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Tardigrade evolution and ecology

Phillip Brent Nichols
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Tardigrade Evolution And Ecology

by

Phillip Brent Nichols

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
Department of Biology
College of Arts and Sciences
University of South Florida

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July 25, 2005

Keywords: ecdysozoa, meiofauna, phylogeny, 18s rna, morphology

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Dedication

For MaMa…
Acknowledgements

There are so many people that I wish to thank and acknowledge for their love, support, and help over the past few years. First of all to the most important person in my life, my wife Tanya, you are my light and my inspiration, thank you for your unwavering love and support. My major advisor, Dr. James R. Garey, has been a wonderful mentor who has helped me see the “Big Picture” on more than one occasion. I will always value both the professional and personal friendship that we have. I would like to thank the members of my Graduate Supervisory Committee, Dr. Richard P. Wunderlin, Dr. Florence M. Thomas, and Dr. Frank A. Romano, III for their participation in my graduate education and training and a special thank you to Dr. Rick Oches for acting as my defense chair. The members of the Garey Lab; Terry Campbell, Hattie Wetherington, Kim Fearne, John Slomba, Mike Robeson, Heather Hamilton, and Stefie Depovic; have been involved with many aspects of this dissertation from collecting field samples to problem solving data collection. Other collaborators that have been influential in the completion of my graduate training include: Dr. Diane Nelson, East Tennessee State University; Dr. Ruth Dewel, Appalachian State University; Dr. Roberto Guidetti, University of Modena, Modena, Italy; Dr. Reinhardt Kristensen, University of Copenhagen, Denmark; Dr. Sandra McInnes, Cambridge University; Mr. Nigel Marley, University of Plymouth; Mr. Ken Hayes, University of Hawaii. I would like to extend my appreciation to the University of South Florida Graduate School and Biology Department not only for financial support throughout my training but for all the “little things” that often go unnoticed. Lastly, I would like to thank my parents for providing me with the opportunities to pursue my own interests.
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1. Arthropoda + Tardigrada: supraesophageal or preoral position of the frontal appendages and their neuromeres (DEWEL & DEWEL 1997).
2. Arthropoda: body with articulated exoskeleton; protocerebrum with compound eyes (NIELSEN 1995).
3. Tardigrada: connective between protocerebrum and ganglion of first pair of legs (DEWEL & DEWEL 1997).
4. Heterotardigrada: Cephalic appendages, legs with digits and/or claws (BARNES & HARRISON 1993).
5. Eutardigrada: Lack of cephalic appendages; legs with claws but not digits (BARNES and HARRISON 1993).
7. Parachela: Without cephalic papillae and with double claws in which primary and secondary branches are joined (SCHUSTER et al. 1980).
8. Macrobiotus: Claw branches with sequence: secondary, primary, primary, secondary; buccal tube with ventral lamina and 10 peribuccal lamellae (SCHUSTER et al. 1980).

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ABSTRACT

A character data set suitable for cladistic analysis of tardigrades at the family level was developed. The data matrix consisted of 50 morphological characters from 15 families of tardigrades and was analyzed by maximum parsimony. Kinorhynchs, loriciferans and gastrotrichs were used as outgroups. The results agree with the currently accepted hypothesis that Eutardigrada and Heterotardigrada are distinct monophyletic groups. Among the eutardigrades, Eoyhypsibiidae was found to be a sister group to Macrobiotidae + Hypsibiidae, while Milnesiidae was the basal eutardigrade family. The basal heterotardigrade family was found to be Oreellidae. Echiniscoideans grouped with some traditional Arthrotardigrada (Renaudarctidae, Coronarctidae + Batillipedidae) suggesting that the arthrotardigrades are not monophyletic. An 18S rRNA phylogenetic hypothesis was developed and supports the monophyly of Heterotardigrada and of Parachela versus Apochela within the Eutardigrada. Mapping of habitat preference suggest that terrestrial tardigrades are the ancestral state. Molecular analysis of a sediment sample with an unusually large population of tardigrades had a higher diversity when compared to manual sorting and counting.
Chapter One

Introduction

Tardigrades were first described by Goeze in 1773 and since then, his "Kleiner Wasser Bärs" have been commonly referred to as “water-bears” because of their bear-like appearance. Spallanzani (1776) termed his similar organism "Il Tardigrado" (slow stepper) recognizing the unique lumbering gate exhibited by tardigrades. Tardigrades are found throughout the world in a variety of freshwater, marine, and terrestrial habitats and are considered cosmopolitan in their distribution. These bilaterally symmetric, hydrophilous micrometazoans are ventrally flattened and dorsally convex. A typical adult tardigrade (Figure 1) is 250-500 micrometers in length and displays limited metamerism with five indistinct segments.

A cephalic segment that is bluntly rounded contains a mouth and may have eyespots and sensory cirri. Four body segments are present, each has a pair of ventrolateral legs terminating in claws or suction discs (Ramazzotti and Maucci, 1983); generally the first three pairs are used for locomotion, the fourth for substrate attachment. Tardigrades have an outer cuticle that may be opaque, white, or
such colors as brown, green, pink, red, orange, or yellow, covering them (Dewel et al., 1993). This color results from pigments in the cuticle, dissolved materials in the body fluids, or from the contents of the digestive tract.

Terrestrial tardigrades mainly feed on algae, cryptogams (mosses, lichens, and liverworts) or animals (rotifers, nematodes, and other small invertebrates). Marine tardigrades are believed to feed primarily on bacteria (R.M. Kristensen, personal communication). Feeding is usually accomplished by piercing the cells with a pair of stylets and “sucking” out their contents, but in some cases whole organisms are ingested. The typical digestive system is comprised of a foregut, midgut, and hindgut. The foregut includes a mouth, buccal tube, salivary glands, stylets, a sucking pharynx (with or without placoids), and an esophagus. The stylets are extended to pierce the cells, and the pharynx pumps the cytoplasmic fluids into the esophagus. The salivary glands are believed to secrete new stylets during ecdysis (Walz, 1982; Ramazzotti and Maucci, 1983).

Food is digested in the midgut and the excretory glands (Malpighian tubules) empty into the junction of the midgut and hindgut. The hindgut can either have a true cloaca (Eutardigrada) or an anus with a separate, preanal gonopore (Heterotardigrada).

Doyere proposed that all tardigrades were hermaphroditic based on what he identified as structures that were two testes and a seminal receptacle (Bertolani, 1979, Nelson, 1982). This belief remained until two structures were identified as malpighian tubules and tardigrades were then considered to be gonochoristic (Bertolani, 1992; Nelson 1982). However, Bertolani (1979) and Bertolani and Manicardi (1986) reported that hermaphroditism exists in some tardigrades.
The major modes of reproduction in tardigrades are amphimixis and parthenogenesis (Ramazzotti and Mauci, 1983; Nelson, 1982; Bertolani, 1992; Kinchin, 1994; Bertolani and Rebecchi, 1998). The females lay eggs outside the body or within the exuvium as they molt. The males then release spermatozoa into the exuvium where external fertilization occurs or into the gonopore or cloaca where they will travel up the oviduct for internal fertilization (Pollock, 1975).

Sexual reproduction in gonochoristic tardigrades (amphimixis) can take place between a female and a single male or several males with the males clinging to the anterior part of the female with their front legs (Kinchin, 1994). There have been limited investigations into the mating behavior of tardigrades. Instead, mating habits have been inferred from anatomical studies (Bertolani, 1992). Males and females are quite similar but can be distinguished from one another by comparing the gonad and the gonoducts. Females have one ovoduct whereas males have 2 vas deferens. Fertilization for terrestrial species usually occurs inside the female’s body while in marine species fertilization is external (Bertolani, 1990).

Parthenogenesis is the development of an egg where there has been no paternal contribution of genes (Futuyma, 1986). This is the common mode of reproduction in unisexual tardigrades found in non-marine habitats (leaf litter, mosses, and freshwater) (Nelson, 1982). Unisexual females are wide spread in tardigrades but recombination may be limited as they will have fewer possibilities for procreation. Parthenogenesis, while limiting genetic variability, may in fact facilitate proliferation in unisexual females (Bertolani, 1987). Polyploidy, possessing more than one entire chromosomal compliment has often been associated with parthenogenesis and several species are known to have polyploid populations (Bertolani, 1982; Nelson, 1982). Also, cytotypes,
or populations with the same morphological characteristics but differing degrees of ploidy (diploidy, triploidy, and tetraploidy), have been observed from samples collected relatively close to one another (Rebecchi and Bertolani, 1988).

The nervous system is composed of a brain with two dorsolateral lobes connected by two circumpharyngeal commissures to a subpharyngeal ganglion, a pair of longitudinal nerve cords, and four ventral ganglia that are united by those nerve strands (Dewel and Dewel, 1996).

The most fascinating feature of some tardigrades is their capacity to enter into a state of suspended animation (cryptobiosis). The water content of the body is reduced from 85% to just 3% and the body becomes barrel-shaped forming a tun. In this state, growth, reproduction, and metabolism are reduced or cease temporarily and resistance to environmental extremes is evident. This resistance allows the tardigrade to survive through cold and dry spells, ionizing radiation, heat, and pollution. Crowe (1975) identified five different types of cryptobiosis (Table 1).

<table>
<thead>
<tr>
<th>MODE</th>
<th>Description</th>
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<tbody>
<tr>
<td>Encystment</td>
<td>Most common among aquatic and soil tardigrades, they encase themselves by molting and remaining inside the old cuticle.</td>
</tr>
<tr>
<td>Anoxybiosis</td>
<td>Induced by low oxygen environments</td>
</tr>
<tr>
<td>Cryobiosis</td>
<td>Induced by low temperatures</td>
</tr>
<tr>
<td>Osmobiosis</td>
<td>Induced by elevated osmotic stress</td>
</tr>
<tr>
<td>Anhydrobiosis</td>
<td>Induced by the removal of water from the tardigrade and its environment through evaporation.</td>
</tr>
</tbody>
</table>
Systematic Research

In 1928 and 1929 Marcus compiled the first reviews of tardigrade morphology, physiology, embryology, and phylogenetic relationships; in 1936, he developed the first basis for classification of the group (Kinchin, 1994). Ramazzotti's first monograph in 1962, a revision in 1972, and a subsequent revision by Ramazzotti and Maucci in 1983 included descriptions of 514 species in three classes, Heterotardigrada, Eutardigrada, and Mesotardigrada. Mesotardigrada was based on a single description of *Thermozodium esakii* from a hot spring near Nagasaki Japan. Type specimens were not preserved and the hot spring where they were found was destroyed in an earthquake (Ramazzotti and Maucci, 1983). Since the publication of the 1983 monograph, the number of described species has increased to over 900.

Modern taxonomy is based on these and a number of other European studies. The history of tardigrade studies is well documented by Ramazotti and Maucci (1983) and Kinchin (1994). Increased interest in tardigrade biology and systematics over the last 25 years is evidenced by numerous publications and 9 international symposia.

Tardigrade systematics has historically been based on a number of morphological characteristics. The current taxonomy of clades within Tardigrada is based on

![Figure 2. Typical tardigrade claws. A. Macrobiotus sp.; B. Milnesium sp.; C. Echiniscus sp.; D. Batillipes sp. Redrawn from Ramazzotti and Maucci (1983).](image-url)
morphological characters that include: claw size, shape, organization, and number (Figure 2); organization of the bucco-pharyngeal apparatus; length of stylets; size, shape, and number of placoids; cuticular patterns and ornamentation; and morphology of eggs. The presence or absence of the Lateral Cirrus A is used to separate the two major classes, Heterotardigrada and Eutardigrada (Figure 3).

The Heterotardigrada are characterized by 4 single claws per leg that may have spurs at the base of the exterior or interior claws. They often have sensory papilla or a spine and/or a dentate collar on the 4th pair of legs. The Heterotardigrada are “armored” and have a thick cuticle, dividing into plates, with species specific pore patterns. Two double claws per leg, which may or may not be similar in size and shape, characterize the Eutardigrada. Each double claw consists of a primary and secondary branch and a base. The sequence of branching and other claw characteristics are taxonomically significant.

Figure 3. Representative head structures of eutardigrades (left) and heterotardigrades (right). A. Cephalic papillae; B. Peribuccal papillae; C. Eye spots; D. Internal cirrus; E. Cephalic papilla; F. External cirrus; G. Lateral Cirrus A; and H. Clava. Redrawn from Ramazzotti and Maucci (1983).
The eutardigrades have a thin cuticle that is not divided into plates, but may be ornamented with spines, reticulations, granules, or pores.

The tardigrade buccal apparatus consists of a pair of piercing stylets, a buccal tube, and a sucking pharynx. Peribuccal papillae, papulae or lobes (Schuster et al., 1980) may be present around the mouth in eutardigrades. The opening of the mouth is followed by the buccal tube which is supported by a ventral lamina in some species. The stylets are found lateral to the buccal tube (Figure 4) and can be extended into the tube and out the mouth by protractor and retractor muscles attached to the stylet supports. The buccal tube connects to the muscular pharyngeal bulb which in most tardigrades is lined with three rows of cuticular thickenings called placoids (macroplacoids and microplacoids). The placoids are posterior to the end of the buccal tube.

![Figure 4. Typical buccal apparatus found in tardigrades. A. Stylet; B. Buccal tube; C. Stylet support; D. Macroplacoid; E. Microplacoid; F. Pharyngeal bulb Redrawn from Ramazzotti and Maucci (1983).](image)
Usually gonochoristic, tardigrades have a single unpaired gonad but in many cases the mode of reproduction is parthenogenic. However, during sexual reproduction the male will deposit spermatozoa into the cloacal opening of the old cuticle while the female is simultaneously molting and laying eggs in the cuticle. Once shed the cuticle is referred to as an exuvium and fertilization takes place inside. Some eutardigrade eggs are freely laid and often ornamental. Both the morphology of the eggs and the spermatozoa is important in the eutardigrades (Bertolani and Rebecchi, 1996; Guidetti and Rebecchi 1996).

The phylogenetic position of Tardigrada has often been debated (for reviews, see Ramazzotti & Maucci, 1983; Kinchin, 1994), and morphology has primarily been used to assess the relationships among some genera within a few families of tardigrades (Renaud-Mornant, 1982; Kristensen, 1987; Pilato, 1989; Pollock, 1995; Bertolani & Biserov, 1996; Jorgensen, 2000; Guidetti & Bertolani, 2001). Tardigrades have been placed along an annelid-arthropod lineage, often closely associated with onychophorans, although there have been some arguments for placing them with a group of pseudocoelomate phyla known as aschelminthes that has since been shown to be polyphyletic (Dewel & Clark, 1973a, 1973b; Kristensen, 1991; Winnepennix et al., 1995). Current evidence suggests, however, that tardigrades, onychophorans, and arthropods form a monophyletic clade known as Panarthropoda (reviewed in Schmidt-Rhaesa et al., 1998).

Garey et al. (1999) found close agreement between molecular and morphology based phylogenies that included five species of tardigrades, suggesting that the characters for the current morphological taxonomy are appropriate. Although tardigrade genera, families, orders, and classes (Table 2) are each well defined by morphological characters,
the relationships among the families and among genera within the families have not been systematically evaluated. One exception is the family Echiniscidae for which several cladistic studies have been published (Kristensen, 1987; Jorgensen, 2000).

**Dispersal**

Tardigrades are dispersed throughout three main environments: terrestrial, freshwater, and marine. Their distributions in each of the habitats may be correlated with physical environmental factors.

The role of moisture, site orientation and altitude on the distribution of terrestrial tardigrade species has been widely investigated. Oxygen availability is the limiting factor in tardigrade distributions in all environments. It is because of oxygen availability that terrestrial tardigrades do not inhabit dense growing thick mosses and the reason that they are found in the top few centimeters of soil around the roots of trees, the soil must also have a degree of porosity to facilitate movement (Ramazzotti and Maucci, 1983). Those in mosses and lichens, which have been categorized into types based on their

<table>
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<th>Class</th>
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<td>Echiniscoidea</td>
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<td>Oreellidae</td>
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*(from Nelson, 2002)*

Table 2*. Higher level Taxonomy of the Tardigrada
moisture content, need alternating periods of wet and dry. The most studied aspect of physical environmental factors on terrestrial tardigrades is that of altitude. Many authors have reported that altitude has a definite effect on tardigrade distribution (Rodriguez-Roda, 1951; Nelson, 1973, 1975; Ramazzotti and Maucci, 1983; Dastych, 1985, 1987, 1988; Beasley, 1988), with most suggesting that species richness increases as altitude increases. Some authors (Ramazzotti and Maucci, 1983; Dastych, 1987, 1988) have even classified tardigrades based on the locality (lowland, upland, montane, etc.). However, others have reported that distributional patterns were not influenced by altitude (Kathman and Cross, 1991).

Very little information exists as to the mode of dispersal of freshwater and marine tardigrades since very few undergo cryptobiosis. They are most likely distributed by way of rapid moving waters during periods of flooding, or by storm surge altering the coastal currents. Tardigrade zonation in littoral habitats, like that of terrestrial species, is limited by the availability of oxygen. Size of the sand grain and circulation of the water may also affect zonation patterns. Both will have an effect on oxygen availability which could account for the distribution of the tardigrades in the first few centimeters of the sand (Pollock 1975). Light availability, salinity, and temperature have been shown to affect species distributions in the marine environment (Pollock 1975).

**Ecological Importance**

Meiofauna are important in many terrestrial and aquatic ecosystems (Wilson, 1992; Palmer *et al.*., 1997). Aquatic communities consist of many diverse and abundant species of benthic invertebrates (including tardigrades). A typical food web often
consists of nematodes, tardigrades, bacteria, algae, rotifers, protozoa, mites, and collembolans (Kinchin 1987). One possible role of terrestrial tardigrades is in the colonizing of new habitats. Tardigrades may play an important role with the ability to move into a newly formed habitat quickly, establishing as a pioneer species and then in turn attracting other meiofaunal groups to the habitat. The habitat then becomes suitable to colonization by macrofaunal species.

Because of the size of tardigrades, limited studies on the ecological role of marine and freshwater species have been performed and it is difficult to assess their importance in ecological functioning. Typically, they are a major player in the meiofaunal assemblages second only to that of nematodes and rotifers. They may play a role in converting plant and animal matter into food for larger organisms and could aid in maintaining aeration and nutrient cycling in the sediments. They may even form distinct functional groups that exists only in a few areas and if lost could be a detriment to the ecosystem.

There are a number of studies of community structure of terrestrial tardigrades, but few (Gaugler, 2003, unpublished thesis) focused on community structure of marine species. Currently there are no models that can accurately measure their role in ecosystem functioning therefore, it would be impossible to assess their importance.

**Significance**

There currently is no phylogenetic hypothesis of tardigrade evolution that treats the group as a whole. Instead, the tardigrade literature consists of a plethora of species descriptions with very few systematic studies. A molecular framework for tardigrade
phylogeny would be very useful and would encourage a wider application of morphological and molecular studies to tardigrade phylogeny. For example, a recent molecular study of nematode phylogeny (Blaxter et al., 1998) was pivotal in encouraging new discussion and a reassessment of nematode phylogeny, particularly in light of the completion of the *Caenorhabditis elegans* genome project. Tardigrada, as a member of Panarthropoda will likely play an important role in the future assessment and discussion of two competing theories of protostome evolution, that of Ecdysozoa (Aguinaldo et al., 1997) and Articulata (Cuvier, 1863) in the same way as onychophorans (de Rosa et al., 1999). Tardigrades, like onychophorans (e.g. Panganiban, et al. 1997, Grenier and Carroll, 2000) will likely become more important in discussions of the evolution of body plans and appendages as well. It has been noted that species or even familial identification of tardigrades can be difficult for untrained investigators. For example, specimens of *Macrobiotus* species have been sold commercially as *Milnesium tardigradum* (Garey et al., 1999). As tardigrades are used more extensively as model organisms for molecular and developmental studies, it becomes very important that specimens are properly identified.
Objectives

1. The present study was designed to provide an overall framework for tardigrade evolution utilizing morphology and molecular based (18S rRNA) phylogenetic hypotheses for tardigrades. Potential morphological characters that are informative at the family level were assembled and a morphology based phylogeny was determined. A data set of 18S rRNA gene sequences was assembled and a molecular based phylogeny was developed. These data were used to investigate the phylogenetic relationships among the tardigrade families and to test the relationships among the families within each tardigrade order to determine if the families are truly monophyletic. Finally, it has been suggested that tardigrades evolved in a marine environment (May 1953) and that some echiniscids adapted to freshwater and then to a terrestrial habitat. The question is asked, what was the ancestral state and how many times did the adaptations occur?

2. The purpose of this study was to investigate the structure (frequency, diversity, similarity) of a meiofaunal community that has an unusually abundant tardigrade population using both morphological species identification and molecular species identification. Individuals were grouped to the lowest practical taxon and the abundance, diversity and similarity between the “morphological community” and “molecular community” was determined.
Chapter 2
Specimen Acquisition and Processing

Specimens were obtained from a network of tardigrade researchers and from field collections. Marine specimens were collected and rinsed with fresh water. The freshwater rinse puts the tardigrades into osmotic shock and they release their grasp on the sand grains. These samples were then rinsed through a set of nested sieves, which have mesh openings from 1600mm to 40mm, with seawater to both collect the individual and stop the osmotic shock (Higgins and Thiel 1988). The specimens were drained into a screw-top jar and preserved by adding 100% ethanol or stored at –80°C for DNA extraction. Dried samples of cryptogams were soaked in tap water in a stoppered funnel for 12-24 hours. The cryptogams were then agitated, removed, and squeezed to drain the remaining water into the funnel. Samples were processed by sieving through nested sieves. The specimens were then sorted, stored in screw-top jars, and preserved. The cryptogams were re-dried and stored in previously labeled paper bags. The preserved samples were placed into a Syracuse dish and searched with a stereoscopic dissecting microscope at a magnification of 7-45X. A pipette was used to extract individual tardigrades to separate glass slides with glass cover slips (18mm circle #1 glass) for identification.
Chapter 3

Morphological Analysis of Tardigrade Phylogeny

Background

Hennig (1979) stated that a classification should express the branching (cladistic) relationships among species, regardless of their degree of similarity or difference. Cladistic analysis is based on common ancestry rather than overall similarity with emphasis placed on character types and the importance of those characters (Forey et al., 1992). For example, a cladistic analysis must determine the polarity of the character states to show which are pleisiomorphic (ancestral) and which are apomorphic (derived).

Cladistic analyses utilize parsimony methods to construct a cladogram. This method implies that the simplest answer is usually correct or in the case of a phylogenetic tree, the shortest tree is usually correct. Thus the advantage of cladistic analysis utilizing polarized character states is that it represents the topology of a phylogenetic tree and can be used to infer evolutionary history of the groups being studied. Cladistic analyses are limited to monophyletic groups, only descendents of a particular ancestor can be included and paraphyletic groups are not allowed. When performed correctly, complications by convergent evolution, parallelism and reversal are avoided. However, differing opinions on character state definitions between researchers and the fact that fossil material may be needed to evaluate a particular missing character can be a disadvantage.
The phylogenetic position of Tardigrada has long been debated (for reviews, see Ramazzotti & Maucci, 1983; Kinchin, 1994), and morphology has been used to assess the relationships among some genera within a few families (Renaud-Mornant, 1982; Kristensen, 1987; Pollock, 1995; Bertolani & Biserov, 1996; Jorgensen, 2000; Guidetti & Bertolani, 2001). Tardigrades have been placed along an annelid-arthropod lineage, often closely associated with the onychophoran-arthropod complex, although there have been some arguments for placing them with the aschelminthes group (Dewel & Clark, 1973a, 1973b; Kristensen, 1991). Current evidence suggests, however, that tardigrades, onychophorans, and arthropods form a monophyletic clade known as Panarthropoda (reviewed in Schmidt-Rhaesa et al., 1998).

The present study was designed to assemble and analyze potential morphological characters that are informative at the family level and to test the relationships among the families within each tardigrade order.

Materials and Methods

Characters

A matrix (Appendix A) consisting of 50 morphological characters (Appendix B) from current literature was scored for 15 tardigrade families (for review, see Schuster et al., 1980; Binda & Kristensen, 1987; Kristensen, 1987; Bertolani & Rebecchi, 1993; Schmidt-Rhaesa et al., 1998; Jorgensen, 2000; Guidetti & Bertolani, 2001). Characters common to most families but with character states that distinguished families from one another were chosen. Three outgroups; two within Ecdysozoa (Loricifer & Kinorhyncha)
and one outside Ecdysozoa (Gastrotricha) were included in the analysis. Ten multi-state characters were present.

Data Analysis

The character data matrix was constructed using Nexus Data Editor (NDE) version 0.5.0 (http://taxonomy.zoology.gla.ac.uk/rod/NDE/nde.html). Phylogenetic Analysis Using Parsimony (PAUP*) version 4.6 (Swofford, 1998) was utilized for maximum parsimony analysis.

Results and Discussion

Morphological Phylogeny

The 50% majority rule tree in Figure 6 was generated from an analysis of the morphological data set and agrees with the consensus that Eutardigrada and Heterotardigrada are distinct monophyletic sister groups as evidenced by a high bootstrap value of 98%. Among the eutardigrades, Eohypsibiidae was a sister group to Macrobiotidae + Hypsibiidae, though the bootstrap support around 60% was minimal. Necopinatidae appears to be basal among the Parachela, which forms a monophyletic sister group to the Apochela and the most basal eutardigrade family, Milnesiidae (Figure 5).

Among the heterotardigrade families, bootstrap values indicate that all branches were moderately or highly supported (Figure 6). Kristensen & Higgins (1984)
peribuccal lamellae. Habitats: Terrestrial (T); Marine (M); or Freshwater (F).

branch. 2. Heterotardigrada: Cephalic appendages, digitate legs with or without claws. 3. Parachela: No cephalic papillae; double claws per leg divided into a secondary and primary branch. 4. Apochela: Six cephalic papillae; two sets of claws per leg, with unbranched primary claw separated from secondary claw; 4 - 6 peribuccal lamellae. Habitats: Terrestrial (T); Marine (M); or Freshwater (F). Maximum parsimony bootstrap values are shown.

Figure 5. Maximum parsimony phylogeny of tardigrade families with mapped habitat preferences. The analysis supports the monophyletic origin of the class Eutardigrada and orders Parachela and Apochela along with the class Heterotardigrada; however, the orders within Heterotardigrada (Arthrotardigrada, Echiniscoidea) may not be monophyletic. Key to Characters: 1. Eutardigrada: No cephalic appendages; lack of dorsal plates; differentiated placoids; double claws with secondary and primary branches. 2. Heterotardigrada: Cephalic appendages, digitate legs with or without claws. 3. Parachela: No cephalic papillae; double claws per leg divided into a secondary and primary branch. 4. Apochela: Six cephalic papillae; two sets of claws per leg, with unbranched primary claw separated from secondary claw; 4 - 6 peribuccal lamellae. Habitats: Terrestrial (T); Marine (M); or Freshwater (F). Maximum parsimony bootstrap values are shown.

established the family Renaudarctidae based on the conclusion that the development of toes and adhesive discs in Halechiniscidae and Batillipedidae were derived characters and that the claw insertion in Stygarctidae was a plesiomorphic state. This assessment agreed with Renaud-Mornant’s (1982) suggestion that Stygarctidae should be the most primitive
heterotardigrade family. The current analysis (Figure 5) suggests instead that Halechiniscidae is more basal than either Stygarctidae or Renaudarctidae and that Oreelidae is the most basal heterotardigrade. This placement supports earlier suggestions by Thulin (1928) and Marcus (1929) that Echiniscidae is derived from arthrotardigrades and that Oreellidae should be considered the most primitive heterotardgrade. The cephalic appendages that define heterotardigrades are present in oreellids, but the dorsal plates, characteristic of other heterotardigrades, are completely absent. The morphology of the various dorsal plates appears to provide important characters in heterotardigrade phylogeny. If the absence of dorsal plates represents a secondary loss within the oreellids, then the basal position of Oreellidae in this study may be an artifact.

The orders Arthrotardigrada and Echiniscoidea have been placed as sister groups based on shared derived characters (Eibye-Jacobsen, 2001). The current results (Figure 5) suggest that these orders are not sister groups, and it appears that Arthrotardigrada is paraphyletic containing some members of Echiniscoidea.

*Habitat Mapping*

Mapping of habitat preferences onto the character tree (Figure 5) suggests that tardigrades have adapted to marine environments twice, to freshwater environments at least three times, and to terrestrial environments twice. The placement of the terrestrial Oreellidae as the basal heterotardigrade suggests that tardigrades were ancestrally terrestrial. However, the placement of Oreellidae in the character tree may be an artifact caused by the secondary loss of dorsal plates.
Chapter 4

Molecular Analysis of Tardigrade Phylogeny

Background

Molecular studies have examined the phylogenetic position of the Tardigrada (Garey et al., 1996; Giribet et al., 1996) placing them in a clade that includes arthropods. There appears to be a consensus that arthropods, onycophorans and tardigrades form a monophyletic clade known as Panarthropoda (reviewed in Schmidt-Rhaesa et al., 1998). An on-going study to use protein coding gene sequences to study arthropod phylogeny utilized several tardigrades as an arthropod outgroup (Regier & Shultz, 2001). Other molecular studies have placed tardigrades within a group of molting animals that includes arthropods, priapulids, nematodes and kinorhynchs (Aguinaldo et al., 1997; Zrzavy et al., 1998). Garey et al. (1999) found close agreement between molecular and morphology based phylogenies that included six species of tardigrades, suggesting that the characters for tardigrade morphological studies are appropriate. This study also suggested that Heterotardigrades, the marine and terrestrial armored species, are the most basal group with the greatest number of plesiomorphic characters.

Garey et al. (1996) showed that tardigrades are closely allied to arthropods and that tardigrades, arthropods and priapulids form a clade. Since priapulids had long been
considered to be members of “aschelminths”, this explained why some tardigrade characters such as cryptobiosis, cuticular structure and the presence of a triradiate pharynx seemed to ally them to aschelminths while other characters such as body muscle specialization, lack of a closed circulatory system, and body segmentation placed tardigrades most closely with arthropods (Garey et al., 1996). A similar molecular study, published the same year by Giribet et al. (1996) also demonstrated that tardigrades are associated with arthropods. There are a number of reports where molecular and morphological data have been evaluated together in studies of rotifer phylogeny, tardigrades, protostomes, urochordates, and deuterostomes (Cameron et al., 2000; Swalla et al., 2000; Garey et al., 1998; Garey et al., 1999; Schmidt-Rhaesa et al., 1998; Zrzavy et al., 1998; Ernissee, 1992; Giribet et al., 2001)

Garey et al. (1999) demonstrated that trees produced by the analysis of 18S rRNA gene sequences of tardigrades are in close agreement to trees based on morphological characters (Fig. 6). This suggests that molecular analyses of tardigrade 18S rRNA gene sequences contain sufficient information to outline the evolutionary relationships among tardigrade orders, among tardigrade families, and even among tardigrade genera.
Materials and Methods

Gene Selection

The near complete 18S rRNA gene sequence (~1800 b; Fig. 7) was used for the molecular analysis. Nuclear protein coding genes were considered and rejected for this study because of the small sample size and the relative difficulty in obtaining both DNA
and poly A+RNA from limited (e.g. size and number) specimens. The 18S rRNA gene was used based upon its recent use to infer intra-phylum relationships (Blaxter et al., 1998) and that it has a long history in determining inter-phylum relationships among metazoans (Field et al., 1988).

**DNA Extraction**

Tardigrades were isolated onto individual glass slides and air dried. The tardigrade cuticle was then mechanically macerated using dental probes previously cleaned with DNA-Away (Molecular BioProducts, Inc., San Diego, CA) and rinsed in deionized water. A 10 µl solution containing 5mg/ml of Proteinase K was added to the macerated cuticle and the sample was treated to 3 freeze/thaw cycles at -20 °C. Proteinase K cleaves peptide bonds and is used for the removal of DNases and RNases during DNA and RNA isolation and has been shown to improve the efficiency of cloning in PCR products (Crowe et al., 1991). The extraction mixture was then transferred to a 200 µl centrifuge tube and incubated at 55 °C for one hour, 5 minutes at 95 °C and then centrifuged at 2000 x g for 5 minutes. Ten µl of the supernatant was then used as template DNA.
**Polymerase Chain Reaction**

PCR amplification of template DNA was carried out using primers specific for the 18S rRNA gene (18S4 5'-GCTTGTCTCAAAGATTAAGCC-3' and 18S5 5'-ACCATACTCCCCCGGAACC-3'). PCR reactions were performed in 200 µl tubes using a Biometra TRIO thermocycler (WhatmanBiometra, Göttingen, Germany). The reaction cycle consisted of an initial denaturing step of 30 sec at 94 °C followed by 35 cycles of 30 seconds denaturing at 94 °C, 30 seconds annealing at 45-55 °C, and 60-120 seconds extension at 72 °C. The PCR reactions consisted of 1X final concentration of 10X PCR buffer (Enzypol, Denver, CO), 2 mM final concentration of magnesium chloride, 0.1 µM final concentration of each primer, 0.25 mM final concentration for each of dATP, dCTP, dTTP, and dGTP, 10 µl of template DNA and 1 unit of EnzyPlus 2000 Taq polymerase (Enzypol, Denver, CO) in a final volume of 50 µl.

**Cloning**

PCR product of 18S rDNA amplified from some of the samples was cloned using the TOPO TA cloning kit for sequencing (Invitrogen Corp., San Diego, CA) following the manufacturer’s instructions. Transformed cells were plated and incubated overnight at 37°C on Luria-Bertani (LB) agar containing 100µg/mL ampicillin and 50µg/mL X-gal (5-Bromo-4-chloro-3-indolyl β-D- galactoside). Colonies were picked and cultured in 96 well microtiter plates for 24 hours. Plasmid extraction from the bacteria was performed using the Eppendorf Perfectprep Plasmid Isolation Kit and then quantified using 0.9% agarose gel electrophoresis. All colonies grown overnight for isolation of plasmid DNA were preserved in 50% glycerol and stored at –80 °C.
**Sequencing**

Template DNA (cloned and direct PCR product) was cycle sequenced using the QuickStart sequencing kit (BeckmanCoulter, La Jolla, CA). The reaction mix contained between 50 and 100 ng of template, 2 µl sequencing reaction mix (SRM), 1 µl 3.2 µM sequencing primer, and water to bring the total reaction volume to 10 µl. All products (clones and PCR) for analysis were sequenced using the 18S4c and 18S2c primer. Sequencing reactions were amplified in the Biometra TRIO thermocycler. A preheat step consisting of only water and template DNA was performed at 96 °C followed by a return to room temperature. The SRM and primers were added and the reaction was cycled as follows: an initial denature at 96 °C for 1 minute; cycled 25 times through a 96 °C, denature step for 15 seconds, a 50 °C annealing step for 30 seconds and an extension step at 60 °C for one minute. Finally, a 60 °C extension step for 7 minutes was performed and a final hold at 4 °C. The cycle sequencing product was purified following the manufacturer’s instructions and analyzed using a CEQ 8000 Genetic Analysis system (BeckmanCoulter, La Jolla, CA).

**Alignments and Sequence Analysis**

Sequences were visually checked and corrected for ambiguous bases (N’s). Data sets from individual tardigrades were aligned according to a secondary structure model (Neefs et al. 1993) using DCSE (De Rijk and De Wachter 1993). Phylogenetic analysis of alignments was performed using MEGA version 2.1 (Kumar, et al. 2001) to produce neighbor-joining trees and Phylogenetic Analysis Using Parsimony (PAUP*) version 4.6 (Swofford, 1998) was utilized for Maximum Parsimony analysis. A variety of arthropod
species were used as outgroups. Trees produced were evaluated by bootstrap analysis (Hillis & Bull 1993).

Results & Discussion

Molecular Phylogeny

Near complete 18S rRNA sequences were amplified and aligned with previously published data (Table 3).

Table 3. List of species, sequence length, origin, and sequence reference.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length</th>
<th>Accesion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arthropoda</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Artemia salina</em></td>
<td>2002</td>
<td>X01723</td>
<td>Nelles <em>et al.</em>, 1984</td>
</tr>
<tr>
<td><em>Meloe proscarabaeus</em></td>
<td>1934</td>
<td>X77786</td>
<td>Chalwatzis <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Okanagana utahensis</em></td>
<td>1918</td>
<td>U06478</td>
<td>Campbell <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Tenebrio molitor</em></td>
<td>2083</td>
<td>X07801</td>
<td>Hendriks <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>Panulirus argus</em></td>
<td>1872</td>
<td>U19182</td>
<td>Trapido-Rosenthal <em>et al.</em>, 1994</td>
</tr>
<tr>
<td><strong>Mollusca</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Placopecten magellanicus</em></td>
<td>1814</td>
<td>X53899</td>
<td>Rice 1990</td>
</tr>
<tr>
<td><strong>Priapulida</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Priapulus caudatus</em></td>
<td>1814</td>
<td>X80234</td>
<td>Winnepenninckx <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><strong>Tardigrada</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Echiniscus viridissimus</em></td>
<td>1824</td>
<td>AF056024</td>
<td>Garey <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Halechiniscus remanei</em></td>
<td>827</td>
<td>AY582118</td>
<td>Jorgensen &amp; Kristensen, 2004</td>
</tr>
<tr>
<td><em>Halobiotus stenostomus</em></td>
<td>1783</td>
<td>AY582121</td>
<td>Jorgensen &amp; Kristensen, 2004</td>
</tr>
<tr>
<td><em>Macrobiotus hufelandi</em></td>
<td>1808</td>
<td>X81442</td>
<td>Garey <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Macrobiotus tonolli</em></td>
<td>1735</td>
<td>U32393</td>
<td>Garey <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Milnesium tardigradum</em></td>
<td>1844</td>
<td>U49909</td>
<td>Aguinaldo <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><em>Milnesium tardigradum</em></td>
<td>1777</td>
<td>AY582120</td>
<td>Jorgensen &amp; Kristensen, 2004</td>
</tr>
<tr>
<td><em>Pseudechiniscus islandicus</em></td>
<td>1820</td>
<td>AY582119</td>
<td>Jorgensen &amp; Kristensen, 2004</td>
</tr>
<tr>
<td><em>Ramazzottius oberhauseri</em></td>
<td>1771</td>
<td>AY582122</td>
<td>Jorgensen &amp; Kristensen, 2004</td>
</tr>
<tr>
<td><em>Thulinius stephaniae</em></td>
<td>1686</td>
<td>AF056023</td>
<td>Garey <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Batillipes mirus</em></td>
<td>1432</td>
<td>Present study</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Hypsibius dujardini</em></td>
<td>1521</td>
<td>Present study</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Calohypsibius schusteri</em></td>
<td>1384</td>
<td>Present study</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Ramazzottius oberhauseri</em></td>
<td>1674</td>
<td>Present study</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Richtersius coronifer</em></td>
<td>1726</td>
<td>Present study</td>
<td>Present study</td>
</tr>
</tbody>
</table>
The NJ and MP trees produced from 18S rRNA gene sequences (Figure 8) were congruent with one another and support the morphological character analysis in indicating that Eutardigrada and Heterotardigrada are each monophyletic as evidenced by bootstrap values of 89 and 84, respectively.

Figure 8. Tardigrade phylogeny based on 18S rRNA gene sequences. Similar trees were recovered for maximum parsimony and maximum likelihood analyses. Branch lengths are drawn to scale (substitutions/site). The numbers above each fork are bootstrap values for the NJ tree. *Specimens were not identified to species in the original study, but have since been verified.
The 18S rRNA gene sequences from *Calohypsibius schusteri, Hypsibius dujardini, Richtersius coronifer, and Ramazzotius oberhouseri* adds 4 additional genera to the previously published eutardigrade dataset. Within eutardigrades the monophyly of Parachela is well supported. Bootstrap values for all branches were high with the lowest value of 90% for the Hypsibiidae + Macrobiotidae node. The *Calohypsibius* sequence indicates that Calohypsibiidae is a sister group to Hypsibiidae (Figure 8). However, in the morphological tree, the relationship between Calohypsibiidae and Hypsibiidae is unresolved (Figure 6). There is more statistical support for relevant nodes within Eutardigrada in the molecular results (82% - 100% bootstrap values) than in the morphological results (54% - 64% bootstrap values). This analysis agrees with the findings of Jorgensen and Kristensen (2004) that the eutardigrades are monophyletic and it is consistent with the morphological analysis in Figure 5. However, the taxon sampling among the Macrobiotidae family is not exhaustive and some questions about the monophyly of this line still exist.

A separate analysis of the Macrobiotidae family by Guidetti *et al.* (2005) used both morphological characters and molecular data from the Cytochrome c Oxidase subunit I gene. Cytochrome c Oxidase subunit I sequences have been shown to be useful in resolving phylogenetic relationships among closely-related taxa among different phyla (Avise 1994, 2000; Brown *et al.* 1994; Lunt *et al.* 1996; Hebert *et al.* 2003a, b). Their findings suggest that Macrobiotidae is not monophyletic and that the subfamily Murrayinae should be elevated to family level Murrayidae. Nearly 40% of the Parachela genera are currently within the family Macrobiotidae and this is clearly an area that needs further study in order to determine an accurate phylogeny.
Within heterotardigrades, the monophyly of Arthrotardigrada and Echiniscoidea is well supported with a bootstrap value of 84%. There is more statistical support for relevant nodes in the molecular results (90% - 100% bootstrap values) than in the morphological tree (60% - 64% bootstrap values). The 18S rRNA gene sequence from *Batillipes mirus* and *Halechiniscus remanae* (Arthrotardigrada) adds an additional heterotardigrade order and two new families (Batillipedidae, HalechiniScidae) to the previously published molecular data set. The addition of *Pseudoechiniscus islandicus* adds an additional genus within the Echiniscidae family to the dataset. The molecular data suggests that heterotardigrades and eutardigrades are both monophyletic.

**Habitat Mapping**

As seen in the morphological dataset, mapping of habitat preferences onto the gene tree (Figure 9A) also suggests that tardigrades have adapted to marine environments twice, to freshwater environments once, and to terrestrial environments twice. The placement of *B. mirus* and *H. remanaei* as the basal heterotardigrade suggest that tardigrades were ancestrally marine. By mapping the habitats preferences on the tree we can trace the number of evolutionary events that occur. If terrestrial tardigrades are considered to be ancestral state then there are four events that occur (Figure 9B) while if we assume that the marine tardigrades are the ancestral tardigrades then there are five events that occur (Figure 9C). This suggests that based on the number of evolutionary changes, terrestrial tardigrades are the ancestral tardigrades. However, more extensive taxon sampling will be required to test this hypothesis.
Figure 9. Tardigrade molecular topology from Fig. 8 with mapped habitat preference. Habitats: Terrestrial (T); Marine (M); or Freshwater (F).
Chapter 5

Meiofaunal Abundance, diversity and Similarity in a
Uniquely Rich Tardigrade Community.

Background

Meiofauna are distributed worldwide and include representatives of 22 of the metazoan phyla (Coull, 1999, 1988). They are found in terrestrial, freshwater and marine habitats and are classified by their small size, generally 50 - 500 µm. Ecologically, meiofauna function as food sources for higher trophic levels and play an important role in the biomineralization of organic matter (Hummon, 1987; Battigelli & Berch, 1993; Coull, 1999). The assemblages can be linked to sediment characteristics (particle size) and bacterial production (Higgins, 1988; Vanreusel et al., 1995; Schratzberger et al., 2000) and are often reported in terms of abundance, distribution and diversity (eg. Hummon, 1987; Trett et al., 2000, Battigelli & Berch, 2004).

Meiofaunal assemblages in marine habitats have been extensively studied and focused on areas from intertidal mud flats and littoral zones (Coull & Wells, 1981; Stoffels et al., 2003) to the bathyal and abyssal depths of the deep sea (Ingole et al., 2000; Tselepides et al., 2004). Vertical and horizontal zonation is affected by an anaerobic-aerobic boundary layer in sediment, increasing depth and salinity gradients. Typically, the highest abundances are found in intertidal zones and decrease as the depth
increases. Salinity, food availability, sediment grain size, and tidal exposure can affect dispersal (Findlay, 1981) which is often patchy with densities changing over a distance of just a few centimeters (Schratzberger et al., 2000).

Surveys on marine tardigrade distributions are primarily descriptions of new species and taxonomical reviews (De Zio Grimaldi & D’Addabbo, 2001). Studies investigating the ecology of marine tardigrades most often do not report information on their abundance and ecology within the context of the meiofaunal community. A few exceptions are from ecological studies from the Mediterranean Sea (for review see, De Zio Grimaldi & D’Addabbo, 2001), psammolittoral tardigrades from North Carolina (Lindgren, 1971), interstitial tardigrades from the Pacific coast of the U.S. and the Galapagos (McKirdy, 1976; Pollock, 1989) and the Faroe Bank (Hansen et al., 2001). An unpublished master’s thesis (Gaugler, 2002) investigated the distribution pattern of meiofaunal at Huntington Beach, SC with particular emphasis on tardigrades.

Numerous reports have investigated meiofaunal assemblages and used them as a measure of the health of the environment (for example, Austen et al., 1989; Schratzberger et al., 2000; Ingole et al., 2000; Schratzberger & Jennings, 2002). The most difficult and prohibitive aspects of these investigations are the high cost of sample processing, difficulty associated with species identifications and the need for broad taxonomic expertise (Warwick, 1988), an area of study that has declined in recent years.

Traditional sorting techniques have involved osmotic shocks and freshwater rinses, Ludox gel isolation, air bubbling, and hand sorting. These methods are time consuming and in some cases can result in damage to the studied organisms (Higgins, 1988). Molecular techniques applied to sediment extracts may provide a more cost
effective and less time consuming method of identifying the structure of a meiofaunal community.

Microbial ecologists often experience similar difficulty when attempting to isolate and culture microbes from environmental samples (Stephen et al., 1996) and those that have been cultured represent only a fraction of the estimated species (Wintzingerode et al., 1997). Techniques developed to extract DNA from sediment samples have allowed microbial ecologists to analyze the species composition of unculturable microbes (Kennedy and Gewin, 1997). The techniques developed for microbial ecology should be applicable to meiofaunal studies and similar investigations have yielded promising yet mixed results (Boenigk et al., 2005; Savin et al., 2004; Blaxter et al., 2003; Hamilton 2003).

Boengk et al. (2005) reported high community diversity with morphological identifications and molecular identification of plankton diversity in the Bay of Fundy. However, even though the community diversity was high, the similarity between the two was low with few species common to both the morphological and molecular analysis (mainly diatom, dinoflagellates, etc.). Savin et al. (2004) isolated flagellates from freshwater sediments and soils to investigate the diversity using rRNA sequence data. They were able to determine groups and identify the groups to closely related taxa. Blaxter et al. (2003) found a high diversity when targeting tardigrade species and utilizing DNA extracts from sediment and moss samples. Hamilton (2003), like Boengk et al. (2005) found discrepancies between the numbers of taxonomical units recovered from sequencing sediment extracts and the traditional methods of performing manual identification and counts. Hamilton (2003) also demonstrated that increasing the size of
the DNA fragment (~230 – 400bp) also increased the percentage of OTU’s that could be identified to a specific taxon from 15% to 70% with bootstrap values to support them.

**Materials and Methods**

*Sample Collection*

Refer to Chapter 2 for general methods on sample collecting and processing.

*DNA Sediment Extraction*

A modification of Hempstead’s protocol (Hempstead *et al.*, 1990) for DNA extraction was used to obtain meiofaunal DNA from marine sediment collected on Dauphin Island, Alabama. Sediment samples were collected from a small sand bar in the salt marsh on the southwest side of the airport runway (30° 15’ 26.11” N/88° 07’ 27.14” W) (Figure 10).

*Figure 10. Location of sediment collection site on Daupin Island, AL (15M Resolution).*

One volume (about 1 mL) of homogenization buffer (3.5% SDS in 1M Tris, pH 8.0, and 100mM EDTA) was added to a 2 mL centrifuge tube containing the sediment sample.
The samples were then homogenized using pre-cleaned Teflon tipped pestles and centrifuged to pellet the sediment. The supernatant was pipetted to a 1.5 mL centrifuge tube and an equal volume of phenol (pH 7.9) was added to each of the tubes. The tubes were mixed gently for 5 minutes and then centrifuged for 5 minutes at 14,000 rpm. The top aqueous layer from the solution was transferred to a new 1.5mL tube and the previous steps were repeated: one more time using phenol (pH 7.9), twice using a 1:1 solution of phenol (pH 7.9): chloroform-isoamyl alcohol (24 parts chloroform to 1 part isoamyl alcohol), and twice with the chloroform-isoamyl solution. The DNA in the final aqueous layer was transferred to a new tube and was precipitated for 24 hours at –20 °C with 2 volumes of 100% ethanol and a 0.1 volume of 3M sodium acetate (pH 6.0). The precipitated DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes, washed with 70% ethanol and suspended in 100 µl of deionized water.

**Polymerase Chain Reaction**

PCR amplification of template DNA was carried out using universal primers specific for the 18S rRNA gene (18S4 5'-GCTTGTCTCAAAGATTAAGCC-3' and 18S5 5'-ACCATACTCCCCCGGAACC-3’). PCR reactions were performed in 200 µl tubes using a Biometra TRIO thermocycler (WhatmanBiometra, Göttingen, Germany). The reaction cycle consisted of an initial denaturing step of 30 sec at 94 °C followed by 35 cycles of 30 seconds denaturing at 94 °C, 30 seconds annealing at 55 °C, and 60-120 seconds extension at 72 °C. The PCR reactions consisted of 1X final concentration of 10X PCR buffer (Enzypol, Denver, CO), 2 mM final concentration of magnesium chloride, 0.1 µM final concentration of each primer, 0.25 mM final concentration for
each of dATP, dCTP, dTTP, and dGTP, 10 µl of template DNA and 1 unit of EnzyPlus 2000 Taq polymerase (Enzypol, Denver, CO) in a final volume of 50 µl.

Cloning

PCR product of was cloned using the TOPO TA cloning kit for sequencing (Invitrogen Corp., San Diego, CA) following the manufacturer’s instructions. Transformed cells were plated and incubated overnight at 37 °C on Luria-Bertani (LB) agar containing 100µg/mL ampicillin and 50µg/mL X-gal (5-Bromo-4-chloro-3-indolyl β-D- galactoside). Colonies were picked and cultured in 96 well microtiter plates for 24 hours. Plasmid extraction from the bacteria was performed using the Eppendorf Perfectprep Plasmid Isolation Kit and then quantified using 0.9% agarose gel electrophoresis. All colonies grown overnight for isolation of plasmid DNA were preserved in 50% glycerol and stored at –80 °C.

Sequencing

Template DNA (cloned and direct PCR product) was cycle sequenced using the QuickStart sequencing kit (BeckmanCoulter, La Jolla, CA). The reaction mix contained between 50 and 100ng of template, 2 µl sequencing reaction mix (SRM), 1µl 3.2 µM sequencing primer, and water to bring the total reaction volume to 10 µl. All products (clones and PCR) for analysis were sequenced using the 18S4 and 18S5 primer. Sequencing reactions were amplified in the Biometra TRIO thermocycler. A preheat step consisting of only water and template DNA was performed at 96 °C followed by a return to room temperature. The SRM and primers were added and the reaction was cycled as follows: an initial denature at 96 °C for 1 minute; cycled 25 times through a 96 °C,
denature step for 15 seconds, a 50 °C annealing step for 30 seconds and an extension step at 60 °C for one minute. Finally, a 60 °C extension step for 7 minutes was performed and a final hold at 4 °C. The cycle sequencing product was purified following the manufacturer’s instructions and analyzed using a CEQ 8000 Genetic Analysis system (BeckmanCoulter, La Jolla, CA).

Alignments and Sequence Analysis

Sequences were visually checked and corrected for ambiguous bases (N’s) and aligned using ClustalX (Thompson, et al. 1997). Phylogenetic analysis of the alignment was performed using MEGA version 2.1 (Kumar et al., 2001) to produce neighbor-joining trees evaluated by bootstrap analysis (Hillis & Bull, 1993). Operational taxonomic units (OTUs) were assigned to sequences from the tree containing all 126 sequences based on the number of differences and the topology of the tree. Assigned OTUs were given to sequences that grouped together as a clade and had less than 5 differences. Sequence misalignments were manually corrected.

Sediment sorting and specimen identification

Meiofaunal samples preserved in 95% ethanol with Rose Bengal stain were manually sorted and counted using a Meiji dissecting scope. Type of species present and frequency of individuals were keyed to the most practical taxonomic level (ie. tardigrade, nematode, and ostracod).

Data Analysis

One sequence from each of the assigned OTUs was randomly selected and added to a data set of reference sequences. This dataset was aligned with ClustalX. A
phylogenetic tree utilizing the neighbor-joining method and the Kimura 2-parameter distance model was generated. The OTUs were identified and numbers of individual clones per OTU were scored.

A rarefaction curve and species diversity indices were plotted using BioDiversity Pro (http://www.sams.ac.uk/). Species diversity was calculated using the Shannon-Wiener index ($H' = -\sum p_i \log p_i$) and Simpson’s index ($C = 1 - \sum p_i^2$), where $p_i$ is the number of individuals of a species divided by the total number of individuals. Community similarity was calculated using the Jaccard coefficient ($CC_j = c / (s_1 + s_2 - c)$); where $c$ is the number of species shared between the communities and $s_1$ and $s_2$ are the number of species in community 1 and 2 respectively; and proportional similarity.

The two communities compared in this study are defined as 1) the OTUs from the sequence analysis and 2) the manual counts from preserved samples.

**Results and Discussion**

The clone library from a sediment sample with a uniquely high concentration of tardigrades at Dauphin Island, Alabama yielded 126 sequences that were used for the analyses. Seventeen OTUs were assigned from the neighbor-joining tree (Figure 12). The tree (Figure 13) generated from a reference data set and unique sequences representing each of the OTUs identified twelve groups (Table 4). Hamilton (2003, unpublished thesis) showed that specific sequences of meiofaunal data could be identified from a reference alignment. The frequencies of individual sequences from each of the 12 groups identified here are reported in Table 4.
Table 4. Sequence groups and the number of sequences per group. These data were used to calculate Shannon diversity, Simpson’s Diversity, Jaccard’s Similarity, and Proportional Similarity.

<table>
<thead>
<tr>
<th>OTU #</th>
<th>Sequence #</th>
<th>Closest Genus</th>
<th>Common Name</th>
<th>Frequency</th>
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<tr>
<td>1</td>
<td>S1140</td>
<td>Echiniscus</td>
<td>Tardigrade</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>B1-10</td>
<td>Ballanus</td>
<td>Barnacle</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>S240</td>
<td>Plectus</td>
<td>Nematode</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>66-18S4</td>
<td>Bugula</td>
<td>Bryozoan</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>6718S4</td>
<td>Bugula</td>
<td>Bryozoan</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>561864</td>
<td>Electra</td>
<td>Bryozoan</td>
<td>28</td>
</tr>
<tr>
<td>7</td>
<td>40-18S4</td>
<td>Vaccinium</td>
<td>Plant</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>6018S4</td>
<td>Vaccinium</td>
<td>Plant</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>4418S4</td>
<td>Vaccinium</td>
<td>Plant</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>S381</td>
<td>Stenostomum</td>
<td>Flatworm</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>B1-9</td>
<td>Wilsonema</td>
<td>Nematode</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>S2B7</td>
<td>Callinectes</td>
<td>Decapod</td>
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<td>14</td>
<td>S39</td>
<td>Caligus</td>
<td>Copepod</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
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<td>Callinectes</td>
<td>Decapod</td>
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</tr>
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<td>S215</td>
<td>Daptonema</td>
<td>Nematode</td>
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</tr>
<tr>
<td>17</td>
<td>B1-8</td>
<td>Enoplus</td>
<td>Nematode</td>
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</tr>
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<td>18</td>
<td>B1-5</td>
<td>Chaetonotus</td>
<td>Gastrotrich</td>
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<td>19</td>
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<td>Abyssothyris</td>
<td>Brachiopod</td>
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<td>20</td>
<td>S1B46</td>
<td>Brachionus</td>
<td>Rotifer</td>
<td>8</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total # of groups: 20</td>
<td>Total # of individuals: 126</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Frequency data of individual OTUs (Table 4) and morphological groups (Table 5) was used to calculate a rarefaction curve to test for saturation of the identified species.

Figure 11. Rarefaction curve calculated for molecular OTUs and morphological groups
(Figure 11). The rarefaction curve shows that molecular OTUs reached a saturation point around 100 individual sequences while the morphological groups reached a saturation point at approximately 70 individuals. These results suggest that the sample size was large enough to adequately assess the communities.
Figure 12 (Continued)
Figure 12 (Continued)
Figure 12 (Continued)

Figure 12. Neighbor-joining phylogenetic tree based on the number of differences between sequences and complete deletion of gaps of all 126 sequences. Groups notated to the right of each group consist of sequences with no more than 5 differences between them. The scale bar on the last page of the tree indicates the number of differences per length of the bar.
Figure 13. Neighbor-joining phylogenetic tree of the unique OTU sequences and a reference data set. The phylogenetic tree was created based on the Kimura 2-parameter distance method and complete deletion of gaps.
Species groups from manual sorting were determined and the frequencies of individuals present are reported in Table 5. Ten groups were found. Tardigrades were the dominant species comprising nearly 35% of the population. Nematodes (18%), polychaetes (12%), rotifers (10%), diatoms (9%) and ostracods (8%) were the other main taxa identified.

There is a striking contrast in the groups represented when compared to the molecular data set. Bryozoans, which were absent in the manual counts, and tardigrades were the most dominant groups in the molecular data set. They represented 24% and 21% respectively of the total sequences while sequences from polychaetes and diatoms are absent from the molecular data set. The presence of the fourteen sequences identified as *Vaccinium*, an asterid plant was probably due to the presence of pollen in the sediment sample. The Frequency of individuals from the molecular OTUs in Table 4 was lumped into the same species groups identified in Table 5 for the analysis. Bryozoans and asterids were omitted from the analyses because because they were not included in the manual sorting efforts and their presence in and the sequences isolated were a result of contamination from

<table>
<thead>
<tr>
<th>Species</th>
<th>Morphological</th>
<th>OTUs</th>
</tr>
</thead>
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<tr>
<td>Tardigrade</td>
<td>128</td>
<td>27</td>
</tr>
<tr>
<td>Nematode</td>
<td>66</td>
<td>17</td>
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<tr>
<td>Barnacle</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Polychaete</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>Flatworm</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Rotifer</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>Diatoms*</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Ostracod</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>Copepod</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Gastrotrich</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Molluscs</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Crustacea</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Brachiopod</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Bryozoan*</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Plant (<em>Vaccinium)</em></td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table 5. Morphological and sequence group (OTUs) frequency of individuals.** These data, with noted omissions (*), were used to calculate Shannon diversity, Simpson’s Diversity, Jaccard’s Similarity and Stander’s Similarity.

**Total # groups: 15**  **Total #: 363**  **Total #: 126**
fragments of bryozoan and pollen from the asterids. The diatoms are not metazoan and were therefore omitted from the analyzed data.

Species Diversity

High species diversity is indicative of a highly complex community and is one expression of the community structure. A series of populations that are equally abundant will have high diversity indices, while those that have few dominant species and many rare species will have low diversity indices. Two diversity indices were calculated to focus on both species richness (number of species) and species evenness (number of individuals among the species). Shannon index (H’) is sensitive to the presence of rare species while Simpson’s index (C) is more sensitive to changes in species richness and species evenness.

The results based on the data from table 5 show that the diversity among the molecular OTUs is higher than the morphological groups (Table 6). The number of species present in OTUs (n=10) and morphological groups (n=9) differs by a count of 1. The distribution of individuals among the OTUs (mean = 6.83) are distributed more equitably and ranged from 0 - 27 while the morphological groups (mean = 27.42) ranged from 0 - 128.

Community Similarity

Similarity between communities has often been questioned by ecologists. A measure of the similarity between two areas can provide an idea of the success of the
Two methods of calculating community similarity are Jaccard’s coefficient and Stander’s coefficient. Jaccard’s coefficient does not take into account relative distribution, but indicates the percentage of species shared between the two communities while Stander’s is a function of the number of species shared and their relative distributions. Both indices will range from 0, when no shared species are found, to 1.0, when all species are shared and have the same relative abundance. For this study, the molecular OTUs and the morphological groups were each treated as a separate community.

There is a noticeable difference in the indices for the two community similarity coefficients. Jaccard’s coefficient which simply accounts for the number of taxa shared between the two communities is 58.3% (Table 7), a decrease of almost 40 points when compared to Stander’s coefficient of 98.6% similarity which is a function of the number of species shared and their relative distributions. The preserved samples contain one less species group, but over 35% of the individuals belonged to one species, *Batillipes mirus* and the second most abundant being nematodes at 10%. Similar findings were seen in the OTUs where tardigrades were the dominant group representing 33% of the community followed by nematodes at 21%.

<table>
<thead>
<tr>
<th>Community Index</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaccard’s</td>
<td>0.583</td>
</tr>
<tr>
<td>Stander’s</td>
<td>0.986</td>
</tr>
</tbody>
</table>

The molecular and phylogenetic methods used here to assess a community of marine tardigrades were successful in discriminating between the meiofaunal groups (OTUs). The results indicate that rarefaction curves can reach saturation and that groups can be identified from small sample sizes. These results can then be used to compare
communities utilizing species diversity, community similarity and other comparative analyses. The capability to identify specific groups utilizing gene sequences may lead to more detailed surveys of meiofaunal communities by lessening the burden on the researcher attempting to identify individuals through traditional techniques.

Tardigrades from the family Batillipedidae are a littoral or sublittoral species with a few records from bathyal depths (Hansen et al., 2001). The findings presented here are from a unique population of meiofauna containing species of Batillipes in an intertidal salt marsh. The abundance of Batillipes from this site is unusually high often with counts nearing 1000 individuals per 10 cm$^2$ core samples (Romano, personal communication) compared to most samples which typically range from zero to several hundred individuals per 10 cm$^2$ (e.g. McGinty & Higgins, 1968; D’Addabbo Gallo et al., 1999). This abundance may be a result of the presence of a sand bar, which is unique when compared to a typical salt marsh. The interaction between the grain size of the sand bar sediment providing the necessary habitat for a population of marine tardigrades and the minimal tidal influence associated with this area may be high enough to facilitate nutrient exchange. Also, the highly dense population of tardigrades may not be unique and could simply be a result of a lack of investigations looking for tardigrades in intertidal salt marshes.
Chapter 6
Summary

Morphological Analysis of Tardigrade Phylogeny

The morphological phylogeny developed here suggests that the class Eutardigrada and Heterotardigrada are both monophyletic. The order Parachela is a monophyletic sistergroup to Apochela within the eutardigrades while the order Parachela is a monophyletic sister group to the Apochela. The phylogeny also suggests that the heterotardigrade orders Arthrotardigrada and Echiniscoidea are not sister groups and it appears that Arthrotardigrada is paraphyletic containing some members of Echiniscoidea. The placement of the terrestrial Oreellidae as the basal heterotardigrade suggests that tardigrades were ancestrally terrestrial. However, the placement of Oreellidae may be an artifact due to the secondary loss of dorsal plates.

Molecular Analysis of Tardigrade Phylogeny

The molecular phylogeny is congruent with the morphological phylogeny in indicating that the class Eutardigrada and Heterotardigrada are each monophyletic. The monophyly of the order Parachela is well supported and the molecular data showed high support for the relationship between the families. The monophyly of the Macrobiotidae family remains in question. The molecular phylogeny, in contrast to the morphological
phylogeny, showed that the order Arthrotardigrada and Echiniscodea are monophyletic groups. Mapping of the habitat preferences suggests that terrestrial tardigrades are the ancestral state. However, more extensive taxon sampling will be required to test this hypothesis.

**Meiofaunal Abundance, diversity and Similarity in a Uniquely Rich Tardigrade Community**

The molecular and phylogenetic methods used here to assess a community of marine tardigrades were successful in discriminating between the meiofaunal groups (OTUs) identifying 17 OTUs in contrast to the 12 morphological groups identified from the manual sorting efforts. Rarefaction curves suggest that the sample sizes were large enough to adequately assess the communities. The two communities were very similar but the OTUs had a higher diversity and eveness than the morphological groups. The dominant species in both communities was tardigrades.
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### Appendix A

**Morphological Data Matrix**

<table>
<thead>
<tr>
<th></th>
<th>Loricifera</th>
<th>Kinorhyncha</th>
<th>Gastrotricha</th>
<th>Macrobiotidae</th>
<th>Eohypsibiidae</th>
<th>Calohypsibiidae</th>
<th>Necopinatidae</th>
<th>Microhypsibiidae</th>
<th>Hypsibiidae</th>
<th>Milnesiidae</th>
<th>Apodibius</th>
<th>Halechiniscidae</th>
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<th>Renaudarctidae</th>
<th>Coronarctidae</th>
<th>Batillipedidae</th>
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Appendix B

List of Morphological Characters

(Unless otherwise stated, 0 = absent, 1 = present)

(1) Molting by ecdysis.

(2) Loss of locomotory cilia; Gastrotrichs have locomotory cilia on their ventral side and they are covered by the cuticle.

(3) Cuticle structure: trilaminate epicuticle, proteinaceous exocuticle and chitinous endocuticle; gastrotrichs do not have a chitinous endocuticle.

(4) Parthenogenesis.

(5) Circumpharyngeal nerve ring.

(6) Complete gut.

(7) Permanent genital pore separate from anus.

(8) Adhesive glands.

(9) Protonephridia.

(10) Adult gut functional.

(11) Triangular shaped pharynx.

(12) Stylets: piercing rods lateral to the buccal tube that are anteriorly pointed and can be protruded out the mouth opening.

(13) Formation of epicuticle: appears over the tip of short microvilli in patches that laterally merge to form a continuous layer. In gastrotrichs the cuticle is brought to the surface by Golgi vesicles in the form of tiny plates.

(14) Terminal mouth.
(15) Cephalic papillae: short, rounded appendages occurring on the head, usually lateral to the mouth opening.

(16) Cephalic appendages.

(17) Peribuccal papillae: short, rounded appendages that surround the mouth opening.

(18) Peribuccal lamellae: Short appendages surrounding the cuticular ring of the buccal opening

(19) Buccal tube: anterior rigid tube without spiral annulations, extending from the mouth opening to the pharyngeal tube or the pharyx.

(20) Buccal tube apophyses: cuticular thickenings at the junction of the buccal tube and pharynx.

(21) Pharyngeal tube: posterior flexible tube with spiral annulations, extending from the buccal tube into the pharynx.

(22) Pharyngeal tube apophyses.

(23) Ventral lamina: a small ventral support that extends from the mouth ring to approximately the middle of the buccal tube.

(24) Stylet supports: structure that attaches the posterior end of the stylet to the buccal tube. Stylet supports: 0 absent, 1 present, 2 either.

(25) Macroplacoid: large, cuticular thickenings that occur in two or three transverse rows in the pharynx; Microplacoid: small, cuticular thickenings located posterior to the macroplacoids. Placoids: 0 absent, 1 microplacoids only, 2 macroplacoids only, 3 both micro and macroplacoids,

(26) Septulum: cuticular thickenings at the base of the buccal tube. Septulum: 0 absent, 1 present, 2 either.
(27) Claw Structure: 0 absent, 1 single, 2 double separated, 3 double connected.

(28) Claw sequence, separated claws of the heterotardigrades were scored 1111 and Milnesiidae were scored 1122: 0 absent, 1 1111, 2 1122, 3 2121, 4 2112.

(29) Transverse cuticular bar: 0 absent, 1 present, 2 either. Located at the base of the claws.

(30) Accessory points: 0 absent, 1 present, 2 either. Located on the tips of the claws.

(31) Lunulae: 0 absent, 1 present, 2 either.

(32) Lateral cirrus A: filamentous appendages occurring at or near the junction of the head plate and the first segmental plate; associated with the clavae.

(33) Median cirrus: filamentous appendages located internally and/or ventrally to the cephalic papillae.

(34) Cuticular armor: cuticular covering as in some heterotardigrades.

(35) Dorsal segmental plates: unpaired plates located behind the head plate, and in the region of the first and second pair legs.

(36) Head plate: the most anterior cuticular plate, which bears the cephalic appendages.

(37) Median plate 1: the plate that is located between the first and second segmental plates.

(38) Median plate 2: the plate that is located between the second and third segmental plates.

(39) Median plate 3: similar to median plates 1 and 2; located between the third segmental plate and the end plate or pseudosegmental plate.

(40) Caudal plate: the most posterior cuticular plate.
(41) Pseudosegmental plate: an unpaired plate that is located immediately posterior to median plate 3 and anterior to the end plate, as in Pseudechiniscus. Pseudosegmental plate: 0 absent, 1 present, 2 either.

(42) Peduncles.

(43) Clavae: short, rounded appendages that occur at or near the junction of the head plate and the first segmental plate; associated with cirri A.

(44) Digitate legs.

(45) Leg 4 morphology: Spines or papilla found on the 4th pair of legs:

Leg 4 morphology: 0 absent, 1 spine, 2 papilla.

(46) Eyespots: 0 absent, 1 present, 2 either.

(47) Cloaca.

(48) Sexual dimorphism of claws

(49) Sexual dimorphism of the gonopore.

(50) Pharyngeal stripes.
About the Author

Phillip Brent Nichols was born in Dyersburg, Tennessee on the eleventh day of January nineteen hundred and seventy two. The only son of Bruce and Sandi Nichols of Moody, Alabama, he attended the Smith county school system and graduated from Smith County High School (Carthage, Tennessee) in May 1990.

Upon completion of his M.S. degree in May 1999, Brent began working toward a Ph.D. at the University of South Florida in the lab of Dr. James R. Garey. While attending USF Brent instructed several courses in biology. He was an active member of the Graduate Assistants Union and was active in re-establishing the Biology Graduate Student Organization and served as its Vice-President from 2002-2003. He has published several papers from both his M.S. and Ph.D. degree work. Brent will enter the private work force upon graduation.