGG-----
TPULL    AGAGCTAATACATGCACT-ATAGCTCCG-ACCCT---------TTCGCG--AGGG-----
ASTRA    AGAGCTAATACATGCACT-ATAGCTCCG-ACCCT---------TTCGCG--AGGG-----
TURBO    AGAGCTAATACATGCACT-ATAGCTCCG-ACCCT---------TTCGCG--AGGG-----
HALIO    AGAGCTAATACATGCACT-A-AGCTCCG-ACCCT---------TTC-TG--AGGG-----
CALLI    AGAGCTAATACATGCACC-ATAGCTCCG-ACCCT---------TCC-----GGGG-----
CITTA    AGAGCTAATACATGCACC-ATAGCTCCG-ACCCT---------TTCGCG--AGGG-----
DASPE    AGAGCTAATACATGCACT-TCGGCTCCG-ACCCT---------TACCCA--AGGG-----
DCAYE    AGAGCTAATACATGCACT-TCGGCTCCG-ACCCT---------TTCCCA--AGGG-----
LEPID    AGAGCTAATACATGAAAC-TCCGCTCC-GACCTCAC----------------GGG-----
ACANT    AGAGCTAATACATGACGT-TCAGCTCC-GACCTTTTT-------GCA-----GGG-----
MOPAL    AGAGCTAATACATGAAAC-TCCGCTCCAGACCTTTA---------CC-----GGG-----
CRYPT    AGAGCTAATACATGAAAC-TCCGCTCC-GACCTTTA---------CC-----GGG-----
ANISO    AGAGCTAATACGTGCAC-TCAAGCCCCG-ACCT-----------CCGCG---AGGGG---
DIAUL    AGAGCTAATACGTGCAC-TCAAGCCCCG-ACCT-----------CCGCG---AGGGGG--
HELIS    AGAGCTAATACATGCTTACCAAGCTCCG-ACCC-----------TCGT----GGAAAG--
LYMNA    AGAGCTAATACATGCTTACCAAGCTCCG-ACCT-----------TTATT---GGAAAG--
HAMIN    AGAGCTAATACATGCCTCTGAAGCTCCG-ACCC-----------TCGT----GGAAAG--
RISSO    AGAGCTAATACATGCCTATGAAGCTCAGTACTC-----------TCGC----AAGAGG--
LIMIC    AGAGCTAATACATGCTTACCAAGCTCCG-ACCC-----------TCGC----GGAAAG--
SIPHO    AGAGCTAATACATGCTTTTGAAGCTCCG-ACCC-----------TCGT----GGAAAG--
LIMAX    AGAGCTAATACATGCTTACCAAGCTCCG-ACCC-----------TCGT----GGAAAG--
APLYS    AGAGCTAATACATGCTTCGCAAGCTCCG-ACCC-----------TCGT----GGAAAG--
FARGO    AGAGCTAATACATGCCTACCAAGCTCCG-ACCC-----------TCGC----GGAAAG--
ONCHI    AGAGCTAATACATGCTATTCAAGCTCCG-ACCC-----------TCTG----GGGAAG--
PHYSA    AGAGCTAATACATGCAATCGAAGCTCCGACCTT-----------TATC----GGGAAG--
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OLIVA      AGAGCTAATACATGCTGA-CCAGCTCCG-ACCC----------CTC-----GGGG-----
THAIS      AGAGCTAATACATGCTGA-CCAGCTCCG-ACCC----------CTC-----CGGG-----
TURBI      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------CTCTCGGCGGGG-----
LAMEL      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
CERAT      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------CCCG-----GG------
OSTRI      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
BUCCI      AGAGCTAATACATGCCGA-ACAGCTCCG-ACCC----------CTCG-----GGG-----
FUSIT      AGAGCTAATACATGCCTA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
SCAPH      AGAGCTAATACATGCCAA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
BSPIR      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TTCG-----GGG-----
BSINI      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TTCG-----GGG-----
SEARL      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TTCG-----GG------
BCARI      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TTCG-----GGG-----
BCANA      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TTCG-----GGG-----
ILYAN      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------TCCG-----GG------
NEPTU      AGAGCTAATACATGCCCA-ACAGCTCCG-ACCC----------TTCG-----GGG-----
POLIN      AGAGCTAATACATGCCCA-CCAGCTTCG-ACCCGTG------CCGCAAGTATGGG-----
CONUS      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC---------CCTCG-----GGG-----
HASTU      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
PHYLO      AGAGCTAATACATGCCGA-ACAGCTCCG-ACCCC------------T-TTGCGGG-----
MUREX      AGAGCTAATACATGCCGA-ACAGCTCCG-ACCCC------------T-TTGCGGG-----
SIRAT      AGAGCTAATACATGCCGA-ACAGCTCCG-ACCCC------------T-TTGCGGG-----
ANNUL      AGAGCTAATACATGCCCA-ACAGCTCCG-ACCCC------------TTTTAGGGG-----
TRUNC      AGAGCTAATACATGCCCA-CCAGCTCCG-ACCCCGGCGCCGTTTCCTTTCGGGGGGGCAG
CORAL      AGAGCTAATACATGCCGA-CCAGCTCCG-ACCCC--------------TCGGGGA-----
ARCTO      AGAGCTAATACATGCTGC-CCAGCTCCG-ACCCC--------------TCGGGGA-----
XENOP      AGAGCTAATAC-TGCCA--CCAGCTCCG-ACCC----------CTCG-----GGG-----
CYPRA      AGAGCTAATACATGCCAA-CCAGCTCCG-ACCC----------CTCG-----GGG-----
CERIT      AGAGCTAATACATGCCAA-CCAGCTCCG-ACCC---------TCAC------GGG-----
BATIL      AGAGCTAATACATGCCAA-CCAGCTCCG-ACCC---------TCAC------GGG-----
POMAC      AGAGCTAATACATGCAAA-CCAGCTCCG-ACCCGG-------TGTCACAGCCGGG-----
MARIS      AGAGCTAATACATGCAAC-CAAGCTCCG-ACCCGG-------TGTCACAGCCGGG-----
CYCLO      AGAGCTAATACATGCGGA-CAAGCTCCG-ACCCGT-------TGTT------GGG-----
NEOCY      AGAGCTAATACATGCGGA-CAAGCTCCG-ACCCTC-------TCGT------GGG-----
CAMPA      AGA-CTAATACATGCGGA-CCAGCTCCG-ACCCGG-------TGTCAAAGCCGGG-----
CIPAN      AGAGCTAATACATGCCCA-ACAGCTCCG-ACCCCGGGCT---TCGCGGTTCGGGG-----
MODUL      AGAGCTAATACATGCTGA-CCAGCTCCG-ACCC----------TTCG-----GGG-----
TECTA      AGAGCTAATACATGCCAA-CCAGCTCCG-ACCT----------CTCG-----GGG-----
EADAN      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
ERUMP      AGAGCTAATACATGTGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
PQUOY      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
PMAUR      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
PLUCA      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
PMIDA      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
PTERE      AGAGCTAATACATGCGAC-CCAGCTCCG-ACCTCTC------CCGCAGGGAAGAGCGCTT
NRITI      AGAGCTAATACGTGCAAG-AAAGCTCCG-ACCT----------CGC----GGG-----
SEPTA      AGAGCTAATACGTGCAAG-AAAGCTCTG-ACCT----------CGC----GGG-----
NRITA      AGAGCTAATACGTGCAAG-AAAGCTCTG-ACCT----------CGC----GGG-----
COCCU      AGAGCTAATACATCAACG--AAGCTCCG-ACTGTA--------GTC------AGT-----
EULEP      AGAGCTAATACATGCAACG-CACCGTGGTCCCCCCCT------CCTTTC--A-----TCC
PARAL      AGAGCTAATACATGCAACG-CACCGTGGTCCCCCCCT------TTCACC--AAAACCTCT
PVULG      AGAGCTAATACATGCAACG-CACCGTGGTCCCCCCTT------T----C--G-----TTT
ACMAE      AGAGCTAATACATGCAACG-CACCGTGGTCCCCCCTC------TTCCCC--A-----TCG
LEPET      AGAGCTAATACATGCAACG-TACCGTGGTCCCCCCTC------TT----------TCC
LOTTI      AGAGCTAATACATGCAACG-CACCGTGGTCCCCGTAG------T----------CT
TECTU      AGAGCTAATACATGCAACG-CACCGTGGTCCCCGTAG------T----------CT
PSACC      AGAGCTAATACATGCAACG-CACCGTGGTCCCCGTAG------T----------CT
NACEL      AGAGCTAATACATGCAACATCACCGTGGAGCCCCTT----------
CELLA      AGAGMTAATACATGCTACG-CAC-GCAACCCCGCTC----------
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NAUTI    TTTTATTAGACCAAGACGATTTAGTCTTCGTTCATAACAA--AG-GCGGTCGCTGT----
NOTOC    CTCC---GGGC--GGGCG----------CGTTTAT--CAG--TT-GAAGCCAGCGG----
TFUNE    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
TPULL    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
ASTRA    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
TURBO    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
HALIO    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
CALLI    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
CITTA    ---------GA-AGAGCG----------CGTTTATC-AG---TTCGAAGCCAGCCG----
DASPE    ---------GA-AGAGCG----------CATTTATC-AGC--TTCGAAGCCAGCCG----
DCAYE    ---------GA-AGAGCG----------CATTTATC-AGC--CTCGAAGCCAGCCG----
LEPID    ----------A-AGAGCG----------CTTTTAT--TTG--ATCAAGATCAACCG----
ACANT    ----------A-AGAGCG----------CTTTTAT--TAG--ATCAAGATCAATCG----
MOPAL    ----------A-AGAGCG----------CTTTTAT--TAG--ATCAAGATCAATAC----
CRYPT    ----------A-AGAGCG----------CTTTTAT--TAG--ATCAAGATCAATAC----
ANISO    ---------AA-GGGGCG----------CTTTTAT--TAG--TTCAAAACCGGTGG-CGC
DIAUL    ---------AA-GGGGCG----------CTTTTAT--TAG--TTCAAAACCGGTGG-CGC
HELIS    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCGT
LYMNA    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCGG
HAMIN    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCG-
RISSO    ---------GT-CGAGCG----------CTTTTAT--TAG--TTCAAAACCAATGGTCGT
LIMIC    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGTCGT
SIPHO    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCGT
LIMAX    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGTCGT
APLYS    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGTCGT
FARGO    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCGT
ONCHI    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGCCGT
PHYSA    --------------AGCG----------CTTTTAT--TAG--TTCAAAACCAATCGGCG-
OLIVA    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
THAIS    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
TURBI    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
LAMEL    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
CERAT    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
OSTRI    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
BUCCI    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
FUSIT    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
SCAPH    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
BSPIR    ---------AA-AGGGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
BSINI    ---------AA-AGGGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
SEARL    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
BCARI    ---------AA-AGGGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
BCANA    ---------AA-AGGGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
ILYAN    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
NEPTU    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
POLIN    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
CONUS    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
HASTU    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
PHYLO    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
MUREX    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
SIRAT    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
ANNUL    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
TRUNC    TGGCCGGGGAA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGAGGG
CORAL    ----------A-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
ARCTO    ----------A-AGAGCG----------CTTTTAT--TAG--TTCCAAACCAGTCG----
XENOP    ---------AA-AGAGCG----------CTTWTAT--YAG--T-CACAACCAGWCG----
CYPRA    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG----
CERIT    ---------AA-AGARCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
BATIL    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
POMAC    ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
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MARIS          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
CYCLO          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
NEOCY          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGC
CAMPA          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGT
CIPAN          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGGG
MODUL          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCGGGTT
TECTA          ---------AA-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAGTCG-G--
EADAN          TTATTAGTTTC-AAAAAA----------CCCGCACG-CGG--ATCGGGCTTCACGGGCGG
ERUMP          TTATTAGTTTC-AAAAAA----------CCCGCACG-CGG--ATTGGGCTTCCTTGGCGG
PQUOY          TTATTAGTTYC-AAAAAA----------CCCGCACG-CGG--TCCGGGYTCCGT-GSCGG
PMAUR          TTATTAGTTTC-AAAAAA----------CCCGCACG-CGG--TCCGGGCTCCGT-GGCGG
PLUCA          TTATTAGTTTC-AAAAAA----------CCCGCACG-CGG--TCCGGGTTCCGT-GGCGG
PMIDA          TTATTAGTTYC-AAAAAA----------CCCGCACG-CGG--GCCGGSCTCCGC-GGCGG
PTERE          TTATTAGTTTC-AAAAAA----------CCCGCACG-CGG--GCCGGKSTCCGC-GGCGG
NRITI          ----------A-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAATCGGGGT
SEPTA          ----------A-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAATCGGG-C
NRITA          ----------A-AGAGCG----------CTTTTAT--TAG--TTCAAAACCAATCGGG-T
COCCU          ----------T-GGAGGG----------CTTTTAT--TAG--T--TGAACCTCCCGARCT
EULEP          TGTGA----GC-GGGTGGGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
PARAL          TTTGGCTCTGC-GGGGCGGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
PVULG          CTCGA-----C-GAGGCGGGG-AAACGGCATTTATTCTCTATACCA-GATCGCCCTAGCC
ACMAE          TGCT------C-GAG--GGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
LEPET          TTCGAGTC--G-GAGGTCGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
LOTTI          TCTA---------GTCTCGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
TECTU          TCTA---------GTCTCGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
PSACC          TCTC---------GTCTCGGGGAAACGGCATTTATTCTCTGGACCA-GATCGCCCTAGCC
NACEL          ---------------CGGGGGACGCGGCATTTATTCTCTAAACCAAGATCGCCCTAGCC
CELLA          ---------------GAAGGGGTGCAGGTATTTATTCTCT--ACAATGACGGCCCGAGCT
                                                        *

NAUTI          -----GATCCTATCTCGTTCG---------------TTTGTTCGTTAGTTCGCAAGCG-
NOTOC          -----GGTTCCA---CGCTC----------------CTTGGGAGTTGGCCTTCATGTGA
TFUNE          -----GGTCGCAAG-----------------------------------GC----------
TPULL          -----GGTCGCAAG-----------------------------------GC----------
ASTRA          -----GGTCGCAAG-----------------------------------GC----------
TURBO          -----GGTCGCAAG-----------------------------------GC----------
HALIO          -----AGCCGCAA------------------------------------------------
CALLI          -----GGCCGCAAG-----------------------------------GC----------
CITTA          -----GGTCGCAAG-----------------------------------GC----------
DASPE          -----GGTCGCAAG-----------------------------------GC----------
DCAYE          -----GGTCGCAAG-----------------------------------GC----------
LEPID          -----GGCTTCG-------------------------------------GC----------
ACANT          -----GGCCTCG-------------------------------------GCC---------
MOPAL          -----GGTTCCGTTCGC--------------------------------GGTC--------
CRYPT          -----G-TGCCG--CAA--------------------------------GGTG--------
ANISO          ----GCGTCCCCCTTCG-------------------------GGCGGGCG---------
DIAUL          ----GCGTCCGGGGCGA-------------------------CTCGGGCG---------
HELIS          -----TGCCC---TTC-------------------------GCGGGGCG---------
LYMNA          -----CGTGCGGGCGA-------------------------CTCGTGCG---------
HAMIN          ----GTCGTCTTCGC---------------------------GGCGGC----------
RISSO          GTGGGTCTTCTTTTCTCGC-----------TCTTCG-------GACGGGAGGGGTTGGTC
LIMIC          -----TGCCCTTCA-----------------------------GCGGGCG---------
SIPHO          -----TGCCCTTCGCAA-------------------------GGGGGGTG---------
LIMAX          -----TACCCTTCGC----------------------------GGGGTG----------
APLYS          -----CTCGCCGTTTTC-------------------------GGGGGGG---------
FARGO          ----GTCTGCTTTC-----------------------------GGGTGGCG---------
ONCHI          ----GTGCTCTTCCC----------------------------GGGGCCG---------
PHYSA          ------CGGCCTCGCA-------------------------AGGGGTTG---------
OLIVA          -----GGTTCT--------------------------------GC----------
THAIS          -----GGTTCT--------------------------------GC----------
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TURBI     -----GGTTCT-----------------------------------GC----------
LAMEL     -----GGTTCG-----------------------------------TC----------
CERAT     -----GGTTCG-----------------------------------TC----------
OSTRI     -----GGTTCG-----------------------------------TC----------
BUCCI     -----GGTTCC-----------------------------------GC----------
FUSIT     -----GGTTCT-----------------------------------GC----------
SCAPH     -----GGTTCT-----------------------------------GC----------
BSPIR     -----GGTTCC-----------------------------------GC----------
BSINI     -----GGTTCC-----------------------------------GC----------
SEARL     -----GGTTCT-----------------------------------GC----------
BCARI     -----GGTTCT-----------------------------------GC----------
BCANA     -----GGTTCT-----------------------------------GC----------
ILYAN     -----GGTTCT-----------------------------------GC----------
NEPTU     -----GGCTCT-----------------------------------GC----------
POLIN     -----GGTTTCTCTTTCG-----------------------GGAGGGC----------
CONUS     -----GGTTC---T--------------------------------GC----------
HASTU     -----GGTTC---T--------------------------------GC----------
PHYLO     -----GGATT------------------------------------TTC----------
MUREX     -----GGATT------------------------------------TTC----------
SIRAT     -----GGATT------------------------------------TTC----------
ANNUL     -GG-TGTCCC----CTCG-----------------------TGGGTTC----------
TRUNC     GGG-CGGCTCGC-GTTCG-----------------------TTCCCTC----------
CORAL     -----GGCCC------------------------------------GTC----------
ARCTO     -----GGTTC------C-----------------------------GGC----------
XENOP     -G---GGT-CC-----------------------------------GCC----------
CYPRA     -G---GGTTCC-----------------------------------GCC----------
CERIT     -----CGCCCCG----------------------------------------------
BATIL     T----CGCCCCG----------------------------------------------
POMAC     CT--CGGCCCCGTC--------------------------------------------
MARIS     CT--CGGCCCCGTC--------------------------------------------
CYCLO     TT--CGGCTCCG----------------------------------------------
NEOCY     TT--CGGCTCCG----------------------------------------------
CAMPA     TT--GGGTTCCGGCCCG-----------------------------CCT----------
CIPAN     TT--TCGGCCCTCG--------------------------------------------
MODUL     T----CGGCCCG----------------------------------------------
TECTA     -----GGTAACC----------------------------------CC----------
EADAN     CGGGGGTCTYGGYTCGTCT-------------GGGC-------TCCCACCACCGCCCGGG
ERUMP     CGGGGGACAGAGTTCGTCT-------------TGGC-------CCCCACCACCGCCCTGG
PQUOY     CGGGGGGTTMAGTSCGAYT---------CGTSGGACKTG-CCCCCCCACCACCGTCGCGG
PMAUR     CGGGGGGTTCAGTSCGACT---------CGTCGGACGTG-SCCCCCCACCACCGTCGCGG
PLUCA     CGGGGGGTTCAGTGCGACT---------CGTCGGACGTG-GCCCCCCACCACCGTCGCGG
PMIDA     CGGGGG-TTCAGTGCGACTTGCCTTGCTCGTCGGACTTGGTGCCCCCACCACCGTCGCGG
PTERE     CGGGGG-TTCAGTGCGACTTGCCTTGCTCGTCGGAC--G-TGCCCCCACCACCGTCGCGG
NRITI     CGCAAGACCC------------------------------------------------
SEPTA     CGCAAGGCCC------------------------------------------------
NRITA     CGCAAGGCCC------------------------------------------------
COCCU     -----GGTCT------------------------------------------------
EULEP     CGCGAAACTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
PARAL     CGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
PVULG     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
ACMAE     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
LEPET     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
LOTTI     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
TECTU     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGCGA
PSACC     TGCGAAGCTACCGTC-------------------------AAAAGTAGCGACGGGGTGA
NACEL     CGCCGAACTGCCA---------------------------AAAGACAGCGAGGGGGTGA
CELLA     CTTCAATTGAATTTT-------------------------KAATTCAGGAGAGTGCCGT


NAUTI     -------TTCGAAATCGCA---AATATTGGTGAC-TCTGGATAACTTTTGTCAGATCGCA
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NOTOC       -------CTCTAGGT-------AACCGTGCCGAT-CGCGGG-AGTTCTTAACAG------
TFUNE       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
TPULL       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
ASTRA       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
TURBO       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCCGA
HALIO       -----------CTCTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
CALLI       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
CITTA       -----------CCGTCT-------CTTTGGTGAC-TCTGGATAACTCTA-GCGGATCGCA
DASPE       -----------CCGTCCAC-----CTTTGGTGAC-TCTGGATAACTGTT-GCGGATCGCA
DCAYE       -----------CCGTCCAC-----CTTTGGTGAC-TCTGGATAACTGTT-GCGGATCGCA
LEPID       ------------GT-CCT------ATTGGTGAT-TCTGAATAACTTTGTGCTGATCGCA
ACANT       ------------CGT-CCT------GTTGGTGAT-TCTGAATAACTTTGTGCTGATCGCA
MOPAL       ------------CGTAGAC------ATTGGTGAT-TCTGAACAACTTTGTGCTGATCGCA
CRYPT       ------------CGTACTC------GTTGGTGAT-TCTGAATAACTTTGTGCTGATCGCA
ANISO       ---------CGCC-TCCCC------CTTGATGAC-TCTGGATAACTTTGAGCTGATCGCA
DIAUL       ---------CGCCCTCCCC------CTTGATGAC-TCTGGATAACTTTGAGCTGATCGCA
HELIS       ---------CGGCGTCCCG------ATTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
LYMNA       ---------CGGCGTCCCC------ATTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
HAMIN       ---------CGGTGTCCCC------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
RISSO       TG-TTTAAGCGGCGTTGTT------TTTGATGAC-TCTGGATAACTTTGAGCTGATCGCA
LIMIC       ---------CGGCGTCCA-------ACTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
SIPHO       ---------CGGCGTCCCC------ACTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
LIMAX       ---------CGGCGTCCCC------ACTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
APLYS       ---------CGGCGTCCCAC-----TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
FARGO       ---------CGGTGTCCCA------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
ONCHI       ----------GGCGTCCCC------CTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
PHYSA       ---------CGTCGTCCCA------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
OLIVA       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
THAIS       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
TURBI       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
LAMEL       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CERAT       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
OSTRI       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BUCCI       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
FUSIT       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
SCAPH       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BSPIR       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BSINI       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
SEARL       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BCARI       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BCANA       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
ILYAN       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
NEPTU       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
POLIN       ---------T--CGTCC--------GTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CONUS       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTTTGCCGATCGCA
HASTU       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
PHYLO       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
MUREX       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
SIRAT       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
ANNUL       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
TRUNC       ---------T--CGTCCCT------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CORAL       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
ARCTO       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACGTTGTGCCGATCGCA
XENOP       ---------C--CGKCC--------TTTGGTGAC-TCTGGATAACTTTGAGCCGATCGCA
CYPRA       ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGAGCCGATCGCA
CERIT       --------------TCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
BATIL       --------------TCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
POMAC       --------------CCT--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
MARIS       --------------CCT--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CYCLO       --------------TCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
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NEOCY  --------------TCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CAMPA  ---------C--GTCCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
CIPAN  --------------TGC--------TTTGGTGAC-TCTGGATAACTTTGAGCCGATCGCA
MODUL  --------------TCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
TECTA  ---------C--CGTCC--------TTTGGTGAC-TCTGGATAACTTTGTGCCGATCGCA
EADAN  TTTCGCTCRCCGCGTCTCTCCAACCCATGGTGAA-TCTGGATAACTTGGATCGGATCGCA
ERUMP  TTTCGCTCGCCGCGTTTCCCCAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
PQUOY  TA-CGCTCTCCGCGTTTTCTAAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
PMAUR  TA-CGCTCTCCGCGTTTTCTAAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
PLUCA  TA-CGCTCTCCGCGTTTTCTAAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
PMIDA  TA-CGCTCTCCGCGTTTTCTAAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
PTERE  TA-CGCTCTCCGCGTTTTCTAAACCCATGGTGAA-TCTGGATAACTTCGATCGGATCGCA
NRITI  -------------GTCC-G------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
SEPTA  -------------GTCC-G------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
NRITA  -------------GTCC-G------TTTGGTGAC-TCTGGATAACTTTGTGCTGATCGCA
COCCU  ------------CGTCG---------ATGGTGAC-TCTGGATAACGGCT-GCTGATCGCT
EULEP  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
PARAL  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
PVULG  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
ACMAE  ----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
LEPET  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
LOTTI  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
TECTU  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
PSACC  A----------GCGACA--------AGTGTTGAAATCCGAATAACTGTG--CCGATCGCG
NACEL  AA---------ACGACA--------AGTGTTGAATTCCGAATAACTGTG--CCGATCGCG
CELLA  G----------GGTA--------AGTGTTGAAATCTGATTAACTTTG--TCGATCACG
                                  **  **     *   *         *

NAUTI  GGGCG-TT-CGCG---CCGGCGACGGGTCTTTCGAACGTCCGCCCCATCA-ACT------
NOTOC  --G---CT-C------CTAGCGACGCGCCCGAAAAACGTCTGCCCTATCACACT------
TFUNE  CGGCCTT---GAG---CCGGCGACGCATCTATCAAGTGTCTGCCCTATCAGACT------
TPULL  CGGCCTT---GAG---CCGGCGACGCATCTATCAAGTGTCTGCCCTATCAGACT------
ASTRA  AGGCCTT---GAG---CCGGCGACGCATCTATCAAGTGTCTGCCCTATCAGACT------
TURBO  TGGCCTT---GAG---CCGGCGACGCATCTATCAAGTGTCTGCCCTATCAGACT------
HALIO  CGGCCTC---GAG---CCGGCGACGCATCTATCAAGTGTCTGCCCTATCAGACT------
CALLI  CGGCCCC---GAG---CCGGCGACGCGTCTATCAAGTGTCTGCCCTATCAGACT------
CITTA  CGCCCTTT--GCG---GCGGCGACGCGTCTATCAAGTGTCTGCCCTATCAAACT------
DASPE  CGGCCCC---GAG---CCGGCGACGCGTCCATCAAATGTCTGCCCTATCAGACT------
DCAYE  CGGCCCC---GAG---CCGGCGACGCGTCCATCAAATGTCTGCCCTATCAGACT------
LEPID  TGGCCC---AGCG---CCGGCGACGTATCTTTCAAGTGTCTGCCCTATCAACTTT-----
ACANT  TGGCCA---CGCG---CCGGCGACGTATCTTTCAAGTGTCTGCCCTATCAACTTT-----
MOPAL  TGGCCT---CGCG---CCGGCGACGTATCTTTCAAGTGTCTGCCCTATCAACTTT-----
CRYPT  GGGCCT---CGCG---CCGGCGACGTATCTTTCAAGTGTCTGCCCTATCAACTTT-----
ANISO  CGGCCTC--TGTG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTCCCGG
DIAUL  CGGCCTC--TGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
HELIS  TGGCCTT---GTG---CTGGCGACCGATCTTTCAAATGTCTGCCCTATCAAATGTC----
LYMNA  TGGCCTT--CGTG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
HAMIN  TGGCCTC--TGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
RISSO  CGCACTCG-TGTG---CCGGCGACACATCTTACAAATGTCTGCCCTATCAAATGTC----
LIMIC  TGGCCTTC-TGTG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
SIPHO  TGGCC--C-TGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
LIMAX  TGGCCTCA-CGTG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
APLYS  TGGCCTTT-TGTG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
FARGO  TGGCCTC--GGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
ONCHI  TGGCCTTT-TGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATTAAATG-C----
PHYSA  TGGCCT---CGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATGTC----
OLIVA  TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
THAIS  TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
TURBI  TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
LAMEL  TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
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CERAT   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
OSTRI   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BUCCI   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
FUSIT   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
SCAPH   GGGCCGT--TGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BSPIR   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BSINI   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
SEARL   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BCARI   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BCANA   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
ILYAN   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
NEPTU   TGGCC-T--CGCG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
POLIN   TGGCC-A--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
CONUS   TGGCC-C--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
HASTU   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
PHYLO   TGGCCT---CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAAAT-------
MUREX   TGGCCT---CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAAAT-------
SIRAT   TGGCCT---CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAAAT-------
ANNUL   TGGCCT---CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
TRUNC   TGGCCTG--TGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
CORAL   TGGCCT---CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
ARCTO   TGGCCT---CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
XENOP   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAA-TGTCTGCCCTATCAAAT-------
CYPRA   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
CERIT   TGGCC-T--TGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
BATIL   TGGCC-T--TGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
POMAC   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
MARIS   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCT-CCCTATCAAAT-------
CYCLO   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
NEOCY   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
CAMPA   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAATT------
CIPAN   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
MODUL   TGGCC-T--TGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
TECTA   TGGCC-T--CGAG---CCGGCGACGCATCTTTCAAATGTCTGCCCTATCAAAT-------
EADAN   CGGGCCGT-CGAR---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
ERUMP   CGGGCCGT-CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
PQUOY   CGGGCCTT-CGAC---CGGCSAACGCGTCTTTCAA-TGTCTTCCCTATCAACTTT-----
PMAUR   CGGGCCTT-CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
PLUCA   CGGGCCTT-CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
PMIDA   CGGGCCTT-CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
PTERE   CGGGCCTT-CGAG---CCGGCGACGCGTCTTTCAAATGTCTGCCCTATCAACTTT-----
NRITI   CGGCCT---CGAG---CCGGCGACGTATCTTTCAAATGTCTGCCCTATCAACTTTA----
SEPTA   CGGCCT---CGAG---CCGGCGACGTATCTTTCAAATGTCTGCCCTATCAACTTTA----
NRITA   GGGCCT---CGAG---CCGGCGACGTATCTTTCAAATGTCTGCCCTATCAACTTTA----
COCCU   AGGCTTT---TGG---CCAGCGACAAATCCAAAAAGTATCTGCCTTATCAGCT-------
EULEP   GGGTCTTCCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
PARAL   GGGTCTTCCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
PVULG   GGGTCTTTCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
ACMAE   GGGTCTTTCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
LEPET   GGGTCTTTCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
LOTTI   GGGTCTTTCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
TECTU   GGGTCTTTCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
PSACC   GGGTCTTTCCGGGCCCCCGGCGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
NACEL   GGGTCCACCCGGGCCCCCGACGACTTTGCCATGAAGTGTCTGTCCCATCAATT-------
CELLA   ----------------CAAGTGACTCCATCATGAAGTGTCTGTCCCATCAATT-------
                              **          *   **   *  ** *

NAUTI   ---------------------------------TTCGACGGTCGGT-TAGGCG-CCGACC
NOTOC   ---------------------------------GTCGATGGTCGGC-CCTGTG-CCCACC
TFUNE   ---------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
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TPULL  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
ASTRA  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
TURBO  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
HALIO  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
CALLI  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
CITTA  ----------------------------------TTCGATGGTATGT-GCTATG-CTTACC
DASPE  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
DCAYE  ----------------------------------GTCGATGGTAAGT-GCTATG-CTTACC
LEPID  ----------------------------------CGATGGTACGT-GCTATG-CCTACC
ACANT  ----------------------------------CGATGGTACGT-GATATG-CCTACC
MOPAL  ----------------------------------CGATGGTACGT-GCTATG-CCTACC
CRYPT  ----------------------------------CGATGGTACGT-GCTATG-CCTACC
ANISO  CGACGCATCTTTCAAATGTCTGCCCTATCAAATGTCGACGGTACGT-GACATG-CCTACC
DIAUL  ----------------------------------GACGGTACGT-GACATG-CCTACC
HELIS  ----------------------------------GATGGTACGT-GATATG-CCTACC
LYMNA  ----------------------------------GATGGTACGT-GATATG-CCTACC
HAMIN  ----------------------------------GATGGTACGT-GATATG-CCTACC
RISSO  ----------------------------------GATGGTAGGT-GATATG-CCTACC
LIMIC  ----------------------------------GATGGTACGT-GACATG-CCTACC
SIPHO  ----------------------------------GATGGTACGT-GACATG-CCTACC
LIMAX  ----------------------------------GATGGTACGT-GACATG-CCTACC
APLYS  ----------------------------------GATGGTACGT-GATATG-C-TACC
FARGO  ----------------------------------GATGGTACGT-GATATG-CCTACC
ONCHI  ----------------------------------GATGGTACGT-GATATG-CCTACC
PHYSA  ----------------------------------GATGGTACGT-GATATG-CCTACC
OLIVA  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
THAIS  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
TURBI  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
LAMEL  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
CERAT  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
OSTRI  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
BUCCI  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
FUSIT  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
SCAPH  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
BSPIR  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
BSINI  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
SEARL  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
BCARI  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
BCANA  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
ILYAN  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
NEPTU  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
POLIN  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
CONUS  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
HASTU  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
PHYLO  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
MUREX  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
SIRAT  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
ANNUL  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
TRUNC  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
CORAL  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
ARCTO  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
XENOP  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
CYPRA  ----------------------------------GACGATGGTACGT-GATCTG-CCTACC
CERIT  ----------------------------------GACGATGGTCGGT-GATCTG-CCTACC
BATIL  ----------------------------------GACGATGGTCGGT-GATCTG-CCTACC
POMAC  ----------------------------------GTCGATGGTACGT-GATAGG-CCTACC
MARIS  ----------------------------------GTCGATGGTACGT-GATAGG-CCTACC
CYCLO  ----------------------------------GACGATGGTACGT-GATAGG-CCTACC
NEOCY  ----------------------------------GACGATGGTACGTTGATAGG-CCTACC
CAMPA  ----------------------------------GAACGAGGGTACGTTGATAGGGCCTACC
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CIPAN  ---------------------------------GACGACGGTACGT-GATCTG-CCTACC
MODUL  ---------------------------------GACGATGGTCGGT-GATCTG-CCTACC
TECTA  ---------------------------------GACGATGGTACGT-GATCTG-CCTACC
EADAN  -----------------------------------CGACGGTACGT-GCCCTG-CCCACC
ERUMP  -----------------------------------CGACGGTACGT-GCCCTG-CCCACC
PQUOY  -----------------------------------CGACGGTACGT--CCCTG-CCCACC
PMAUR  -----------------------------------CGACGGTACGT-ACCCTG-CCCACC
PLUCA  -----------------------------------CGACGGTACGT-ACCCTG-CCCACC
PMIDA  -----------------------------------CGACGGTACGT-GCCCTG-CCCACC
PTERE  -----------------------------------CGACGGTACGT-GCCCTG-CCCACC
NRITI  -----------------------------------GATGGTACGT-GATATG-CCTACC
SEPTA  -----------------------------------GATGGTACGT-GATATG-CCTACC
NRITA  -----------------------------------GATGGTACGT-GATATG-CCTACC
COCCU  -----------------------------------AGTAGGTGGTCGACCTGACCACCT
EULEP  ---------------------------------TGCGATGGTCGGC-GACCTG-CCTACC
PARAL  ---------------------------------TGCGATGGTCGGC-GACCTG-CCTACC
PVULG  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
ACMAE  ---------------------------------TGCGATGGTCGGC-GACCTG-CCTACC
LEPET  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
LOTTI  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
TECTU  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
PSACC  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
NACEL  ---------------------------------GGCGATGGTCGGC-GACCTG-CCTACC
CELLA  ---------------------------------GACGATGGTCGGC-GCCCTG-CCTACC
                                           *   *       * *   *

NAUTI  GTGGT-GATAACGGGTAACGGGG--ATCG-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
NOTOC  ATGGT-GACAACGGGTGACGGGG-GATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TFUNE  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TPULL  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ASTRA  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TURBO  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
HALIO  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CALLI  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CITTA  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
DASPE  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
DCAYE  ATGGT-GATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
LEPID  ATGGT-TGTAACGGGTAACGGAG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ACANT  ATGGT-TGTAACGGGTAACGGAG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
MOPAL  ATGGT-TGTAACGGGTAACGGAG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CRYPT  ATGGT-TGTAACGGGTAACGGAG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ANISO  GTGTT-GGCGACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCACGAGAA
DIAUL  GTGTT-GGCGACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCACGAGAA
HELIS  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
LYMNA  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
HAMIN  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
RISSO  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
LIMIC  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
SIPHO  ATGTT-TATAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
LIMAX  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
APLYS  ATGTT-TGTAACGGGTAACGGGGTAATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
FARGO  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ONCHI  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
PHYSA  ATGTT-TGTAACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
OLIVA  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
THAIS  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TURBI  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
LAMEL  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CERAT  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
OSTRI  ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
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BUCCI       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
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SCAPH       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
BSPIR       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
BSINI       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
SEARL       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
BCARI       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
BCANA       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ILYAN       ATGTT-AGCAACGGGTAGCGGTG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
NEPTU       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
POLIN       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CONUS       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
HASTU       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
PHYLO       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
MUREX       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
SIRAT       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ANNUL       ATGTTTAGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TRUNC       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CORAL       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
ARCTO       ATGTT-GGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
XENOP       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CYPRA       ATGTT-AGCAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CERIT       ATGTTT-ACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
BATIL       ATGTTT-ACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
POMAC       ATGTTG-ACTACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
MARIS       ATGTTG-ACTACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CYCLO       ATGTTT-ACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
NEOCY       ATGTTT-ACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CAMPA       ATGTTTGACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
CIPAN       GTGTTG-ACTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
MODUL       ATGTTG-GCTACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
TECTA       ATGTT-AACAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
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ERUMP       GTGGT-CGCAACGGGTGACGGGG-AATCA-GGGTTCGGTTCCGGAGAGGGAGCATGCGAA
PQUOY       GTGGT-CGCAACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
PMAUR       GTGGT-CGCAACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
PLUCA       GTGGT-CGCAACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
PMIDA       GTGGT-CGCAACGGGTGACGGG--AATCA-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
PTERE       GTGGT-CGCAACGGGTGACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGCGAA
NRITI       ATGGT-TATAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
SEPTA       ATGGT-TATAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
NRITA       ATGGT-TATAACGGGTAGCGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCATGAGAA
COCCU       AGGCT--ACCACGGGTAGCGGGG-AATCATGGGTTTGATTCCGGAGAGGGAGCATGAGAA
EULEP       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
PARAL       ACGGT-GTTGACGG-TAACG----------------------------------------
PVULG       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
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LEPET       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
LOTTI       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
TECTU       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
PSACC       ACGGT-GTTGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
NACEL       ACGGT-GATGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
CELLA       ACGGT-GACGACGGGTAACGGGG-AATCA-GGGTTCGATTCCGGAGAGGGAGCCTGCGAA
              * *      **** *  **

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NOTOC       ACGGCTACCACC-------TCTATGG-AAGGCAGCAGGCGC-CAACTTACCCAATCTCGA
TFUNE       ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
TPULL       ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
ASTRA       ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
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HALIO    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
CALLI    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
CITTA    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
DASPE    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
DCAYE    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
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MOPAL    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAAATTACCCAATCTCGA
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DIAUL    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCCGG
HELIS    ACGGCTACCAC-----------------------GGCGCGCAACTTACCCACTCC-GG
LYMNA    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCCGG
HAMIN    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCCGG
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TRUNC    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
CORAL    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
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MARIS    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCCAG
CYCLO    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
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CIPAN    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
MODUL    ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
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PMAUR          ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
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SEPTA          ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
NRITA          ACGGCTACCACA-------TCCAAGG-AAGGCAGCAGGCGCGCAACTTACCCACTCCTGG
COCCU          ACGGCTACCACG-------TCCAAGG-AAGGCAGCAGGCACGCAACTTACCCAATCCCGA
EULEP          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
PARAL          ------------------------------------------------------------
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ACMAE          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
LEPET          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
LOTTI          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
TECTU          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
PSACC          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
NACEL          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA
CELLA          ACGGCTACCACC-------TCCAAGG-AGGGCAGCAGGCGCGCAACTTACCCAATCCCGA

NAUTI          -CACGGGGAG-TAGTGACGAAAAATATCGGTG-CGGGTCT
NOTOC          -CTCGAGGAGGTAGTG-CGAAAAATATCG-TA-GGGGACT
TFUNE          -CACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
TPULL          -CACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
ASTRA          -CACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
TURBO          -CACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
HALIO          -TACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
CALLI          -CACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
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MOPAL          -TACGAGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
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DIAUL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
HELIS          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
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HAMIN          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
RISSO          -CACGGGGAGGTAGTGACGAAAAATAACAATC-CGGGACT
LIMIC          -CACGGAGGGGTAGTGACGAAAAATAACAATA-CGGGACT
SIPHO          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
LIMAX          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
APLYS          -CACGGGGAGGTAGTGA-GAAAAATAACAATAACGGGACT
FARGO          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
ONCHI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
PHYSA          -CACGGGGAG-TAGTGACGAAAAATAACAATA-CGGGACT
OLIVA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
THAIS          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
TURBI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
LAMEL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CERAT          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
OSTRI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
BUCCI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
FUSIT          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
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BSINI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
SEARL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
BCARI          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
BCANA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
ILYAN          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
NEPTU          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
POLIN          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
CONUS          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
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PHYLO          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
MUREX          --ACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
SIRAT          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
ANNUL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
TRUNC          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CORAL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
ARCTO          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
XENOP          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CYPRA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
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MARIS          -CTCGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CYCLO          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
NEOCY          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CAMPA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
CIPAN          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
MODUL          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
TECTA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGAACT
EADAN          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
ERUMP          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
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PMAUR          -CACGGGGAG-TAGTGACGAAAAATAACAATA-CGGGACT
PLUCA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
PMIDA          -CACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
PTERE          -CACGGGGAG-TAGTGACGAAAAATAACAATA-CGGGACT
NRITI          -TACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
SEPTA          -TACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
NRITA          -AACGGGGAGGTAGTGACGAAAAATAACAATA-CGGGACT
COCCU          -CACGGGGAGGTAGTGACGAAAAATACCAATA-CGGGACT
EULEP          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
PARAL          ----------------------------------------
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ACMAE          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
LEPET          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
LOTTI          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
TECTU          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
PSACC          -CACGGGGAGGTAGTGACGAAAAATAACG-TGGCGGGGCC
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APPENDIX III: Glossary of terms

- **Allogastropoda** A grade of gastropods, which include the fossil Nerineoidea, and the recent Architectonicoidea/Omalogyridae, Rissoelloidea, Glacidorboidea and Pyramidelloidea. These groups represent a step-by-step evolutionary path towards the euthyneuran level of organization. (Haszprunar, 1988)
- **Allogastropoda** This taxon is considered to be the earliest clade of the Heterobranchia (Van Den Biggelaar & Haszprunar, 1996). It is thought that the Allogastropoda gave rise to the Euthyneurans.
- **Apogastropoda** A relatively modern subclass grouping that contains two subclades Caenogastropoda and the allogastropod Heterobranchia (Harasewych *et al.* 1997)
- **Apogastropoda** A relatively modern term for a group of higher streptoneurans that used to be part of the Archaeogastropoda. (Haszprunar, 1988)
- **Archaeogastropoda** A very conservative and early taxon that has shown to be very polyphyletic. The organisms in this group all show architaenioglossate radulae, however two distinct nervous system patterns (hypoathroid and dystenoid) are thought to be orthophyletic. Recently brought back into use by Haszprunar to describe everything that is not an apogastropod or an euthyneuran (Ponder & Lindberg, 1997)
- **Architaenioglossa** An early group of freshwater and terrestrial gastropods that is often included at the base of Caenogastropoda or previously the Mesogastropoda (Harasewych *et al.* 1998). This order includes organisms such as *Pomacea*.
- **Caenogastropoda** A current superorder that contains the majority of living shelled, marine gastropods (Harasewych *et al.*, 1998) Functionally containing all of the Neogastropoda and the Mesogastropoda (Van Den Biggelaar & Haszprunar, 1996) except for the Valvatoidea, which are now considered heterobranchs (Ponder & Lindberg, 1996). It currently contains two clades, the Architaenioglossa and the Eucaenogastropoda (Haszprunar, 1988) [=Hypsogastropoda (Ponder& Lindberg, 1997)].
- **Chelating compound** A heterocyclic compound having a metal ion attached by coordinate that bonds to at least two nonmetal ions.
- **Clade** A group of biological taxa or species that share features inherited from a common ancestor as opposed to a grade.
- **Cocculinimorpha** A subclass defined by Haszprunar (1988) that includes the Cocculinidae and the Lepetelloidea.
- **Columellar muscle** The musculature originating on the columella of the gastropod shell and inserting on the ventral wall of the foot or interdigitating with the tarsos muscles (depending on species). This muscle is generally used for strong large scale movements.
- **Cyclobranchia** Term put forward by Golikov and Starobogatov (1975) based on gill morphology that is synonymous with Patellogastropoda, not in current use. See also Scutibranchia and Pectinibranchia.

- **Docoglossa** This term is thought to be nearly synonymous with the term Patellogastropoda (Bieler, 1992, Van Den Biggelaar & Haszprunar, 1996). Considered by many (Haszprunar, 1988) to be the earliest gastropod offshoot, they have retained very ancestral characteristics such as stereoglossate radulae and their symmetrical limpet-like form.
- **Eogastropoda** Ponder and Lindberg (1996) found that the Patellogastropoda and some related hot vent species (Neolepetopsina) were so distinct that they recognized it as a group equivalent to the rest of the gastropoda (termed the Orthogastropoda).
- **Eucaenogastropoda** A taxon put forward by Haszprunar (1988) to describe the higher Caenogastropoda, the lower Caenogastropoda are represented by the Architaenioglossa.
- **Euthyneura** Taxon put forward by Spengel (1881). A longstanding monophyletic group that includes the Opisthobranchia and the Pulmonata. (Haszprunar, 1988).
- **Grade** A relative position or degree of value in a graded group.
- **Heterobranchia** This grouping includes the Allogastropoda, Opisthobranchia and the Pulmonata (Van Den Biggelaar & Haszprunar, 1996). The group is considered to be the most derived of the gastropods.
- **Heterostropha** A group of families (Pyramidellidae and Architectonicidae) that displays both typical prosobranch and opisthobranch characters. Also called the Allogastropoda (Bieler, 1992). The Heterostropha and the Euthyneura are grouped together as the Heterobranchia.
- **Hydrostatic cavity** A hydrostatic skeleton in which a fluid filled cavity is bounded by muscles. The muscles act against the incompressible fluid to effect desired movements. Often seen in areas where quick generalized movement is needed. See also Muscular hydrostat and Hydrostatic skeleton.
- **Hydrostatic skeleton** A type of skeleton in which antagonistic force to the action of muscles is present in the incompressible nature of fluid in a cavity. The muscle/fluid cavity arrangement can range from muscular hydrostats to hydrostatic cavities resembling muscle bound cylinders.
- **Hypsogastropoda** Another name for Eucaenogastropoda, one of two subclades within the Caenogastropoda (Ponder & Lindberg, 1997).
- **Mesogastropoda** One of three conservative taxa below the Prosobranchia (the others being Archaeogastropoda and Neogastropoda).
- **Muscular Hydrostat** Term put forward by Kier (1985) denoting a hydrostatic skeleton in which the fluid under pressure is actually the internal fluid pressure within the muscle cells. A hydrostatic skeletal element often seen in areas where fine control is needed. See also Hydrostatic cavity and Hydrostatic skeleton.
- **Neogastropoda** Order put forward by Wenz (1943). There is quite a bit of anatomical and genetic data that supports the monophyly of this taxon. (Harasewych *et al.*, 1997)
- **Neritimorpha** A subclass defined by Golikov and Starobogatov (1975), which include all of the Neritimorpha and some of the Cocculinimorpha (the Cocculinidae).
- **Neritimorpha** Formerly considered an Archaeogastropod (Golikov & Starobogatov, 1975, Haszprunar, 1988) now is considered as being a problematic and primitive off

shoot between the Patellogastropoda and the Vetigastropoda. May also contain the problematic family Cocculinidae or even the subclass Cocculinimorpha.

- **Opisthobranchia** This taxon and the pulmonates form the highly derived Euthyneura. This group includes the sea slugs.
- **Orthogastropoda** Term used by Ponder and Lindberg (1996) to separate the class gastropoda in order to recognize the trenchant grouping of the Patellogastropoda.
- **Patellogastropoda** A subclass that includes organisms such as the limpets. This group is thought to be quite ancient. Paired with the Vetigastropoda, these groups were previously known as the Archaeogastropoda.
- **Pectinibranchia** Term put forward by Golikov and Starobogatov (1975) based on single gill morphology that includes all gastropods that are not Cyclobranchia, Scutibranchia or Euthyneura.
- **Pentaganglionata** Another term synonymous with Euthyneura (Salvini-Plawen & Haszprunar, 1987)
- **Prosobranchia** The largest traditional subclass is now known to be extremely paraphyletic
- **Pulmonata** Together with the Opisthobranchia this group forms the Euthyneura. The Pulmonata is considered to be the most derived of all the gastropods and is often termed the "Crown" group (Haszprunar, 1988). This group contains snails with pulmonary sacs such as *Lymnaea* and terrestrial slugs.
- **Rachiglossa** Put forward by Gray (1853). This taxon is more recently considered as three superfamilies, the Muricoidea, the Buccinoidea and the Volutoidea. Together with the Toxoglossa, they form the Stenoglossa. They differ in radula morphology. (Harasewych *et al.* 1997) They are now considered subclades of the Neogastropoda.
- **Radula, Ptenoglossate** Highly developed radular condition found in certain caenogastropods (eg.. *Janthina*) where the radula is limited to a sheet of curved teeth designed to pull the whole prey into the gut.
- **Radula, Rachiglossate** Radular condition shown by many advanced caenogastropods (neogastropod) such as Muricids and Naticids. They lack all marginal teeth and use the remaining medial teeth for boring and carnivorous feeding.
- **Radula, Rhipidoglossate** Radular condition of primitive Patellogastropods and Vetigastropods (archaeogastropod) that shows brush-like marginal teeth used for scraping biofilms from substrata.
- **Radula, Taenioglossate** Radula is similar to Rhipodoglossate condition, but has reduced marginal teeth and elaborated odontophore complex muscles designed to scrape off surface cell layers of algae. This is a condition shown by many early caenogastropods (mesogastropoda) such as the Littorinids.
- **Radula, Toxoglossate** Extremely modified radula that is reduced to a few poison injecting teeth. Found in the Conoidea, a higher Caenogastropoda (neogastropod)
- **Scutibranchia** Term put forward by Golikov and Starobogatov (1975) based on paired gill morphology that includes many of the Vetigastropoda, not in current use. See also Cyclobranchia and Pectinibranchia.

- **Stenoglossa** Taxon put forward by Bouvier (1887) was an earlier name for the Neogastropoda. (Harasewych *et al.* 1997)
- **Streptoneura** Older term put forward by Spengel (1881). The subclass contained everything that was not a Euthyneuran (classically all of the Prosobranchia) (Bieler, 1992). This term is generally not used since the breakup of the Archaeogastropoda.
- **Synapomorphy** A character upon which a monophyly is based. Organisms within a monophyly that share a unique derived characteristic are said to share a synapomorphy.
- **Tarsos musculature** The fine musculature of the gastropod foot responsible for fine movements. These muscles originate on and interdigitate with the columellar muscle and insert on the foot wall. One can differentiate between this musculature and the columellar muscle by its less dense nature. The term "tarsos" was put forward by Voltzow (1985).
- **Toxoglossa** Put forward by Troschel (1847). They are now considered as the superfamily Conoidea. Together with the Rachiglossa for the Stenoglossa. They differ in radula morphology. (Harasewych *et al.* 1997) They are now considered subclades of the Neogastropoda.
- **Vetigastropoda** A recent subclass that includes organisms such as *Haliotis*. This group was once paired with Patellogastropoda in the taxon Archaeogastropoda.

APPENDIX IV: Gastropod lists and numbers

IV.1 Percentages and number of species with respect to various higher taxa

The following is a breakdown of number of species belonging within major subclass taxa as percentages. These numbers include an exhaustive list of all North American species and were adapted from Turgeon *et al.* (1988).

Polyplacophorans	121	2.85%
Gastropods	4126	97.15%
Total	**4247**	**100.00%**

Prosobranchs	2234	54.14%
Opisthobranchs	786	19.05%
Pulmonates	1106	26.81%
Total	**4126**	**100.00%**

prosobranchs	Archaeogastropoda	290	12.98%
	Mesogastropoda	1119	50.09%
	Neogastropoda	825	36.93%
	Total	**2234**	**100.00%**
opisthobranchs	Pyramidelloidea	298	37.91%
	Cephalaspidae	110	13.99%
	Nudibranchia	243	30.92%
	Others	135	17.18%
	Total	**786**	**100.00%**
pulmonates	Archaeopulmonata	18	1.63%
	Basommatophora	171	15.46%
	Stylommatophora	906	81.92%
	Systellommatophora	11	0.99%
	Total	**1106**	**100.00%**

IV.2 Familial list and numbers of North American species

Complete current classification scheme for the classes Polyplacophora and Gastropoda As put forward by Zoological Records, 1998 and Turgeon *et al.* (1988) (The American Fisheries Society)

Subclass	Order	Family	# Species	Species of Specimen
Class Polyplacophora				
	Neoloricata		121	
		Lepidopleura	5	
		Hanleyidae	1	
		Ischnochitonidae	67	
				Lepidozona mertensii
		Chaetopleuridae	6	
		Mopaliidae	27	
				Mopalia muscosa
		Katharinidae	1	
		Chitonidae	4	
		Acanthochitonidae	10	*Acanthopleura japonica*
Class Gastropoda				
Prosobranchia			2234	
	Archaeogastropoda		290	
		Pleurotomariidae	3	*Etemnotrochus adansonianus*
				Etemnotrochus rumphii
				Perotrochus quoyanus
				Perotrochus lucaya
				Perotrochus maureri
				Perotrochus midas
				Perotrochus teremachii
		Scissurellidae	7	
		Haliotididae	9	*Haliotis rufescens*
		Fissurellidae	62	*Diodora cayenensis*
				Diodora aspera
		Acmaeidae	31	*Acmaea mitra*
				Cellana nigrolineata
		Lepetidae	5	*Notocrater houbricki*
		Cocculinidae	4	*Cocculina messingi*
		Addisoniidae	2	
		Trochidae	94	*Astraea caelata*
				Calliostoma canaliculatum
				Cittarium pica
				Tegula brunnea
				Tegula funebralis
		Seguenziidae	3	
		Cyclostrematidae	18	
		Skeneidae	4	
		Turbinidae	24	*Turbo castanea*
		Phasianellidae	9	
		Neritidae	9	*Nerita versicolor*
				Meritina reclivata
		Phenacolepadidae	1	
		Helicinidae	5	
	Mesogastropoda		1119	
		Valvatidae	11	
		Viviparidae	27	
		Pilidae	3	*Marisa cornuarietis*
				Pomacea bridgesi
		Bithyniidae	1	
		Hydrobiidae	170	
		Pomatiopsidae	6	

Subclass	Order	Family	# Species	Species of Specimen
		Thiaridae	3	
		Pleuroceridae	145	
		Annulariidae	1	
		Lacunidae	12	
		Littorinidae	19	
		Rissoidae	68	
		Barleeiidae	15	
		Assimineidae	3	
		Falsicingulidae	1	
		Pelycidiidae	1	
		Elachisinidae	1	
		Truncatellidae	7	
		Rissoellidae	2	
		Skeneopsidae	2	
		Omalogyridae	1	
		Vitrinellidae	68	
		Tornidae	3	
		Caecidae	28	
		Turritellidae	17	
		Siliquariidae	1	
		Vermetidae	10	
		Planaxidae	2	
		Modulidae	1	
		Potamididae	6	
		Cerithiidae	40	*Cerithium atratum*
		Cerithiopsidae	41	
		Mathildidae	3	
		Architectonicidae	12	
		Triphoridae	21	
		Janthinidae	5	
		Epitoniidae	63	
		Aclididae	13	
		Eulimidae	49	
		Entoconchidae	3	
		Aporrhaididae	1	
		Strombidae	6	
		Hipponicidae	4	
		Fossaridae	8	
		Vanikoroidae	1	
		Capulidae	3	
		Trichotropidae	11	
		Calyptraeidae	23	
		Xenophoridae	3	*Xenophora exutum*
		Lamellariidae	25	
		Triviidae	14	
		Cypraeidae	6	
		Ovulidae	28	
		Atlantidae	9	
		Carinariidae	5	
		Pterotracheidae	4	
		Naticidae	40	*Polinices lewisii*
		Cassidae	8	
		Ranellidae	20	

Subclass	Order	Family	# Species	Species of Specimen
		Bursidae	7	
		Tonnidae	4	*Fusitriton oregonense*
		Oocorythidae	2	
		Ficidae	2	
	Neogastropoda		825	
		Muridicae	111	*Nucella lamellosa*
				Nucella ostrina
				Ceratostoma foliatum
		Coralliophilidae	8	
		Columbellidae	59	
		Buccinidae	154	*Searlesia dira*
		Colubrariidae	2	
		Melongenidae	9	
		Nassariidae	18	
		Fasciolariidae	29	
		Olividae	24	*Oliva sayana*
		Harpidae	3	
		Mitridae	7	
		Costellariidae	16	
		Volutomitridae	2	
		Turbinellidae	5	
		Volutidae	5	
		Marginellidae	38	
		Cancellariidae	19	
		Conidae	26	*Hastula cinerea*
		Terebridae	22	
		Turridae	268	
Opisthobranchia			786	
	Pyramidelloida		298	
		Pyramidellidae	298	*Fargoa bushiana*
	Cephalaspidae		110	
		Acteonidae	10	
		Bullinidae	1	
		Hydatinidae	2	
		Ringiculidae	2	
		Scaphandridae	19	
		Cylichnidae	9	
		Aglajidae	7	
		Philinidae	13	
		Gastropteridae	4	
		Diaphanidae	5	
		Runcinidae	1	
		Bullidae	5	
		Atyidae	13	*Haminoea antillarum*
		Retusidae	19	
	Acochlidioidea		1	
		Microhedylidae	1	
	Thecosomata		35	
		Limacinidae	6	
		Cavoliniidae	17	
		Peraclididae	4	
		Cymbuliidae	6	
		Desmopteridae	2	

Subclass	Order	Family	# Species	Species of Specimen
	Gymnosomata		13	
		Clionidae	3	
		Cliopsidae	1	
		Hydromylidae	1	
		Notobranchaeidae	2	
		Pneumodermatidae	5	
		Thilptodontidae	1	
	Anaspidae		24	
		Akeridae	1	
		Aplysiidae	23	*Aplysia dactylomela*
	Sacoglossa		45	
		Boselliidae	3	
		Caliphyllidae	5	
		Costasiellidae	1	
		Cylindrobullidae	2	
		Elysiidae	13	
		Hermaeidae	6	
		Juliidae	1	
		Lobigeridae	1	
		Oxynoidae	2	
		Stiligeridae	11	
	Notaspidae		17	
		Tylodinidae	3	
		Umbraculidae	1	
		Pleurobranchidae	9	
		Pleurobranchaeidae	4	
	Nudibranchia		243	
		Corambidae	4	
		Goniodorididae	17	
		Onchidorididae	21	
		Triophidae	4	
		Heterodorididae	1	
		Aegiretidae	1	
		Gymnodorididae	1	
		Polyceridae	17	
		Cadlinidae	10	
		Chromodorididae	10	
		Asteronotidae	2	
		Actinocyclidae	1	
		Conualeviidae	1	
		Calycidorididae	1	
		Aldisidae	3	
		Rostangidae	1	
		Doridiidae	3	
		Dendrodorididae	7	
		Phyllidiidae	1	
		Archidorididae	4	
		Discodorididae	17	*Anisodoris nobilis*
				Diaulula sandiegensis
		Kentrodorididae	1	
		Platydorididae	2	
		Tritoniidae	7	
		Hancockiidae	1	

Subclass	Order	Family	# Species	Species of Specimen
		Dendronotidae	9	
		Tethyidae	1	
		Lomanotidae	1	
		Scyllaeidae	1	
		Phylliroidae	2	
		Dotoidae	11	
		Arminidae	2	
		Dironidae	3	
		Janolidae	1	
		Coryphellidae	13	
		Eubranchidae	9	
		Cumanotidae	1	
		Tergipedidae	25	
		Fionidae	1	
		Babkinidae	1	
		Facelinidae	14	
		Aeolidiidae	4	
		Spurillidae	5	
		Claucidae	1	
Pulmonata			1106	
	Archaeopulmonata		18	
		Melampodidae	18	
	Basommatophora		171	
		Acroloxidae	1	
		Lymnaeidae	57	*Lymnaea stagnalis*
		Physidae	38	*Physa heterostropha*
		Planorbidae	45	*Helisoma trivolvis*
		Ancylidae	13	
		Carychiidae	9	
		Siphonariidae	6	
		Trimusculidae	2	
	Stylommatophora		906	
		Cochlicopidae	4	
		Pupillidae	82	
		Valloniidae	11	
		Strobilopsidae	5	
		Ceriidae	6	
		Ferussaciidae	2	
		Subulinidae	8	
		Spiraxidae	5	
		Achatinidae	1	
		Streptaxidae	1	
		Haplotrematidae	14	
		Urocoptidae	25	
		Bulimulidae	13	
		Punctidae	8	
		Charopidae	2	
		Helicodiscidae	24	
		Discidae	23	
		Arionidae	35	
		Philomycidae	18	
		Succineidae	43	
		Helicarionidae	9	

Subclass	Order	Family	# Species	Species of Specimen
		Zonitidae	99	
		Vitrinidae	2	
		Limacidae	11	*Limax maximus*
		Milacidae	1	
		Testacellidae	1	
		Polygyridae	205	
		Sagdidae	2	
		Thysanophoridae	4	
		Camaenidae	2	
		Ammonitellidae	8	
		Oreohelicidae	45	
		Bradybaenidae	1	
		Helminthoglyptidae	171	
		Helicellidae	6	
		Helicidae	9	
	Systellommatophora		11	
		Onchidiidae	3	*Onchidella celtica*
		Veronicellidae	8	

APPENDIX V: Software

V.1 A description of the supplemental software accompanying this thesis

The supplemental software accompanying this thesis, entitled SnailView: A Three-Dimensional Gastropod Atlas is a multiplatform and multiviewer Java (trademark of the Sun Computer Corporation) applet. It was authored by my brother, Mr. Keith Uyeno of Critical Mass in Calgary, Alberta for the purpose of easily viewing the orthogonal slices developed by Dr. Bradley Smith at the University of Michegan at Ann Arbor. The raw data for the orthogonal slices were provided by the Duke University's Center for *In Vivo* Microscopy with the assistance of Dr. Bradley Smith, Mr. Gary Cofer and Dr. Al Johnson. The images were adapted for SnailView with the help of Dr. Doug Phillips.

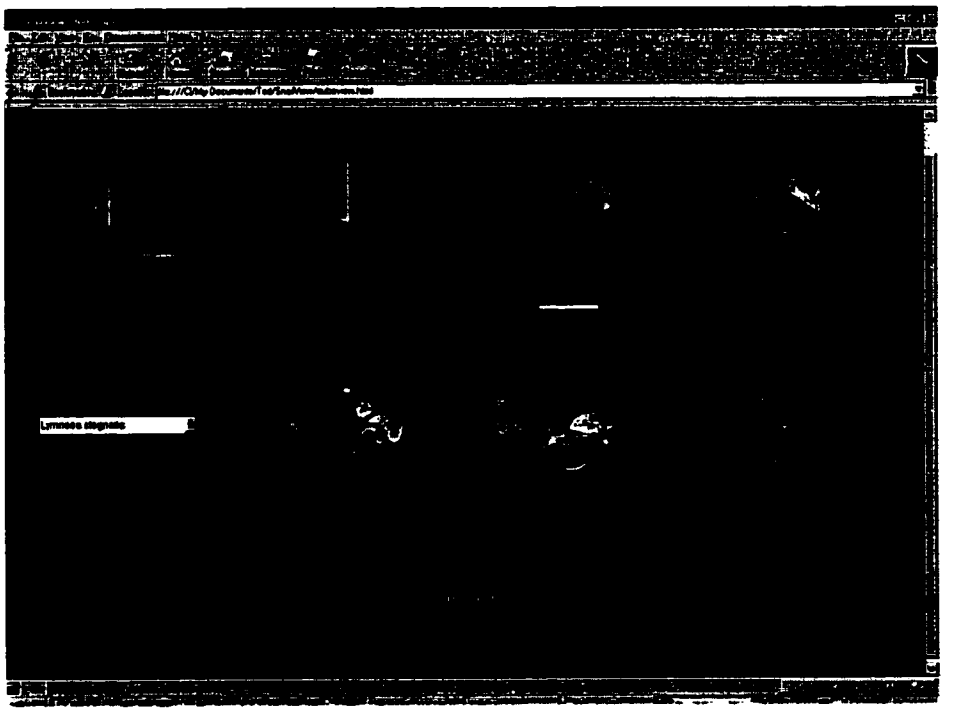

Figure 47. Screenshot of SnailView, an interactive slice manipulator.

The program offers a three-quarter position whole animal image enclosed within a cube. This is the interactive slice manipulator. Clicking on one of its three exposed faces causes an orthogonal plane to be chosen. Notice the three whole animal pictures to the right. The leftmost is an image of the front view. The center is an image of the snail's side aspect and the right most is a top view. When the corresponding cube face is clicked, a line appears over the snail image and the corresponding slice is displayed directly below. The front view displays the bar indicating which sagittal slice is being selected. The side view shows the corresponding frontal section and the top view shows the corresponding transverse section. In this way, all the sections describing each of the X, Y and Z axis are intuitively displayed.

Finally, the snails are ordered in alphabetical order with respect to genus name. You can select which gastropod you wish to view by clicking your mouse over the genus name box located directly below the slice manipulator cube. This will cause the rest of the pull down menu to appear and allow a different gastropod to be selected.

V.2 Installation and procedures to run

The accompanying compact disc contains the images and the Java program. Testing indicates that the following hardware and software are preferred for the optimal presentation:

Operating system: MS Windows 9X/NT (Intel Pentium II 300 or better preferred), UNIX (Including Linux), Macintosh OS 7.5+.

Hard Drive space: 50 Mbytes

Memory: 32 Mbytes, more preferred

Display resolution: 1024 by 768 pixels, 1152 by 864 pixels preferred

Colour resolution: High colour (16 bit), True colour (32 bit) preferred

Internet Browser: A Java enabled browser: MS Internet Explorer 4.0 or better, Netscape Communicator 4 or better.

To run this program, create a directory on your hard drive named SnailView. Transfer all the directories and files on the CD to this SnailView directory. Next, open one of the above mentioned Java enabled browsers and open the file X:/SnailView/cubeview.html. (X:/ being the name of the drive to which you copied all the information on the CD.) Please wait while the browser load the applet and the images (This may take a while since whole image sets for each snail is being loaded directly to memory. Depending on your browser, a status bar may show the loading progress). If you are using Windows 98 and find that your monitor is too small and have the accesibility options installed, you may elect to use the magnifier tool, which can be found under Start/Programs/Accessories/Accessibility/Magnifier. If you wish to install this option, choose Start/Settings/Control Panel and click on Add/Remove Programs and simply install it under the Windows Setup tab.

V.3 About the CubeView author

Mr.Dave Rose and I conceived the CubeView software while discussing how best to present this massive amount of visual data. Mr. Rose (of the Geological Survey of Canada – Calgary) originally wrote some of the code in the PERL (PerlTK) language, however, the inherent capabilities of Java and Keith Uyeno's expertice in this field made this latter language a better choice. The original idea is closely based on the MacOS software publication of Dr. Brad Smith *et al.* (1995) entitled "Digital Atlas of Mouse Embryology".

Keith Uyeno is a 23 year old database architect with the Calgary Internet company Critical Mass. He graduated from the University of Calgary with a degree in Computer Sciences in early 1999. SnailView represents the successful completion of his first Java program.

APPENDIX VI: Biographical information

VI.1 Biography

Theodore (Ted) Akira Uyeno was born (July 25th, 1974) and raised in Calgary, Alberta, Canada. He received a B.Sc. in Zoology from the University of Calgary in June of 1997. During his research for his M.Sc. degree, he received a Tuition fees scholarship as well as a University of Calgary Teaching Assistanceship, a Research Assistanceship, A Graduate Students Association Academic Project grant and a Department of Graduate Studies Travel grant. He is also grateful to the University of Calgary Learning Commons as well as the Department of Biological Sciences for considering his research as a useful teaching tool in providing a curriculum enhancement research grant to offset the MRM data collection costs. The Duke University's Center for In Vivo Microscopy is also thanked for their generous contribution in providing expertise and microscope time at no cost to me.

Ted Uyeno is a member of the Society for Integrative and Comparative Biology (formerly the American Society of Zoologists) in the sections of Invertebrate Zoology and Evolution and Systematics. Ted is also a member of the American Microscopical Society.

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