TARDIGRADE PHYLOGENETIC SYSTEMATICS AT THE FAMILY LEVEL USING MORPHOLOGICAL AND MOLECULAR DATA

TARDIGRADE PHYLOGENETIC SYSTEMATICS AT THE FAMILY LEVEL USING MORPHOLOGICAL AND MOLECULAR DATA

By

CARMEN M. CHEUNG, Hons. BSc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2012) McMaster University
(Biology) Hamilton, Ontario

TITLE: TARDIGRADE PHYLOGENETIC SYSTEMATICS AT THE FAMILY LEVEL USING MORPHOLOGICAL AND MOLECULAR DATA

AUTHOR: Carmen M. Cheung, Hons. B.Sc. (McMaster University)

SUPERVISOR: Dr. Jonathon R. Stone

NUMBER OF PAGES: ix, 137

ABSTRACT

Tardigrade phylogenetic systematic analyses have been conducted using morphological and molecular data; however, incongruencies between results obtained independently with the data types have been found. This thesis contains new morphological and molecular phylogenetic systematic analyses of tardigrades at the family level, building on previous research. The first part involves morphological data, the second part involves molecular data, and the third part involves combined morphological and molecular data. The morphological data include 50 characters for 15 tardigrade families. The molecular data include updated 18S rRNA, 28S rRNA, and COI gene sequences, in two sets; the first set provides the most-extensive representation of tardigrade families and comprises 18S rRNA sequences; the second set provides the most-complete representation of molecular data per species, where available, and involves the concatenation of 18S rRNA, 28S rRNA, and COI gene sequences. Finally, the combined data involves a supermatrix containing morphological and molecular data. The analyses are used to test results from previous systematics research and to contribute more information to tardigrade systematics.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jon Stone for being my patient and supportive supervisor. Thank you for fostering my love for evolutionary biology, and for providing me with countless opportunities to get involved with the research community.

I am deeply indepted to Dr. W.R. Miller, Dr. H. Dastych, Dr. A. Jorgensen, Dr. C. B. Beasley, and Dr. R. Bertolani for their tardigrade expertise, advice about characters, and their willingness to help. Thank you Dr. R.Hochberg for his knowledgeable expertise on Gastrotricha reproduction.

I would like to extend my gratitude to Dr. Brian Golding (McMaster University) for computer cluster access and access to PAUP* 4.0b10 (Swofford, 2003) software for Maximum Parsimony and Neighbor-Joining analyses. I would also like to thank Dr. Brian Golding and Dr. Ben Evans (McMaster University) for inviting me to your weekly lab meetings and for welcoming me as a member of their labs.

Special mentions go to: Patrica Pak, Safiah Mai, and Samantha I. Feder (and the Feder Family) for being my 'cheering section' through my entire journey (I am very thankful for your support and friendship); Dr. Nathan Cooper and Hazel Ann Francis for being great listeners; Dr. Lovaye Kajiura for her inspirational pep talks; iGNITE (John P. Nathan, Dona Nathan, and Brian B.) for the opportunity to lead.

Honourable mentions go to Craig S. Philips for giving me a reason to stay positive.

A huge thank you to my family for their love, encouragement and support. Thank you for being my greatest supporters.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1 :
GENERAL INTRODUCTION

1.1 **An Overview of Tardigrade Biology**

Individuals in the invertebrate phylum Tardigrada, known colloquially as 'water bears' or 'slow-walkers', were recorded first by German priest J. A .E. Goeze in 1773. The phylum was proposed by Ramazzotti in 1962 (Nelson and Marley 2000; Romano 2003). Taxonomically, more than 900 species of tardigrades have been described. Ecologically, they can be found in environments ranging from marine to freshwater to semi-terrestrial, across the globe (Ramazzotti and Maucci 1983; Kinchin 1994; Nelson 2001; Nelson 2002; Jorgensen and Kristensen 2004). Habitually, tardigrades are found in water-filled moss cushions that help facilitate gas exchange for respiration and assist in locomotion (Kinchin 1994). Morphologically, tardigrades appear 'bear-like' and exhibit a slow plodding gait, with a body length typically ranging from 0.25 mm to 0.5 mm (Dewel *et al.* 1993; Nelson and Marley 2000); they are characterized by a thick, cylindrical, bilaterally symmetrical body with four trunk segments, including a head segment with eyes and four pairs of stub-like lobopodic legs that terminate distally in claws or digits (Romano 2003).

Tardigrades are classified into two main classes, Heterotardigrada and Eutardigrada, by the morphological presence or absence of body armor plates, sensory appendages, and claw structures (Kristensen, 1987). A potential third class of tardigrades, Mesotardigrada, contains only an extinct taxon, as the single documented species, *Thermozodium esakii*, was eradicated with the destruction of their only known habitat, in Nagasaki, Japan (Nelson 2002; Romano 2003; Jorgensen, Mobjerg *et al.* 2011). The two tardigrades classes are further classified into 4 orders. The class Heterotardigrada comprises the Orders Arthrotardigrada and Echiniscoidea, and the class Eutardigrada comprises the Orders Apochela and Parachela (Nelson 2002; Romano 2003). Tardigrades are further

subdivided into 21 families and 106 genera (Nelson *et al.*, 2010).

1.2 **Morphology and Classification**

The classification of tardigrades is based on the use of morphological characters, such as cuticle structure and body armor, sensory structures, buccal-pharyngeal apparatus, claw structure or branching, and sexually dimorphic gonopores. The following section presents an introduction to these morphological characters and description of differences among tardigrade classes, orders, families, and genera.

1.2.1 Cuticle Structure and Body Armour

The tardigrade cuticle and its structures often are used in identifying species and for classifying tardigrades to classes, orders, and genera. Each tardigrade possesses a trilayered cuticle, consisting of epicuticle, exocuticle, and endocuticle. Each also undergoes molting (shedding or ecdysis), in which the old layer of epicuticle is removed and a new layer of epicuticle is formed to replace it (Aguinaldo *et al.*, 1997). The new epicuticle layer is formed by the secretion of material from short microvilli, forming separated patches that eventually fuse together to form a continuous layer (Schmidt-Rhaesa *et al.*, 1998). The exocuticle layer is free of chitin and is composed of a layer of cross-linking proteins (Schmidt-Rhaesa *et al.*, 1998). The endocuticle layer is composed of chitin (Kristensen & Neuhaus, 1999; Greven *et al.*, 2005).

Although Heterotardigrada and Eutardigrada species possess cuticle, only heterotardigrades possess an armored cuticle that can be differentiated by the presence of dorsal armor plates (Figure 1.1, left). Eutardigrades possess a thin, smooth, or sculptured (textured) cuticle without any armored plates (Figure 1.1, right).

Heterotardigrade cuticle armor appears as thickened dorsal plates that may be paired and vary in shape,

number, and sculpture (Kristensen, 1987). Dorsal plates among heterotardigrades develop from different layers of the cuticle. Most members in the Echiniscidae possess plates that are sclerotized (hardened with scelortin), while members in the Renaudarctidae and Stygarctidae possess plates that are not (Kristensen, 1987; Jorgensen, 2000). Species in the genus *Pseudoechiniscus*, within the Echiniscidae, constitute an exception, wherein their dorsal plates are not sclerotized (Kristensen, 1987; Nelson *et al.*, 2010).

Heterotardigrade armored plates are used for classification to family, genus, and species (Ramazzotti and Maucci, 1983; Kristensen, 1987; Jorgensen *et al.*, 2011). These plates are differentiated based on their location on the dorsal surface and are referred to as head plate, segmental plates, median plates, and pseudosegmental plates (Figure 1.2, left). The head plate (or cephalic plate) is the anterior-most cuticular plate and often bears cephalic appendages (Kristensen, 1987). Dorsal segmental plates are located posterior to the head plate and are numbered according to their succession, from I-IV (Jorgensen, 2000; Nelson *et al.*, 2010). They can appear paired or unpaired and often are followed by intersegmental ridges or folds and comprise median plates and pseudosegmental plates (Kristensen, 1987). In tardigrade classification, the first trunk dorsal segmental plate is also called the scapular plate, while segmental plate IV is referred to as the caudal or terminal plate (Kristensen, 1987; Jorgensen *et al.*, 2011). Median plates I-III are located between dorsal segmental plates and sometimes appear flanked by pseudosegmental plates when present (Bello & de Zio Grimaldi, 1998; Jorgensen, 2000; Jorgensen *et al.*, 2011). Pseudosegmental plates II-IV appear unpaired and are situated between segmental plates, usually flanking median plates when present. These plates are used to distinguish among genera within the Echiniscidae (Kristensen, 1987, Jorgensen, 2000).

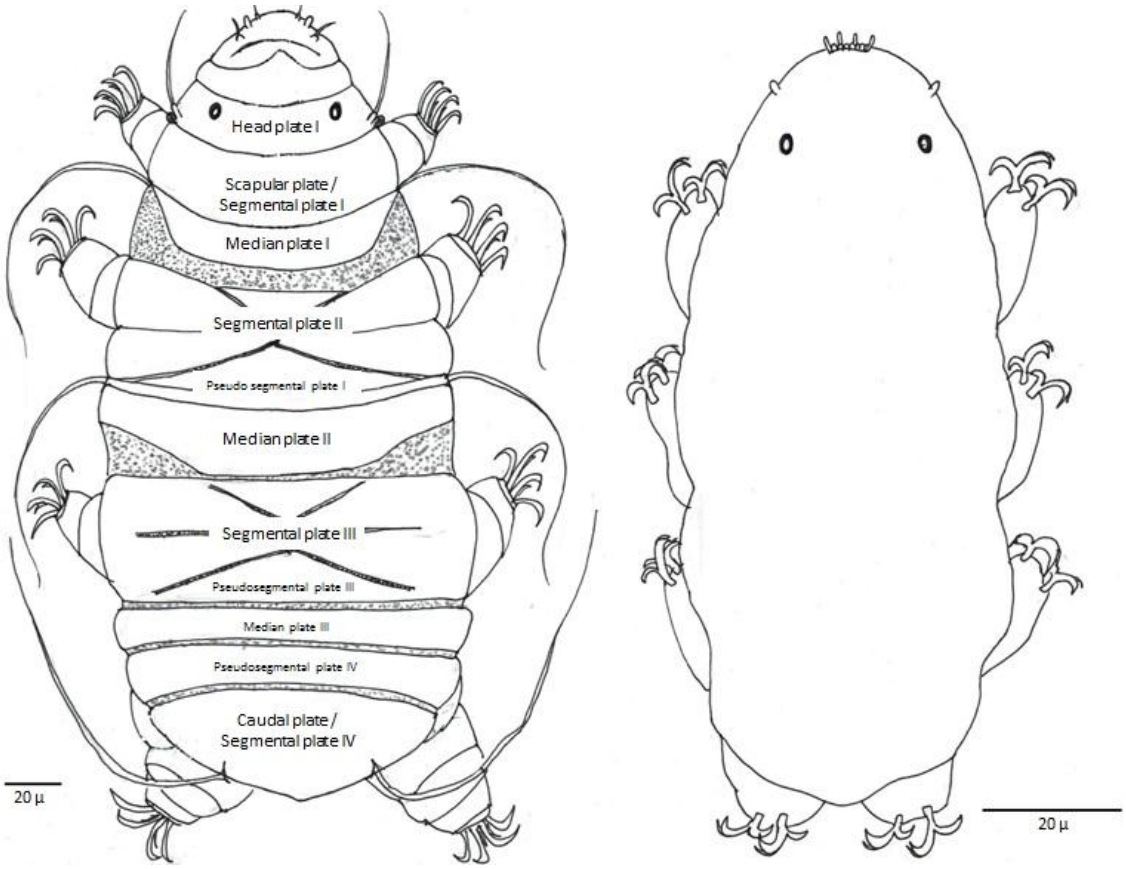


Figure 1.1: Dorsal view of Heterotardigrada and Eutardigrada species. Left: Dorsal view of cuticle armor of *Proechiniscus hanneae* (Echiniscidae, Heterotardigrada). Right: Dorsal view of smooth unarmoured *Hypsibius dujardini* (Hypsibiidae, Eutardigrada). Images modified from Jorgensen *et al.* (2011), Kristensen (1987), Nelson *et al.* (2010), Nelson and Marley (2000).

1.2.2 Cephalic Sensory Appendages

Cephalic sensory appendages are used in classifying tardigrades into heterotardigrade and eutardigrade classes. Heterotardigrades possess cephalic appendages, whereas eutardigrades possess cephalic papillae and peribuccal structures (Figure 1.2). Cephalic appendages, cephalic papillae, and peribuccal structures are sensory appendages that aid in environmental perception and have been proposed as being homologous (see Table 3 for definitions; Schuster *et al.*, 1980; Nelson, 2001; Marley *et al.*, 2011).

In heterotardigrade systematics, cephalic appendages serve as an umbrella-term to describe anteriorly located projections, which include internal buccal cirri, external buccal cirri, clavae, and lateral cirri (or cirri A) (Kristensen, 1987; Nelson, 2001). Buccal cirri are filament-like projections found near the mouth. The lateral cirrus A is a long filamentous projection located between the head and scapular plate (Kristensen, 1987; Jorgensen, 2000; Nelson, 2001). The median cirrus is a short projection found on the anterior end of tardigrades within the order Arthrotardigrada but absent within the order Echiniscoidea (Horning *et al.*, 1978; Kristensen & Higgins, 1984b; Villora-Moreno, 1996; Jorgensen *et al.*, 2011). Three types of clavae are identified: primary clavae, secondary clavae, and tertiary clavae, each appearing short and broad and found in between the head plate and the first segmental plate (Kristensen, 1987; Nelson *et al.*, 2010). Primary clava can be found on the scapular plate, arising from a cirrophore located near the lateral cirrus, while secondary and tertiary clavae can be found on the cephalic plate (Kristensen, 1987).

Cephalic papillae are stub-like cuticular projections that appear on each lateral side of the head (Pilato & Binda, 2010; Nelson, 2001). Peribuccal structures are cuticular structures surrounding the mouth (Schuster *et al.*, 1980; Guidetti *et al.*, 2005). Depending on their shape, they are named peribuccal pappilae, peribuccal

lamellae, peribuccal lobes, or peribuccal papulae. Peribuccal papillae are elongated cuticular projections unique to the family Milnesiidae (Schuster *et al.*, 1980). Peribuccal lamellae form a thickened cuticular ring surrounding the mouth (Schuster *et al.*, 1980; Pilato, 1982; Guidetti *et al.*, 2005). Peribuccal lobes are flat peribuccal structures that extend posteriorly from the buccal orifice (Schuster *et al.*, 1980; Pilato, 1982). Peribuccal papulae are lower profile peribuccal structures that extend posteriorly from the buccal orifice (Schuster *et al.*, 1980; Pilato, 1982).

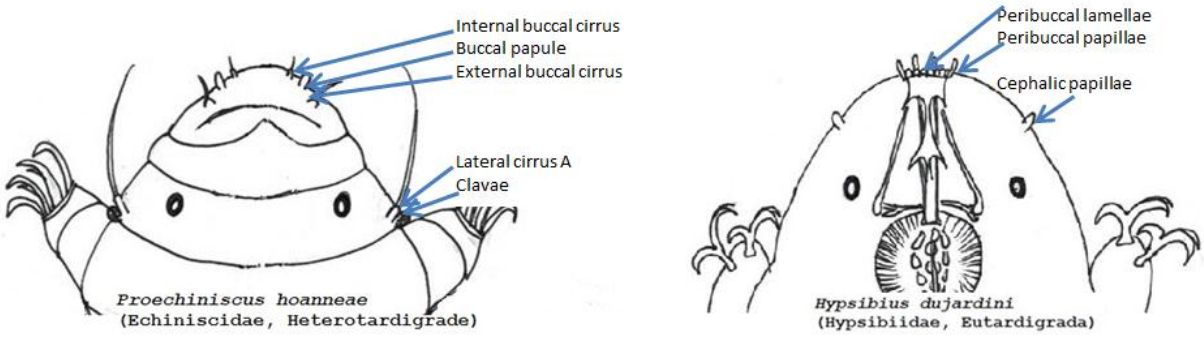


Figure 1.2: Tardigrade head sensory appendages. Left: Heterotardigrade sensory appendages. Right: Eutardigrade sensory appendages. Modified from Nelson et al. (2010)

1.2.3 Buccal-Pharyngeal Apparatus

The buccal-pharyngeal apparatus is the anterior part of the digestive tract, which is followed by the esophagous and stomach and terminates with a cloaca or anus (Miller, 2011). The buccal-pharyngeal apparatus contains the mouth, buccal tube, pharyngeal bulb/tube, and other taxonomically useful ultrastructures (See Figure 1.4) (Guidetti *et al.*, 2005; Nelson, 2002; Jorgensen *et al.*, 2011).

Stylets are supportive structures that span from the mouth along the length of the buccal tube and terminate at club-like furcae (Nelson & Marley, 2000; Balian, 2008). The stylets are connected to stylet supports that attach at the furcae, which connect to the posterior end of the

buccal tube (Schuster *et al.*, 1980; Guidetti *et al.*, 2005). Along the buccal tube, the ventral lamina (or ventral crest) acts as a support structure that runs from the buccal ring to the midregion of the tube (Nelson & Marley, 2000). The pharynx is supported by cuticular structures known as placoids. Depending on the tardigrade class, placoids may appear fused, as in heterotardigrades, or unfused, as in eutardigrades, wherein they appear as microplacoids and macroplacoids (Eibye-Jacobsen, 2001; Marley *et al.*, 2011). Within the pharyngeal bulb and posterior to the placoids is the septulum, a thickened cuticular structure used in classification, although not consistently described in literature (Schuster *et al.*, 1980; Nelson, 2001; Nelson and Marley, 2000). Along the buccal tube and pharyngeal tube are cuticular thickenings called apophyses. An apophysis is an insertion structure for stylet muscles in the buccal-pharyngeal apparatus. They appear as hooks, ridges, or in combination and have been used recently as characters in morphology-based systematics (Pilato and Binda, 2010; Marley *et al.*, 2011).

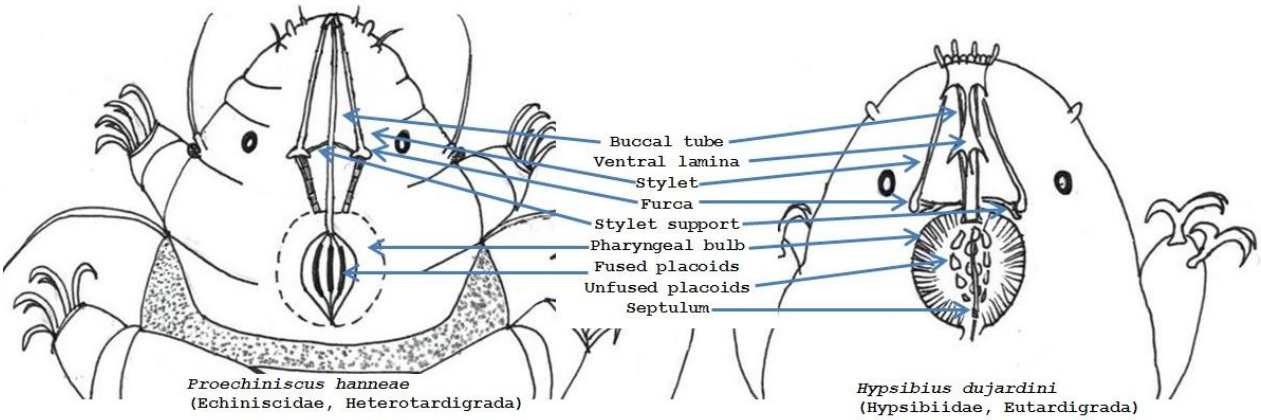


Figure 1.3: Structures within the buccal-pharyngeal apparatus. Left: Heterotardigrade. Right: Eutardigrade. Modified from Kristensen (1987), Nelson *et al.* (2010), and Jorgensen *et al.* (2011).

1.2.4 Claw Structure and Morphology

Claws are used often to classify tardigrades into classes, families, and species. At the class level, heterotardigrade claws appear as multiple, single-branches terminating in digits or toes or directly from the leg, while most eutardigrades possess two double-branched claws that terminate directly from the leg (Nelson & Marley, 2000; Pilato & Binda, 2010). While no systematically-significant claw trends have been described within heterotardigrades, claw features, such as structure and branching sequence, are used to identify eutardigrades.

Each eutardigrade claw comprises a longer primary branch, a shorter secondary branch, and a basal tract in which the two branches join together to connect at the distal end to the leg (Figure 1.4). In some genera, the primary branch may contain accessory points that appear as spikes at the distal end of claw, while the secondary branch does not (Schuster *et al.*, 1980; Nelson & Marley, 2000; Nichols, 2005; Pilato & Binda, 2010). In some species, a simple claw (single-branched claw) may exist, containing a primary branch with accessory points (Schuster *et al.*, 1980; Nelson & Marley, 2000). Other claw features include the lunule, peduncle, and cuticular bar. The lunule appears in some eutadigrades as a cuticular thickening near at the base of the claw, appearing either smooth or dentated (toothed) (Nelson & Marley, 2000; Pilato & Binda, 2010). The peduncle is a trait that can be found within heterotardigrades and eutardigrades, appearing as a narrow stem connecting the basal tract of the claw to the leg and is differentiated by a septum (Horning *et al.*, 1978; Kristensen & Hallas, 1980; Nelson *et al.*, 2010). A claw does not contain a peduncle if the claw has a continuous basal section that is followed by a primary or secondary branch or when the claw separates directly into a primary and secondary branch without a basal section (Nelson & Marley, 2000; Hansen, 2007; Pilato & Binda, 2010). A cuticular bar is a long straight and rigid

structure that may appear in between claws or off to the side (Kristensen, 1987; Bertolani & Rebecchi, 1996; Pilato *et al.*, 2002).

Eutardigrade claw arrangement has been used to describe different genera. The arrangement is based on how the two primary- and secondary-branched claws rest according to the midline of the extended legs. The 2-1-2-1 claw sequence occurs when claws alternate in arrangement according to secondary-primary-secondary-primary branches, while 2-1-1-2 claw sequence occurs when two primary branches are adjacent to one another (Schuster, 1980; Nelson & Marley, 2000). Researchers also have described claw symmetry according to the arrangement of the claws, in reference to the median plane dividing each leg pair. A symmetrical claw arrangement is represented as 2112, while an asymmetrical claw arrangement is represented as 2121 (Guidetti *et al.*, 2005; Pilato & Binda, 2010; Marley *et al.*, 2011).

Eutardigrade claws have been shown to exhibit sexual dimorphism in some species, in which the claw appears modified in the first leg pair in mature males. This modification usually appears after the final molt before sexual maturity and serves the purpose of grasping onto a female during copulation (Pollock, 1970; Rebecchi & Nelson, 1998; Claxton, 1999).

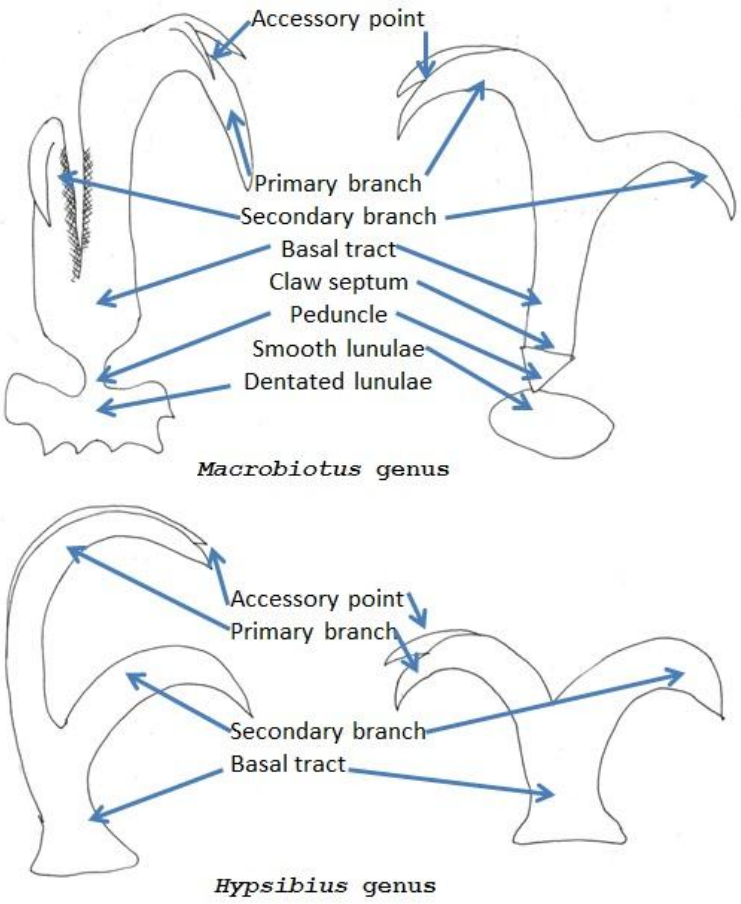


Figure 1.4: Diagrammatic representations of eutardigrade claw structures.
Modified from Nelson and Marley (2000), Nelson *et al.* (2010), and image from W.R. Miller.

1.2.5 Sexual Dimorphic Gonopores

Tardigrades possess secondary sex characters, most notably the difference between the appearances of the male and female gonopore within heterotardigrades. This feature is not found within eutardigrades, as they possess a cloaca that appears similar between the two genders. In heterotardigrades, the male gonopore appears as a small rounded tube, while the female gonopore consists of six cuticular valves that form the shape of a rosette (Figure 1.6; Rebecchi & Nelson, 1998; Nelson *et al.*, 2010).

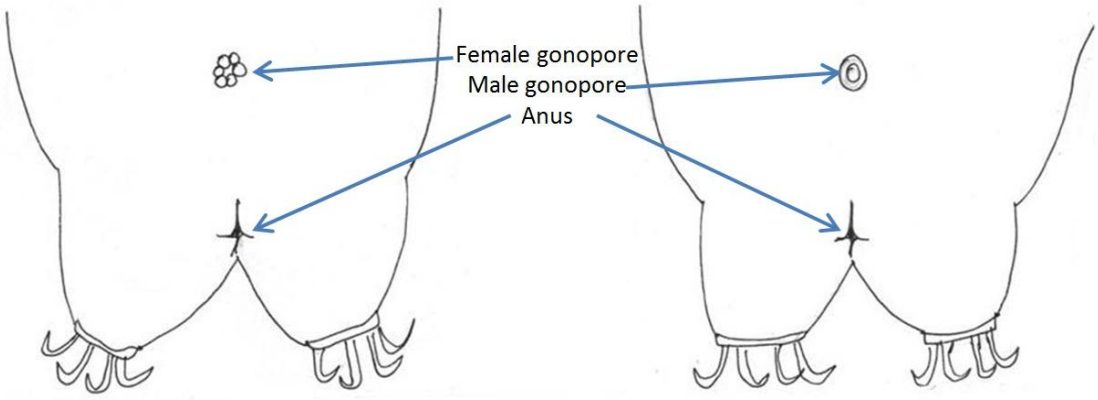


Figure 1.5: Ventral view of heterotardigrade female (L) and male (R) gonopore.
Diagram drawn from photos in Nelson *et al.* (2010).

1.3 **Phylogenetic Systematic Analyses: From Morphology to Molecules**

Tardigrade systematics originated from family-level and species-level taxonomic descriptions based on morphological data. Morphological characters, analyzed with phylogenetic systematic analysis methods, have contributed to our current understanding of evolution within the phylum. Recent systematic studies using molecular 18S rRNA, 28S rRNA, and cytochrome oxidase subunit I (COI) gene sequences have provided complementary data to further resolve tardigrade systematics. The following section will describe studies on tardigrade systematics from morphological data to molecular data.

1.3.1 Morphology-Based Analyses

The use of morphological characters for tardigrade systematics began with Pilato (1969), who revised eutardigrade systematics, first suggested by Ramazzotti (1972), by proposing the use of claw structures to classify eutardigrades into genera and families (Schuster *et al.*, 1980).

Early contributions to heterotardigrade systematics involved species descriptions and proposals of new hierarchal tiers. It began with Kristensen and Higgins (1984a) contribution to the phylogenetic systematics of genera within the subfamily Styraconyxinae (family Stygarctidae), involving morphological descriptions of the clavae, cirri, and claw structures among ten species. Their study described two new species, *Styraconyx nanoqsunguak* and *Styraconyx qwitoq*, with redescriptions of the type species *S. craticulus*, *S. hallasi*, *S. haploceros*, *S. k. kristtnseni*, *S. k. neocaledoniensis*, *S. kristenseni*, *S. paulae*, and *S. sargassi*. Their study postulated evolutionary lines within the genus *Styraconyx*, derived on the basis of sense organs synapomorphies.

Another study by Kristensen and Higgins (1984b) established a new family, Renaudarctidae, within the order Arthrotardigrada, based on the presence of toes with claws and cuticularized dorsal plates. Their study included the description of the type species *Renaudarctus psammocryptus* as well as a discussion of phylogenetic relationship of Renaudarctidae among other heterotardigrade families. Kristensen and Higgins proposed that Renaudarctidae may be allied distinctly with Halechiniscidae and Stygarctidae, based on the plesiomorphic traits it possesses. They also conducted a character-based phylogenetic systematic analysis to infer relationship among families of Heterotardigrades (Figure 1.6).

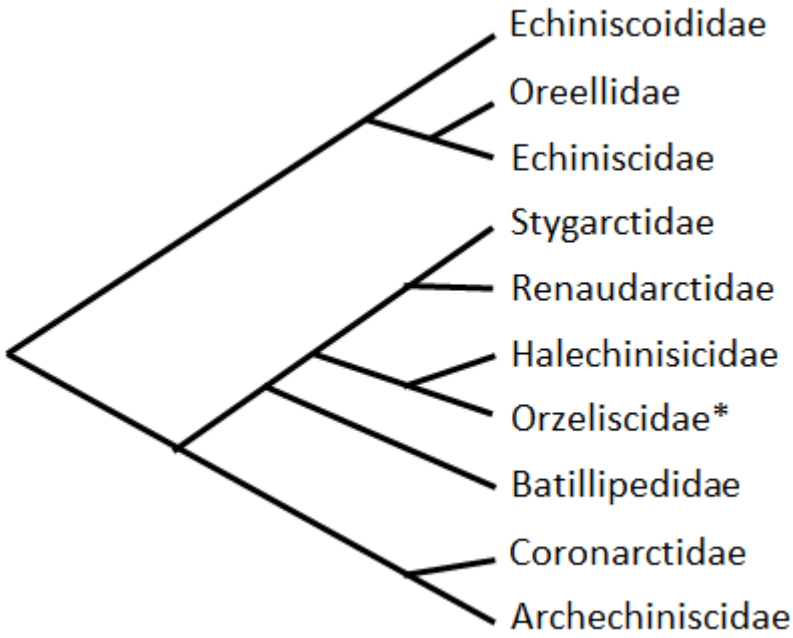


Figure 1.6: Proposed systematics of Heterotardigrada based on morphological characters with addition of Renaudarctidae. *- First proposed by Schulz 1963 but now renamed as the subfamily Orzeliscinae within Halechiniscidae. Reproduced from Kristensen and Higgins (1984b).

Kristensen (1987) presented a cladogram of 12 genera within the heterotardigrade family Echiniscidae, with *Oreella* (Family Oreellidae) as the outgroup. The cladogram was constructed manually using evolutionary lines postulated primarily on dorsal plate structures and the assumption that plates evolved within the Oreellidae family. The cladogram was redrawn three times to visualize distributions of states for three multistate characters (segmentation plate, leg morphology, and buccal apparatus), and 20 morphological apomorphic character states (derived from the sense organs, buccal apparatus, cuticle, and reproductive system). Kristensen suggested that Echniscidae is monophyletic, represented by two main lines consisting of the genera *Echiniscus* and *Pseudechiniscus*. Kristensen postulated that dorsal and ventral plates present in the Heterotardigrada are plesiomorphic character states and that their absences are derived character states. Kristensen further suggested that claw morphology is a conserved character within Echiniscidae, while the flexible buccal tube

probably was developed by convergence at least two times in the family.

Pollock (1995) proposed a new subfamily, Dipodarctidae, within the family Halechiniscidae through the morphological description of two new species, *Dipodarctus borrori* and *Dipodarctus anaholiensis*. Pollock described character states found within Halechiniscidae subfamilies, which were summarized in a manually constructed cladogram. The analysis identified two main groups within Halechiniscidae, comprising Halechinidcinae + Orzeliscinae and Dipodarctinae + Florarctinae + Tanarctinae + Styraconyxinae + Archechiniscinae + Euclavarctinae. The two clades were distinguished by the presence and absence of toe-length patterns, claw features, and the shape of cephalic appendages.

A study by Bello and de Zio Grimaldi (1998) investigated the phylogeny of genera within the family Stygarctidae as well as family-level relationships among Stygarctidae, Renaudarctidae, and Neostygarctidae. They manually constructued a parsimony cladogram, using 30 morphological characters and one outgroup species representing the genus *Halechiniscus* (Halechiniscidae). Characters used involved cuticle plates, cuticle structures (spikes and spines), sensory structures (clavae and cirri), head shape and form, claw structures, leg arrangements, and gender-associated structures. Results indicated monophyly for Stygarctidae when the genus *Neoarctus* was removed. Megastygarctides appeared as the sister-group to all stygarctids, which suggested that they may represent a new subfamily in the Megastygarctidinae. Results also indicated that *Neoarctus* should be placed in a new family, named Neoarctidae. Renaudarctidae appeared as a sister group to Stygarctidae. This suggests that some morphological characters shared between *Renaudarctus* and *Stygarctus* may be the result of convergent evolution. The cladistic analysis of Stygarctidae revealed similar results to Kristensen's (1987) study on Echiniscidae, revealing a number of homoplasous and atavistic character states. Bello and de Zio Grimaldi suggested that homoplasous character states may indicate similar selection operating among closely-

related organisms. The researchers also suggested for future studies to investigate relationships among genera in Halechiniscidae and Batillipedidae, the position of Neoarctidae and Coronarctidae, and the phylogenetic relationships among the Arthrotardigrade families Neostygarctidae, Stygarctidae, and Renaudarctidae.

A study by Jorgensen (2000) followed-up on Kristensen's (1987) study by completing a cladistics analysis of Echiniscidae, using parsimony and a branch-and-bound algorithm. Jorgensen reanalyzed the Kristensen 1987 cladogram (20 characters and Oreellidae outgroup) as well as a cladogram constructed from parsimony, using 35 characters from 12 genera within Echiniscidae, with *Orella* (Oreellidae), *Halechiniscus* (Halechiniscidae), and *Renaudarctus* (Renaudarctidae) as outgroups. The reanalysis resulted in three equally parsimonious cladograms with a consensus tree length of 100 steps. Jorgensen built three cladograms, differing by outgroup combination, using 35 informative characters consisting mainly of data for cuticle plates, sensory structures, claw morphology, and buccal-pharyngeal structures. Two cladograms constructed using two different outgroup combinations, Oreella and Oreella + Halechiniscus, respectively, resulted in two equally parsimonious trees and consensus trees with similar topologies and a length of 91 steps and 104 steps. The analysis with *Oreella*, *Halechiniscus*, and *Renaudarcticus* as outgroups resulted in 7 equally parsimonious cladograms with a consensus tree with length 115 steps. Results from the preferred among the three cladograms showed that Echiniscidae is monophyletic, the *Pseudechiniscus* and *Echiniscus* lines are paraphyletic, and the development of plates from the epicuticle and coloured eyespots are autapomorphic character states for Echiniscidae.

Following Pilato (1969) paper on eutardigrades, Schuster *et al.* (1980) updated the systematic criteria for distinguishing eutardigrade families by introducing characters from the buccal-pharyngeal apparatus. Their study involved the description of 25 characters, including structures from the cuticle, claws, eyes, buccal apparatus, peribuccal opening, buccal tube, and

pharynx, represented by 16 type species from 16 eutardigrade genera belonging to three families. Their observations were summarized in a matrix containing the 25 characters and a key to families and genera of the Eutardigrada (Schuster *et al.*, 1980).

A study by Guidetti, Rebecchi, and Bertolani (2000) investigated cuticle structures, using light and electron microscopy for 11 species within the Macrobiotidae. The researchers found pillars in the epicuticle for the genera *Murrayon* and *Dactylobiotus*, while species within the genera *Macrobiotus*, *Richtersius*, and *Xerobiotus* lacked pillars. Observations suggested that the lack of epicuticle pillars were an atavistic synapomorphy for the Macrobiotidae, which lead to the proposal of two new subfamilies, Macrobiotinae and Murrayinae. Observations were visualized with a two-branched diagram depicting the assumed phyletic lines of the two proposed subfamilies and their associated major morphological characters (i.e., claw symmetry, loss of pillars in the epicuticle, claw shape, and ventral hook on the buccal tube).

A follow-up study on Macrobiotidae was published by Guidetti and Bertolani (2001), which included additional morphological characters from the genera *Pseudodiphascon* and *Calcarobiotus*. Their study was the first to combine cladistics and morphological apomorphies to investigate eutardigrade systematics. Results from their manually constructed parsimony cladogram identified two phyletic lines within Macrobiotidae, supporting the subfamilies Macrobiotinae and Murrayinae. Their investigation found that relationships among genera within Murrayinae were resolved, while relationships among the genera in Macrobiotinae were unresolved. Their paper suggested for future studies on eutardigrade systematics applying cladistic methods and including additional morphological characters (Guidetti and Bertolani, 2001).

1.3.2 Molecular-Based Analyses

While morphological descriptions were being used for tardigrade systematics, another group of studies was

emerging: investigating tardigrade systematics using molecular sequences. Early molecular analyses involved investigating the monophyly of the tardigrade phylum and its placement among other invertebrate phyla, using 18S rRNA. Later, studies on tardigrade systematics involving additional molecular sequences, such as 28S rRNA and cytochrome oxidase subunit I (COI), expanded phylogenetic applications at the class, order, and genus levels.

Molecular-based phylogenies at the phyla-level

Morphological observations had indicated that tardigrades shared close affinity with arthropods, in particular to their shared possession of a cuticle, lobopodia terminating distally in claws, terminal mouths, and a caudal segment associated with the last pair of legs (Kinchin 1994; Romano 2003). Other studies suggested that onychophorans were closely related to tardigrades, due to shared morphological similarities with the fossilized Cambrian lobopod *Aysheaia* (Garey *et al.*, 1996).

The monophyly of the phylum Tardigrada and a close affinity to the phylum Arthropoda was first suggested in a study by Giribet *et al.* (1996), involving 18S rRNA and 18S rDNA sequences from *Macrobiotus hulfelandi* and 24 metazoan taxa (mainly protozoa). Giribet *et al.* constructed phenograms and cladograms, using neighbour-joining (NJ) and maximum parsimony (MP) techniques. Results from both analyses showed a close relationship between *M. hulfelandi* and the Arthropoda.

Another study investigating the tardigrade-arthropod association was completed by Garey *et al.* (1996), involving 18S rRNA sequences from eutardigrade *Macrobiotus aerolatus* and metazoan species from Arthropoda, Annelida, Gastrotricha + Platyhelminthes, Mollusca, Nematoda, Rotifera, and some Deuterostomes. Phenograms and cladograms were constructed using neighbour-joining (NJ) and maximum parsimony (MP) techniques. Results from both analyses showed that the Arthropoda and Tardigrada were sister taxa, which reinforced the morphological similarities shared between them.

A similar study on tardigrade-arthropod associations was completed by Moon and Kim (1996), using 18S rRNA sequences from tardigrades (*Hypsibius* sp.), nematodes (*Caenorhabditis elegans*), arthropods (*Eurypelma californica*, *Artemia salina*, *Tenebrio molitor*), annelids (*Chaetopterus* sp.), molluscs (*Cryptochiton stelleri*), sipunculids (*Golfingia gouldii*), and nemertines (*Cerebratulus lacteus*). Results from neighbour-joining and maximum parsimony techinques suggested that tardigrades diverged before protostomes, therefore suggesting that tardigrades share close affinity with neither annelids nor arthropods.

A study by Aguinaldo *et al.* (1997) provided 18S rRNA support for an ecdysozoan (molting) clade composed of arthropods, tardigrades, onychophorans, nematodes, nematomorphs, kinorhynchs, and priapulids. Aguinaldo *et al.* used four reconstruction techniques, including Jukes-Cantor distances, Kimura two-parameter distances, paralinear (LogDet) distances, and maximum parsimony (MP). Results from all showed two monophyletic groups, one contained molting animals, while the other contained non-molting, articulate, lophotrochozoans, such as brachipods, molluscs, oligiochaetse, polychaetes, and rotifers. The monophyly of an ecdysozoan clade suggested that the ability to undergo ecdysis may have evolved once within the protostomes.

Despite molecular evidence suggesting an ecdyosozoan origin for tardigrades and arthropods in Aguinaldo *et al.* (1997), a follow-up morphological-based paper by Schmidt-Rheasa *et al.* (1998) contained a discussion about an ecdysozoan (panarthropod+cycloneurolia) and articulate (panarthropod+annelida) hypothesis. Although molecular and morphological evidence supported an ecdysozoan origin for arthropods and tardigrades, some morphological evidence also supported arthropod and tardigrade origins within articulates. Their paper concluded that further morphological- and molecular-based studies were needed to investigate the origins of Panarthropoda (Euarthropoda, Onychophora and Tardigrada).

A study by Mallatt *et al.* (2004) investigated ecdysozoan phylogeny, using 18S and 28S rRNA sequences from 35 taxa. The analytical techniques used were minimum-evolution analysis of LogDet-transformed distances and likelihood-based Bayesian inference. Analyses suggested monophyly of the clade Panarthropoda within Ecdysozoa, while the divergence between arthropods, onychophorans, and tardigrades remained unresolved.

Campbell *et al.* (2011) used expressed sequence tags (ESTs) and microRNAs (miRNAs) to resolve the phylogenetic position of the Tardigrada within Ecdysozoa. The EST analysis, involving 49023 amino acid sites from 255 proteins and miRNA libraries, was analyzed in a Bayesian framework, using a site heterogeneous mixture model (CAT-GTR+Γ). Site-stripping was used to estimate the substitution rate at various sites, then fast-evolving sites were removed, and, finally, the remaining sites were aligned. A signal dissection analysis was completed to compare the signal at slow- and fast-evolving sites. Campbell *et al.* also conducted a taxon-pruning experiment to evaluate the robustness of their EST results and to see whether long-branch attraction (LBA) could be reduced or emphasized between Tardigrada, Onychophora, and Nematoda. Results supported a monophyletic Panarthropoda including Tardigrada and suggested a sister group relationship between Arthropoda and Onychophora. Campbell *et al.* concluded that past molecular studies showing a Tardigrada + Nematoda group were hampered by long-branch attraction.

Molecular-based phylogenies at the class-level

While some studies were conducted in an attempt to resolve whether the Tardigrada is positioned within Ecdysozoa or Articulata, other tardigrade systematists were trying to determine monophyly within its two classes, Heterotardigrada and Eutardigrada. Heterotardigrada and Eutardigrada were first described as taxonomic classes based on two genera, in the 1830s, *Echiniscus* (Heterotardigrade) and *Macrobiotus* (Eutardigrade), distinguished by the absence or presence of cuticle armor (Marley *et al.* 2011).

Cladistic evidence of the two classes was seldom studied, until Jorgensen and Kristensen (2004) published a paper on the monophyly of the class Heterotardigrada, using 18S rRNA sequences from three heterotardigrade and eight eutardigrade species. They constructed phylogenies using maximum parsimony (MP) and maximum likelihood (ML) techniques with a General Time Reversal + Gamma + Proportion Invariant (GTR+G+I) model of evolution, which was estimated with 100 bootstrap replicates. Both MP and ML analyses resulted in similar topologies supporting a monophyletic Heterotardigrada and eutardigrade families Macrobiotidae and Hypsibiidae. Jorgensen and Kristensen suggested for future studies to resolve phylogenetic relationships within the eutardigrade order Parachela with additional taxon and gene sampling from more families.

Molecular-based phylogenies at the order-level

Sands *et al.* (2008b) investigated monophyly of the tardigrade families Hypsibiidae and Macrobiotidae, by finding support for dividing the Order Parachela into three superfamilies, Isohypsibiodea, Macrobiotoidea, and Hypsibioidea (which was complemented by a study by Marley *et al.*, 2011). Their study used 18S rDNA sequences from 343 individuals, which were analyzed to construct cladograms, using maximum parsimony (MP) and Bayesian inference (Bi) techniques with GTR+I+Γ, resampling 5000 trees. Both MP and Bi produced similar consensus topologies, with rooted phylogenies containing two distinct tardigrade classes, Heterotardigrada and Eutardigrada. Results also supported the division of Heterotardigrada into the orders Arthrotardigrada and Echiniscoidea. However, the analysis yielded a low support (posterior probability, pp 0.77) for the break-down of Echiniscoidea into its families, involving Echiniscoididae and the sister-clades Oreellidae and Echiniscidae. Results within Eutardigrada revealed that the classes Apochela and Parachela are reciprocally monophyletic. Their results also supported the three superfamilies within Parachela.

Molecular-based phylogenies at the family-level

Kiehl *et al.* (2007) investigated relationships within the Eutardigrade family Hypsibiidae and the taxonomic uncertainty of *Hypsibius klebelsbergi*, using 18s rDNA sequences from seven newly sequenced tardigrade species (*Hypsibius klebelsbergi*, *Hypsibius* cf. *convergens* 1, *Hypsibius* cf. *convergens* 2, *Hypsibius scabropygus*, *Hebensuncus conjungens*, *Isohypsibius cambrensis*, and *Isohypsibius granulifer*) and ten previously published sequences from eutardigrade species and species groups. The researchers used Neighbour Joining (NJ), Minimum Evolution (ME), and Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and Maximum Parsimony (MP). All four techniques divided Hypsibiidae into three groups: *Ramazzottius-Hebesuncus* clade, *Isohypsibius* clade, and *Hypsibius* clade. Results from NJ, ME, and MP could not resolve relationships among the three clades, while UPGMA suggested sister group relationships between the *Isohypsibius* and *Macrobiotus* + *Ramazzottius* + *Hebesuncus* clades (bootstrap value, bv 83%) and the *Ramazzottius* – *Hebesuncus* and *Macrobiotus* clades (bv 99%). Kiehl *et al.* suggested that the close relationship between the clades *Macrobiotus* and *Ramazzottius* + *Hebesuncus* was not consistent with morphological systematics between the two genera, in which characters from the bucco-pharyngeal apparatus suggested instead a close affinity between *Macrobiotus* and *Isohypsibiius*. Although researchers for a previously published paper on *Hypsibius klebelsbergi* had concluded taxonomic uncertainty for its possession of characters intermediate to both genera, *Isohypsibius* and *Hypsibius*, results from Kiehl *et al.* confirmed its placement within the *Hypsibius* genus. Despite including additional 18s rDNA sequences of Hypsibiidae species, the results still suggested polyphyly within Hypsibiidae.

Molecular-based phylogenies at the genera-level

A study by Guidetti *et al.* (2009) involved 18S rDNA sequences (five new) from 19 species and COI mtDNA sequences (15 new) from 20 species, representing a total of seven families. The 18S rDNA data were analyzed using minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) techniques. The COI mtDNA analysis was performed by first translating mtDNA

sequences into amino acid sequences, then protein-based analyses were conducted by neighbour-joining, MP, and ML techniques. The 18s rDNA and COI protein analyses produced similar similar topologies, thus two diagrams were used to summarize the results. Results corresponded with current systematics of Echiniscidae and confirmed the orders Apochela and Parachela as sister-groups. Results also showed that the *Ramazzottius* genus, traditionally classified within the family Hypsibiidae, was more related to genera in the family Macrobiotidae than to genera in the Hypsibiidae, and the family Macrobiotidae and genus Macrobiotus were not monophyletic. The 18S rDNA and COI mtDNA analyses showed a new lineage within *Macrobiotus*, corresponding to the 'richtersi-areolatus group', as well as the identification of a new genus, *Paramacrobiotus*.

Jorgensen *et al.* (2010) conducted a study on Arthrotardigrada, using 18S and 28S rRNA sequences from 29 taxa, consisting of 16 arthrotardigrades (ten subfamilies and six families) and three species from Echiniscoidea (*Echiniscus sigismundi*, *Echiniscus testudo*, *Pseudechiniscus islandicus*). Analyses were conducted using maximum parsimony (MP) and Bayesian inference (Bi). Results suggested monophyly between the classes Heterotardigrada and Eutardigrada; however, the heterotardigrade order Echiniscoidea appeared monophyletic while nested within the paraphyletic Arthrotardigrada. The eutardigrades were divided into the orders Apochela and Parachela, which supported previous molecular analyses. Results from the study did not support the classification of the family Halechiniscidae, which was polyphyletic. The Bi analyses did not support the subfamily Styraconyxinae as part of Halechiniscidae, while *Archechiniscus* was sister group to the remaining halechiniscids and *Orzeliscus* was placed in an unresolved relationship, basal to the remaining halechiniscids. Results included affinity between *Echiniscus* and *Pseudechiniscus* and affinity between the halechiniscid subfamilies Florarctinae and Dipodarctinae but excluding Tanarctinae. Results showed low support for Halechiniscidae clades and incongruences among different

methods. Results also supported the division of Parachela into four superfamilies, one newly proposed superfamily Eohypsibiidae, and three that had been suggested originally by Sands *et al.* (2008).

The most-recent molecular-based analyses, conducted by Guil and Giribet (2012), involved the use of three markers 18S rRNA, 28S rRNA, and COI, for 42 individuals in 16 species, from 12 genera, and five families from the classes Heterotardigrada and Eutardigrada, as well as additional sequences from Genbank. They completed five sets of analyses: (1) 18S rRNA; (2) 28S rRNA; (3) 18S + 28S rRNA; (4) 18S rRNA, 28S rRNA, and COI sequences; (5) combined (18S rRNA, 28S rRNA, 18S rRNA + 28S rRNA, and 18SrRNA + 28S rRNA + COI sequences). Parsimony and maximum-likelihood (ML) analyses were conducted using a General Time Reversal model for nucleotide substitution with the Γ correction for rate heterogeneity (GTR+Γ), with a primary search for 20 ML trees, and nodal support evaluated with 100 bootstrap replicates. Results supported the monophyly of both classes, Heterotardigrada and Eutardigrada. In the class Heterotardigrada, the order Arthrotardigrada and family Echiniscidae were not monophyletic because the genus *Oreella* (Oreellidae, Echiniscoidea) appeared closely related to some echiniscid genera (i.e., *Pseudechiniscus*, *Testechiniscus*, and *Corechiniscus*), and the arthrotardigrade subfamilies and genera did not form a clade. The order Echiniscoidea was well supported, but several genera of Echiniscidae were not monophyletic, in which species *Pseudechiniscus islandicus* appeared closest to *Cornechiniscus lobatus*, or *Echiniscus* sp. appeared as sister group to *Pseudechiniscus facettalis*. The remaining *Echiniscus* species formed a sister clade to *Testechiniscus spitbergensis*. Eutardigrade monophyly was controversial because the order Apochela was represented by several Milnesium species and was sister group to the class Heterotardigrada in many outgroup combinations. The order Parachela was monophyletic in all analyses, with high bootstrap support. The Parachela superfamilies (Hypsibioidea, Isohypsibioidea, and Macrobiotoidea) were supported well. Among eutardigrade families, only three,

Milnesiidae, Calohypsibiidae, and Murrayidae, out of six families analyzed, were monophyletic. Neither Hysibiidae nor its subfamilies were monophyletic. *Richtersius coronifer* appeared basal to Macrobiotoidea and Isohypsibiodea and *Macrobiotus hufelandi* were basal to Isohypsibiodea. Results from the five data combinations differed little from each other. In the analysis involving 18SrRNA + 28SrRNA + COI, results showed that parsimony and ML agreed in topology. Results showed that Heterotardigrades and Eutardigrades were monophyletic. Within heterotardigrades, the order Echiniscoidea and family Echiniscidae were monophyletic, while the order Arthrotardigrada and its families were polyphyletic. The composition of Hypsibioidea was expanded to include Astatumen. Within Macrobiotoidea, the family Murrayidae was monophyletic. The genus *Bertolanius* was sister to or nested within Macrobiotoidea, depending on the information used. Within Heterotardigrada, no family was strictly monophyletic, as genera from different families and orders were allied closely. Within Arthrotardigrada, only Echiniscidae was monophyletic in the 28S rRNA data cladogram. The rest of the analysis placed *Oreella* with echiniscid genera, disrupting the monophyly of the family. Among eutardigrades, only Milnesiidae and Murrayidae were monophyletic. Macrobiotidae also was paraphyletic with respect to Murrayidae. The subfamilies within Hypsibiidae were not monophyletic (Hypsibiinae, Diphasconinae, and Itaquasconinae), suggesting that their taxonomy should be re-examined. Results showed that many genera were not monophyletic, which may be caused by low genetic variability of genes selected at this taxonomic level. The study aimed to evaluate the relationships and monophyletic status at higher taxonomic levels and the influence of data completeness in the measures of stability and support for different clades.

1.3.3 Analyses combining morphology and molecular data

As researchers started to construct molecular-derived systematics and compare results to morphologically-

derived systematics, they found inconsistencies and a lack of resolution at the family and genera levels. New studies, involving the combination of morphological and molecular data, started to be published in attempts to reconcile and resolve problems.

Garey *et al.* (1999) compared the congruency between morphological and molecular data, by analysing 18S rRNA from two newly sequenced species, *Thulinia stephaniae* and *Echiniscus viridissimus*, combined with sequences from four tardigrade groups and eight outgroup species. Garey *et al.* used Neighbor Joining (NJ), Maximum Parsimony (MP), and Maximum Likelihood (ML), which produced similar topologies, supporting the monophyly of tardigrades and placing them as sister group to arthropods. Tardigrades also appeared to be allied closely to the two ecdysozoan phyla Priapulida and Nematomorpha. Results confirmed Parachela and Apochela as sister groups and the one heterotardigrade representative, *Echiniscus*, appeared as the most basal tardigrade lineage, which agreed with previous morphological analyses.

To compare congruency between morphological and molecular data, morphological character states were distributed onto the phenograms and cladograms. Results indicated that molecular analyses were congruent with previous morphological analyses, in particular with the absence or presence of sensory appendages, structure of buccal aperture, and claw branching morphology.

In another study involving combined data, Guidetti *et al.* (2005) compared morphological- and molecular-derived topologies to resolve relationships within the Eutardigrade family Macrobiotidae. The study involved a maximum parsimony (MP) morphological analysis using 15 taxa and 17 characters, and molecular analyses using cytochrome oxidase subunit 1 (COI) mtDNA sequences constructed using neighbour joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (Bi) techniques, involving seven species from Macrobiotidae and one Eohypsibiidae species as the outgroup. Results from the morphological and molecular analyses returned a monophyletic subfamily Murrayinae,

while relationships among the genera within the subfamily Macrobiotinae were unresolved. Guidetti *et al.* also noticed a lack of synapomorphic character states between Macrobiotinae and Murrayinae, the uncertain position of *Amphibolus*, the presence of multiple synapomorphic character states for Murrayinae, and a close relationship between the genera *Dactylobiotus* and *Murrayon*. All of these observations led Guidetti *et al.* to propose a division of the Macrobiotidae family into a new family, the Murrayidae. Guidetti *et al.* also discussed that the lack of resolution within *Macrobiotus* and other unresolved nodes may be influenced by large genetic distance between species, resulting in topologies/classifications different from the proposed systematics.

Following Guidetti *et al.* (2005), Nichols *et al.* (2006) studied tardigrade phylogeny at the family-level by conducting morphological and molecular analyses. The morphological analysis was conducted for 15 tardigrade families (seven Eutardigrada and eight Heterotardigrada) and three outgroup species (Kinorhyncha, Gastrotricha, and Loricifera), using 50 morphological characters and Maximum Parsimony (MP) techniques. The molecular analysis was constructed for eight tardigrade species (seven Eutardigrade, one Hetertardigrade) representing five families and seven outgroup species, using 18S rRNA sequences and Neighbor Joining (NJ) and MP techniques, estimated with an unknown number of bootstrap replicates. Results showed that Heterotardigrada and Eutardigrada are monophyletic sister groups (18S rRNA analysis; bootstrap value, bv 98%). Within the heterotardigrades, Oreellidae was the most basal family, followed by Halechiniscidae, Stygarctidae, and Renaudarctidae. The heterotardigrade orders Arthrotardigrada and Echiniscoidea appeared as non sister groups, and the Arthrotardigrada was paraphyletic with members of Echiniscoidea. Within the eutardigrades, Eohypsibiidae was sister clade to Macrobiotidae + Hypsibiidae (bv 60%). Necopinatidae appeared basal to the monophyletic Parachela, forming a monophyletic sister clade to Apochela, with Milnesiidae as the most basal eutardigrade family. The only inconsistency between the

analyses was that Calohypsibiidae appeared as sister group to Hypsibiidae in the 18S rRNA analyses, while the morphological analysis suggested that the relationship was unresolved.

In 2011, Jorgensen *et al.* published a paper evaluating the phylogeny and evolution of the Heterotardigrade family Echniscidae by comparing the congruencies between morphological and molecular data. The morphological analysis used 51 characters modified from Jorgensen (2000) and Kristensen (1987), representing 23 species. The molecular analysis was constructed using ten species representing 17 genera from Echiniscidae and four genera (*Batillipes*, *Florarctus*, *Echiniscoides* and *Oreella*) representing outgroup taxa. Multiple data sets were created using 18S, 28S rRNA, COI mtDNA sequences and analyzed with maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (Bi). Results confirmed Echiniscidae as a monophyletic clade with the combined data set excluding COI data (i.e., morphology, 18S, and 28S). Five species of *Echniscus* appeared monophyletic and as a sister group to *Testechiniscus* (the COI analysis returned *Echiniscus* as paraphyletic). *Parechiniscus* was inferred to be sister group to all other echinsicid taxa, a phylogenetic position corresponding well to its weakly sclerotized doral plates. Echiniscoidea did not appear monophyletic, which contradicted results from previous studies involving the phylogenetic systematic analyses of Arthrotardigrades (Jorgensen *et al.*, 2010; Sands *et al.*, 2008b). Results from the morphological analysis returned an unresolved cladogram with low bootstrap values. Echiniscoidea was inferred to be monophyletic; however, the genus *Echiniscoides* appeared as sister group to Echiniscidae. Within Echiniscidae, the genera *Echniscus* and *Hypechiniscus* were monophyletic, except for *Pseudechiniscus*, which appeared polyphyletic. *Parechiniscus* and *Bryodelphax* were situated close to the root in the echiniscid lineage, and the higher nodes were unresolved except for *Echiniscus* + *Testechiniscus* (according to the Bi analysis). Analyses did not yield monophyly of Echiniscoidea, however they supported an *Oreella* + Echiniscidae clade from combined mixed

morphology and molecule analyses. Echiniscidae appeared monophyletic in morphological analysis, 18S, 28S, and combined 18S and 28S rRNA data analyses. The COI analysis returned an unresolved phylogeny. In the morphological analysis, synapomorphic character states of Echiniscidae from character tracing included unpaired dorsal segmental plates I and IV, undivided median plate, and black eye spots. *Pseudechiniscus* was polyphyletic (two sequences of *P.islandicus*, one from Faroe Islands and one from Iceland, did not group together with *P. suillus*).

Marley *et al.* (2011) utilized morphological characters, such as claw morphology and apophysis insertion of stylet muscle (AISM; Table 2.2), to reassess paraphyly in the class Parachela (Eutardigrada) and find morphological evidence supporting current molecular-based systematics. Although morphological apomorphic character states were lacking in previous studies, sufficient molecular evidence was available from studies by Sands *et al.* (2005), Jorgensen *et al.* (2010), and Guil and Giribet (2012). Marley *et al.* re-evaluated morphological and molecular data and found support for six new taxa within Parchela: the families Isohypsibiidae and Ramazzottidaeand superfamilies Eohypsibioidea, Hypsibioidea, Isohypsibioidea, and Macrobiotoidea. The revision of the higher taxa in Parachela and the introduction of the four superfamilies was based on support from combinations of structural differences in claws and the AISM, from which Marley *et al.* identified three characters: claw branch symmetry versus asymmetry; the basal claw section rigidly joined to secondary and primary branches versus a rigid basal section or secondary branch and a flexible primary branch; and the development of ride- or hook-like AISM which may be modified via a ventral lamina. The current proposed systematics of Parachela is presented in Figure 1.7, with the presence of four new superfamilies and two new families.

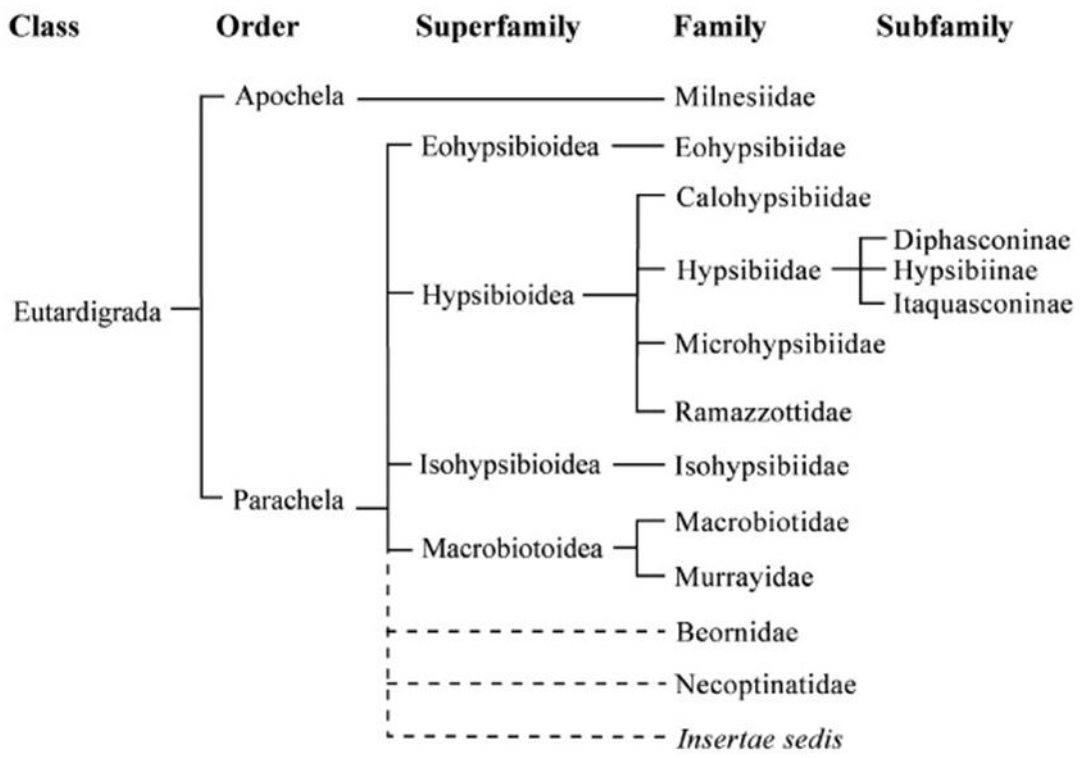


Figure 1.7: Current proposed systematics of Parachela with the additiona of new taxa.
Reproduced from Marley et al., 2011.

1.4 Research Goals, Hypotheses and Projects

The goals of this thesis are to conduct phylogenetic systematic analyses of the phylum Tardigrada at the family-level, using three data sets, morphological, molecular, and combined morphological & molecular, to evaluate current published tardigrade systematics. The three projects were modelled after the three studies Nichols *et al.* (2006), Jorgensen *et al.* (2011), and Guil and Giribet (2012).

The three projects are aimed to: (1) test for strict monophyly of families within Tardigrada, by providing greater taxon sampling at genera and species levels; (2) enable proposals for phylogenetic relationships among families and compare congruency to current systematics; and (3) enable character mapping for combined data analysis and test congruency between current systematics and taxonomic keys.

Formally, these aims were carried out as the following:

1. The first project (Chapter II) involved a comprehensive review & summary of tardigrade morphological data. Characters used by Nichols *et al.* (2006) were evaluated and modified if necessary. Analyses were conducted to compare results obtained using a revised taxon-character matrix to results obtained by Nichols *et al.*

2. The second project (Chapter III) involved molecular analyses of two data sets: (1) concatenated 18S rRNA + COI mtDNA + 28SrRNA seqences and (2) 18S rRNA. Analyses were conducted to compare the results using a revised taxon-character matrix to results obtained by Jorgensen *et al.* (2011) and Guil & Giribet (2012).

3. The third project (Chapter IV) involved phylogenetic systematic analyses of combined morphological and molecular (18S rRNA + 28S rRNA + COI mtDNA sequences) data. Character state distributions were examined to test the congruency between molecular-based systematics and the taxonomic keys used to classify tardigrades at the species level. Analyses were conducted to compare results obtained using a revised taxon-character matrix to results obtained by Jorgensen *et al.* (2011) and Marley *et al.* (2011).

Chapter 2 :

MORPHOLOGICAL PHYLOGENETIC SYSTEMATICS OF TARDIGRADES AT THE FAMILY-LEVEL

2.1 **Abstract**

A table of morphological characters compiled from a published systematic study has been reviewed and details therein revised to form a data matrix, which was analyzed to construct one family-level phenogram and three family-level cladograms. Results included monophyletic Heterotardigrada and Eutardigrada; a non sister group relationship between the heterotardigrade orders Arthrotardigrada and Echiniscoidea; a paraphyletic Parachela (Echiniscidae), requiring the exclusion of Milnesiidae (order Apochela; Echiniscidae); Oreellidae + Echniscoididae as the most basal heterotardigrade clade (all four analyses); eutardigrade clades composed of **(**Eohypsibiidae + Macrobiotidae + Milnesiidae**)**, **(**Calohypsibiidae + Microhypsibidae**)** (neighbour-joining and Bayesian inference analyses), and an unresolved placement of Hypsibiidae.

2.2 **Introduction**

Nichols *et al.* (2006) presented the most-recent evaluation of tardigrade systematics at the family-level, using 50 morphological characters from 15 families (eight heterotardigrades and seven eutardigrades), one species incertae sedis, and three outgroup members (summarized in Table 2.1). The researchers constructed morphological-based cladograms using maximum parsimony (MP) and summarized results with one majority rule consensus tree. Their study showed that Heterotardigrada and Eutardigrada are monophyletic sister groups (bootstrap value, bv 98%). Within Heterotardigrada, Oreellidae appeared as the most basal family followed by the divergence of Halechiniscidae, Stygarctidae, and Renaudarctidae. The Heterotardigrade classes, Arthrotardigrada and Echiniscoidea, did not appear as sister groups, and Arthrotardigrada appeared paraphyletic, containing some families from Echiniscoidea. Within Eutardigrada, Eohypsibiidae appeared as a sister group to Macrobiotidae + Hypsibiidae (bv 60%). Necopinatidae appeared as the most basal family within the Parachela, which together formed a sister group to Apochela (with its only family Milnesiidae). Although the molecular component of their study suggested that Calohypsibiidae is a sister group to

Hypsibiidae, the relationship between the two families using morphological data remained unresolved.

In this study, we reassessed the 50 characters used in Nichols *et al.* (2006). We constructed a table in which 56 characters were summarized and defined, including six new characters, six original characters (separate genital pore & anus; buccal tube apophyses; pharyngeal tube apophyses; eyespots; cloaca; pharyngeal stripes) that were identified to be omitted from subsequent analysis, 15 original characters that were retained outright, and 29 characters that were recoded (Table 2.2). These data were translated into a data matrix, which was analyzed to construct three branching-diagram topologies, using neighbor-joining (NJ), maximum parsimony (MP), and Bayesian inference (Bi) techniques.

In contrast to Nichols *et al.* (2006), our analysis included constructing phylogenies using distance (e.g., NJ) and Bayesian (e.g., Bi) methods, using Phylogenetic Analysis Using Parsimony (PAUP*) 4.0b10 (Swofford, 2003) and mrbayes-3.1.2 (Ronquist *et al.*, 2003) software, in addition to the parsimony method.

Table 2.1: Summary of Tardigrade families (and Outgroups) and their classifications

No.	Family	Order	Class
1	Macrobiotidae	Parachela	Eutardigrade
2	Eohypsibiidae		
3	Calohypsibiidae		
4	Necopinatidae		
5	Microhypsibiidae		
6	Hypsibiidae		
7	*Apodibius (Incertae sedis)*		
8	Milnesiidae	Apochela	
9	Halechiniscidae	Arthrotardigrada	Heterotardigrade
10	Stygarctidae		
11	Renaudarctidae		
12	Coronarctidae		
13	Batillipedidae		
14	Echiniscoididae	Echiniscoidea	
15	Echiniscidae		
16	Oreellidae		
17	Loricifera	Outgroup	
18	Kinoryncha		
19	Gastrotricha		

2.3 **Material and Methods**

2.3.1 Glossary of Morphological Characters

A table was created to contain 56 morphological characters that were used in Nichols *et al.* (2006), ordered numerically, and described according to the headings: Action, Information, References, and Coding (Table 2.2). The Action category presented the character status within the current study, in which the character was labelled as retained, recoded, removed, or novel. Characters were retained if the coding within the Nichols *et al.* (2006) data matrix was confirmed in literature review. Characters were recoded if inconsistencies were found between the Nichols *et al.* data matrix and information in literature, where coding was changed to reflect conclusions from literature. Characters were removed when insufficient or lack of literature support was available to confirm data matrix coding. Novel characters were introduced if sufficient literature-based evidence supported their inclusion. The Information category contained a description of the character. The References category listed references consulted for the literature review. The Coding category contained the character states that were input into a data matrix for subsequent analyses (Table 2.3).

Table 2.2: Table of morphological characters

Character	Nichols *et al.*	Current study	Comments
Molting by ecdysis	1	1	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: Shedding of the cuticle marking discrete bouts of growth References: Aguinaldo *et al.*,1997; Wallace *et al.*, 1996; Schmidt-Rhaesa *et al.*, 1998 Coding: 0-absence, 1-presence
Loss of locomotory cilia	2	2	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: Ectodermal cilia used for locomotion. References: Martin, 1978; Brusca & Brusca, 1990; Aguinaldo *et al.*,1997; Schmidt-Rhaesa *et al.*, 1998; Valentine & Collins, 2000 Coding: 0-absence, 1-presence
Cuticle structure	3	3	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: Cuticle structure is described in taxa as either trilayered cuticle or a bilayered cuticle. Cuticle consisting of three layers (epicuticle, exocuticle and endocuticle). The Kinorhyncha, Lorcifera and Tardigrada also possess an endocuticle composed of chitin. The Gastrotricha possess a two layered cuticle consisting of an epicuticle and a basal layer, neither of which contain chitin. References: Neuhaus *et al.*, 1997; Kristensen & Neuhaus, 1999; Schmidt-Rhaesa *et al.*, 1998; Greven *et al.*, 2005; Rieger and Riger 2009 Coding: 0-absence, 1-presence
Parthenogenesis	4	4	Action: Recoded; References confirmed parthenogenesis within the tardigrade families Macrobiotidae, Hypsibiidae and Milnesiidae, and the Gastrotricha outgroup. Lorcifera and Kinorhyncha have been recoded to reflect conclusions from references. No references were found for the remaining tardigrade families, therefore recoded as unknown (?). Information: First described by Kinchin (1994) as an asexual reproductive behaviour found in many tardigrade taxa. Bertolani (2001) mentions that it is a behaviour not yet known in Heterotardigrades, most which inhabit marine habitats. Marine tardigrades have been described as bisexual, containing both male and females individuals, possessing sexually dimorphic gonopore structures. No parthenogenetically (asexual) reproducing individuals have been observed in populations that usually are sexually reproducing. Jorgensen *et al.* (2007) further resolves the assumption that genus Echiniscus (Heterotardigrade, Echiniscidae) parthenogenetic reproductive ability is still unknown. Parthenogenesis is most commonly found in nonmarine limnic (Rebecchi *et al.*, 2003) amd terrestrial tardigrades, specifically those species that inhabit moss (Nelson *et al.*, 2001). References: Rebecchi & Bertolani, 1988; Kinchin, 1994; Bertolani, 1994; Wallace *et al.*, 1996; Bertolani, 2001; Nelson *et al.*, 2001; Rebecchi *et al.*, 2003; Jorgensen *et al.*,2007 Coding: 0-absence, 1-presence, ?-unknown.
Circumpharyngeal nerve ring	5	5	Action: Retained; References confirmed previous matrix coding for all members of the ingroup and outgroup. Information: A ring of nerves and ganglia (the brain) that form a circular structure surrounding the pharynx. References: Brusca & Brusca, 1990; Wallace *et al.*, 1996

			Coding: 0-absence, 1-presence
Complete gut	6	6	Action: Retained; References confirmed previous matrix coding for all members of the ingroup and outgroup. Information: A complete digestive tract. References: Wallace *et al.*, 1996; Nelson, 2002 Coding: Coding: 0-absence, 1-presence
Separate genital pore & anus	7	-	Action: Removed; Reference confirmed coding for all members of the ingroup and most members of the outgroup. Gastrotricha should be recoded to reflect absence of character, please see "Reproductive pore" for more details. Character has been removed, along with 'Cloaca' to contribute to a new character 'Reproductive pore', as the two former characters were not mutually exclusive of each other. Information: The genital pore, also called a gonopore, is a hole through which eggs or embryos are released. The genital pore opens ventrally and is located anterior to the anus. It is a character found in all adult heterotardigrades. References: Wallace *et al.*, 1996; Rebecchi & Nelson, 1998 Coding: N/A
Reproductive pore	-	7	Action: Novel; Added to replace 'Separate genital pore & anus' and 'Cloaca' as a new character. Information: Gastrotricha possess reproductive pores depending on their life-phase. During the male phase, they possess a midbody male pore, a caudal organ pore and an anus. Gastrotricha in the male phase pass sperm (from the seminal receptacle) externally from their midbody pore to their caudal organ, in which sperm is stored. They do this by bending the body. The caudal organ then transfers the sperm into a spermatophore, functioning to transfer it to a female partner. There is no formal female pore in most species, and it is thought that the spermatophore is hypodermically injected into the partner. Then, fertilized eggs pass out of the partner via a breakage in the body wall. See 'Separate genital pore & anus' and 'Cloaca' for more information. References: Hochberg & Litvaitis, 2000; Correspondence with Dr.R.Hochberg for Gastrotricha; See 'Separate genital pore & anus' and 'Cloaca' for more information. Coding: 0-neither, 1-genital pore & anus, 2-cloaca, 3-life-phase dependant reproductive pores (gastrotricha)
Adhesive glands	8	8	Action: Recoded; Confirmed within the outgroups and recoded as present for tardigrade family Batillipedidae Information: Cement glands used for attachment to surfaces. (Wallace, Ricci *et al.* 1996) (Wallace, Ricci *et al.* 1996) References: Kristensen & Higgins, 1984b; Wallace *et al.*, 1996; Coding: 0-absence, 1-presence.
Protonephridia	9	9	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: An osmoregulatory organ. References: Wallace *et al.*, 1996; Nielsen, 2001 Coding: 0-absence, 1-presence.
Adult gut	10	10	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: functional digestive system in the adult References: Ruppert & Barnes 1994; Wallace *et al.*, 1996

			Coding: 0-absence, 1-presence.
Triangular pharynx	11	11	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: A part of the digestive tract. In tardigrades, it is as part of the buccal-pharyngeal apparatus. References: Wallace *et al.*, 1996; Schuster *et al.*, 1980, Nelson *et al.*, 2010 Coding: 0-absence, 1-presence.
Stylets	12	12	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: Supportive structures found as part of the bucco-pharyngeal apparatus that protrude from the mouth References: Nelson & Marley, 2000; Balian, 2008 Coding: 0-absence, 1-presence.
Formation of the epicuticle	13	13	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: After each molt, ecdysozoans (lorcifera, kinorhyncha and tardigrades) form a new epicuticle layer from material secreted through short microvilli, this forms separated patches that eventually fuse together to form a continuous layer. The gastrotricha epicuticle does not form by this method. References: Schmidt-Rhaesa *et al.*, 1998 Coding: 0-absence, 1-presence.
Terminal mouth	14	14	Action: Retained; References confirm coding for all members of the ingroup and outgroup. Information: A mouth appearing on the terminal or subterminal end of the animal. References: Kisielewski, 1987; Todaro & Kristensen, 1998; Schmidt-Rhaesa *et al.*, 1998; Telford *et al.*, 2008 Coding: 0-absence, 1-presence.
Cephalic papillae	15	15	Action: Recoded; References confirmed coding for (eutardigrade) presence in Order Apochela (family Milnesiidae) and absence in Order Parachela. No references found to confirm presence or absence within heterotardigrade families, therefore recoded as unknown (?). Information: Cuticular projections located on the head (anterior) region of the class Eutardigrade References: Pilato & Binda, 2010; Marley *et al.*, 2011; Nelson, 2001 Coding: 0-absence, 1-presence, ?-unknown.
Cephalic appendages	16	16	Action: Retained; References confirm previous matrix coding for all members of the ingroup and outgroup. Information: Cephalic appendages served as an umbrella-term to describe anteriorly located projections in class Heterotardigrada, including internal buccal cirri, external buccal cirri, clavae, and lateral cirri (or cirri A). References: Kristensen, 1987; Nelson, 2001 Coding: 0-absence, 1-presence.
Peribuccal pappilae	17	17	Action: Recoded; References confirmed coding for Milnesiidae, Microhypsibiidae and Hypsibiidae, while coding for Macrobiotidae, Eohypsibiidae, Calohypsibiidae and Necopinatidae have been changed to reflect conclusions from references. Information: Cuticular structures surrounding the mouth of Eutardigrades. Peribuccal papillae are elongated cuticular projections unique to the family Milnesiidae. References: Schuster *et al.*, 1980; Guidetti *et al.*, 2005

			Coding: 0-absence, 1-presence.
Peribuccal lamellae	18	18	Action: Recoded; References confirmed coding for Eohypsibiidae, Calohypsibiidae, Necopinatidae, Microhypsibiidae, Hypsibiidae and Milnesiidae, while coding for Macrobiotidae have been changed to reflect observation from references. Information: Cuticular structures surrounding the mouth of Eutardigrades. Peribuccal lamellae is a thickened cuticular ring surrounding the mouth. References: Schuster *et al.*, 1980; Pilato, 1982; Guidetti *et al.*, 2005; Nichols *et al.*, 2006 Coding: 0-absence, 1-presence.
Buccal tube	19	19	Action: Retained; References confirm previous matrix coding for all members of the ingroup. Information: A section of digestive tract in between the mouth and pharyngeal bulb. Typically described in literature as a part of the bucco-pharyngeal apparatus. References: Kristensen, 1987; Guidetti *et al.*, 2005; Nelson, 2002; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Buccal tube apophyses	20	-	Action: Removed; References confirmed coding for Macrobiotidae, while the other 15 tardigrade families could not be confirmed. Character has been removed, along with 'Pharyngeal tube apophyses' to contribute to a new character 'Apophyses Insertion Stylet Muscle' (AISM) (see character for more details). Information: Cuticular thickenings on the buccal-pharyngeal apparatus for the insertion of the stylet muscles for eutardigrades References: Guidetti *et al.*, 2005; Pilato & Binda, 2010; Marley *et al.*, 2011 Coding: N/A.
Peribuccal lobe	-	20	Action: Novel; Added as an addition to the peribuccal structures not previously discussed in Nichols *et al.* (2006). Information: Peribuccal lobes are flat peribuccal structures that extend posteriorly from the buccal orifice. References: Schuster *et al.*, 1980; Pilato, 1982 Coding: 0-absence, 1-presence
Pharyngeal tube	21	21	Action: Recoded; No description of character states was provided by Nichols *et al.* (2006), and pharyngeal tube has been coded as an apomorphy within select tardigrade families (specifically eutardirgades). This could not be confirmed from references. As the pharyngeal tube is often described in literature as the bucco-pharyngeal apparatus, which is possessed by all members of the ingroup, matrix has been recoded to reflect conclusions from references. Information: A section of digestive tract following after the mouth and buccal tube. In some species it terminates into a pharyngeal bulb. Both terms used synonymously. References: Guidetti *et al.*, 2005; Nelson & Marley, 2000; Guidetti *et al.*, 2005; Nelson, 2002; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Pharyngeal tube apophyses	22	-	Action: Removed; References confirmed coding for Macrobiotidae, while coding for the other 15 tardigrade families could not be confirmed. Character has been removed, along with 'Buccal tube apophyses' to contribute to a new character 'Apophyses Insertion Stylet Muscle' (AISM) (see character for more details). Information: Cuticular thickenings on the buccal-pharyngeal apparatus for the insertion of the stylet muscles for eutardigrades. References: Pilato & Binda, 2010; Marley *et al.*, 2011 Coding: N/A.

Peribuccal papulae	-	22	Action: Novel; Added as an addition to the peribuccal structures not previously discussed in Nichols *et al.* (2006). Information: Peribuccal papulae are lower profile peribuccal structures that extend posteriorly from the buccal orifice. References: Schuster *et al.*, 1980; Pilato, 1982 Coding: 0-absence, 1-presence
Ventral lamina	23	23	Action: Recoded; Reference confirmed coding for Macrobiotidae and Milnesiidae, while coding for Eohypsibiidae, Calohypsibiidae, Necopinatidae, Microhypsibiidae and Hypsibiidae have been changed to reflect conclusions from references. Information: A buccal tube support structure found along the buccal ring to the midregion of tube. Also called ventral crest. References: Nelson & Marley, 2000 Coding: 0-absence, 1-presence, 2-varied (not observed in all genera).
Stylet support	24	24	Action: Recoded; References confirmed coding for Macrobiotidae, Hypsibiidae and Milnesiidae, while Eohypsibiidae, Calohypsibiidae, Hypsibiidae and Milnesiidae have been recoded to reflect conclusions from references. No references could be found to confirm coding for Necopinatidae, Microhypsibiidae and Apodibius; therefore, these have been recoded to unknown (?). Reference confirmed stylet supports within all Heterotardigrade, so matrix has been recoded to reflect observation. Information: A flexible lateral extension that attaches the furca of the stylet to the buccal tube. References: Schuster *et al.*, 1980; Biserov, 1992; Kristensen & Higgins, 1984b; Guidetti *et al.*, 2005; Nelson *et al.*, 2010 Coding: 0-absent, 1-presence, 2-varied (depending on genera)
Placoids	25	25	Action: Recoded; Description of character states from Nichols *et al.* (2006) did not correspond with coding in matrix. Matrix has been recoded to reflect observations from references. Information: Cuticular supportive structures in the pharynx. Fused placoids are found in class Heterotardigrade, while microplacoid and macroplacoids are found in class Eutardigrade. Heterotardigrades possess a continuous placoid structure, while eutardigrades have differentiated placoid structure. References: Eibye-Jacobsen, 2001; Nichols *et al.*, 2006; Marley *et al.*, 2011 Coding: 0-absent, 1-fused placoid, 2-micro/macroplacoid.
Septulum	26	26	Action: Recoded; References confirmed coding for Calohypsibiidae, Necopinatidae and Milnesiidae, while Macrobiotidae and Hypsibiidae coding did not match the observations from reference. No references found to confirm coding for 11 tardigrade families, therefore recoded as unknown (?). Information: A thickened cuticle structure found within the pharyngeal bulb. References: Schuster *et al.*, 1980; Nelson, 2001; Nichols *et al.*, 2006, Nelson and Marley, 2000 Coding: 0-absent, 1-presence, 2-varied (not in all genera).
Claw structure	27	27	Action: Recoded; References confirmed coding for Macrobiotidae, Eohypsibiidae, Calohypsibiidae, Microhypsibiidae, Hypsibiidae and Milnesiidae, while Necopinatidae, Echiniscidae and Oreelidae have been changed to reflect conclusions from references. The remaining tardigrade coding could not be confirmed, therefore recoded as unknown (?). Information: Eutardigrades typically possess two double-branching claws. Each double-branch claw contains a basal tract, a longer primary branch with accessory points and a shorter secondary branch without accessory points. Heterotardigrade claws have not been extensively described or used for phylogenetic purposes.

			References: Schuster *et al.*, 1980; Pilato, 1998; Nelson & Marley, 2000; Pilato & Binda, 2010 Coding: 0 - claws absent, 1 -single claw, 2 - double-claw separated, 3 - double-claw connected
Claw sequence	28	28	Action: Recoded; Nichols *et al.* (2006) coded Heterotardigrades with a 1111 claw sequence for their separated claws, however it does not match the definition of claw sequence offered by references. Milnesiidae had also been coded with a claw arrangement of 1122, however references did not confirm this coding. Both heterotardigrades and Milnesiidae taxa have been recoded and the coding legend has been changed to reflect conclusions from references. Information: The arrangement of the primary- and secondary-branched claws of individuals within class Eutardigrada according to the midline of the extended legs. The 2-1-2-1 claw sequence occurs when claws alternate in arrangement according to secondary-primary-secondary-primary, while 2-1-1-2 claw sequence occurs when two primary branches are adjacent to one another. References have also described character as claw symmetry according to the arrangement of the claws in reference to the median plane dividing each leg pair. A symmetrical claw arrangement is numerated as 2112, while claw asymmetry is represented by the 2121 arrangement. References: Schuster, 1980; Nelson & Marley, 2000. Claw Symmetry - Guidetti *et al.*, 2005; Pilato & Binda, 2010; Marley *et al.*, 2011 Coding: 0=absent, 1=2121, 2=2112.
Transverse cuticular bar	29	29	Action: Recoded; References confirmed coding for Hypsibiidae, Macrobiotidae and Necopinatidae, while the coding for Milnesiidae and Calohypsibiidae have been recoded to reflect conclusions from references. The coding for Eohypsibiidae, Microhypsibiidae and the other tardigrade taxa could not be confirmed, therefore recoded as unknown (?). Information: Cuticle thickening appearing slender or broad, located at the base of the claw, either in between claws or off to the side. Described first by Kristensen (1987) as bar-shaped cuticular structure within heterotardigrades (however no confirmed observations found). Character has been described in some species within the genera Dactylobiotus and Macroversum, however Murrayidae family was not included in study. Character not to be confused with pharyngeal cuticular bar, defined as a structure located at the end of the buccal tube aligned with macroplacoids in eutardigrades. References: Schuster, 1980; Manicardi, 1989; Biserov, 1992; Utsugi & Ohyama, 1993; Kendall-Fite & Nelson, 1996; Bertolani & Rebecchi, 1996; Nelson & Marley, 2000; Pilato *et al.*, 2002; Pilato *et al.*, 2004; Nichols *et al.*, 2006; Li *et al.*, 2008; Meyer & Hinton, 2010; Correspondence with William R. Miller. Coding: 0 = absence, 1 = presence, 2 = varied (not in all genera), ? – unknown.
Accessory point	30	30	Action: Recoded; References confirmed coding for most families within eutardigrades and heterotardigrades. Necopinatidae and *Apodibius*, both which possess small forceps or absent claws, have been recoded to reflect conclusions from references. Information: A eutardigrade claw character often associated with the double claws found on each leg. Each claw usually bifurcates into two branches (a primary and secondary branch), in which the longer primary branch contains two accessory points at the distal end, while the secondary branch does not. In some cases, a simple claw may exist containing a primary branch with accessory point(s). References: Schuster *et al.*, 1980; Nelson & Marley, 2000; Nichols, 2005; Pilato & Binda, 2010 Coding: 0-absence, 1-presence, 2-varied (not in all genera).
Lunulae	31	31	Action: Recoded; References confirmed coding for most families within eutardigrades and heterotardigrades. Macrobiotidae has been recoded to reflect conclusions from references.

			Information: A cuticular thickening located at the base of the eutardigrade claw. It may appear smooth or dentated (toothed). References: Nelson & Marley, 2000; Pilato & Binda, 2010; Marley *et al.*, 2011; correspondence with W.R. Miller Coding: 0-absence, 1-presence, 2-varied (not in all genera).
Lateral cirrus A	32	32	Action: Retained; References confirmed coding for all members of the ingroup and outgroup. Information: A long filamentous projection located between the scapular plate and head plate found in heterotardigrades. References: Kristensen, 1987; Jorgensen, 2000; Nelson, 2001 Coding: 0-absence, 1-presence.
Median cirrus	33	33	Action: Retained; References confirmed coding for presence of character within arthrotardigrades and absence of character within echiniscoideans, eutardigrades and outgroup. Information: A short projection found on the anterior end of tardigrades within the suborder arthrotardigrades and not echiniscoidea families. References: Horning *et al.*, 1978; Kristensen & Higgins, 1984b; Villora-Moreno, 1996; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Cuticular armor	34	34	Action: Recoded; References confirmed coding for Stygarctidae, Renaudarctidae, Echiniscoididae, Echiniscidae and Oreellidae. Halechiniscidae, Coronarctidae and Batillipedidae have been recoded to reflect conclusions from references. Information: Heterotardigrades possess a cuticle armor, which appears as thickened dorsal cuticle plates. These plates may appear paired, vary in shape and number within a genera or species, and have a species-specific sculpture. Unarmored tardigrades lack plates, but possess a thin, smooth or sculptured cuticle. A sculptured cuticle can contain pores, granulation, reticulation, tubercles, papillae, or spines. Within the family Echiniscidae, the genus Pseudoechiniscus constitute an exception, wherein their dorsal plates are not sclerotized. References: Kristensen & Hallas,1980; Kristensen & Higgins, 1984b; Kristensen, 1987; Jorgensen *et al.*, 2000; Nelson, 2001; Nelson, 2002; Nelson *et al.*, 2010; Correspondence with W.R. Miller. Coding: 0-absence, 1-presence.
Dorsal segmental plates	35	35	Action: Recoded; References confirmed coding for Stygarctidae, Renaudarctidae, Echiniscidae and most of the tardigrades, while Batillipedidae has been recoded to reflect conclusions from references. Information: Dorsal plates located posterior to the head plate. They can appear paired or unpaired. Intersegmental ridges or folds, median plates and pseudosegmental plates are found in between the segmental plates. Dorsal segmental plates are more commonly referred to as segmental plates I-IV. The first trunk dorsal segmental plate is also called the scapular plate, while segmental plate IV is referred to as the caudal plate. In this study, only segmental plates I-III are considered for the "Dorsal segmental plate" character, while "Caudal plate" is a separate character. References: Kristensen & Hallas, 1980; Kristensen & Higgins, 1984b; Kristensen, 1987; Bello & de Zio Grimaldi, 1998; Dastych *et al.*, 1998; Jorgensen, 2000; Nelson *et al.*, 2010; Jorgensen *et al.*, 2011; Correspondence with W.R. Miller and A. Jorgensen. Coding: 0-absence, 1-presence.
Head plate	36	36	Action: Recoded; References confirmed coding within Echiniscidae, Renaudarctidae, Stygarctidae and for most tardigrade families, while Batillipedidae has been recoded to reflect conclusions from references.

			Information: An armored tardigrade character that appears as the most anterior cuticular plate. It often bears the cephalic appendages, and is also called a cephalic plate. References: Kristensen & Higgins, 1984b; Kristensen, 1987; Bello & de Zio Grimaldi,1998; Dastych *et al.*, 1998 Coding: 0-absence, 1-presence.
Median plate I	37	37	Action: Recoded; References confirmed coding for Echiniscidae, the remaining tardigrades and members of the outgroup. Information: Cuticular plate located in between dorsal segmental plates I and II. References: Bello & de Zio Grimaldi, 1998; Jorgensen, 2000; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Median plate II	38	38	Action: Recoded; References confirmed coding for Echiniscidae, Renaudarctidae and most tardigrades, and members of the outgroup. Coronarctidae and Batillipedidae have been recoded to reflect conclusions from the references. Information: Cuticular plate located between dorsal segmental plates II and III, appearing after the pseudosegmental plate II when present. References: Bello & de Zio Grimaldi,1998; Jorgensen, 2000; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Median plate III	39	39	Action: Recoded; References confirmed coding for Echiniscidae, Renaudarctidae, most tardigrades, and members of the outgroup. Coronarctidae and Batillipedidae have been recoded to reflect conclusions from the references. Information: Cuticular plate located in between dorsal segmental plates III and IV, or when present, it may also appear flanked by pseudosegmental plates III and IV, which all together are flanked by segmental plates III and IV. References: Bello & de Zio Grimaldi,1998; Jorgensen, 2000; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Caudal plate	40	40	Action: Recoded; References confirmed coding for Echiniscidae, Renaudarctidae, most tardigrades, and members of the outgroup. Stygarctidae, Coronarctidae and Batillipedidae have been recoded to reflect conclusions from the references. Information: The most posterior cuticular plate found in armored tardigrades. Also called segmental plate IV or terminal plate. References: Kristensen & Higgins, 1984b; Bello & de Zio Grimaldi,1998; Jorgensen, 2000; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence.
Pseudosegmental plates	41	41	Action: Recoded; References confirmed coding for Echiniscidae and the remaining tardigrades and outgroups. Information: Pseudosegmental plates II-IV appear between segmental plates, ususally flanking the median plates when available. Plates are used to distinguish between the genera within the Echiniscidae tardigrade family. They only appear unpaired. References: Kristensen, 1987, Jorgensen, 2000; Nichols *et al.*, 2006; Jorgensen *et al.*, 2011 Coding: 0-absence, 1-presence, 2-either.
Peduncles	42	42	Action: Recoded; References confirmed coding for most eutardigrades and heterotardigrade families Coronarctidae, Batillipedidae and Oreellidae. The eutardigrade family Macrobitidae and most heterotardigrade families have been recoded to reflect conclusions from references. Information: Commonly, a eutardigrade claw character that has also been described in some heterotradigrade literature. Alternatively called a stem, it is observed when an obvious septum

			divides the basal section of the claw into two separate parts, the peduncle and distal section. The claw does not contain a peduncle when the claw has a continuous basal section that is followed by a primary or secondary branch or when the claw separates directly into a primary and secondary branch without a basal section. In some references it has also been defined as the septum dividing the basal tract from the rest of the claw. References: Horning *et al.*, 1978; Kristensen & Hallas, 1980; Kristensen & Higgins, 1984b; de Zio Grimaldi *et al.*, 1987; Villora-Morena and de Zio, 1996; de Zio Grimaldi *et al.*, 1999; Nelson & Marley, 2000; Hansen, 2007; Pilato & Binda, 2010; Correspondence with Dr. W.R. Miller Coding: 0-absence, 1-presence, 2-varied (not in all genera)
Clava	43	43	Action: Recoded; References confirmed coding for most heterotardigrades families, except for Halechiniscidae and Oreellidae, which have been recoded to reflect conclusions from references. Information: A heterotardigrade character that appears as a short and broad paired sensory appendage. Three types of clavae exist: primary clava, secondary clava, and tertiary clava. They are located on the lateral sides of the scapular plate, in between the headplate and the first segmental plate. The primary clava can be found on the scapular plate arising from a cirrophore, and near the lateral cirrus A. The secondary and tertiary clavae can be found on the cephalic plate, alongside with the medial cirrus, internal and external cirri. Secondary clavae have been described as H-shaped, dome-shaped. References: Kristensen, 1987; Kristensen & Higgins, 1984b; de Zio Grimaldi *et al.*, 1992; Villora-Moreno, 1996; Nichols, 2005; Nelson *et al.*, 2010; Calloway *et al.*, 2011; Jorgensen *et al.*, 2011 Coding: 0-absent, 1-posssesson one or more clavae.
Digitate legs	44	44	Action: Recoded; References confirmed coding for Halechiniscidae, Stygarctidae, Coronarctidae, Batillipedidae and Echiniscoididae. Renaudarctidae, Echiniscidae and Oreellidae have been recoded from unknown to reflect conclusions from references. Information: A heterotardigrade character described as digits found on the proximal end of the leg, which may terminate into claws, adhesive disks or nothing; alternatively named digit, toes, or digit glands. References: Pollock, 1970; Kristensen & Higgins, 1984b; Villora-Moreno, 1996; D'Addabbo Gallo *et al.*, 1999, Nelson, 2002 Coding: 0-absence, 1-presence.
Leg 4 morphology	45	45	Action: Recoded; No references were found to confirm coding within eutardigrades and some heterotardigrades, but through correspondence with W.R. Miller, confirmed that all eutardigrades do not possess leg 4 morphology and Echiniscoididae possessed spines, therefore recoded to reflect conclusion. Stygarctidae could not be confirmed, therefore have been recoded to unknown (?). References have been found to confirm observation of leg 4 morphology in the following species: *Bathyechiniscus tetronyx, B. craticulus, B. hallasi, Styraconyx paulae, Dipodarctus anaholiensis, D. borrori, Halechiniscus greveni* (Halechiniscidae), *Renaudarctus psammocryptus* (Renaudarctidae), *Coronarctus verrucatus, Coronarctus stylisetus, Coronarctus fastigus* (Coronarctidae), *Batillipes noerrevangi* (Batillipedidae), *Bryodelphax parvulus, Pseudechiniscus brevimontanus, P. Razmazzotti, P. brevimontaus, Echiniscus corrugicaudatus, Pseudechiniscus suillus* (Echiniscidae) and *Oreella chugachii* (Oreellidae). The corresponding families have been recoded to reflect conclusions from references. The descriptions of the sensory organs were species specific and the spines or papilla description interchangeably in references, therefore the original Nichols *et al.* (2006) coding of character has been modified. Information: The fourth leg-pair morphology is used to describe the shape of the sensory organs

			appearing on the fourth leg of tardigrades. They appear as spines or papillae depending on the species and are systematically important in classifying tardigrades from a genus to species level. References: Kristensen, 1981, Pollock, 1983; Kristensen & Higgins, 1984b; Miller *et al.*, 1994; Kendall-Fite & Nelson, 1997; Kacmarek & Michalcyzk, 2004; Hansen, 2007; de Zio-Grimaldi *et al.*, 2000; McInnes, 2010; Calloway *et al.*, 2011; Correspondence with Dr. W.R. William (November, 2011). Coding: 0-absence, 1-presence, 2-varied (not in all genera), ?-unknown.
Eyespots**	46	–	Action: Removed; References confirmed coding using type species for Macrobiotidae, Eohypsibiidae, Hypsibiidae, Milnesiidae, Halechiniscidae, Batillipedidae, and Echiniscoidea. Calohypsibiidae and Oreellidae have been recoded to reflect conclusions from references. The type species used are listed as the following (absence of eyespots indicated): **Echiniscidae**: *Echiniscus ollantaytamboensis, Echiniscus cf. jenningsi* (absent), *Echiniscus cf. spiniger* (absent), *Pseudechiniscus suillus, Echiniscus tenuis,* **Hypsibidae**: *Hebesuncus cf. schusteri, Diphascon chilenense langhovdense* (absent), *Diphascon pingue* (absent), *Diphascon cf. rugosum , Diphascon puniceum* (absent), *Diphascon prosirostre* (absent), *Ramajendas heatwolei, Isohypsibius cf. sattleri* (absent), *Hypsibius dujardini, Isohypsibius archangajensis, Diphascon linzhiensis* (absent), *Diphascon gordonense* (absent), *Diphascon greveni* (absent), *Isohypsibius malawiensis* (absent), *Isohypsibius myrops* (absent), *Isohypsibius kristenseni, Hypsibius tetradactyloide, Hypsibius scoticus* (absent), *Hypsibius convergens, Hypsibius sattleri, Hypsibius pallidus, Acutuncus antarcticus,* **Macrobitidae**: *Macrobiotus hastatus, Macrobiotus fliucatus, Macrobiotus areolatus, Macrobiotus echinogenitus, Macrobiotus trunovae, Macrobiotus richtersi* (absent), *Macrobiotus areolatus, Macrobiotus harmsworthi* (present/absent), *Minibiotus asteris, Macrobiotus intermedius, Macrobiotus richtersi,* **Milnesidae**: *Milnesium tardigradum* (present/absent), *Macrobiotus cf. furciger*, **Halechiniscidae**: *Archechiniscus symbalanus, Styraconyx craticuliformis,* **Echiniscoididae**: *Echiniscoides andamanensis, Echiniscoides horningi* **Eohypsibiidae**: *Amphibolus weglarskae,* **Calohypsbiibdae**: *Calohypsibius ornatus* (absent), *Calohypsibius maliki* (absent), *Calohypsibius ornatus* (absent), **Oreellidae**: *Oreella chugachii, Oreella gen.* (absent), **Batillipedidae**: *Batillipes spinicauda* (absent). No references were found to confirm Necopinatidae, Microhypsibiidae, *Apodibius*, Stygarctidae, Renaudarctidae and Coronarctidae, therefore were recoded to as unknown (?). Information: A cluster of pigment granules often present in the cephalic area of some species, but always are absent in others. Eyes have been noted in some species of all genera of Eutardirgada, and therefore no value for defining genera. Also called eyes, eye-pigment or ocelli. Eye spots appear either black, red, or brown-black. References: Beasley, 1968; Mehlen, 1969; Schuster *et al.*, 1980; Kristensen, 1987; Chang & Rho, 1998; Kathman, 1990; Jorgensen, 2001; Miller *et al.*, 2001; Nickel *et al.*, 2001; D'Addabbo Gallo et l., 2005; Gabriel *et al.*, 2007; Greven, 2007; Li, 2007; Hohberg & Trunsperger, 2009; Biserov *et al.*, 2011; Calloway *et al.*, 2011 Coding: N/A **Correspondence with W. R. Miller and C. B. Beasley, both advised not using the character because organisms usually viewed after fixing on slide. The slide fixing solution has been known to dissolve the eyes, however are not always noted in literature. Although some observations are done with live specimens, most are not, therefore becomes an unreliable character to use.
Dorsal plate development	-	46	Action: Novel; Added to differentiate between the dorsal plates. Information: Dorsal plates in some heterotardigrades developed from different layers of the cuticle. Echiniscidae plates are sclerotized, while Renaudarctidae and Stygarctidae are not.

			References: Kristensen, 1987; Jorgensen, 2000 Coding: 0-absence, 1-epicuticle, 2-procuticle.
Cloaca	47	-	Action: Removed; Reference confirmed coding for all members of the ingroup and most members of the outgroup (please see "Reproductive pore" for more details). Character has been removed, along with 'Separate genital pore and anus' to contribute to a new character 'Reproductive pore', as the two former characters were not mutually exclusive of each other. Information: The cloaca is a single canal used for excretory and reproductive purposes. It is a character found in all eutardigrades. References: Margulis & Schwartz, 1998; Rebecchi & Nelson, 1998 Coding: N/A
Apophyses Insertion Stylet Muscle (AISM)	-	47	Action: Novel; Added to reflect new character suggested by Marley *et al.* (2011) to reflect observations first described by Pilato & Binda (2010). Character replaces 'Buccal tube apophyses' and 'Pharyngeal tube apophyses' as a new character. Information: Cuticular thickenings on the buccal-pharyngeal apparatus for the insertion of the stylet muscles for eutardigrades References: Guidetti *et al.*, 2005; Pilato & Binda, 2010; Marley *et al.*, 2011 Coding: 0-absence, 1-presence, 2-varied (occur in some genera).
Sexual dimorphism of claws	48	48	Action: Recoded; References confirmed coding for Milnesiidae and most tardigrade families. Since the presence of the character has been observed in select species, the matrix coding has been redefined from 1=presence (in all species in family) to represent 1=observed in some species. Hypsibiidae has been recoded to reflect observations concluded from references. The type species used are listed as the following: **Milnesiidae**: *Milnesium tardigradum*, *Milnesioides exertum*, **Hypsibiidae**: *Pseudobiotus megalonyx, Pseudobiotus augusti.* Information: Claw modification of the first leg pair observed in mature male eutardigrades. The modification of the male claw usually appears after the final molt before sexual maturity, and serves the purpose of grasping onto the female during copulation. References: Pollock, 1970; Rebecchi & Nelson, 1998; Claxton, 1999; Nelson & Marley, 2000 Coding: 0-absence, 1-observed in some species.
Sexual dimorphism of gonopore	49	49	Action: Recoded); References confirmed coding for all members of heterotardigrades, eutardigrades and outgroup. Information: A tardigrade specific character, where heterotardigrades possess a gonopore (also called a genital pore) that appears different between males and females, while eutardigrades possess a cloaca that appears similar between the two sexes. In heterotardigrades, the male gonopore appears as a small rounded tube, while the female gonopore consists of six cuticular valves that form the shape of a rosette. References: Rebecchi & Nelson, 1998, Nelson *et al.*, 2010 Coding: 0-absence, 1-presence.
Pharyngeal stripes	50	-	Action: Removed; No corresponding coding for the character exists in the matrix. No references were found to confirm character. Information: N/A References: N/A Coding: N/A
Cleavage Pattern	-	50	Action: Novel; Added as an addition to the original 50 characters in Nichols *et al.*, 2006 Information: Tardigrades, Lorciferans and Kinorhyncha undergo spiral cleavaging during development. Gastrotricha undergo radial or modified radial cleavaging during development.

			References: Wallace *et al.*, 1996; Valentine, 1997 Coding: 0-absence, 1- spiral, 2- radial

2.3.2 Data Matrix & Phylogenetic Inferences

Character states for the 50 characters labelled as retained, recoded, or novel (Table 2.2) were input into a taxon-character matrix for 15 families (seven eutardigrades and eight heterotardigrades), one species *incertae sedis*, and three outgroup members, using the software Mesquite 2.75 (Maddison and Maddison 2011), and saved as a Nexus file (Appendix A).

Analyses were conducted using neighbor-joining (NJ) (PAUP* 4.0b10; Swofford 2003), maximum parsimony (MP) (PAUP* 4.0b10; Swofford 2003), and Bayesian Inference (Bi) (mrbayes-3.1.2; Ronquist 2003) techniques. The Nexus-formatted data matrix was uploaded onto the McMaster University EVOL server for access by the software Phylogenetic Analysis Using Parsimony (PAUP*) 4.0b10 (Swofford 2003) for the NJ and MP analyses, for the Bi analysis it was uploaded onto a Windows OS platform to access mrbayes-3.1.2 (Ronquist, 2003) software. The NJ analysis was performed using a total distance model (where distance between taxon pairs = total number of pairwise differences across character states, ? codings were ignored; Figure 2.2) with no bootstrapping, thus producing a phenogram representing similarities among species. The MP topology was produced using a bootstrap heuristic search, 200 replicates of pseudorandom sequence entry, and tree-bisection-reconnection (TBR) branch swapping, as performed previously by Jorgensen *et al.* (2011). The bootstrap values produced from resampling represent the proportion out of the 200 tree replicates in which possessed the exact branching distribution as the resultant tree. The Bi analysis was performed using a JC69 (all rates the same) substitution model [lset nst = 1] with an equal rate distribution. Starting from pseudorandom topologies, four Markov chains (one cold, three heated) ran in parallel to sample topologies using a Markov Chain Monte Carlo (MCMC) protocol [MCMC savebrlens = yes] for 1000000 generations with a sample frequency of 1000; 900 trees were discarded for burnin, in which convergence of the four chains was reached at the 100^{th} sample, determined by plotting likelihood scores (LnL) versus time (Gen) from the 'dot-p" files created by the mrbayes-3.1.2 software (Ronquist, 2003); 100 tree samples

were analyzed after burnin, representing posterior probabilities for the entire analysis.

The MP bootstrap replicates were summarized using majority rule consensus trees option on PAUP* 4.0b10 (Swofford, 2003), and saved as Nexus-formated tree file. Support for monophyletic groups is considered strong when the value of bootstrap or posterior probability is 95% or above.

The NJ phenogram, MP cladogram, and Bi tree were visualized using FigTree v.1.3.1 (Rambaut, 2009) and labelled using Microsoft PowerPoint v.14 (2010) and Paint v.6.1 (2009) (Figures 2.1, 2.3-2.5). NJ phenogram tree branches were modified by using the 'proportional' option through FigTree v.1.3.1 software (Rambaut, 2009); scale bars were modified by to compensate for the branch length changes.

Table 2.3: Matrix of morphological characters used in current study.
(See Table 3.2 for coding summary)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	Molting by ecdysis	Loss of locomotory cilia	Cuticle structure	Parthenogenesis	Circumpharyngeal nerve ring	Complete gut	Reproductive pore	Adhesive glands	Protonephridia	Adult gut	Triangular pharynx	Stylets	Formation of the epicuticle	Terminal mouth	Cephalic papillae	Cephalic appendages	Peribuccal papillae	Peribuccal lamellae	Buccal tube	Peribuccal lobe	Pharyngeal tube	Peribuccal papulae	Ventral lamina	Stylet support	Placoids
Loricifera	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Kinorhyncha	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Gastrotricha	0	0	0	1	1	1	3	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Macrobiotidae	1	1	1	1	1	1	2	0	0	1	1	1	1	1	0	0	0	1	1	2	1	1	1	1	2
Eohypsibiidae	1	1	1	?	1	1	2	0	0	1	1	1	1	1	0	0	0	1	1	?	1	?	0	1	2
Calohypsibiidae	1	1	1	?	1	1	2	0	0	1	1	1	1	1	0	0	0	0	1	2	1	2	2	1	2
Necopinatidae	1	1	1	?	1	1	2	0	0	1	1	1	1	1	0	0	0	0	1	?	1	2	2	?	2
Microhypsibiidae	1	1	1	?	1	1	2	0	0	1	1	1	1	1	0	0	0	0	1	?	1	0	0	?	2
Hypsibiidae	1	1	1	1	1	1	2	0	0	1	1	1	1	1	0	0	0	2	1	2	1	2	2	2	2
Milnesiidae	1	1	1	1	1	1	2	0	0	1	1	1	1	1	1	0	1	1	1	0	1	?	1	2	2
Apodibius	1	1	1	?	1	1	2	0	0	1	1	1	1	1	0	0	0	0	1	0	1	0	1	?	2
Halechiniscidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Stygarctidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Renaudarctidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Coronarctidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Batillipedidae	1	1	1	?	1	1	1	1	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Echiniscoididae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Echiniscidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1
Oreellidae	1	1	1	?	1	1	1	0	0	1	1	1	1	1	?	1	0	0	1	0	1	0	0	1	1

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
	Septulum	Claw structure	Claw sequence	Transverse cuticular bar	Accessory point	Lunulae	Lateral cirrus A	Median cirrus	Cuticular armor	Dorsal segmental plates	Head plate	Median plate I	Median plate II	Median plate III	Caudal plate	Pseudosegmental plates	Peduncles	Clava	Digitate legs	Leg 4 morphology	Dorsal plate development	Apophyses Insertion Stylet Muscle (AISM)	Sexual dimorphism of claws	Sexual dimorphism of gonopore	Cleavage Pattern
Loricifera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Kinorhyncha	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Gastrotricha	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Macrobiotidae	0	3	2	2	2	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	?	0	0	1
Eohypsibiidae	?	3	3	?	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Calohypsibiidae	0	3	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1
Necopinatidae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1
Microhypsibiidae	?	3	1	?	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Hypsibiidae	2	3	1	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
Milnesiidae	0	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1
Apodibius	?	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	1
Halechiniscidae	?	?	0	?	0	0	1	1	0	0	0	0	0	0	0	0	2	1	1	2	0	0	0	1	1
Stygarctidae	?	?	0	?	0	0	1	1	1	1	1	0	0	0	1	0	2	1	0	?	2	0	0	1	1
Renaudarctidae	?	?	0	?	0	0	1	1	1	1	1	0	1	1	1	0	0	1	1	2	2	0	0	1	1
Coronarctidae	?	?	0	?	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	1	1
Batillipedidae	?	?	0	?	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	1	1
Echiniscoididae	?	?	0	?	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	1	1
Echiniscidae	?	1	0	?	0	0	1	0	1	1	1	1	1	1	1	2	0	1	1	2	1	0	0	1	1
Oreellidae	?	1	0	?	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	1	1

2.4 **Results**

Analyses produced three results: one NJ FITCH-inferred phenogram (Phylip-3.69; Felsenstein, 1993), one MP consensus cladogram, and one Bi tree. All inferred monophyly for the phylum Tardigrada, with distinct monophyly between the two tardigrade clases, Heterotardigrada and Eutardigrada. The NJ phenogram and Bi tree exhibited similar topologies.

2.4.1 Neighbor Joining Fitch-Margoliash Phenogram

Results from the Fitch-Margoliash analysis (Figure 2.4) suggested great divergence between the two distinctly monophyletic classes Heterotardigrada and Eutardigrada. Within Heterotardigrada, two distinct groups related to the classes Arthrotardigrada and Echiniscoidea were revealed but with Echiniscidae appearing among other arthrotardigrades. Stygarctidae appeared as a sister group to the Renaudarctidae + Echiniscidae clade. Halechiniscidae appeared sister group to Coronarctidae, and, together, they were sister group to Batillipedidae. Echiniscoididae appeared as a sister group to Oreellidae. Within Eutardigrada, Milnesiidae (Class Parachela) and Macrobiotidae (Class Apochela) appeared as the most recently diverged clade, while *Apodibius* appeared as the most basal branch.

```
19
Lorcifera
Kinorhynch   0
Gastrotric   8   8
Macrobioti  18  18  23
Eohypsibii  13  13  19   4
Calohypsib  14  14  20   7   5
Necopinati  10  10  16   9   7   3
Microhypsi  10  10  16   6   3   3   6
Hypsibiida  20  20  25   8   6   7   9   5
Milnesiida  18  18  23   8   8  11  10   7   9
Apodibius    7   7  13   8   6   6   3   4   9   7
Halechinis  14  14  21  16  15  16  13  13  20  18  11
Stygarctid  17  17  24  19  18  19  16  16  23  21  14   6
Renaudarct  20  20  27  24  21  22  19  19  26  24  17   8   4
Coronarcti  13  13  20  17  14  15  12  12  19  17  10   1   7   7
Batilliped  12  12  19  18  15  16  13  13  20  18  11   2   8   8   1
Echiniscoi  12  12  19  16  13  14  11  11  18  16   9   2   8   8   1   2
Echiniscid  22  22  29  26  23  24  20  21  28  26  19  11   8   4  10  11   9
Oreellidae  13  13  20  17  14  15  11  12  19  17  10   2   8   8   1   2   0   9
```

Figure 2.1: Distance matrix created for the Neighbor-Joining phenogram using TOTAL distance calculation in PAUP* 4.0b10 (Swofford, 2003)

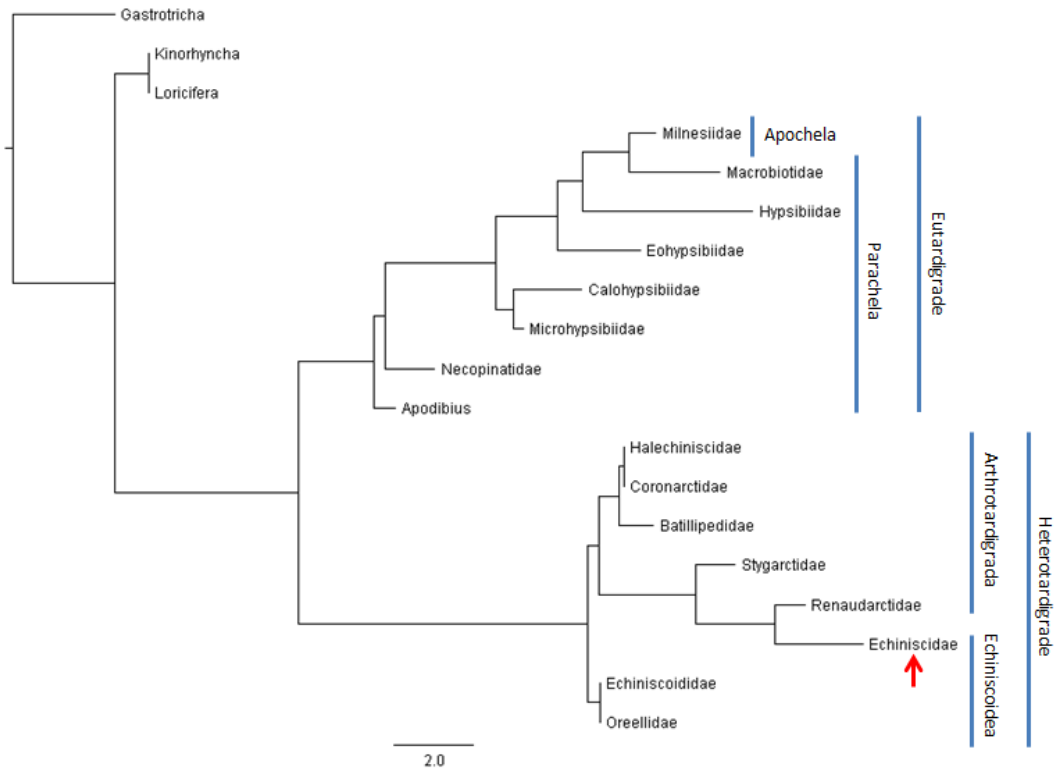


Figure 2.2: Neighbor-Joining phenogram of morphological characters. Arrow representing Echiniscidae, suggesting paraphyly within the order Echiniscoidea.

2.4.2 Maximum Parsimony Consensus Phylogeny

The MP consensus cladogram (Figure 2.1) contained monophyletic groups for the two tardigrade classes Heterotardigrada (bootstrap value, bv 99.15%) and Eutardigrada (bv 64.77%). However, relationships among most families remained unresolved. Within Eutardigrada, *Apodibius* appeared as the most basal group, followed by Necopinatidae, and then the other six families. Relationships among Macrobiotidae, Eohypsibiidae, Calohypsibiidae, Microhypsibiidae, Hypsibiidae, and Milnesiidae were unresolved; the orders Apochela and Parachela were not distinctly monophyletic. Within Heterotardigrada, the Class Arthrotardigrada appeared paraphyletic, with Echiniscidae (Echiniscoidea) forming a sister group with arthrotardigrade Renaudarctidae (bv 90.09%). The bootstrap value for the outgroup appeared as 0 as result of designating it as monophyletic , using ReTree (Phylip-3.69; Felsenstein, 1993).

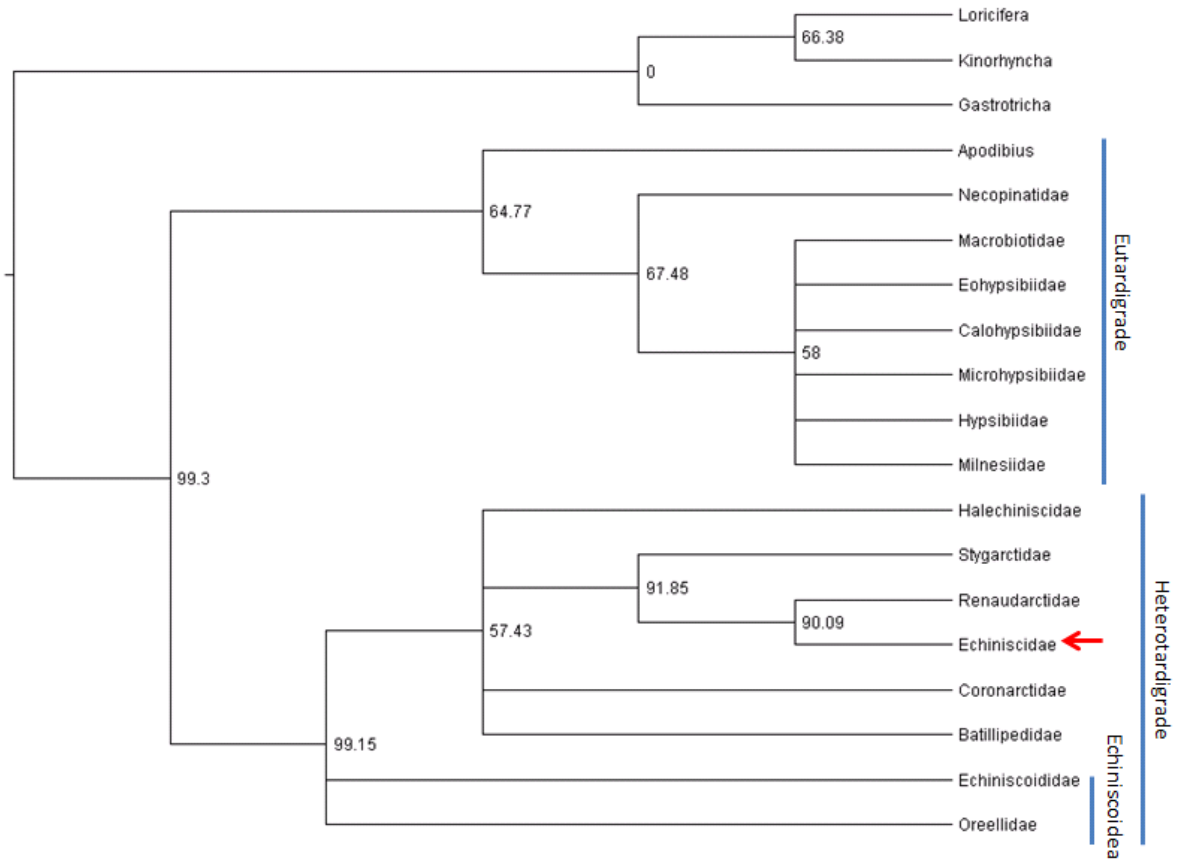


Figure 2.3: Maximum parsimony cladogram of morphological characters with 200 bootstrap* replicates.
Arrow shows position of Echiniscidae, suggesting paraphyly within the order Echiniscoidea.
***- Bootstrap values did not appear as an expected proportion of 200 replicates due to bugs within PAUP* 4.0b10. Bootstrap values may have been misrecorded because the heuristic search involved with bootstrapping using random sequence addition may have saved more trees than should be saved (Carmen Cheung, personal communication, September 22, 2012).**

2.4.3 Bayesian Inference Phylogeny

The Bi analysis (Figure 2.10) returned monophyletic Heterotardigrada and Eutardigrada (posterior probabilities 100% and 80%). Within the heterotardigrades, the two Classes Echiniscoidea and Arthrotardigrada did not appear monophyletic, with Echiniscidae sharing a close affinity with families within Arthrotardigrada. Stygarctidae grouped with Renaudarctidae + Echinsicidae, while relationships among Halechiniscidae, Coronarctidae,

and Batillipedidae remained unresolved. The relationship between Echiniscoididae and Oreellidae remained unresolved, however, both appeared as basal families within Heterotardigrada.

Milnesiidae (Class Apochela) and Macrobiotidae appeared as the least basal clade of eutardigrades. Eohypsibiidae appeared as a sister group to Macrobiotidae + Milnesiidae. Relationships among this group and Microhypsibiidae and Hypsibiidae were unresolved. *Apodibius* appeared as the most basal eutardigrade group, followed by Necopinatidae and Calohypsibiidae.

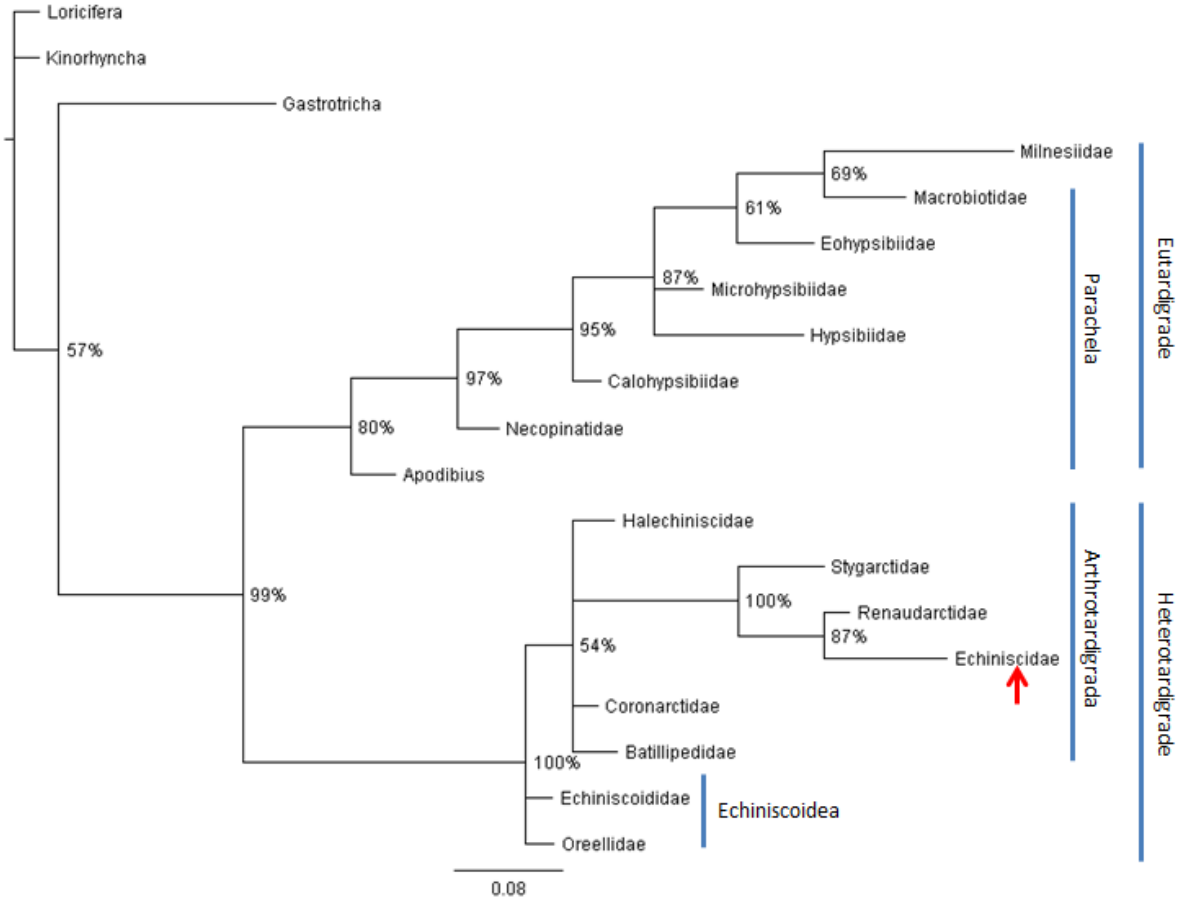


Figure 2.4: Bayesian inference tree of morphological characters with posterior probabilities.
Arrow representing Echiniscidae, suggesting paraphyly within the order Echiniscoidea.

2.5 **Discussion**

All three analytical methods suggested monophyly for taxa within Tardigrada, with two distinct classes, Heterotardigrada and Eutardigrada. This finding is consistent with results reported by Nichols *et al.* (2006). Within the heterotardigrades, no resolved relationships could be determined among Oreellidae + Echniscoididae, Halechiniscidae, Coronarctidae, and Batillipedidae. However, Oreellidae + Echniscoididae appeared as the most basal group among heterotardigrades. This conclusion contradicts conclusions drawn by Nichols *et al.* (2006), who suggested Oreellidae as the most basal family, followed by the divergence of Halechiniscidae, Stygarctidae, and Renaudarctidae. The current analysis entails that Arthrotardigrada and Echiniscoidea are non sister groups because Echiniscidae appeared among families within Arthrotardigrada. This conclusion agrees with one drawn by Nichols *et al.* (2006), however, it contradicts one drawn by Eibye-Jacobsen (2001). The NJ phenogram and Bi tree supported close affinity of Eohypsibiidae to a Macrobiotidae + Milnesiidae clade, which contradicts findings of Nichols *et al.* (2006), who proposed affinity of Eohypsibiidae to Macrobiotidae + Hypsibiidae. In the NJ and Bi analyses, the positioning for Hypsibiidae remained unresolved. No analyses returned Necopinatidae as the most basal member in the Parachela. Instead, Parachela appeared paraphyletic with Milnesiidae, which, itself, formed close affinity to Macrobiotidae. Within the NJ phenogram and Bi tree, Calohypsibiidae formed close affinity with Microhypsibiidae or was the third most basal eutardigrade family, while Hypsibiidae formed close affinity with Macrobiotidae or formed a polytomy with the clades Microhypsibiidae and Eohypsibiidae + Macrobiotidae + Milnesiidae. This conclusion helped determine the branching order between Calohypsibiidae and Hypsibiidae from Nichols *et al.* (2006). The current study entails that *Apodibius* is the most basal group among eutardigrades; this contradicts conclusions drawn by Garey *et al.* (1999), who suggested that Milnesiidae is considered as the sister group to other eutardigrades, and Nichols *et al.* (2006), who suggested that Necopinatidae is the most-basal.

Chapter 3 :

MOLECULAR PHYLOGENETIC SYSTEMATICS OF TARDIGRADES AT THE FAMILY-LEVEL

3.1 **Abstract**

Molecular-based studies on tardigrade systematics have been used to confirm conclusions drawn from morphological-based tardigrade systematics. In the current study, analyses using 18S rRNA, 28S rRNA, and COI mtDNA sequences were conducted to produce one neighbor-joining (NJ) phenogram, one maximum parsimony (MP) cladogram, and one Bayesian inference (Bi) cladogram. Results from a combined data analysis included a monophyletic Heterotardigrada and Eutardigrada, with the exception of the MP analysis, in which the Stygarctidae (Heterotardigrada) comprised a polytomy with the two classes; paraphyletic heterotardigrade Orders Echiniscoidea (Echinsicidae, Oreellidae, and Echiniscoididae) and Arthrotardigrada (Halechiniscidae, Batillipedidae and Stygarctidae); monophyletic Echiniscidae, except in the MP analysis, in which it comprised a polytomy with Oreellidae; monophyletic eutardigrade orders Apochela and Parachela and families Murrayidae, Hypsibiidae, and Isohypsibiidae, with the exception of Macrobiotidae; Milnesiidae appearing as the most basal eutardigrade. Results from an 18S rRNA analysis included a monophyletic heterotardigrade order Echiniscoidea (Echiniscidae, Echiniscoididae, and Oreellidae), except in the NJ analysis, in which it was paraphyletic; monophyletic Echiniscidae, Milnesiidae, Calohypsibiidae, Murrayidae, and Ramazzottidae; and nonmonophyletic Hypsibiidae.

3.2 **Introduction**

Nichols *et al.* (2006) presented the only family-level analysis of tardigrade systematics. They analyzed 18S rRNA sequences from eight species (one Hetertardigrade, seven Eutardigrade), representing five families. Guil & Giribet (2012) analyzed two data sets containing sequences from both classes, Heterotardigrada and Eutardigrada, one 18S rRNA and the other combined 18S rRNA + 28S rRNA + COI mtDNA, from 42 individuals representing 16 species, 12 genera, and five families. Jorgensen *et al.* (2011) analyzed multiple datasets for Echiniscidae (Heterotardigrada), one containing 18S rRNA

sequences and the other containing combined 18SrRNA + 28SrRNA + COI mtDNA sequences, representing ten genera of echiniscids and four genera (*Batillipes*, *Florarctus*, *Echiniscoides*, and *Oreella*) as outgroups.

Nichols *et al.* (2006) used neighbor joining (NJ) and maximum parsimony (MP) techniques, involving seven outgroup species. Results from the 18S rRNA analysis showed that Heterotardigrada and Eutardigrada are monophyletic sister groups (bootstrap value, bv 100%). Within the heterotardigrades, the orders Echiniscoidea (Echiniscus viridissimus) and Arthrotardigrada (Batillpes mirus) appeared as sister groups (bv 99% for NJ and 100% for MP). Within the eutardigrades, Milnesiidae appeared as the most basal family, forming a sister group to the monophyletic families Macrobiotidae and Hypsibiidae (bv 100%). Although the 18S rRNA analysis suggested a close affinity between Calohypsibiidae and Hypsibiidae; this relationship was inconsistent with results from a morphological analyses, in which their taxonomic relationship remained unresolved.

Jorgensen *et al.* (2011) analyzed data using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (Bi) techniques. Analyses on 18S and combined (18S, 28S and COI) sequence analyses produced similar topologies. Results for the 18S analysis suggested a close affinity between *Oreella* + Echiniscidae, a monophyletic Echiniscidae clade, and a paraphyletic Echiniscoidea, which contradicted findings by Jørgensen *et al.* (2010) and Sands *et al.* (2008b). Results for the combined data analysis did not always confirm inferences from the 18S data analyses.

Guil and Giribet (2012) conducted analyses involving parsimony and maximum-likelihood (ML) techniques, using a General Time Reversal model for nucleotide substitution with the Γ correction for rate heterogeneity (GTR+Γ), with a primary search for 20 ML trees and nodal support estimated with 100 bootstrap replicates. Results revealed few monophyletic tardigrade families. Specifically within Heterotardigrada, no family was monophyletic, as genera from different families and orders appeared closely

related to each other. Within Arthrotardigrada, only the Echiniscidae was monophyletic according to 28S rRNA data. Other anayses revealed *Oreella* (Oreellidae) grouping with echiniscid genera, producing paraphyly within Echiniscidae. Within Eutardigrada, Milnesiidae and Murrayidae were monophyletic. Macrobiotidae appeared paraphyletic due to the position of Murrayidae.

In this study, we re-evaluated tardigrade systematics, building on research by Nichols *et al.* (2006), Jorgensen *et al.* (2011), and Guil & Giribet (2012), using 18S rRNA sequence data and combined gene (18S rRNA + 28S rRNA + COI) sequence data. The combined data analysis involved three outgroup species (Gastrotricha, Priapulida, and Kinorhyncha) represented by one sequence each, and 47 tardigrade species, representing 31 genera and 15 families. The 18S rRNA dataset involved seven outgroup species (*Artemia salina*, *Placopecten magellanicus*, *Priapulus caudatus*, *Tenebrio molitor*, *Meloe proscarabaeus*, *Okanagana utahensis*, and *Panulirus argus*) and 80 tardigrade species, represented by 286 sequences, which, in turn, represented 36 tardigrade genera and 14 families. Data were analyzed using NJ, MP, and Bi methods. Techniques involved estimated branch lengths, using Fitch-Margoliash for NJ, estimated bootstrap replicates for MP, and a tree topology evaluated using posterior probability for Bi.

3.3 **Materials and Methods**

3.3.1 Species & Classification

A list of tardigrade species and the sequences used in this study were compiled from Nichols *et al.* (2006), Jorgensen *et al.* (2011), Guil & Giribet (2012) and supplemented with 78 tardigrade sequences and seven outgroup sequences from the National Center for Biotechnology Information (NCBI). Sequences from publications were confirmed for accurate species labelling by cross-referencing accession numbers to the NCBI. All accession numbers were sorted according to species and catergorized according to tardigrade species

checklists created by Bertolani & Guidetti (2005) and Degma *et al.* (2010). A complete list of species and their classification is presented in Appendix B.

3.3.2 Sequences

Sequences were found through keyword searches in the NCBI, between November 2010 and January 2011, which resulted in a collection of 253 18S rRNA sequences, 100 COI mtDNA sequences, and 70 28S rRNA sequences. Sequences from the NCBI were obtained by keyword searches in all databases, using 'Tardigrada', 'Heterotardigrada', and 'Eutardigrada'. The NCBI database returned over 63000 nucelotide sequence hits and 133 PopSet database hits. Sequences from Nucelotide database were restricted to 18S rRNA, 28S rRNA, and COI mtDNA, based on their availability for most species, and accession numbers were downloaded and compiled into Appendix B. PopSet results were refined into 18S rRNA, 28S rRNA, and COI mtDNA sequences, then accession numbers were downloaded into Appendix B. Accession numbers were cross-referenced with their identification in published papers through the Web of Science® database and saved as .pdf files; citations were imported into EndNote X4, and references were compiled in Appendix B. Accession numbers were sorted by species, and duplicates were removed manually. A revision of the sequence data sets occurred in May 2011, when Jorgensen *et al.* (2011) published a paper on the molecular analysis for the Echiniscidae family, which provided an additional 50 sequences to the study, of which 16 were 18S rRNA, 17 28S rRNA, and 17 COI mtDNA. In July 2011, a preprint by Guil & Giribet (2012) provided information about an additional 11 sequences of 18S rRNA, 16 sequences of COI, and one sequence of 28S rRNA.

This study involved consideration of a total of 501 sequences: 280 sequences of 18S ribosomal RNA (rRNA), 133 sequences of cytochrome c oxidase I gene (COI), and 88 sequences of 28S ribosomal RNA (rRNA), representing 15 tardigrade families. Among the 501 sequences, 78 sequences were unpublished, 267 were obtained information from other published papers, 48 sequences were sequenced

by Jorgensen *et al.* (2011), and 105 sequences were sequenced by Guil & Giribet (2012). Three novel sequences from Nichols *et al.* (2006) were unrecoverable and, so, ultimately were exempted from the current study. A complete list of species seqences and their accession numbers may be found in the Appendix B.

3.3.3 Phylogenetic Analyses

Sequences were sorted into two datasets, one representing species for which all three sequence types were available (18S rRNA, COI mtDNA, and 28S rRNA) and one representing all species for which 18S rRNA sequences were available. The 18S rRNA, COI mtDNA, and 28S rRNA sequences were concatenated to form 25 sequences representing 25 tardigrade species from five families, with *Priapulus caudatus* as an outgroup (Table 3.2). This concatenated data set represented the most-complete molecular data available to date, with more than 5800 nucleotides per species. The 18S rRNA dataset represented 69 tardigrade species from 11 families and seven outgroups (*Artemia salina*, *Placopecten magellanicus*, *Priapulus caudatus*, *Tenebrio molitor*, *Meloe proscaraboeus*, *Okanagana utahensis*, and *Panulirus argus*)(Table 3.3). This data set represented the most-widely distributed taxon sampling, 15 out of 23 extant tardigrade families identified by Degma *et al.* (2011), with sequences upwards of 2700 nucleotides.

3.3.3.1 Concatenated 18S rRNA, COI mtDNA and 28S rRNA Phylogenetic Analysis

The 18S rRNA, COI mtDNA, and 28S rRNA sequences were downloaded and edited using the software SeaView 4.0 (Gouy, 2010). The sequences were imported from accession numbers through the Genbank database, using the SeaView (Gouy, 2010) option "import from dbs". Ribosomal RNA and genes with multiple accession numbers for each species-group were aligned using MUSCLE (Edgar, 2004) and then condensed into a single sequence using SeaView 4.0 (Gouy, 2010) option "consensus sequence" and saved in Nexus

format. Sequences were concatenated manually using a text editor, to create a string of 18S rRNA, COI mtDNA, and 28S rRNA sequences, and finally reopened in SeaView 4.0 (Gouy, 2010) and aligned with MUSCLE (Edgar, 2004). The concatenated data set contained sequences from 48 tardigrade species from 31 genera and 15 families and three outgroup species, which were saved in Nexus format for analyses.

Three analyses were conducted, using neighbor-joining (NJ), maximum parsimony (MP), and Bayesian inference (Bi) techniques. The NJ and MP analyses were completed using PAUP* 4.0b10 (Swofford, 2003), accessed from the McMaster EVOL server (maintained by G.B. Golding). The Bayesian inference analysis was completed using mrbayes-3.1.2 (Ronquist, 2003) on a Windows OS platform.

The NJ derived phenogram was constructed using a Kimura-2-parameter (K2P) model for distance with no bootstrapping. The resulting phenogram compared similarities between.

The parsimony analysis was conducted using 100 bootstrap replicates with a heuristic search. The heuristic search began with a stepwise method, using a tree bisection-reconnection (TBR) branch-swapping algorithm, and sequences were added pseudorandomly (nreps=10). The bootstrap values produced from resampling represent the proportion out of the 100 tree replicates that contained the identified clades. Results from the parsimony analysis were condensed into a single tree, using majority rule consensus.

The Bayesian inference analysis for the concatenated sequence dataset was completed using a general-time reversible substitution model with gamma-distributed rate variation for invariable sites (GTR+I+G), which was originally used and suggested by Jorgensen *et al.* (2011). The Markov chain Monte Carlo (MCMC) analysis was completed for 5 000 000 generations with four chains (one cold + three hot), a 25% burnin, and a sampling frequency of 100. After 12500 trees were tossed for burnin, 37500 trees were analyzed, representing a homogenous distribution sampling of posterior probabilities from the entire analysis. Support for monophyletic groups is

considered strong when the value of bootstrap or posterior probability is 95% or above. All three diagrams were visualized using FigTree v.1.3.1 (Rambaut, 2009) and labelled using Microsoft PowerPoint v.14 (2010) and Paint v.6.1 (2009). NJ phenogram branches were modified through FigTree v.1.3.1 software (Rambaut, 2009), using 'proportional' option; scale bars were modified by the software to reflect changes to the branch lengths.

Table 3.1: List of taxa and their associated families used in the 18S rRNA + COI gene + 28S rRNA analysis

Taxon	Genus	Family
Macrobiotus hufelandi	*Macrobiotus*	Macrobiotidae
Macrobiotus pallarii		
Paramacrobiotus richtersi	*Paramacrobiotus*	
Richtersius coronifer	*Richtersius*	
Minibiotus furcatus	*Minibiotus*	
Minibiotus gumersindoi		
Dactylobiotus_sp.	*Dactylobiotus*	Murrayidae
Murrayon c.f. dianeae	*Murrayon*	
Murrayon pullari		
Hypsibius convergens	*Hypsibius*	Hypsibiidae
Diphascon pingue	*Diphascon*	
Astatumen trinacriae	*Astatumen*	
Thulinius stephaniae	*Thulinius*	Isohypsibiidae
Eremobiotus alicatai	*Eremobiotus*	
Isohypsibius_sp.	*Isohypsibius*	
Isohypsibius granulifer		
Isohypsibius prosostomus		
Calohypsibius_sp.	*Calohypsibius*	Calohypsibiidae
Ramazzottius oberhaeuseri	*Ramazzottius*	Ramazzottiidae
Bertolanius nebulosus	*Bertolanius*	Eohypsibiidae
Milnesium c.f. tardigradum	*Milnesium*	Milnesiidae
Florarctus_sp.	*Florarctus*	Halechiniscidae
Stygarctus_sp.	*Stygarctus*	Stygarctidae
Batillipes mirus	*Batillipes*	Batillipedidae
Echiniscoides sigismundi	*Echiniscoides*	Echiniscoididae
Bryodelphax parvulus	*Bryodelphax*	Echiniscidae
Echiniscus blumi Greenland	*Echiniscus*	
Echiniscus blumi Chile		
Echiniscus bigranulatus		
Echiniscus canadensis		
Echiniscus merokensis merokensis		
Echiniscus spiniger		
Echiniscus testudo		
Echiniscus trisetosus		
Echiniscus viridissimus		
Cornechiniscus lobatus	*Cornechiniscus*	
Pseudechiniscus facettalis	*Pseudechiniscus*	
Pseudechiniscus islandicus Faroe Isl.		
Pseudechiniscus islandicus Iceland		
Pseudechiniscus novaezelandiae		

Testechiniscus spitsbergensis	*Testechiniscus*	
Mopechiniscus granulosus	*Mopechiniscus*	
Antechiniscus lateromamillatus	*Antechiniscus*	
Proechiniscus hanneae	*Proechiniscus*	
Parechiniscus chitonides	*Parechiniscus*	
Hypechiniscus exarmatus	*Hypechiniscus*	
Hypechiniscus gladiator		
Oreella mollis	*Oreella*	Oreellidae
Priapulida	Outgroup	
Kinorhyncha		
Gastrotricha		
Total:51	31	14

3.3.3.2 18S rRNA Dataset Analysis

18S rRNA sequences were downloaded and edited using SeaView 4.0 (Gouy, 2010). The sequences were imported from accession numbers, through the Genbank database using the SeaView (Gouy, 2010) option "import from dbs". Species and genus sequence groups possessing multiple accession numbers for 18S rRNA sequences were aligned using the MUSCLE (Edgar, 2004) software application within SeaView 4.0 (Gouy, 2010) and then condensed into a single sequence, with the SeaView 4.0 (Gouy, 2010) option "consensus sequence". The 286 sequences representing 80 tardigrade species from 14 families and seven outgroups were aligned using MUSCLE (Edgar, 2004) and saved as a Nexus format for phylogenetic analyses.

Three analytical methods were used NJ, MP, and Bi, with parameters identical to the concatenated dataset analysis, except with the Bi analysis, for which the MCMC analysis was completed for 8 000 000 generations with four chains (one cold + three hot), and a sampling frequency of 100. After the analysis, 20000 trees were tossed with a 25% burn-in, resulting in a posterior probability sampling of 60000 trees from the entire distribution. All phylogenetic trees were visualized using FigTree v.1.3.1 (Rambaut, 2009) and labelled using Microsoft PowerPoint v.14 (2010) and Paint v.6.1 (2009).

Table 3.2: List of taxa and their associated families used in 18S rRNA analysis

Taxa	No. of individuals	Genera	Family
Macrobiotus_sp.	30	*Macrobiotus*	Macrobiotidae
Macrobiotus furciger	3		
Macrobiotus hufelandi	6		
Macrobiotus pallarii	1		
Macrobiotus sapiens	1		
Macrobiotus tonollii	2		
Paramacrobiotus areolatus	1	*Paramacrobiotus*	
Paramacrobiotus richtersi	6		
Richtersius coronifer	4	*Richtersius*	
Minibiotus_sp.	4	*Minibiotus*	
Minibiotus furcatus	2		
Minibiotus gumersindoi	1	*Dactylobiotus*	Murrayidae
Dactylobiotus_sp.	7		
Dactylobiotus ambiguus	6		
Dactylobiotus octavi	1		
Murrayon dianeae	1	*Murrayon*	
Murrayon pullari	1		
Hypsibius_sp.	7	*Hypsibius*	Hypsibiidae
Hypsibius convergens	2		
Hypsibius cf.convergens	2		
Hypsibius klebelsbergi	1		
Hypsibius scabropygus	1		
Acutuncus antarcticus	6	*Actuncus*	
Diphascon_sp.	15	*Diphascon*	
Diphascon maucci	1		
Diphascon pingue	3		
Diphascon puniceum	4		
Astatumen trinacriae	3	*Astatumen*	
Halobiotus crispae	2	*Halobiotus*	Isohypsibiidae
Halobiotus stenostomus	1		
Thulinius stephaniae	5	*Thulinius*	
Eremobiotus alicatai	2	*Eremobiotus*	
Isohypsibius_sp.	3	*Isohypsibius*	Isohypsibiidae
Isohypsibius asper	4		
Isohypsibius granulifer	2		
Isohypsibius papillifer	1		
Isohypsibius prosostomus	1		
Calohypsibius_sp.	3	*Calohypsibius*	Calohypsibiidae
Hebesuncus_sp.	2	*Hebesuncus*	Ramazzottiidae
Hebesuncus conjugens	1		
Hebesuncus ryani	1		
Ramazzottius oberhaeuseri	23	*Ramazzottius*	
Ramazzottius_sp.	1		
Bertolanius nebulosus	1	*Bertolanius*	Eohypsibiidae
Milnesium_sp.	9	*Milnesium*	Milnesiidae

Milnesium c.f. tardigradum	20		
Florarctus_sp.	1	*Florarctus*	Halechiniscidae
Halechiniscus perfectus	1	*Halechiniscus*	
Halechiniscus remanei	1		
Orzeliscus_sp.	1	*Orzeliscus*	
Raiarctus colurus	1	*Raiarctus*	
Batillipes mirus	1	*Batillipes*	Batillipedidae
Echiniscoides sigismundi	3	*Echiniscoides*	Echiniscoididae
Bryodelphax_sp.	4	*Bryodelphax*	Echiniscidae
Bryodelphax parvulus	1		
Echiniscus_sp.	23	*Echiniscus*	
Echiniscus blumi	2		
Echiniscus bigranulatus	1		
Echiniscus canadensis	3		
Echiniscus granulatus	1		
Echiniscus jenningsi	1		
Echiniscus merokensis merokensis	1		
Echiniscus spiniger	1		
Echiniscus testudo	2		
Echiniscus trisetosus	3		
Echiniscus viridissimus	1		
Cornechiniscus lobatus	3	*Cornechiniscus*	
Pseudechiniscus facettalis	3	*Pseudechiniscus*	
Pseudechiniscus islandicus Faroe Isl.	1		
Pseudechiniscus islandicus Iceland	1		
Pseudechiniscus_sp.	2		
Pseudechiniscus novaezelandiae	1		
Testechiniscus spitsbergensis	3	*Testechiniscus*	
Mopechiniscus granulosus	1	*Mopechiniscus*	
Antechiniscus lateromamillatus	1	*Antechiniscus*	
Proechiniscus hanneae	1	*Proechiniscus*	
Parechiniscus chitonides	1	*Parechiniscus*	
Hypechiniscus exarmatus	1	*Hypechiniscus*	
Hypechiniscus gladiator	1		
Oreella mollis	1	*Oreella*	Oreellidae
Artemia salina (brine shrimp)	1	Outgroup	
Placopecten magellanicus (Mollusca)	1		
Priapulus caudatus (Priapulida)	2		
Tenebrio molitor (darkling beetle)	1		
Meloe proscarabaeus (European oil beetle)	1		
Okanagana utahensis (cicada)	1		
Panulirus argus (Caribbean spiny lobster)	1		
Total:87	286	36	13

3.4 Results

3.4.1 Combined 18S rRNA, COI mtDNA and 28S rRNA analyses

3.4.1.1 Neighbor Joining Fitch-Margoliash Phenogram

The Neigbor-joining (NJ) phenogram (Figure 3.1) contained a nonmonophyletic Heterotardigrada and a monophyletic Eutardigrada. Within Heterotardigrada, most families appeared outside their assumed taxonomic group, specifically the families Stygarctidae and Batillipedidae, which shared close affinities to the outgroup species. Within the monophyletic Echiniscidae family, the genus *Testechiniscus* nested within the genus *Echiniscus*. Oreellidae appeared as a sister group to Echiniscidae, followed by the divergence of Echiniscoididae. Within Eutardigrada, Macrobiotidae and Murrayidae did not appear monophyletic, as *Richtersius coronifer* shared close affinity with *Murrayon* c.f. *dianeae* and *Murrayon pullari*, and *Dactylobiotus* sp. appeared as the second most basal eutardigrade. Hypsibiidae appeared paraphyletic, with Calohypsibiidae nested within the family. Isohypsibiidae appeared monophyletic, forming a clade with *Ramazzottius oberhaeuseri* (Ramazzottidae). The Isohypsibiidae + Ramazzottidae clade shared a close affinity to the Hypsibiidae + Calohypsibiidae clade, both diverging from the Macrobiotidae + Murrayidae clade. Eohypsibiidae, represented by *Bertolanius nebulosus*, appeared as the basal-most eutardigrade family.

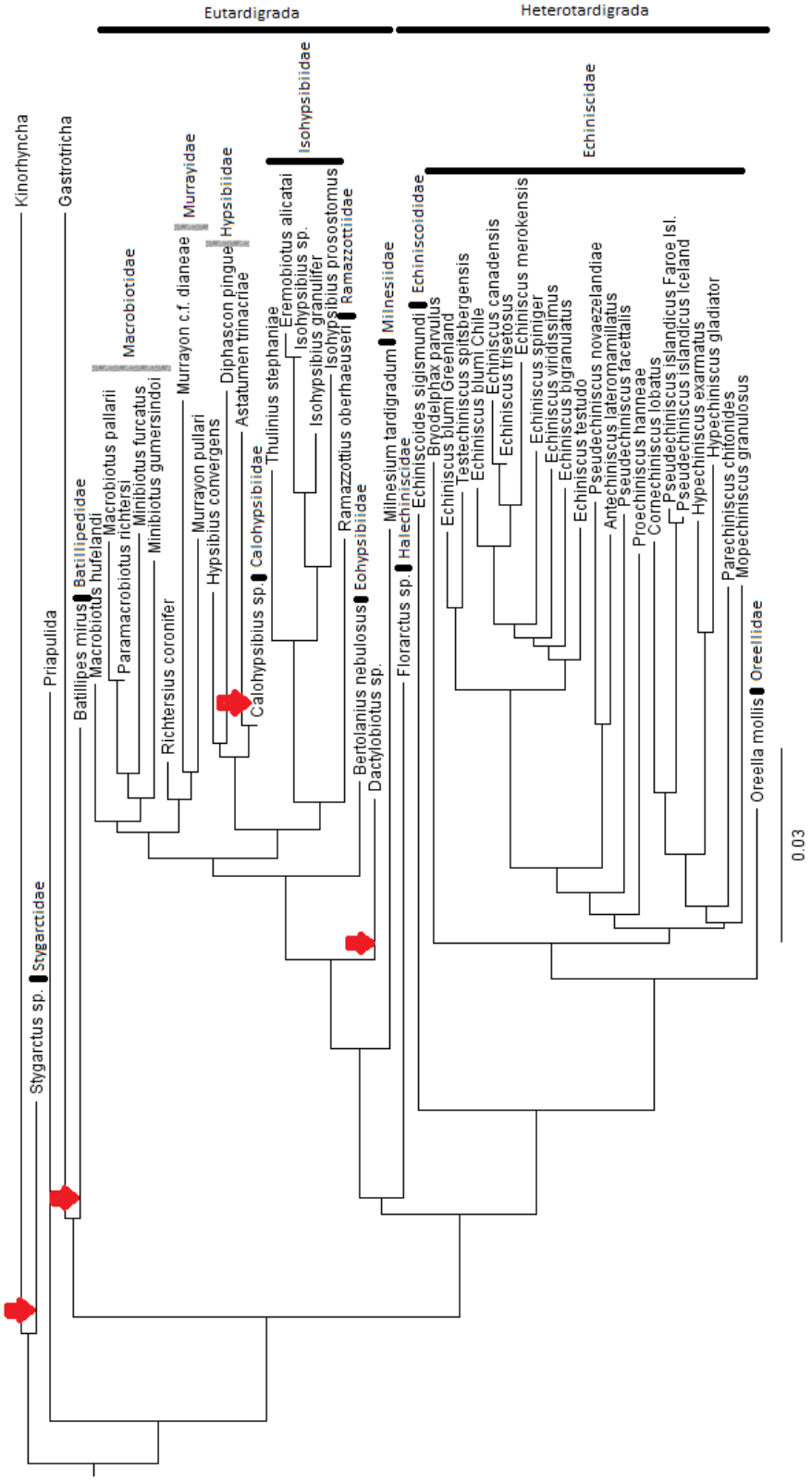


Figure 3.1: Neighbor-Joining phenogram for concatenated 18S rRNA+ 28S rRNA + COI mtDNA sequences.

Arrows indicating Stygarctidae (Heterotardigrada), which shares close affinity to the outgroup; Batillipedidae, which shares close affinity to the outgroup; Calohypsibiidae, which is nested within the Hypsibiidae family; and *Dactylobiotus*, which does not share affinity to other species within Murrayidae.

3.4.1.2 Maximum Parsimony Consensus Cladogram

The maximum parsimony (MP) cladogram (Figure 3.2) contained a nonmonophyletic Heterotardigrada because the family Stygarctidae formed a trichotomy with the two classes and a monophyletic Eutardigrada (bootstrap value, bv 94.9%). Within Heterotardigrada (bv 77.48%), with Stygarctidae exempted, the families Halechiniscidae + Batillipedidae + Echiniscoididae formed a polychotomy with Echinisicidae + Oreellidae (bv 74%). Within Eutardigrada, Murrayidae appeared monophyletic (bv 98.7%), sharing close affinity to a nonmonophyletic Macrobiotidae (bv 57.25%). The species *Richtersius coronifer*, an assumed macrobiotid, formed a trichotomy with Macrobiotidae and Murrayidae (bv 84.62%). Eohypsibiidae, represented by *Bertolanius nebulosus*, formed a clade with Macrobiotidae + Murrayidae + *R. coronifer* (bv 55.34%). Hypsibiidae appeared as a monophyletic sister group (bv 91.88%) to Calohypsibiidae (bv 95.33%), both of which, together, formed a tricotomy with Ramazzottidae and the Macrobiotidae + Murrayidae + *R. coronifer* clade (bv 81.73%). A monophyletic Isohypsibiidae formed a clade with a Macrobiotidae + Murrayidae + Eohypsibiidae + Hypsibiidae + Calohypsibiidae + Ramazzottidae clade, and Milnesiidae appeared as the most basal family in the Eutardigrada class (bv 100%). Macrobiotidae appeared monophyletic, with the exception of *Richtersius coronifer*; its evolutionary relationship with Macrobiotidae and Murrayidae remained unresolved.

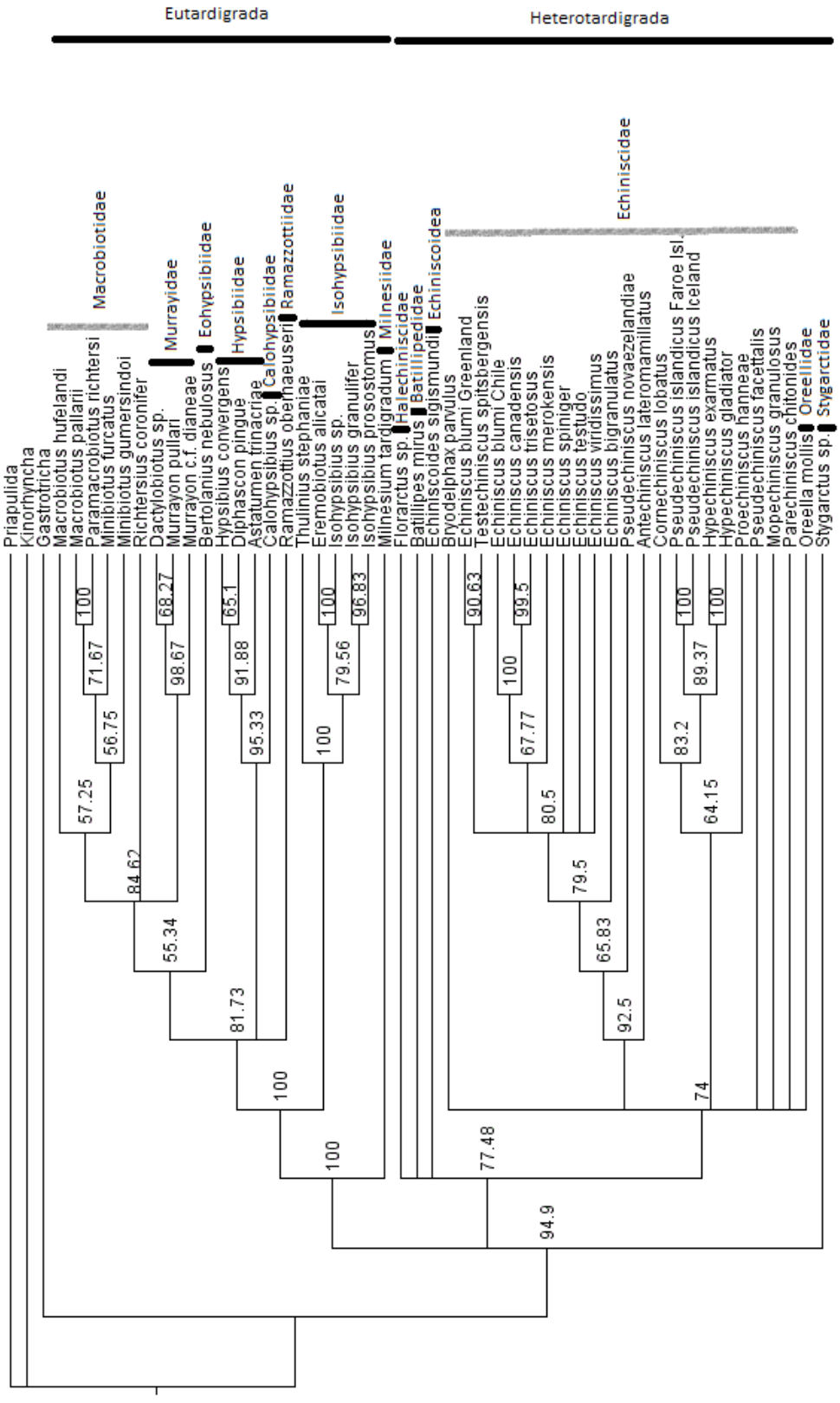

Eutardigrada
Heterotardigrada
Macrobiotidae
Murrayidae
Eohypsibiidae
Hypsibiidae
Calohypsibiidae
Ramazzottiidae
Isohypsibiidae
Milnesiidae
Halechiniscidae
Batillipedidae
Echiniscoidea
Echiniscidae
Priapulida
Kinorhyncha
Gastrotricha
Macrobiotus hufelandi
Macrobiotus pallarii
Paramacrobiotus richtersi
Minibiotus furcatus
Minibiotus gumersindoi
Richtersius coronifer
Dactylobiotus sp.
Murrayon pullari
Murrayon c.f. dianeae
Bertolanius nebulosus
Hypsibius convergens
Diphascon pingue
Astatumen trinacriae
Calohypsibius sp.
Ramazzottius oberhaeuseri
Thulinius stephaniae
Eremobiotus alicatai
Isohypsibius sp.
Isohypsibius granulifer
Isohypsibius prosostomus
Milnesium tardigradum
Florarctus sp.
Batillipes mirus
Echiniscoides sigismundi
Bryodelphax parvulus
Echiniscus blumi Greenland
Testechiniscus spitsbergensis
Echiniscus blumi Chile
Echiniscus canadensis
Echiniscus trisetosus
Echiniscus merokensis
Echiniscus spiniger
Echiniscus testudo
Echiniscus viridissimus
Echiniscus bigranulatus
Pseudechiniscus novaezelandiae
Antechiniscus lateromamillatus
Cornechiniscus lobatus
Pseudechiniscus islandicus Faroe Isl.
Pseudechiniscus islandicus Iceland
Hypechiniscus exarmatus
Hypechiniscus gladiator
Proechiniscus hanneae
Pseudechiniscus facettalis
Mopechiniscus granulosus
Parechiniscus chitonides
Oreella mollis
Oreellidae
Stygarctidae
57.25
100
71.67
56.75
84.62
68.27
98.67
55.34
65.1
91.88
95.33
81.73
100
100
79.56
96.83
100
90.63
99.5
100
67.77
80.5
79.5
65.83
92.5
83.2
100
89.37
64.15
100
74
77.48
94.9

Figure 3.2: Parsimony cladogram of concatenated sequences with 100 bootstrap* replicates.
***- Bootstrap values did not appear as an expected proportion of 100 replicates due to bugs within PAUP* 4.0b10. Our bootstrap values may have been misrecorded because the heuristic search applied for bootstrapping using random sequence addition may have saved more trees than should be saved (Carmen Cheung, personal communication, September 22, 2012).**

3.4.1.3 Bayesian Inference Cladogram

The Bayesian inference (Bi) cladogram (Figure 3.3) contained monophyletic Heterotardigrada and Eutardigrada. Within Heterotardigrada, Echiniscidae appeared paraphyletic, with Oreellidae nested within the family (pp 100). Arthrotardigrada formed a monophyletic clade (pp 99.64), with Echiniscidae + Oreellidae (pp 100) as sister groups and Echiniscoididae diverging from Echiniscidae + Oreellidae clade. Batillipedidae diverged from Arthrotardigrada (pp 92.53), and *Florarctus* sp., representing Halechinisicidae diverged from the Batillipedidae + Arthrotardigrada clade (pp 97.85). Stygarctidae appeared as the most basal family within the Heterotardigrade class (pp 76.34). Within Eutardigrada, Macobiotidae was nonmonophyletic, as *Richtersius coronifer* showed a close affinity to species within the monophyletic Murrayidae (posterior probability 65.37%). Sister group to the Macrobiotidae + Murrayidae clade was the Eohypsibiidae (pp 99). Sister group to the Macrobiotidae + Murrayidae + Eohypsibiidae clade was the clade composed of Hypsibiidae + Calohypsibiidae + Ramazzottidae (pp 99.91). The monophyletic Hypsibiidae appeared as a sister group to Calohypsibiidae, together they were sister group to Ramazzottidae (pp 99.9). Isohypsibiidae appeared monophyletic and formed a sister group for the clade composed of Macrobiotidae + Murrayidae + Eohypsibiidae + Hypsibiidae + Calohypsibiidae + Ramazzottidae (pp 99.9). Milnesiidae appeared as the basal-most eutardigrade family (pp 99.88).

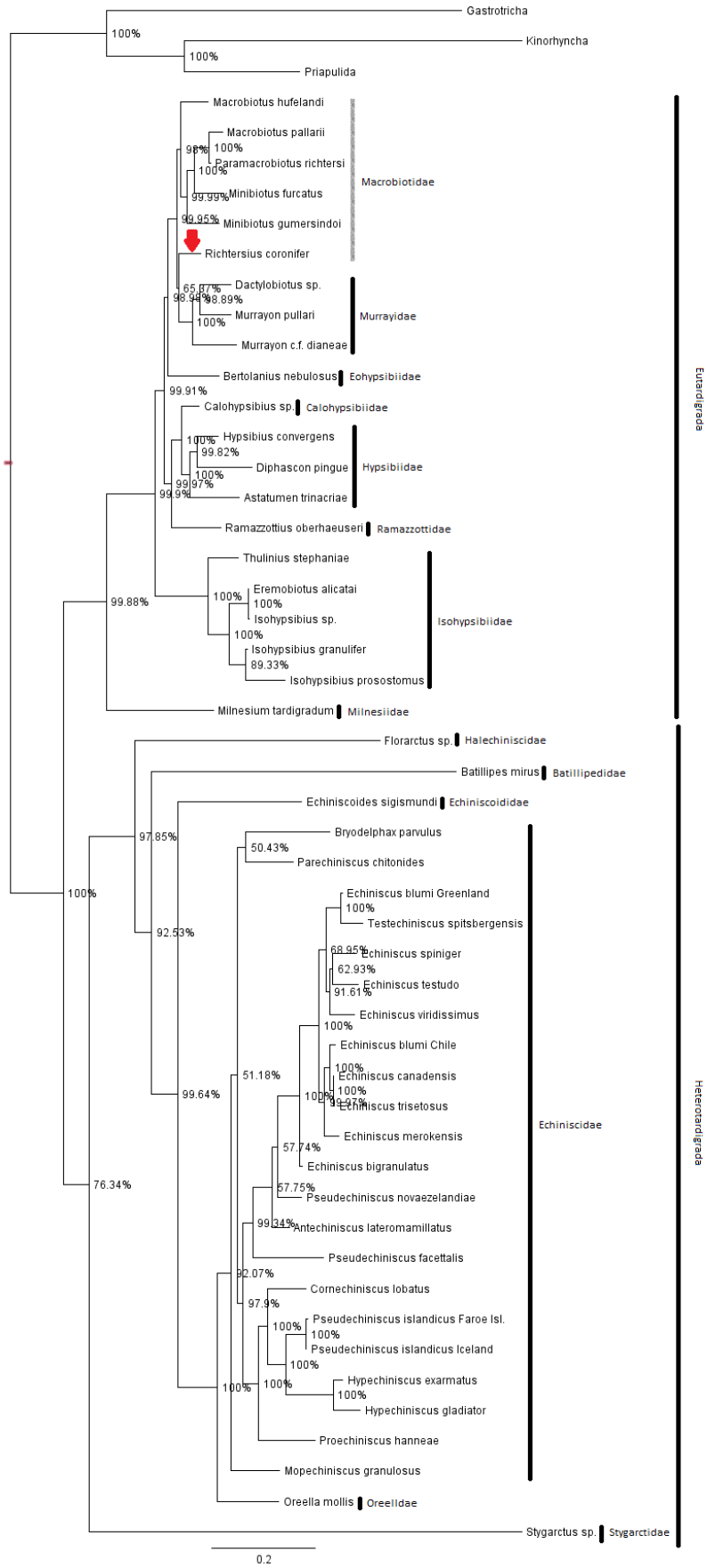

Gastrotricha
Kinorhyncha
Priapulida
Macrobiotus hufelandi
Macrobiotus pallarii
Paramacrobiotus richtersi
Minibiotus furcatus
Minibiotus gumersindoi
Richtersius coronifer
Macrobiotidae
Dactylobiotus sp.
Murrayon pullari
Murrayon c.f. dianeae
Murrayidae
Bertolanius nebulosus
Eohypsibiidae
Calohypsibius sp.
Calohypsibiidae
Hypsibius convergens
Diphascon pingue
Astatumen trinacriae
Hypsibiidae
Ramazzottius oberhaeuseri
Ramazzottidae
Thulinius stephaniae
Eremobiotus alicatai
Isohypsibius sp.
Isohypsibius granulifer
Isohypsibius prosostomus
Isohypsibiidae
Milnesium tardigradum
Milnesiidae
Eutardigrada
Florarctus sp.
Halechiniscidae
Batillipes mirus
Batillipedidae
Echiniscoides sigismundi
Echiniscoididae
Bryodelphax parvulus
Parechiniscus chitonides
Echiniscus blumi Greenland
Testechiniscus spitsbergensis
Echiniscus spiniger
Echiniscus testudo
Echiniscus viridissimus
Echiniscus blumi Chile
Echiniscus canadensis
Echiniscus trisetosus
Echiniscus merokensis
Echiniscus bigranulatus
Pseudechiniscus novaezelandiae
Antechiniscus lateromamillatus
Pseudechiniscus facettalis
Cornechiniscus lobatus
Pseudechiniscus islandicus Faroe Isl.
Pseudechiniscus islandicus Iceland
Hypechiniscus exarmatus
Hypechiniscus gladiator
Proechiniscus hanneae
Mopechiniscus granulosus
Echiniscidae
Oreella mollis
Oreellidae
Stygarctus sp.
Stygarctidae
Heterotardigrada
0.2

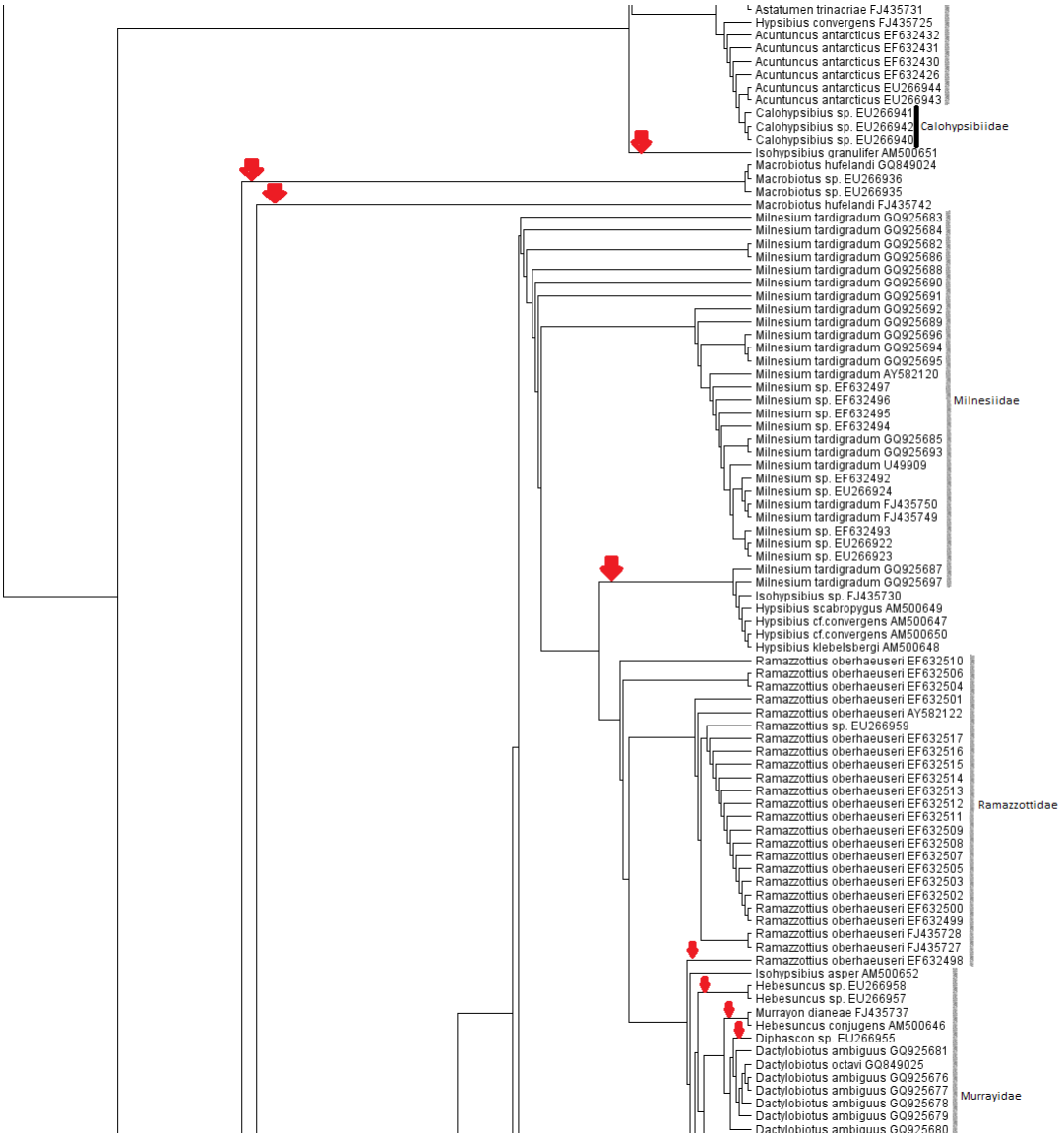
Figure 3.3: Bayesian inference tree for concatenated sequences with posterior probabilities.
Arrows represent *Richtersius coronifer*, which shares close affinity to species within Murrayidae.

3.4.2 18S rRNA Analyses

3.4.2.1 Neighbor-Joining Fitch-Margoliash Estimated Phenogram

The neighbor-joining phenogram (Figure 3.4) did not support a monophyletic sister group relationship between Heterotardigrada and Eutardigrada; instead, families within both classes appeared in discordance with previously generated taxonomies. Within Heterotardigrada, Echiniscoididae appeared paraphyletic, branching from Eutardigrada; Halechiniscidae appeared paraphyletic, with *Batillipes mirus* (Batillipedidae) nested within; Echiniscidae taxa appeared paraphyletic and separated into two clades, one clade consisted of the genera *Pseudechiniscus*, *Cornechiniscus*, *Bryodelphax*, and *Hypechiniscus*, with Halechiniscidae and Oreellidae nested within, and the second clade consisted of the genus *Echinsicus*. Within Eutardigrada, a monophyletic Calohypsibiidae appeared nested within a paraphyletic Hypsibiidae; Macrobiotidae appeared nonmonophyletic, separating into two clades, one clade consisting of *Macrobiotus* sp. (EF632478 – EF632491; EU266938) and *M. hufelandi* (FJ43538 – FJ43540), and the other clade branching from *Bertolanius nebulosus* (Eohypsibiidae) and consisting of *R. coronifer*, the *Minibiotus* genus, the *Paramacrobiotus* genus, and the remainder of the *Macrobiotus* genus, with *Raiarctus colorus* (Halechiniscidae) nested within; Milnesiidae appeared mostly monophyletic, with the exception of *Milnesium tardigradum* (GQ925687 – GQ925697) branching from Ramazzottidae, which was monophyletic with the exception of *R. oberhaeuseri* EF632498 and *Hebesuncus ryani*, which branched from a paraphyletic Murrayidae; Isohypsibiidae appeared paraphyletic with *Hypsibius* sp. Z93337 nested within.

Continued on next page…



Continued on next page…

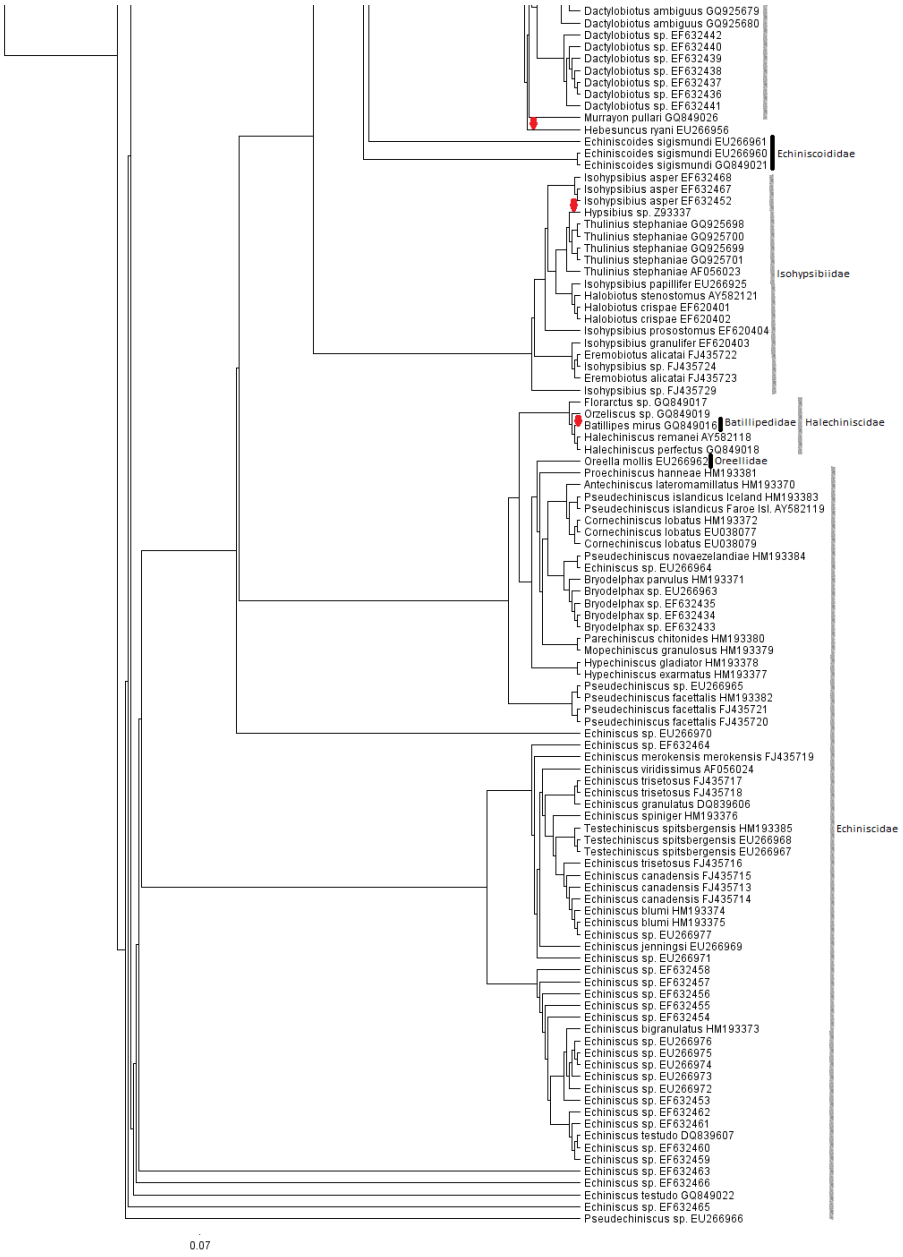
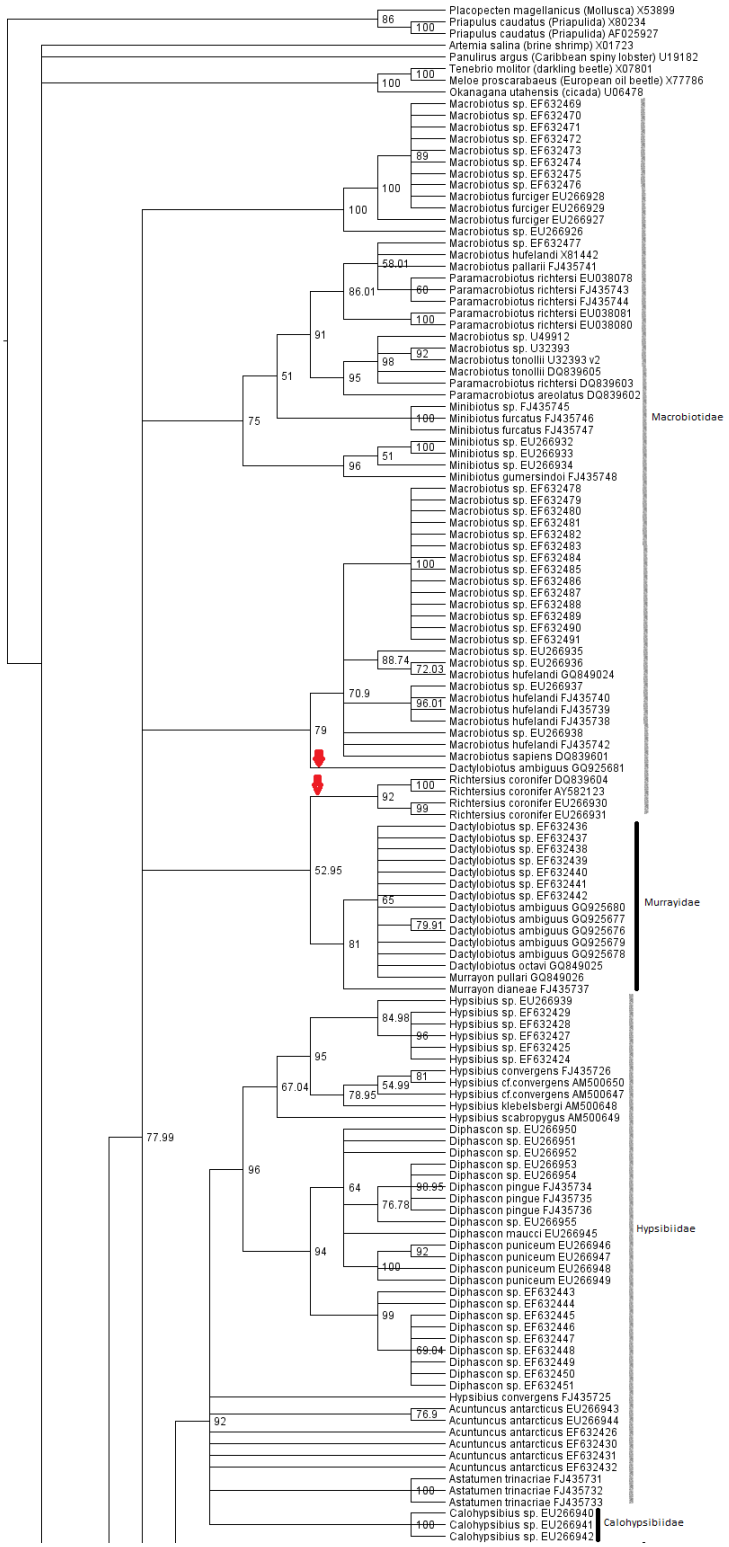


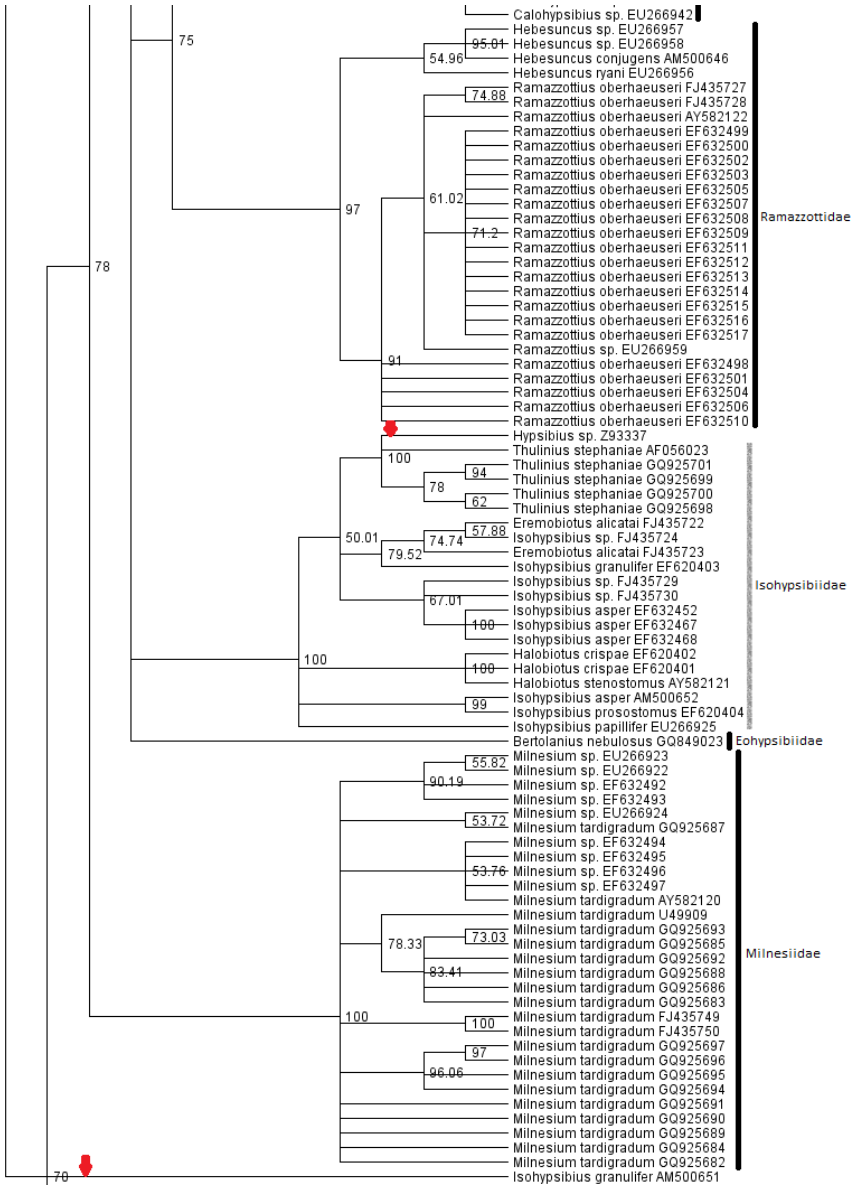
Figure 3.4: Neighbor-Joining phenogram for 18S rRNA sequences. Arrows representing Batillipedidae, which nested within Halechiniscidae; *Hypsbius* sp., which shared close affinity to *Isohypsbius* species; *Hebesuncus ryanii*, which shared close affinity to Murrayidae; *Diphascon* sp. (Hypsibiidae), which shared close affinity to Murrayidae; and *Hebesuncus conjugens*, *Hebesuncus* sp., and *Ramazzottius oberhaeuseri*, which shared close affinity to Murrayidae; *Milnesium tardigradum* diverged from Ramazzottidae; *Macrobiotus* sp. and *M. hufelandi* do not associate with other members of *Macrobiotus*; *Isohypsibius granulifer* share close affinity to Hypsibiidae; *Raiarctus colurus* (Halechiniscidae) nested within Macrobiotidae.

3.4.2.2 Maximum Parsimony Consensus Cladogram

The maximum parsimony (MP) cladogram (Figure 3.5) contained a Eutardigrada group (bootstrap value, bv 78%) that included *Isohypsibius granulifer* (AM500651) and a Heterotardigrada that was not monophyletic because the families Halechiniscidae, Batillipedidae, and Echiniscoididae formed a poltomy with Eutardigrada and the remaining families in Heterotardigrada (bv 70%). Within Heterotardigrada, only Echiniscoididae appeared monophyletic, and Echiniscidae + Oreellidae formed a clade, in which *Oreella mollis* (Oreellidae) nested within the Echiniscidae (bv 87%). Within Eutardigrada, Macrobiotidae did not appear monophyletic, instead separated into three clades that consisted of (1) *Macrobiotus* sp. + *Macrobiotus furciger*, (2) *Macrobiotus* sp. + *Paramacrobiotus richtersius* + *Paramacrobiotus areolatus* + *Minibiotus* sp. + *Minibiotus furcatus* + *Minibiotus gumersindoi*, and (3) *Macrobiotus* sp. + *Macrobiotus hufelandi* + *Macrobiotus sapiens*. The three Macrobiotidae clades formed a polytomy with Murrayidae group (bv 77.99%), with the species *Dactylobiotus ambigus* sharing close affinity to clade 3 in the macrobiotids. Some species of *Richtersius coronifer* did not group within the three macrobiotid clades, instead showing close affinity to Murrayidae. The nonmonophyletic Hypsibiidae appeared as a sister group to the monophyletic Calohypsibiidae (bv 92%), with hypsibid genera *Acutuncus* and *Astatumen* sharing a polytomic relationship with the two families. Diverging from Hypsibiidae + Calohypsibiidae was the monophyletic Ramazzottidae (bv 97%). An Isohypsibiidae group appeared, but *Hypsibius* sp. (Z93337) grouped with *Thulinius stephaniae*, which, together with the only representative of the Eohypsibiidae family, *Bertolanius nebolosus*, formed a polytomy with the Macrobiotidae, Murrayidae, Hypsibiidae + Calohypsibiidae + Ramazzottidae clades. Milnesiidae appeared monophyletic and was the most basal Eutardigrada family.



Continued on next page…



Continued on next page…

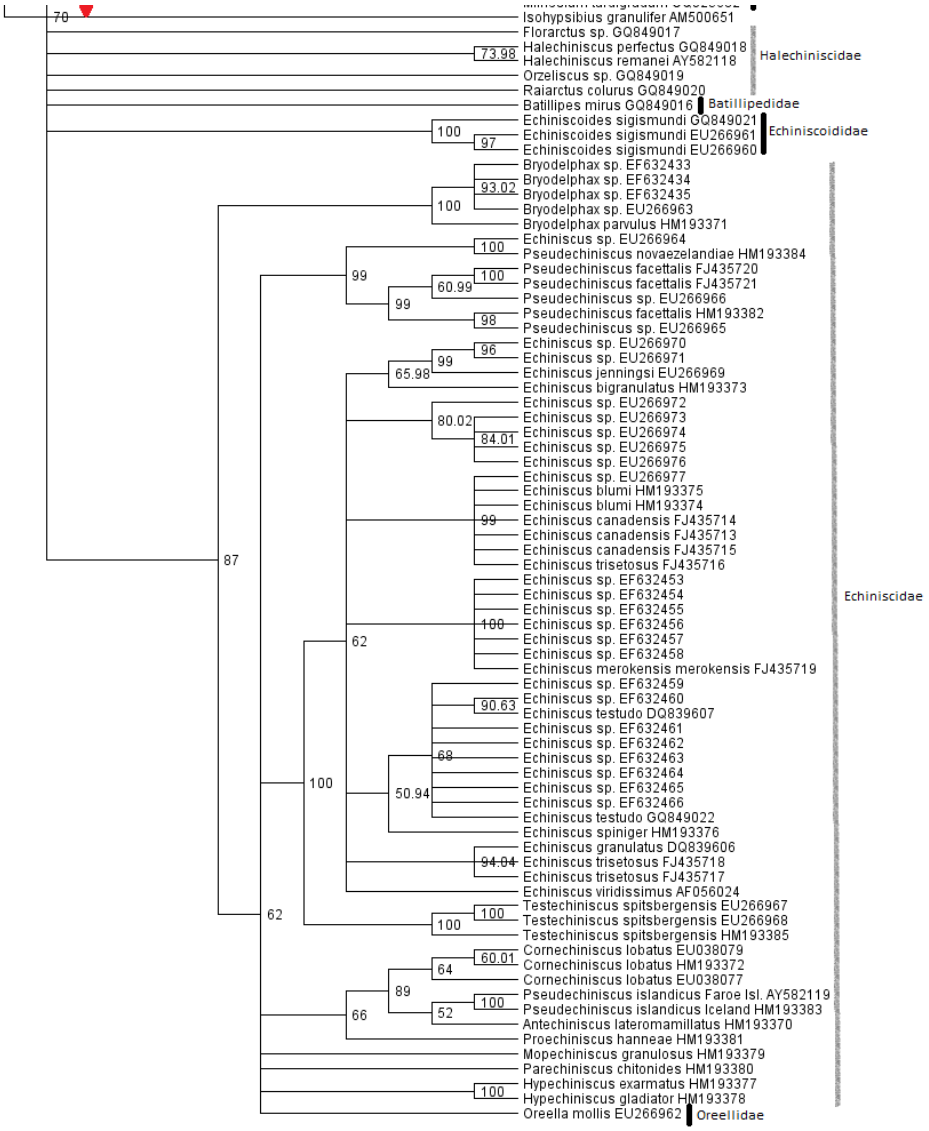
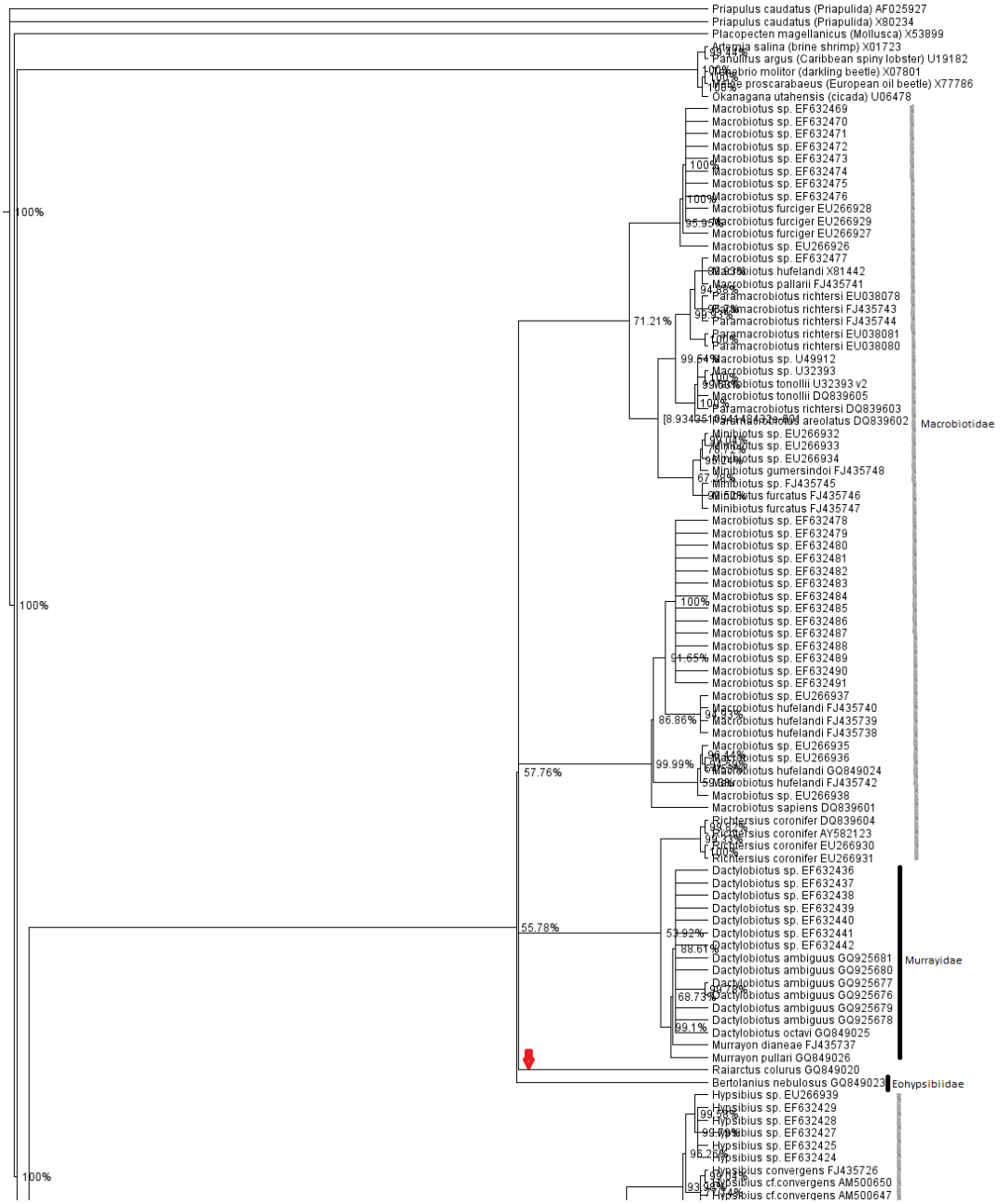


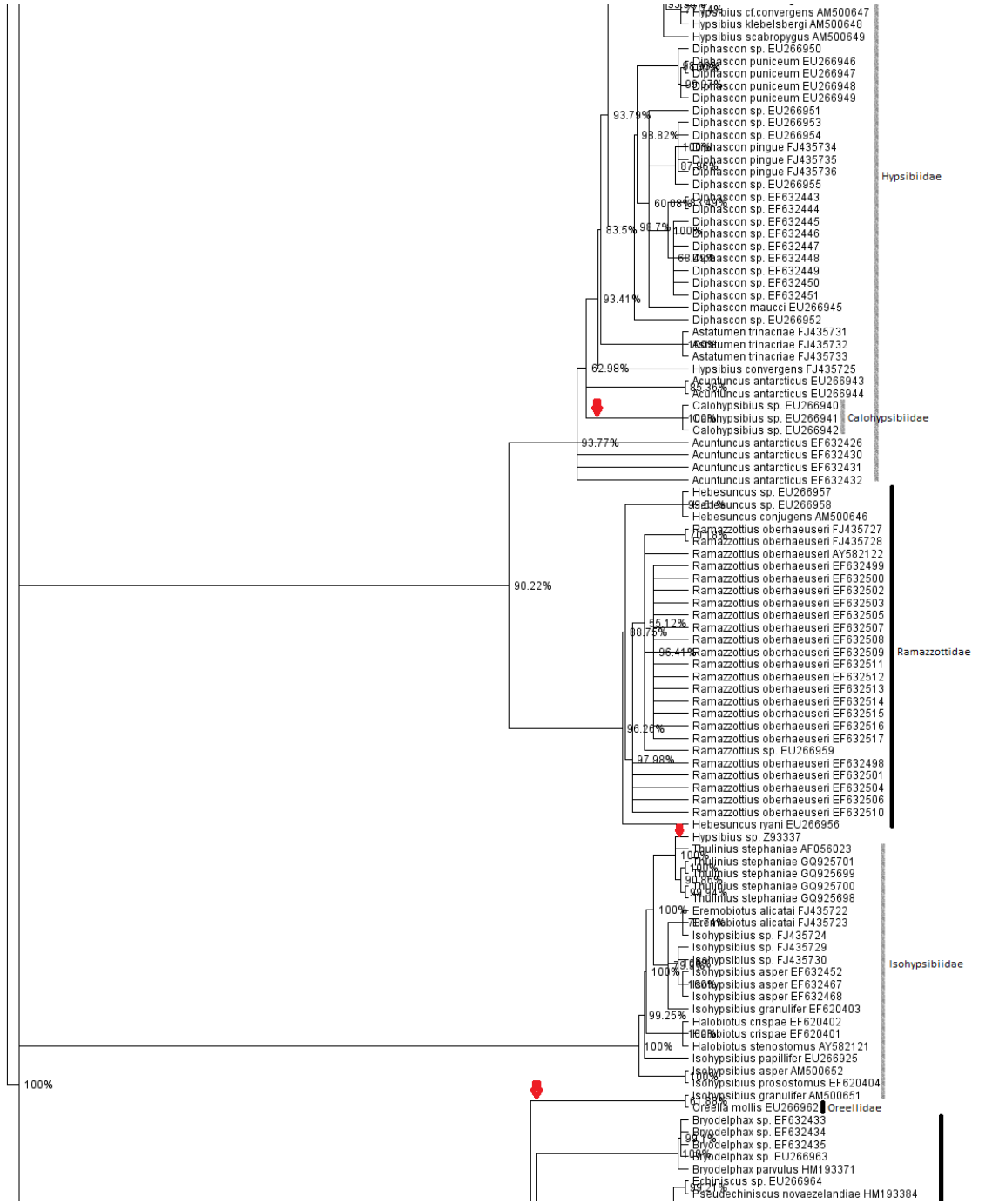
Figure 3.5: Maximum parsimony cladogram for 18S rRNA sequences, analyzed with 100 bootstrap* replicates.
Arrows representing *Isohypsibius granulifer* does not share close affinity to other *Isohypsibius* species; *Hypsibius* sp. shares close affinity with species within Isohypsibiidae; *Dactylobiotus ambiguus* shares close affinity to Macrobiotidae species; *Richtersius coronifer* shares close affinity to Murrayidae.
***- Bootstrap values did not appear as an expected proportion of 100 replicates due to bugs within PAUP* 4.0b10. Our bootstrap values may have been misrecorded because the heuristic search applied for bootstrapping using random sequence addition may have saved more trees than should be saved (Carmen Cheung, personal communication, September 22, 2012).**

3.4.2.3 Bayesian inference 18S rRNA Cladogram

The Bayesian inference cladogram (Figure 3.6) contained a monophyletic Heterotardigrada and a non monophyletic Eutardigrada with (posterior probability, pp 100%). Within Heterotardigrada, the monophyletic Echiniscidae appeared as a sister group to Oreellidae (pp 61.49), themselves, together forming a sister group to the monophyletic Echiniscoididae (pp 54.47). The Halechiniscidae and Batillipedidae were monophyletic and situated more basal relative to the Echiniscidae + Oreellidae + Echiniscoididae clade (pp 62.88). Within the eutardigrades, families formed a four clade polytomy, sister with the Heterotardigrada clade. The first eutardigrade clade contained Macrobiotidae divided into two clades (pp 57.76) sharing a polytomic node with the monophyletic Murrayidae. Sister group to Macrobiotidae + Murrayidae was *Bertolanius nebolosus* from the family Eohypsibiidae. The second eutardigrade clade contained the nonmonophyletic Hypsibiidae (with Calohypsibiide nested within the family) as sister group to the monophyletic Ramazzottidae (pp 90.22). The third eutardigrde clade contained the nonmonophyletic Isohypsibiidae. And, finally, the fourth eutardigrade clade contained members of the monophyletic Milnesiidae family (pp 100%).



Continued on next page…



Continued on next page…

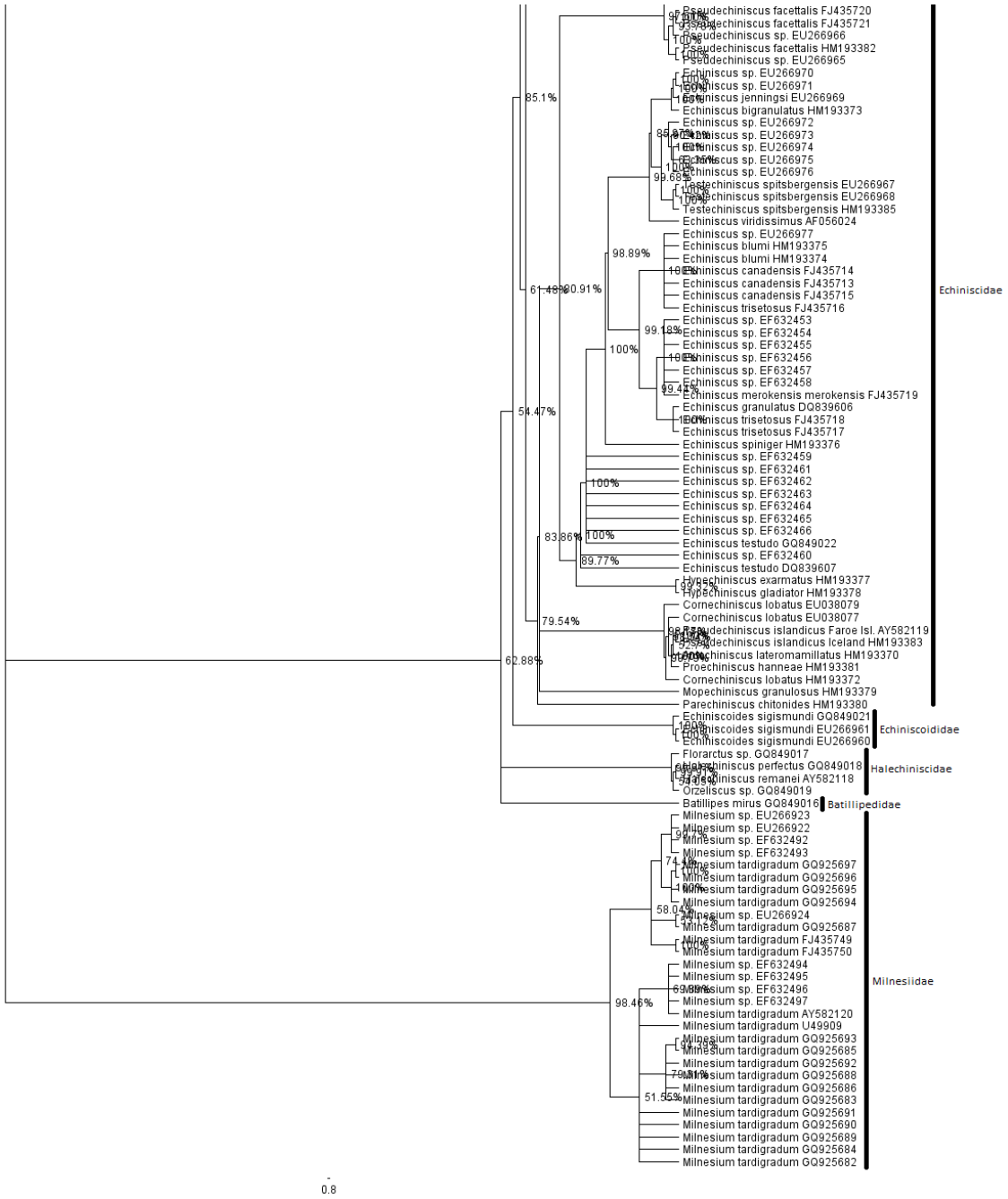


Figure 3.6: Bayesian inference tree for 18S rRNA sequences with posterior probabilities.
Arrows representing *Isohypsbius granulifer*, which shares close affinity to Oreellidae; *Hypsibius* sp. share close affinity to Isohypsibiidae, Calohypsobiidae nested within Hypsibiidae; *Raiarctus colurus* (Halechiniscidae) appeared to diverge from Macrobiotidae + Murrayidae.

3.5 **Discussion**

Analysing the concatenated 18S rRNA, 28S rRNA, and COI mtDNA sequences confirmed that the classes Heterotardigrada and Eutardigrada are monophyletic sister groups (bootstrap value, bv 94.9% and posterior probability, pp 100%), with the exception of the parsimony analysis, in which Stygarctidae appeared in a polytomy with Heterotardigrada and Eutardigrada. Within Heterotardigrada, the order Echiniscoidea, represented by Echinsicidae, Oreellidae, and Echiniscoididae, did not appear monophyletic. The family Echiniscidae was monophyletic in the NJ and Bi analyses but formed a polytomy with Oreellidae in the parsimony analysis. Results from the current study are inconsistent with results obtained by Guil and Giribet (2012), who combined 18S rRNA, 28S rRNA, and COI mtDNA sequences and performed a maximum-likelihood analysis; they concluded that the order Echiniscoidea (represented by the single family Echiniscidae) was monophyletic. Our study also was inconsistent with results obtained by Jorgensen *et al.* (2011); they concluded that Echiniscoidea was monophyletic. Observations from the current study entail that the Arthrotardigrada (Halechiniscidae, Batillipedidae, and Stygarctidae) is paraphyletic. This result is inconsistent with results obtained by Guil and Giribet (2012), in which the Arthrotardigrada and its families were polyphyletic. Within Eutardigrada, the orders Apochela and Parachela (MP bv 100%; Bi pp 99.88) and the families represented by more than one species, Murrayidae, Hypsibiidae, Isohypsibiidae, were monophyletic, with the exception of the polyphyletic Macrobiotidae. In all analyses, Milnesiidae was the most basal family, which is consistent with results obtained by Nichols *et al.* (2006) and Guil and Giribet (2012).

Analyzing the 18S rRNA sequences confirmed that the heterotardigrade order Echiniscoidea (Echiniscidae, Echiniscoididae, and Oreellidae) was monophyletic, with the exception of the NJ analysis, in which the Echiniscoididae shared close affinity to some

eutardigrade families (Milnesiidae, Ramazzottidae, and Murrayidae). The monophyly of Echiniscoidea is consistent with results from Nichols *et al.* (2006), Sands *et al.* (2008b), and Jorgensen *et al.* (2010); however, it contradicts results from Jorgensen *et al.* (2011). From the three different analysis methods, only Bayesian inference resulted in the monophyly of the five heterotardigrade families investigated (Echiniscidae, Echiniscoididae, Halechiniscidae – Oreellidae and Batillipedidae had only one sequence to represent them). Within the eutardigrada, Hypsibiidae appeared nonmonophyletic, which was consistent with the results onbtained by Guil & Giribet (2012). Also consistent with results obtained by Guil & Giribet was the finding that the eutardigrade families Milnesiidae, Calohypsibiidae, and Murrayidae were monophyletic. In the current study, the family Ramazzottidae is monophyletic, consistent with conclusions drawn by Marley *et al.* (2011). The current study suggested a monophyly of Echiniscidae, which is consistent with findings by Jorgensen *et al.* (2011).

Chapter 4 :

PHYLOGENETIC SYSTEMATICS OF TARDIGRADES AT THE FAMILY-LEVEL USING COMBINED MORPHOLOGICAL AND MOLECULAR DATA

4.1 **Abstract**

Tardigrade systematics has been studied at a variety of taxonomic levels using morphological and molecular techniques. In the current study, the first total evidence analysis for the phylum level, combining morphological characters and molecular sequences was analyzed for 53 tardigrade species representing 18 families. The total evidence supermatrix of morphological and 18S, 28S, and COI sequence data was analyzed using maximum parsimony (MP) and Bayesian inference (Bi) techniques, and results were compared to results obtained in the morphological and molecular analyses in Chapters II and III. Results obtained from the combined data analysis were inconclusive at the class and order levels from the MP analysis. The Bi analysis returned monophyletic Heterotardigrada and Eutardigrada; monophyletic Arthrotardigrada and Echiniscoidea; paraphyletic Parachela and Apochela; monophyletic Echiniscidae and Murrayidae; polyphyletic Macrobiotidae and Hypsibiidae; Necopinatidae forming a trichotomy with Eutardigrada and Heterotardigrada; Microhypsibiidae appearing as the most basal family within Eutardigrada; *Apodibius* appearing as the basal-most clade among all tardigrades. Incongruences between total evidence and single data type evidence (using either morphology or molecules) analyses are discussed.

4.2 **Introduction**

Jorgensen *et al.* (2011) presented the first total evidence analysis of the Echiniscidae family (Heterotardigrade) by combining 18S, 28S, and COI sequences into a matrix containing 34 morphological characters, involving 19 species from Echiniscidae and four outgroup species (*Batillipes mirus*, *Florarctus* sp., *Echniscoides sigismundi*, and *Oreella mollis*). Cladograms constructed from maximum parsimony (MP) and Bayesian inference (Bi) techniques, in which the combined data set

was analyzed by partitions in a single analysis. Results revealed a monophyletic Echiniscoidea (Bremer index: MP 68 and Bi 88; decay index 0), an Echiniscidae + *Oreella* clade (100, 100, 8), and a monophyletic Echiniscidae (88, 100, 4); however, relationships among genera within the Echiniscidae family were unresolved.

In this study, we performed total evidence analyses of tardigrades at the family level. The study involved 14 families, including three outgroup species (Gastrotricha, Kinorhyncha, Priapulida), 52 known species, and one species *incertae sedis*. The total evidence analyses involved 50 morphological characters (Table 4.1) and 18S, 28S, and COI sequences, and phylogenies were constructed using MP and Bi techniques. Results returned a monophyletic Murrayidae and superfamilies Hypsibioidea, Isohypsibioidea, and Macrobiotoidea and relationships among tardigrade families and genera within Echiniscidae, Murrayidae, Hypsibiidae, and Macrobiotidae.

4.3 **Materials and Methods**

A list of 53 tardigrade taxa was compiled to represent 18 families, one species *incertae sedis*, and 47 species (from Chapters II and III; Tables 4.1 and 4.2). Taxa were chosen based on the availability of 18S, 28S, and COI sequences. The families Batillipedidae, Halechiniscidae, Echiniscoididae, Oreellidae, Eohypsibiidae, Calohypsibiidae, and Stygarctidae are represented by one species, whereas no sequences were found to represent Necopinatidae, Microhypsibiidae, Renaudarctidae, Coronarctidae, and *Apodibius*. Multiple species represented the families Echiniscidae, Hypsibiidae, Murrayidae, and Macrobiotidae. Fifty morphological characters (from Chapter II; Table 4.1) and 140 sequences of 18S, 28S, and COI (Table 4.2) genes, each representing one among 56 taxa were concatenated into strings and combined with the morphological characters to create a supermatrix. Gastrotricha, Kinorhyncha, and Priapulida were chosen as outgroups based on the availability of 18S rRNA, 28S rRNA, and COI sequences (and their previous use as outgroups in chapters II and III).

A single sequence was selected to represent each of 18S rRNA, 28S rRNA, and COI genes for each species. In situations where multiple copies of the same gene existed, a multiple sequence alignment using MUSCLE (Edgar, 2004) was conducted. One gene was chosen from the multiple copies on the basis of length (longest possible), variable sites (least), gaps (fewest) in the alignment, and overall consistency with the other sequences in the MUSCLE (Edgar, 2004) alignment. The selected sequence then was verified by a Basic Local Alignment Search Tool (BLAST) analysis, where a nucleotide-based search for highly similar sequences (megablast) was used to demonstrate correspondence between gene accession number and the appropriate species. If BLAST results returned queries identical to sequences with the corresponding accession numbers used in the alignment, represented in descending order of best-matched query results, then sequences were retained. If BLAST results returned best-matched queries for another species or gene, then the sequence was discarded and another sequence was chosen. In situations where different accession numbers were assigned to identical sequences, one accession number was chosen arbitrarily. The alignment for *Echiniscus trisetosus* was either gapped or had a close affinity to *Echiniscus canadensis*.

The 18S, 28S, and COI sequences were downloaded using accession numbers (summarized in Table 4.1) from the Genbank database using the software SeaView 4.0 (Gouy, 2010) option “import from dbs”. Sequences were saved in a text file and concatenated manually in a text editor to the 50 associated morphological characters. The supermatrix manually was converted into an interleaved format and saved as a Nexus file. That file was analyzed using two methods, maximum parsimony (MP) (PAUP* 4.0b10 (Swofford, 2003), run on the McMaster EVOL server [maintained by G.B. Golding]), and Bayesian inference (Bi) (mrbayes-3.1.2 (Ronquist, 2003)) on a Windows OS platform. The MP analysis treated the mixed data as ‘standard type’ in which the symbols "0123ACGTMSWYRKHDBVN" represented both molecular and morphological characters. A parsimony analysis was completed using 100 bootstrap replicates

with a heuristic search, which was used to estimate support at each node. The heuristic search began by a stepwise method using a tree bisection-reconnection (TBR) branch-swapping algorithm, and sequences were added pseudorandomly (nreps=10). Results were condensed into a single tree using majority rule consensus.

The Bayesian inference analysis was completed by separating the combined data into four partitions: morphology, 18S, 28S, and COI. The morphology partition was treated as standard data and analyzed using the JC69 substitution model (all rates the same; nst=1) with an equal rate distribution (rates=equal). The 18S, 28S, and COI partitions were analyzed as DNA data and underwent a general-time reversible substitution with gamma-distributed rate variation for invariable sites (GTR+I+G) (nst=6) (Jorgensen *et al.*, 2011). The partitioned data were subjected to a Markov chain Monte Carlo (MCMC) analysis for 4000000 generations with four chains (one cold + three hot), a 25% burnin and a sampling frequency of 100. After 10000 trees were tossed for burnin, 30000 sampled posterior probability trees were analyzed from the entire analysis. Support for monophyletic groups is considered strong when the value of bootstrap or posterior probability is 95% or above. The two cladograms were visualized using the software FigTree v.1.3.1 (Rambaut, 2009) and labelled using the softwares Microsoft PowerPoint v.14 (2010) and Paint v.6.1 (2009).

Table 4.1: List of Morphological Characters

Morphological Characters	
Molting by ecdysis	Septulum
Loss of locomotory cilia	Claw structure
Cuticle structure	Claw sequence
Parthenogenesis	Transverse cuticular bar
Circumpharyngeal nerve ring	Accessory point
Complete gut	Lunulae
Reproductive pore	Lateral cirrus A
Adhesive glands	Median cirrus
Protonephridia	Cuticular armor
Adult gut	Dorsal segmental plates
Triangular pharynx	Head plate
Stylets	Median plate I
Formation of the epicuticle	Median plate II
Terminal mouth	Median plate III
Cephalic papillae*	Caudal plate
Cephalic appendages*	Pseudosegmental plates
Peribuccal pappilae	Peduncles
Peribuccal lamellae	Clava

Buccal tube	Digitate legs
Peribuccal lobe	Leg 4 morphology
Pharyngeal tube	Dorsal plate development
Peribuccal papulae	Apophyses Insertion Stylet Muscle (AISM)
Ventral lamina	Sexual dimorphism of claws
Stylet support	Sexual dimorphism of gonopore
Placoids	Cleavage Pattern

Table 4.2: Accession list of tardigrades

Species	18S	28S	COI	Genus	Family
Macrobiotus hulfelandi	FJ435739	FJ435755	FJ435805	*Macrobiotus*	Macrobiotidae
Macrobiotus pallarii	FJ435741	FJ435756	FJ435807		
Paramacrobiotus richtersi	EU038078	FJ435757	EU244597	*Paramacrobiotus*	
Richtersius coronifer	EU266930	GQ849048	EU244606	*Richtersius*	
Minibiotus furcatus	FJ435746	FJ435759	FJ435802	*Minibiotus*	
Minibiotus gumersindoi	FJ435748	FJ435761	FJ435803		
Dactylobiotus_sp.	EF632436	GQ849049	EF632525	*Dactylobiotus*	Murrayidae
Murrayon c.f. dianeae	FJ435737	FJ435762	FJ435801	*Murrayon*	
Murrayon pullari	GQ849026	GQ849050	AY598772		
Hypsibius convergens	FJ435726	FJ435771	FJ435798	*Hypsibius*	Hypsibiidae
Diphascon pingue	FJ435734	FJ435776	FJ435794	*Diphascon*	
Astatumen trinacriae	FJ435732	FJ435773	FJ435790	*Astatumen*	
Thulinius stephaniae	AF056023	EF620407	EF620417	*Thulinius*	Isohypsibiidae
Eremobiotus alicatai	FJ435722	FJ435766	FJ435796	*Eremobiotus*	
Isohypsibius_sp.	FJ435724	FJ435764	FJ435797	*Isohysibius*	
Isohypsibius granulifer	EF620403	EF620405	EF620415		
Isohypsibius prosostomus	EF620404	EF620406	EF620416		
Calohypsibius sp.	EU266940	-	-	*Calohypsibius*	Calohypsibiidae
Ramazzottius oberhaeuseri	EF632498	FJ435769	FJ435800	*Ramazzottius*	Ramazzottidae
Bertolanius nebulosus	GQ849023	GQ849046	-	*Bertolanius*	Eohypsibiidae
Milnesium tardigradum	AY582120	FJ435779	EU244603	*Milnesium*	Milnesiidae
Florarctus sp.	GQ849017	GQ849034	-	*Florarctus*	Halechiniscidae
Stygarctus sp.	GQ849041	-	-	*Stygarctus*	Stygarctidae
Batillipes mirus	GQ849016	GQ849027	-	*Batillipes*	Batillipedidae
Echiniscoides sigismundi	GQ849021	GQ849042	HM193403	*Echiniscoides*	Echiniscoididae
Bryodelphax parvulus	HM193371	HM193387	HM193405	*Bryodelphax*	Echiniscidae
Echiniscus blumi Greenland	HM193375	HM193391	EF620382	*Echinsicus*	
Echiniscus blumi Chile	HM193374	HM193390	HM193407		
Echiniscus	HM193373	HM193389	HM193406		

bigranulatus					
Echiniscus canadensis	FJ435714	FJ435784	FJ435814		
Echiniscus merokensis	FJ435719	FJ435787	FJ435813		
Echinicus spiniger	HM193376	HM193392	HM193408		
Echiniscus testudo	GQ849022	GQ849043	EF620378		
Echiniscus trisetosus	FJ435717	FJ435781	FJ435816		
Echiniscus viridissimus	AF056024	HM193393	HM193409		
Cornechiniscus lobatus	HM193372	HM193388	EU244602	*Cornechiniscus*	
Pseudechiniscus facettalis	HM193382	HM193399	HM193415	*Pseudechiniscus*	
Pseudechiniscus islandicus Faroe Isl.	AY582119	GQ849044	HM193416		
Pseudechiniscus islandicus Iceland	HM193383	HM193400	HM193417		
Pseudoechiniscus novaezelandiae	HM193384	HM193401	HM193418		
Testechiniscus spitsbergensis	HM193385	HM193402	HM193419	*Testechiniscus*	
Mopechiniscus granulosus	HM193379	HM193396	HM193412	*Mopechiniscus*	
Antechiniscus lateromamillatus	HM193370	HM193386	HM193404	*Antechiniscus*	
Proechiniscus hannae	HM193381	HM193398	HM193414	*Proechiniscus*	
Parechiniscus chitonides	HM193380	HM193397	HM193413		
Hypechiniscus exarmatus	HM193377	HM193394	HM193410	*Hypechiniscus*	
Hypechiniscus gladiator	HM193378	HM193395	HM193411		
Oreella mollis	EU266962	-	-	*Oreella*	Oreellidae
Necopinatidae	-	-	-	-	Necopinatidae
Microhypsibiidae	-	-	-	-	Microhypsibiidae
Apodibius	-	-	-	-	-
Renaudarctidae	-	-	-	-	Renaudarctidae
Coronarctidae	-	-	-	-	Coronarctidae
Priapulus caudatus (Priapulida)	X80234	AY210840	DQ087502	Outrgroup	
Pycnophyes sp. (Kinorhyncha)	AY859598	AY859597	-		
Diplodasys meloriae (Gastrotricha)	JF357640	JF357680	JF432031		
Total: 56	51	48	44	30	18

4.4 **Results**

Analyses of total evidence data returned two cladograms, one maximum parsimony (MP) and one Bayesian inference

(Bi). The MP cladogram contained no resolved relationships at a higher level, while the Bi tree revealed resolved relationships throughout the phylum.

The cladogram for the MP analysis contained non monophyletic classes Eutardigrada and Heterotardigrada. The tardigrade orders Parachela and Apochela did not appear monophyletic, nor did the heterotardigrade orders Echniscoidea and Arthrotardigrada.

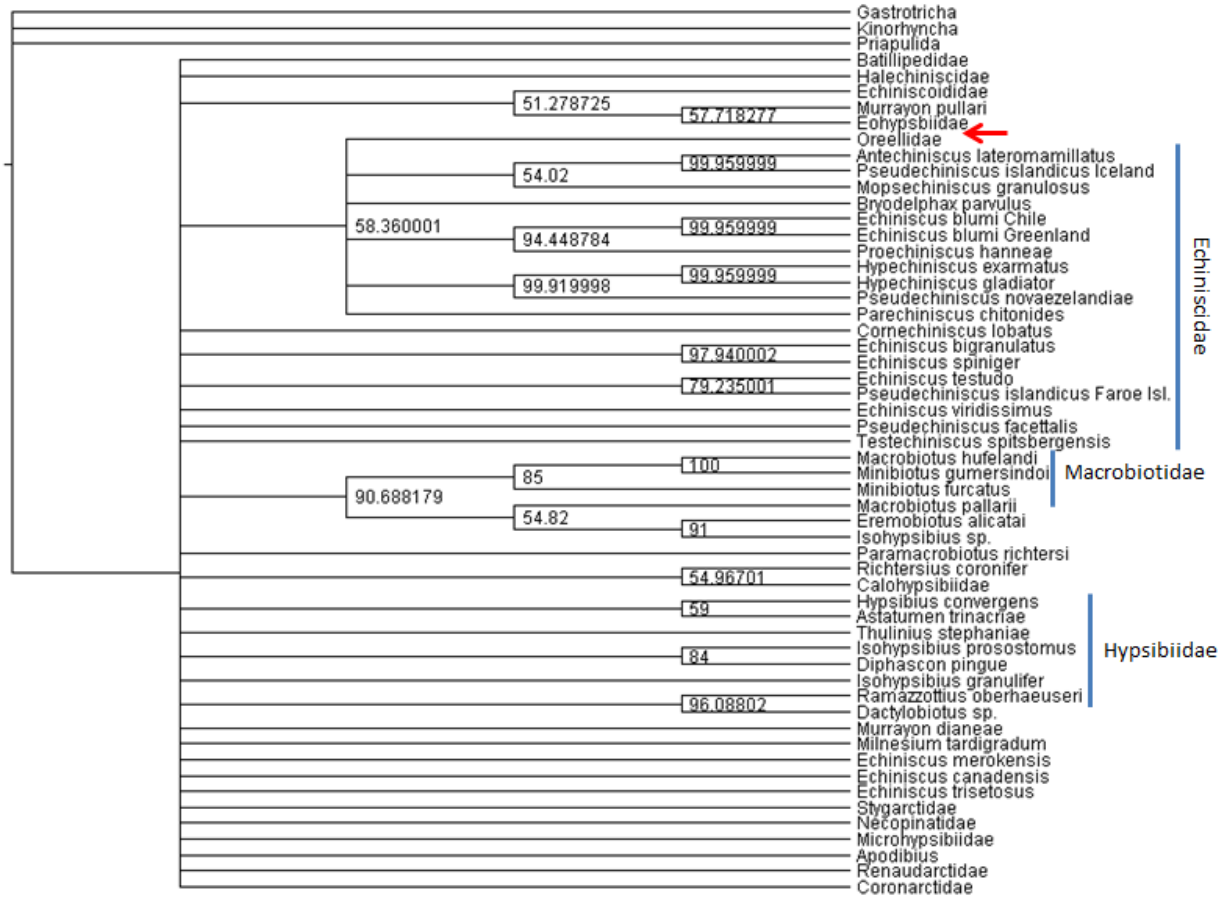


Figure 4.1: Parsimony cladogram returned by analysis of combined morphological and molecular data using 100 bootstrap* replicates. Arrow indicating Oreellidae as a member in a clade with Echiniscidae species. *- Bootstrap values did not appear as an expected proportion of 100 replicates due to bugs within PAUP* 4.0b10. Our bootstrap values may have been misrecorded because the heuristic search applied for bootstrapping using random sequence addition may have saved more trees than should be saved (Carmen Cheung, personal communication, September 22, 2012).

Analysis of the Bi total evidence analysis returned a sister group relationship between the tardigrade classes Heterotardigrada and Eutardigrada (posterior probability 57%), with the species *incertae sedis* in *Apodibius* appearing as the most basal taxon (posterior probability, pp 98%). Within the heterotardigrades, families within the order Arthrotardigrada did not group into a clade among its members, whereas Echiniscoidea appeared monophyletic. Within Arthrotardigrada, Stygarctidae and Renaudarctidae appeared as sister groups, and the evolutionary relationships among Batillipedidae, Halechiniscidae, and Coronarctidae was unresolved. Within Echiniscoidea, Oreellidae (represented by the species *Oreella mollis*) appeared as a sister group to the family Echiniscidae. Echiniscoididae appeared as a sister group to the Echiniscidae + Oreellidae clade. Echiniscidae was monophyletic (pp 59%), with the genera *Echiniscus* (pp 85%) and *Hypechiniscus* (pp 82%) each monophyletic, while *Pseudechiniscus* appeared polyphyletic. *Mopechiniscus* granulosus appeared as the most basal species of echiniscids (pp 60%), followed by the divergence of the monophyletic *Bryodelphax parvulus* and *Parechiniscus chitonides* (pp 53%). Within the eutardigades, the orders Parachela and Apochela did not appear monophyletic. At the family level, Murrayidae appeared monophyletic and nested within Macrobiotidae, sharing close affinity to *Richtersius coronifer*. Macrobiotidae did not appear monophyletic, instead bifurcating into two separate clades (pp 100%). One clade contained the species *Richtersius coronifer* and *Macrobiotus hufelandi*, which shared a close affinity to Murrayidae, whereas the other clade consisted of the species *Macrobiotus pallarii*, *Macrobiotus richtersi*, *Minibiotus furcatus*, and *Minibiotus gumersindoi*. Murrayidae and Macrobiotidae formed a monophyletic clade, which supported the superfamily Macrobiotoidea (pp 100%). Eohypsibiidae was represented by the species *Bertolanius nebulosus*, which diverged at the base of the Macrobiotoidea clade (pp 100%). Hypsibiidae was not monophyletic and diverged from the Eohypsibiidae + Macrobiotoidea clade in three separate branches. One Hypsibiidae clade diverged from Eohypsibiidae + Macrobiotoidea and consisted of the

genera *Hypsibius*, *Diphascon*, and *Astatumen*, whereas the second clade consisted of the species *Ramazzotius oberhaeuseri*, and the third clade consisted of *Thulinius*, *Eremobiotus*, and *Isohypsibius*. Calohypsibiidae shared close affinity with the hypsibid clade *Hypsibius* + *Diphascon* + *Astatumen*. The separation of Hypsibiidae provided support for the two superfamilies Hypsibioidea and Isohypsibioidea.

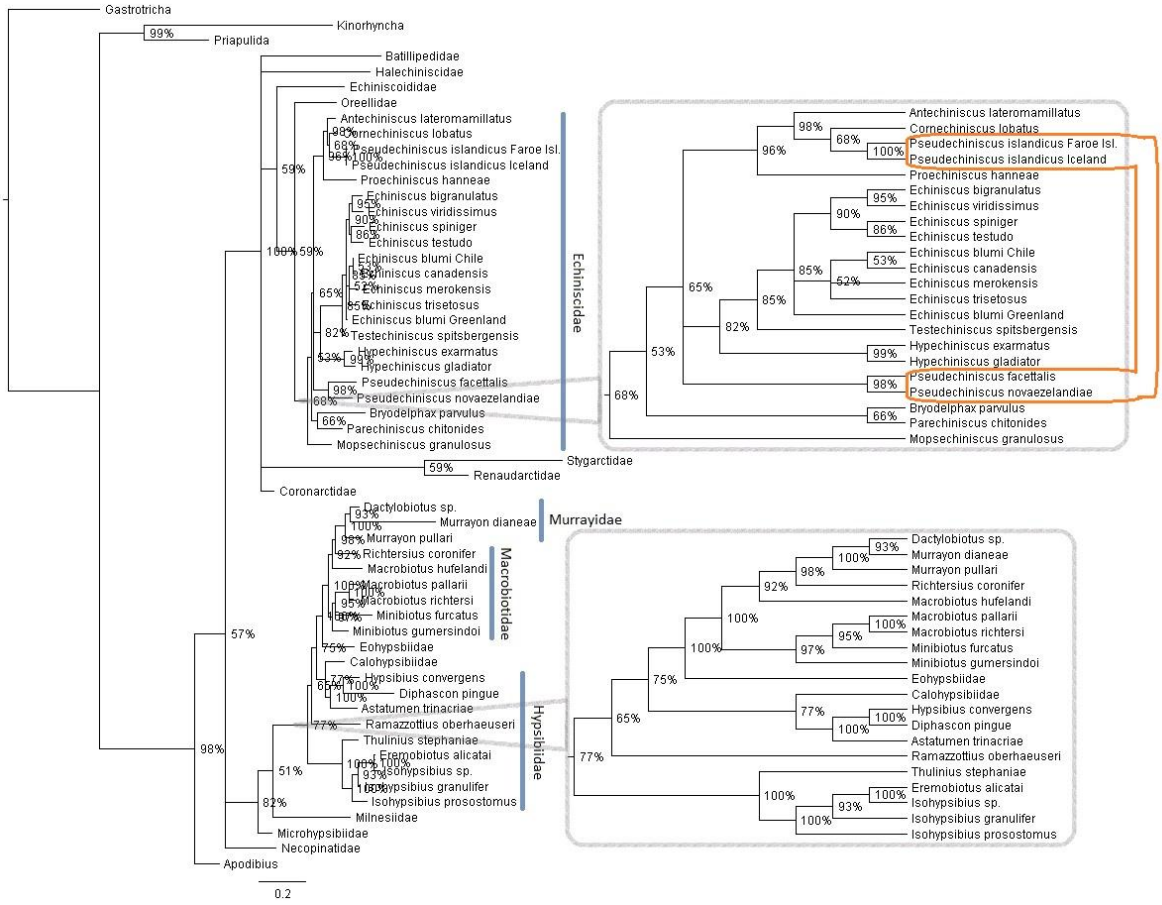


Figure 4.2: Bayesian inference tree returned by analysis of combined morphological and molecular data.
***Pseudechiniscus* genus appeared polyphyletic.**

4.5 **Discussion**

The maximum parsimony (MP) and Bayesian inference (Bi) analyses constructed from a supermatrix of morphologicaland 18S, 28S, and COI sequence data yielded different evolutionary relationships. Results from the MP analysis mostly were inconclusive at the order and class levels, whereas results from the Bi analysis revealed a monophyletic Heterotardigrada and Eutardigrada, a

monophyletic Arthrotardigrada and Echiniscoidea, and paraphyletic eutardigrade classes Parachela and Apochela. The monophyly observed in Echiniscoidea is consistent with results from Jorgensen *et al.* (2011). Within the Bi tree, the families Echiniscidae and Murrayidae were represented by multiple species and each was monophyletic, whereas Macrobiotidae and Hypsibiidae were polyphyletic. Macrobiotidae appeared as two separate groups, one paraphyletic, consisting of the species of *Richtersius coronifer* and *Macrobiotus hufelandi* diverging from the Murrayidae family, the other (*Macrobiotus pallari* + *Macrobiotus richtersi* + *Macrobiotus furcatus* + *Minibiotus gumersindoi*) formed a monophyletic clade. Hypsibiidae appeared as two clades, one clade consisted of the genera *Hypsibius* + *Diphascon* + *Astatumen*, while the second clade consisted of *Thulinius* + *Eremobiotus* + *Isohypsibius*. The *Hypsibius* + *Diphascon* + *Astatumen* clade supported the establishment of the superfamily Hypsibioidea, the *Thulinius* + *Eremobiotus* + *Isohypsibius* clade supported the Isohypsiobioidea, and the Murrayidae + Macrobiotidae clade formed the superfamily Macrobioitoidea. Eohypsibiidae shared close affinity to Macrobioitoidea. Calohypsibiidae, represented by the single species *Bertolanius nebulosus*, shared close affinity to Hypsibioidea. *Ramazzottius oberhauseri* diverged from the clade [(Macrobioitoidea, Eohypsibiidea), (Calohypsibiidae, Hypsibioidea)]. Milnesiidae (*Milnesium tardigradum*), the only family representing the class Apochela, nested among genera within Parachela. The *incertae sedis* species in *Apodibius* appeared as the basal-most tardigrade taxa, while Necopinatidae did not group within an order and formed a trichotomy with Eutardigrada and Heterotardigrada. Microhypsibiidae appeared as the most basal eutardigrade. Within the heterotardigrades, no relationships were resolved for Batillipedidae, Halechiniscidae, and Coronarctidae. Stygarctidae appeared as a sister group to Renaudarctidae. The class Echiniscoidea appeared monophyletic, with the families Oreellidae and Echiniscidae appearing as sister groups, while the monophyletic Echiniscoididae appeared as the basal-most member in the Echiniscoidea. These conclusions were consistent with observations made by Jorgensen *et al.*

(2011). Although three additional species of Echiniscidae were analyzed in addition to the 19 Echiniscidae taxa from Jorgensen *et al.* (2011), no relationships among the genera were resolved. Species within Echiniscidae did not sort according to genera, which may suggest that a reorganization of the species at the genus level is needed.

Chapter 5 :

CONCLUSION

Most previous studies on tardigrade systematics have used either morphological or molecular data to construct classifications at the class, order, or family levels, however, little research has been done to investigate incongruencies among results obtained with the two different data types at the family-level. In the current study, we provided an updated analysis of tardigrade systematics at the family-level, using morphological, molecular, and combined data. Our morphological study involved the re-evaluation of tardigrade characters from Nichols *et al.* (2006) and the construction of a cladogram at the family-level using 50 characters, which provided support for Oreellidae + Echniscoididae as the most basal heterotardigrades. This conclusion contradicts the conclusion in Nichols *et al.* (2006), who suggested that Oreellidae was the basal-most heterotardigrade family. Our study supported the conclusion drawn by Nichols *et al.* (2006) that Arthrotardigrada and Echiniscoidea are non sister groups. From our morphological analyses, we note that Milnesiidae (Apochela) shared plesiomorphic characters with members within Parachela, and speculate that, over time, diverged from Parachela by acquiring Apochela-specific apomorphies. Our molecular study involved the use of a combined gene (18S rRNA, 28S rRNA, and COI mtDNA) sequence data set and an 18S rRNA data set. Phylogenies constructed from the concatenation of 18S rRNA, COI mtDNA, and 28S rRNA sequences confirmed the classes Heterotardirgada and Eutardigrada as sister groups. Within Heterotardigrada, the order Echiniscoidea did not appear monophyletic, and Echiniscidae appeared monophyletic within the neighbor-joining and Bayesian inference analyses, both observations contradicting observations from Jorgensen et al (2011) and Guil and Giribet (2012). Results from both data sets included Milnesiidae as the most basal eutardigrade family; this conclusion was consistent with studies by Nichols *et al.* (2006) and Guil and Giribet (2012). Phylogenies constructed from 18S rRNA sequences supported a nonmonophyletic Hypsibiidae as well as monophyletic Milnesiidae, Calohypsibiidae, and Murrayidae. Our study also supported the Marley *et al.* (2011) conclusion of grouping the genera Ramazzottius and Hebesuncus as their own family Ramazzottidae. The monophyly of the order Echiniscoidea also was supported in the 18S rRNA data set, as was a monophyly of the family Echiniscidae (Jorgensen *et al.*, 2011).

We investigated incongruencies between morphological and molecular topologies in our combined analyses by combining the two different data types into a single supermatrix for analysis. Some taxonomic revisions may be needed for the Apochela clade of Milnesiidae, in which that class appeared nested within Parachela. We suggest for future studies in tardigrade systematics to continue the use of combined data supermatrices to conduct phylogenetic systematic analyses. The use of cladograms returned by combined data analyses also may be used for character mapping, to help understand the origins of tardigrade behaviours (i.e. parthenogenesis [virgin-birth] and cryptobiosis). Future studies also should include additional research within Heterotardigrade families, specifically Halechiniscidae, Renaudarctidae, Stygarctidae, and Batillipedidae.

Future directions for the study will involve modifications to the maximum parsimony (MP) and Bayesian inference (Bi) analyses, as well as the combined data analyses from Chapter 4. For the MP analysis, a Dollo parsimony model will be used to assign weight to the morphological characters, instead of an equal rates of change assumption, in which all characters can only evolve once. For the BI analysis, the convergence of the four chains will be evaluated using the software AWTY (Are We There Yet?) as opposed to arbitrary setting a burnin of 25% or by plotting on the likelihood scores (LnL) versus time (Gen) from the "dot-p" files from mrbayes-3.1.2 (Ronquist, 2003). The discrepancies between morphological and molecular data may be modified by combining the morphological and molecular trees through the use of supertrees, instead of using a supermatrix in Chapter 4.

Chapter 6 :

REFERENCES

Aguinaldo, A. M. A., *et al.* (1997). "Evidence for a clade of nematodes, arthropods and other moulting animals." Nature **387**(6632): 489-493.

Balian, E. V., *et al.* (2008). " The Freshwater Animal Diversity Assessment: an overview of the results." Developments in Hydrobiology **198** 627-637.

Beasley, C. W. (1967). "Tardigrades from Kansas." Transactions of the Kansas Academy of Science (1903-) **70**(4): 464-470.

Bello, G. and G. S. de Zio (1998). "Phylogeny of the Genera of the Stygarctidae and Related Families (Tardigrada: Heterotardigrada)." Zoologischer Anzeiger **237**: 171-183.

Bertolani, R. (1981). "The taxonomic position of some eutardigrades." Bolletino di zoologia **48**(2): 197-203.

Bertolani, R. (2001). "Evolution of the Reproductive Mechanisms in Tardigrades — A Review." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 247-252.

Bertolani, R. and J. Kristensen (1987). New records of Eohypsibius nadjae Kristensen,1982, and revision of the taxonomic position of two genera of Eutardigrada (Tardigrada). Biology of Tardigrades, Select Symposium Monographs. R. Bertolani. Mucchi, Modena, U.Z.I. **1:** 359-372.

Bertolani, R. and L. Rebecchi (1996). "The tardigrades of Emilia (Italy). II. Monte Rondinaio. A multihabitat study on a high altitude valley of the northern Apennines." Zoological Journal of the Linnean Society **116**(1-2): 3-12.

Bertolani, R. and L. Rebecchi (1999). Tardigrada Encyclopedia of Reproduction. E. Knobil and D. J. Neill. San Diego, California, Academic Press. **4:** 703-717.

Biserov, V., *et al.* (2011). "Macrobiotus trunovae sp.n., a new species of tardigrade from Russia." Invertebrate Zoology **8**(1): 57-62.

Biserov, V. I. (1992). "A new genus and three new species of tardigrades (Tardigrada: Eutardigrada) from the USSR." Bolletino di zoologia **59**(1): 95-103.

Brusca, R. C. and G. J. Brusca (1990). Invertebrates. Sunderland, Mass., Sinauer Associates

Calloway, S., *et al.* (2011). "Tardigrades of North America: Oreella chugachii, a new species (Heterotardigrada, Echiniscoide, Oreellidae) from Alaska." Proceedings of the Biological Society of Washington **124**(1): 28-39.

Campbell, B. C., et al. (1994). "Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences." Insect Mol Biol 3(2): 73-88.

Campbell, L. I., *et al.* (2011). "MicroRNAs and phylogenomics resolve the relationships of Tardigrada and suggest that velvet worms are the sister group of Arthropoda." Proceedings of the National Academy of Sciences **108**(38): 15920-15924.

Chalwatzis, N., et al. (1995). "Strongly expanded 18S rRNA genes correlated with a particular morphology in the insect order Strepsiptera." Zoology (98): 115-126.

Chang, C. Y. and H. S. Rho (1998). "Three new tardigrade species associated with barnacles from the Thai coast of Andaman sea." Korean Journal of Biological Sciences **2**(3): 323-331.

Claxton, S. (1999). Milnesioides exsertum gen. n. sp., a new tardigrade from Australia (Tardigrada: Milnesiidae). Proceedings of the seventh international symposium on the Tardigrada. H. Greven. Du¨sseldorf, Germany, Zoologischer Anzeiger. **238:** 183-190.

Claxton, S. K. (1996). "Sexual dimorphism in Australian Echiniscus (Tardigrada, Echiniscidae) with descriptions of three new species." Zoological Journal of the Linnean Society **116**(1-2): 13-33.

Cohen, B. L., et al. (1998). "Molecular phylogeny of brachiopods and phoronids based on nuclear-encoded

small subunit ribosomal RNA gene sequences." Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 353(1378): 2039-2061.

D'Addabbo Gallo, M., *et al.* (1999). "Diversity and dynamics of an interstitial Tardigrada population in the Meloria Shoals, Ligurian Sea, with a redescription of Batillipes similis (Heterotardigrada, Batillipedidae)." Italian Journal of Zoology **66**(1): 51-61.

D'Addabbo Gallo, M., *et al.* (2005). "A new Batillipedidae (Tardigrada, Heterotardigrada) from the Orosei Gulf, Sardinia, Tyrrhenian Sea." Zoologischer Anzeiger **243**: 219-225.

Dastych, H., *et al.* (1998). "Oreela mollis Murray, 1910 (Tardigrada): a redescription and revision of Oreella." Mitteilungen Hamburgisches Zoologisches Museum und Institut **95**: 89-113.

De Laet, J. E. (2005). Parsimony and the problem of inapplicables in sequence data. Parsimony, Phylogeny, and Genomics. V. A. Albert. Oxford, Oxford University Press: 81–116.

de Zio Grimaldi, S., *et al.* (1992). "Neoarctus primigenius n. g., n. sp., a new Stygarctidae of the Tyrrhenian Sea (Tardigrada, Arthrotardigrada)." Bolletino di zoologia **59**(3): 309-313.

de Zio Grimaldi, S., *et al.* (2000). "Two new sub-Antarctic Echiniscoididae from Marion Island (Heterotardigrada, Echiniscoidea)." Italian Journal of Zoology **67**(2): 221-228.

de Zio Grimaldi, S., *et al.* (1987). "Adaptive radiation and phylogenesis in marine Tardigrada and the establishment of Neostygarctidae, a new family of Heterotardigrada." Bolletino di zoologia **54**(1): 27-33.

de Zio Grimaldi, S., *et al.* (1999). "Florarctinae of Asdhu Island, Maldives, Indian Ocean (Tardigrada, Heterotardigrada)." Italian Journal of Zoology **66**(4): 383-391.

Degma, P., *et al.* (2009-2011) "Actual checklist of Tardigrada species (2009-2011, Ver. 19: 31-05-

2011)." Laboratory of Evolutionary Zoology - Department of Biology - University of Modena and Reggio Emilia.

Dewel, R., *et al.* (1993). Tardigrada. Microscopic Anatomy of Invertebrates - Onychophora, Chilopoda, and lesser Protostomata. F. W. Harrison and M. E. Rice. New York, Wiley-Liss. **12:** 143-183.

Dewel, R. A. D. a. W. C. (1997). The place of tardigrades in arthropod evolution Arthropod Relationships R. A. F. a. R. H. Thomas. London, Chapman & Hall. **55:** 109-124.

Edgar, R. C. (2004). "MUSCLE: multiple sequence alignment with high accuracy and high throughput." Nucleic Acids Research **32**(5): 1792-1797.

Eibye-Jacobson, J. (2001). "A New Method for Making SEM Preparations of the Tardigrade Buccopharyngeal Apparatus." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 309-319.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.: Distributed by the author.

Gabriel, W. N., *et al.* (2007). "The tardigrade Hypsibius dujardini, a new model for studying the evolution of development." Developmental Biology **312**(2): 545-559.

Gadagkar, S. R., *et al.* (2005). "Inferring species phylogenies from multiple genes: Concatenated sequence tree versus consensus gene tree." Journal of Experimental Zoology Part B: Molecular and Developmental Evolution **304B**(1): 64-74.

Gallo D'Addabbo, M., *et al.* (2005). "A new Batillipedidae (Tardigrada, Heterotardigrada) from the Orosei Gulf, Sardinia, Tyrrhenian Sea." Zoologischer Anzeiger - A Journal of Comparative Zoology **243**(4): 219-225.

Garey, J. R., *et al.* (1996). "Molecular Analysis Supports a Tardigrade-Arthropod Association." Invertebrate Biology **115**: 79-88.

Garey, J. R., *et al.* (1999). "Tardigrade phylogeny: Congruency of morphological and molecular evidence " Zoologischer Anzeiger: 238:205-210.

Giribet, G., *et al.* (1996). "First molecular evidence for the existence of a Tardigrada + Arthropoda clade." Molecular Biology and Evolution **13**(1): 76-84.

Giribet, G., G.D. Edgecombe and W.C. Wheeler (2001). "Arthropod phylogeny based on eight molecular loci and morphology." Nature **413**: 157-161.

Gouy, M., *et al.* (2010). "SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building." Molecular Biology and Evolution **27**(2): 221-224.

Greven, H. (2007). "Comments on the eyes of tardigrades." Arthropod Structure & Development **36**(4): 401-407.

Greven, H., *et al.* (2005). "Notes on the integument of the glacier-dwelling tardigrade Hypsibius klebelsbergi MIHELCIC, 1959 (Tardigrada)." Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut: (102):111-120.

Guidetti, R., *et al.* (2011). "On dormancy strategies in tardigrades." Journal of Insect Physiology **57**(5): 567-576.

Guidetti, R. and R. Bertolani (2001). "Phylogenetic Relationships in the Macrobiotidae (Tardigrada: Eutardigrada: Parachela)." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 371-376.

Guidetti, R. and R. Bertolani (2005). "Tardigrade taxonomy: an updated check list of the taxa and a list of characters for their identification." Zootaxa: 845:841-846.

Guidetti, R., *et al.* (2005). "Phylogenetic analysis of Macrobiotidae (Eutardigrada, Parachela): a combined morphological and molecular approach." The Norwegian Academy of Science and Letters: 235-244.

Guidetti, R., *et al.* (2009). "New molecular data for tardigrade phylogeny, with the erection of Paramacrobiotus gen. nov Neue molekulare Daten zur

Tardigraden-Phylogenie mit der Einführung von Paramacrobiotus gen. nov." Journal of Zoological Systematics and Evolutionary Research **47**(4): 315-321.

Guil, N. and G. Giribet (2012). "A comprehensive molecular phylogeny of tardigradesuadding genes and taxa to a poorly resolved phylum-level phylogeny." Cladistics **28**(1): 21-49.

Hansen, J. G. (2007). "The deep sea elements of the Faroe Bank tardigrade fauna with a description of two new species." J. Limnol. **66**(1): 12-20.

Hendriks, L., et al. (1988). "Primary and secondary structure of the 18 S ribosomal RNA of the insect species Tenebrio molitor." FEBS Lett 232(1): 115-120.

Hochberg, R. and M. Litvaitis (2000). "Phylogeny of Gastrotricha: a morphology-based framework of gastrotrich relationships." The Biological Bulletin **198**(2): 299-305.

Hohberg, K. and W. Traunspurger (2009). "Foraging theory and partial consumption in a tardigrade–nematode system." Behavioral Ecology **20**(4): 884-890.

Horning, D. S., *et al.* (1978). "Tardigrada of New Zealand." New Zealand Journal of Zoology **5**: 185-280.

Jorgensen, A. (2000). "Cladistic analysis of the Echiniscidae Thulin, 1928 (Tardigrada: Heterotardigrada: Echiniscoidea)." Steenstrupia: 25:11-23.

Jörgensen, A. (2001). "Graphical Presentation of the African Tardigrade FaunaUsing GIS with the Description of Isohypsibius malawiensis sp. n. (Eutardigrada: Hypsibiidae) from Lake Malawi." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 441-449.

Jorgensen, A. and R. M. Kristensen (2004). "Molecular phylogeny of Tardigrada - investigation of the monophyly of Heterotardigrada." Molecular Phylogenetics and Evolution 32:666-670.

Jorgensen, A., *et al.* (2007). "Molecular study of the tardigrade Echiniscus testudo (Echiniscidae) reveals

low DNA sequence diversity over a large geographical area." Journal of Limnology **66**: 77-83.

Jørgensen, A., *et al.* (2010). "Molecular phylogeny of Arthrotardigrada (Tardigrada)." Molecular Phylogenetics and Evolution **54**(3): 1006-1015.

Jorgensen, A., *et al.* (2011). "Phylogeny and evolution of the Echiniscidae (Echiniscoidea, Tardirgrada) - an investigation of the congruence between molecules and morphology." Journal of Zoological Systematics and Evolutionary Research: 6-16.

Kaczmarek, L. and L. Michalczyk (2004). "A new species Bryodelphax asiaticus (Tardigrada: Heterotardigrada: Echiniscidae) from Mongolia (Central Asia)." The Raffles Bulletin of Zoology **52**(2): 599-602.

Kathman, R. D. (1990). "Eutardigrada from Vancouver Island, British Columbia, Canada, including a description of Platicrista cheleusis n.sp." Canadian Journal of Zoology **68**(9): 1880-1895.

Kendall-Fite, K. and D. R. Nelson (1996). "Two new species of tardigrades from Short Mountain, Tennessee, U.S.A." Zoological Journal of the Linnean Society **116**(1-2): 205-214.

Kiehl, E., H. Dastych, J. D'Haese and H. Greven (2007). "The 18S rDNA sequences support polyphyly of the Hypsibiidae (Eutardigrada)." J. Limnol. **66**(Suppl. 1): 21-25.

Kinchin, I. M., Ed. (1994). The biology of tardigrades. London ; Chapel Hill, NC, U.S.A. , Portland Press.

Kisielewski, J. (1987). "Two new interesting genera of Gastrotricha (Macrodasyida and Chaetonotida) from the Brazilian freshwater psammon." Hydrobiologia **153**: 23-30.

Kristensen, R. M. (1981). "Sense Organs of Two Marine Arthrotardigrades (Heterotardigrada, Tardigrada)." Acta Zoologica **62**(1): 27-41.

Kristensen, R. M. (1987). Generic revision of the Echiniscidae (Heterotardigrada), with a discussion of the origin of the family. Biology of Tardigrades,

Selected Symposia and Monographs No. 1. Mucchi, Modena: 261-335.

Kristensen, R. M. and T. E. Hallas (1980). "The Tidal Genus Echiniscoides and Its Variability, with Erection of Echiniscoididae fam.n. (Tardigrada)1." Zoologica Scripta **9**(1-4): 113-127.

Kristensen, R. M. and R. P. Higgins (1984a). "Revision of Styraconyx (Tardigrada: Halechiniscidae) with Descriptions of Two New Species from Disko Bay, West Greenland." Smithsonian Contributions to Zoology **391**: 1-40.

Kristensen, R. M. and R. P. Higgins (1984b). "A New Family of Arthrotardigrada (Tardigrada: Heterotardigrada) from the Atlantic Coast of Florida." U.S.A. Transactions of the American Microscopical Society **103**: 295-311.

Kristensen, R. M. and B. Neuhaus (1999). "The ultrastructure of the tardigrade cuticle with special attention to marine species " Zoologischer Anzeiger: 261-281.

Li, X. (2007). "A new species and a newly recorded species of tardigrade (Eutardigrada: Hypsibiidae) from China (Asia)." Proceedings of the Biological Society of Washington **120**(2): 189-196.

Li, X. and H. Li (2008). "Tardigrades from Taiwan, with the Description of a New Species of Doryphoribius (Tardigrada, Hypsibiidae)." Zoological Science **25**(5): 554-559.

Maddison, W. P. and D. R. Maddison. (2011). "Mesquite: a modular system for evolutionary analysis. Version 2.75 http://mesquiteproject.org."

Mallatt, J. M., *et al.* (2004). "Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin." Molecular Phylogenetics and Evolution **31**(1): 178-191.

Mallatt, J. and G. Giribet (2006). "Further use of nearly complete 28S and 18S rRNA genes to classify

Ecdysozoa: 37 more arthropods and a kinorhynch." Molecular Phylogenetics and Evolution 40(3): 772-794.

Manicardi, G. C. (1989). "Two new species of soil moss eutardigrades (Tardigrada) from Canada." Canadian Journal of Zoology **67**(9): 2282-2285.

Margulis, L. and K. V. Schwartz (1998). Five Kingdoms. An Illustrated Guide to the Phyla of Life on Earth. New York, W.H. Freeman.

Marley, N. J., *et al.* (2011). "Phylum Tardigrada: A re-evaluation of the Parachela " Zootaxa: 2819:2851-2864.

Martin, G. G. (1978). "Ciliary gliding in lower invertebrates." Zoomorphology: 249-261.

McInnes, S. (2010). "Echiniscus corrugicaudatus (Heterotardigrada; Echiniscidae) a new species from Ellsworth Land, Antarctica." Polar Biology **33**(1): 59-70.

McInnes, S. J. and P. J. A. Pugh (1998). "Biogeography of limno-terrestrial Tardigrada, with particular reference to the Antarctic fauna." Journal of Biogeography **25**(1): 31-36.

Mehleh, R. H. (1969). "Tardigrada: Taxonomy and Distribution in Costa Rica " Transactions of the American Microscopical Society **88**(4): 498-505.

Meyer, H. A. and J. G. Hinton (2010). "Milnesium zsalakoae and M. jacobi, two new species of Tardigrada (Eutardigrada: Apochela: Milnesiidae) from the southwestern United States." Proceedings of the Biological Society of Washington **123**(2): 113-120.

Michalczyk, L. and L. Kaczmarek (2005). "The first record of the genus Calohypsibius Thulin, 1928 (Eutardigrada: Calohypsibiidae) from Chile (South America) with a description of a new species Calohypsibius maliki." New Zealand Journal of Zoology **32**(4): 287-292.

Miller, W. R. (2011). "Tardigrades." American Scientist **99**(Sept.-Oct.): 384-391.

Miller, W. R., *et al.* (1994). "Tardigrades of the Australian Antarctic Territories: The Larsemann Hills, East Antarctica." Transactions of the American Microscopical Society **113**(2): 142-160.

Miller, W. R., *et al.* (2001). "Tardigrades of the Australian Antarctic: Macquarie Island, sub-Antarctica." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 475-491.

Mobjerg, N., *et al.* (2007). "New records on cyclomorphosis in the marine eutardigrade Halobiotus crispae (Eutardigrada : Hypsibiidae)." Journal of Limnology **66**: 132-140.

Moon, S. and W. Kim (1996). "Phylogenetic position of the Tardigrada based on the 18S ribosomal RNA gene sequences." Zoological Journal of the Linnean Society **116**: 61-69.

Munoz, J., et al. (2008). "Phylogeography and local endemism of the native Mediterranean brine shrimp Artemia salina (Branchiopoda: Anostraca)." Mol Ecol 17(13): 3160-3177.

Nelles, L., et al. (1984). "Nucleotide sequence of a crustacean 18S ribosomal RNA gene and secondary structure of eukaryotic small subunit ribosomal RNAs." Nucleic Acids Res 12(23): 8749-8768.

Nelson, D. R. (2001). Tardigrada. Ecology and classification of North American freshwater invertebrates. J. H. Thorp and A. P. Covich. San Diego, CA, Academic Press: 527-550.

Nelson, D. R. (2002). "Current Status of the Tardigrada: Evolution and Ecology." Integrative and Comparative Biology **42**(3): 652-659.

Nelson, D. R. and N. J. Marley (2000). "The biology and ecology of lotic Tardigarda." Freshwater Biology **44** 93-108.

Nelson, D. R., *et al.* (2010). Tardigrada. Ecology and Classification of North American Freshwater Invertebrate. J. H. Thorp and A. P. Covich. San Diego, CA, Academic Press: 455-484.

Neuhaus, B., *et al.* (1997). "Ultrastructure of the cuticle of Loricifera and demonstration of chitin using gold-labelled wheat germed agglutinin." Acta Zoologica: 215-225.

Nichols, P. B. (2005). Tardigrade Evolution and Ecology Department of Biology University of South Florida. **Doctor of Philosophy** 103.

Nichols, P. B., *et al.* (2006). "A family level analysis of tardigrade phylogeny." Hydrobiologia: 558:553-560.

Nickel, K. e. n., *et al.* (2001). "Tardigrades of South America: Machu Picchu and Ollantaytambo, Peru." Zoologischer Anzeiger - A Journal of Comparative Zoology **240**(3-4): 505-509.

Nielsen, C. (2001). Animal evolution: interrelationships of the living phyla. New York Oxford University Press.

Nielsen, C., *et al.* (1996). "Cladistic analyses of the animal kingdom." Biological Journal of the Linnean Society **57**(4): 385-410.

Peterson, K. J. and N. J. Butterfield (2005). "Origin of the Eumetazoa: testing ecological predictions of molecular clocks against the Proterozoic fossil record." Proc Natl Acad Sci U S A 102(27): 9547-9552.

Pilato, G. (1969). "Evoluzione e nuova sistemazione degli Eutardigrada." Bollettino di Zoologia **36**: 327-345.

Pilato, G. (1982). "The systematics of Eutardigrada." Journal of Zoological Systematics and Evolutionary Research **20**(4): 271-284.

Pilato, G. (1998). "Microhypsibiidae, new family of eutardigrades, and description of the new genus Franctonotus (Tardigrada). ." Spixiana **21**(2): 129-134.

Pilato, G. and M. G. Binda (2010). "Definition of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification " Zootaxa: 2404:2401-2454.

Pilato, G., *et al.* (2004). "Three new species of eutardigrades from the Seychelles." New Zealand Journal of Zoology **33**(1): 39-48.

Pilato, G., *et al.* (2002). "Remarks on some species of tardigrades from South America with the description of two new species." Journal of Natural History **38**(9): 1081-1086.

Pollock, L. W. (1970). "Reproductive Anatomy of Some Marine Heterotardigrada." Transactions of the American Microscopical Society **89**(2): 308-316.

Pollock, L. W. (1983). "A Closer Look at Some Marine Heterotardigrada II. The Morphology and Taxonomy of Bathyechiniscus, with a Description of B. Craticulus n. sp. from the Caribbean." Bulletin of Marine Science **33**(1): 109-117.

Pollock, L. W. (1995). "New Marine Tardigrades from Hawaiian Beach Sand and Phylogeny of the Family Halechiniscidae." Invertebrate Biology **114**(3): 220-235.

Ramazzotti, G. M. and W. Maucci (1983). "Il Phylym Tardigrada. Ill edizione riveduta e aggiornata. English translation by CW Beasley." Memorie dell'Istituto Italiano di Idrobiologia **41**: 1-1012.

Rambaut, A. (2009). "FigTree 1.3.1. [http://tree.bio.ed.ac.uk/software/figtree/] webcite." 2010.

Rebecchi, L., *et al.* (2008). "A new discovery of Novechiniscus armadilloides (Schuster, 1975) (Tardigrada, Echiniscidae) from Utah, USA with considerations on non-marine Heterotardigrada phylogeny and biogeography." Organisms Diversity & Evolution **8**(1): 58-65.

Rebecchi, L. and R. Bertolani (1988). "New cases of parthenogenesis and polyploidy in the genus Ramazzottius (Tardigrada, Hypsibiidae) and a hypothesis concerning their origin." International journal of invertebrate reproduction and development **14**(2-3): 187-196.

Rebecchi, L. and D. R. Nelson (1998). "Evaluation of a Secondary Sex Character in Eutardigrades." Invertebrate Biology **117**(3): 194-198.

Rebecchi, L., *et al.* (2003). "Reproductive Modes and Genetic Polymorphism in the Tardigrade Richtersius coronifer (Eutardigrada, Macrobiotidae) " Invertebrate Biology **122**: 19-27.

Rice, E. L. (1990). "Nucleotide sequence of the 18S ribosomal RNA gene from the Atlantic sea scallop Placopecten magellanicus (Gmelin, 1791)." Nucleic Acids Res 18(18): 5551.

Rieger, G. E. and R. M. Riger (2009). "Comparative fine structure study of the Gastrotrich cuticle and aspects of cuticle evolution within the Aschelminthes." Journal of Zoological Systematics and Evolutionary Research 81-124.

Romano, F. A. (2003). "On water bears." Florida Entomologist: 134-137.

Ronquist, F. a. J. P. H. (2003). "MRBAYES 3: Bayesian phylogenetic inference under mixed models." Bioinformatics **19**: 1572-1574.

Ruppert, E. E. and R. D. Barnes (1994). Invertebrate Zoology Orlando, Florida, Saunders College Publishing, Harcourt Brace and Company.

Sands, C. J., P. Convey, K. Linse and S. J. McInnes (2008a). "Assessing meiofaunal variation among individuals utilising morphological and molecular approaches: an example using the Tardigrada." BMC Ecology **8**: 1-11.

Sands, C. J., S.J McInnes, N.J. Marley, W.P. Goodall-Copestake, P. Convey, K. Linse (2008b). "Phylum Tardigrada: an "individual" approach." Cladistics 24:861-871.

Schill, R. O. and G. Steinbrueck (2007). "Identification and differentiation of Heterotardigrada and Eutardigrada species by riboprinting." Journal of Zoological Systematics and Evolutionary Research **45**(3): 184-190.

Schmidt-Rhaesa, A., *et al.* (1998). "The position of the Arthropoda in the phylogenetic system." Journal of Morphology **238**(3): 263-285.

Schuster, R. O., *et al.* (1980). "Systematic criteria of the Eutardigrada." Transactions of the American Microscopical Societ: 99(93):284-393.

Swofford, D. L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. . Sunderland, Massachusetts, Sinauer Associates.

Tamura K, P. D., Peterson N, Stecher G, Nei M, and Kumar, S. (2011). "MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. ." Molecular Biology and Evolution **28**: 2731-2739.

Telford, M. J., *et al.* (2008). "The evolution of the Ecdysozoa." Philosophical Transactions of the Royal Academy **363**: 1529-1537.

Todaro, M. A., *et al.* (2011). "Phylogeny of Thaumastodermatidae (Gastrotricha: Macrodasyida) Inferred from Nuclear and Mitochondrial Sequence Data." PLoS ONE **6**(3): e17892.

Todaro, M. A. and R. M. Kristensen (1998). "A new species and first report of the genus nanaloricus (loricifera, nanaloricida, nanaloricidae) from the mediterranean sea." Italian Journal of Zoology **65**(2): 219-226.

Utsugi, K. and Y. Ohyama (1993). "Antarctic Tardigrada III. Fildes Peninsula of King George Island." Proceedings of the NIPR Symposium on Polar Biology **6**: 139-151.

Valentine, J. W. (1997). "Cleavage patterns and the topology of the metazoan tree of life." Proceedings of the National Academy of Sciences **94**(15): 8001-8005.

Valentine, J. W. and A. G. Collins (2000). "The significance of moulting in Ecdysozoan evolution." Evolution & Development **2**(3): 152-156.

Villora-Moreno, S. (1996). "A new genus and species of the deep-sea family Coronarctidae (Tardigrada) from a submarine cave with a deep-sea like condition." Invertebrate Taxonomy **81**(4): 275-283.

Villora-Moreno, S. and S. de Zio Grimaldi (1996). "New records of marine Tardigrada in the Mediterranean Sea." Zoological Journal of the Linnean Society **116**(1-2): 149-166.

Wainberg, R. H. and W. D. Hummon (1981). "Morphological Variability of the Tardigrade Isohypsibius saltursus." Transactions of the American Microscopical Society **100**(1): 21-33.

Wallace, R. L., *et al.* (1996). "A Cladistic Analysis of Pseudocoelomate (Aschelminth) Morphology." Invertebrate Biology **115**(2): 104-112.

Winnepenninckx, B., et al. (1995). "18S rRNA data indicate that Aschelminthes are polyphyletic in origin and consist of at least three distinct clades." Mol Biol Evol 12(6): 1132-1137.

Chapter 7 :

APPENDICES

Appendix A

```
#NEXUS
begin data;
dimensions ntax=19 nchar=30;
FORMAT DATATYPE = STANDARD GAP = - MISSING = ? symbols = "0 1 2 3" ;
matrix
        Lorcifera_          111110000000000000000000000000
        Kinoryncha_         111110000000000000000000000000
        Gastrotricha_       021100000000000000000000000000
        Macrobiotidae       120010021011?1232100000000000
        Eohypsibiidae       1200100??011110233100000000000
        Calohypsibiidae     120010022001122231100000000000
        Necopinatidae       1200100?200112221000000000000
        Microhypsibiidae_   1200100?000111023110000000200
        Hypsibiidae         120010022021112231100000000000
        Milnesiidae         12001100?111111222100000000000
        Apodibius           12001000?001101200?00000000000
        Halechnisicidae     110011100001100100011100000211
        Stygarctidae        110011100001100100011111?1?110
        Renauarctidae       110011100001100100011111210011
        Coronarctidae       110011100001100100011?1???011
        Batillipedidae_     110011100001100100011101210011
        Echiniscoididae     110010100001100100010101000?10
        Echiniscidae        110010100001100100010111112010
        Oreellidae          110010100001100100010101000?10
;
end;
```

Appendix B

CLASS	ORDER	SUPERFAMILY	FAMILY	SPECIES/ ACCESION NAME	REF. NO.	PAPER USED			SEQUENCE ACCESSION NO.		
						1	2	3	18S	COI	28S
EUTARDIGRADA Richters, 1926 PARACHELA Schuster *et al.*, 1980 MACROBIOTOIDEA Thulin, 1928 Macrobiotidae Thulin, 1928						Nichols [illegible]	Guil & [illegible]	Jorgensen [illegible]			
				Macrobiotus sp	Sb		*		EF632469	-	-
					Sb		*		EF632470	-	-
					Sb		*		EF632471	-	-
					Sb		*		EF632472	-	-
					Sb		*		EF632473	-	-
					Sb		*		EF632474	-	-
					Sb		*		EF632475	-	-
					Sb		*		EF632476	-	-
					Sb		*		EF632477	-	-
					Sb		*		EF632478	-	-
					Sb		*		EF632479	-	-
					Sb		*		EF632480	-	-
					Sb		*		EF632481	-	-
					Sb		*		EF632482	-	-
					Sb		*		EF632483	-	-
					Sb		*		EF632484	-	-
					Sb		*		EF632485	-	-
					Sb		*		EF632486	-	-
					Sb		*		EF632487	-	-
					Sb		*		EF632488	-	-
					Sb		*		EF632489	-	-
					Sb		*		EF632490	-	-
					Sb		*		EF632491	-	-
					A		*		U49912	-	-
					Ga96		*		U32393	-	-
					Sa		*		EU266926	-	-
					Sa		*		EU266935	-	-
					Sa		*		EU266936	-	-
					Sa		*		EU266937	-	-
					Sa		*		EU266938	-	-

					Macrobiotus furciger	Sa		*		EU266927	-	-
						Sa		*		EU266928	-	-
						Sa		*		EU266929	-	-
					Macrobiotus hufelandi	Gi96	*	*		X81442	-	-
						GG		*		FJ435742	-	-
						GG		*		FJ435740	-	-
						GG		*		FJ435739	-	-
						GG		*		FJ435738	-	-
						J10		*		GQ849024	-	-
						GG		*		-	*FJ435806*	-
						GG				-	*FJ435805*	-
						GG				-	*FJ435804*	-
						Gu05		*		-	*AY598773*	-
						Gu05		*		-	*AY598774*	-
						GG				-	-	FJ435755
						GG				-	-	FJ435754
						GG		*		-	-	FJ435753
						GG				-	-	FJ435752
						GG		*		-	-	FJ435751
						J10		*		-	-	GQ849047
					Macrobiotus pallarii	GG		*		FJ435741	-	-
						GG		*		-	FJ435807	-
						GG		*		-	-	FJ435756
					Macrobiotus persimilis	UP				-	EU244608	-
					Macrobiotus sapiens	SS		*		DQ839601	-	-
					Macrobiotus terminalis	G05		*		-	AY598775	-
					Macrobiotus tonolli	SS		*		DQ839605	-	-
						Ga96	*			U32393	-	-
						UP				-	EU244609	-
					Paramacrobiotus areolatus	SS		*		DQ839602	-	-
					Paramacrobiotus richtersi	SS		*		DQ839603	-	-
						Gu09		*		EU038081	-	-
						Gu09		*		EU038080	-	-
						Gu09		*		EU038078	-	-
						GG		*		FJ435743	-	-

						GG		*		FJ435744	-	-
						UP				-	EU244605	-
						UP				-	EU244597	-
						UP				-	EU244598	-
						UP				-	GU339056	-
						GG		*		-	FJ435808	-
						GG		*		-	FJ435809	-
						G05		*		-	AY598778	-
						G05		*		-	AY598779	-
						GG		*		-		FJ435757
					Richtersius sp.	UP				-	EU244610	-
						UP				-	EU244611	-
					Richtersius coronifer	SS		*		DQ839604	-	-
						JK		*		AY582123	-	-
						Sa		*		EU266930	-	-
						Sa		*		EU266931	-	-
						UP				-	EU244606	-
						UP				-	EU244607	-
						Gu05		*		-	AY598780	-
						Gu05		*		-	AY598781	-
						UP				-	GU237485	-
						UP				-	EU244607	-
						J10		*		-	-	GQ849048
					Minibiotus sp.	Sa		*		EU266932	-	-
						Sa				EU266933	-	-
						Sa		*		EU266934	-	-
					Minibiotus furcatus	GG		*		FJ435745	-	-
						GG		*		FJ435746	-	-
						GG		*		FJ435747	-	-
						GG		*		-	FJ435802	-
						GG		*		-		FJ435758
						GG		*		-		FJ435759
						GG		*		-		FJ435760
					Minibiotus gumersindoi	GG		*		FJ435748	-	-
						GG		*		-	FJ435803	-
						GG		*		-	-	FJ435761
					Xerobiotus pseudohufelandi	Gu05		*		-	AY598776	-

						Gu05		*		-	AY598777	-
Murrayidae Guidetti *et al.* 2005												
					Dactylobiotus sp.	Sb		*		EF632436	-	-
						Sb		*		EF632437	-	-
						Sb		*		EF632438	-	-
						Sb		*		EF632439	-	-
						Sb		*		EF632440	-	-
						Sb		*		EF632441	-	-
						Sb		*		EF632442	-	-
						UP				-	EF632529	-
						UP				-	EF632528	-
						UP				-	EF632527	-
						UP				-	EF632526	-
						UP				-	EF632525	-
						UP				-	EF632524	-
						UP				-	EF632523	-
					Dactylobiotus ambiguus	UP		*		GQ925681	-	-
						UP		*		GQ925680	-	-
						UP		*		GQ925677	-	-
						UP		*		GQ925676	-	-
						UP				GQ925679	-	-
						UP				GQ925678	-	-
					Dactylobiotus octavi	J10		*		GQ849025	-	-
						J10		*		-	-	GQ849049
					Dactylobiotus parthenogeneticus	Gu05		*		-	AY598771	-
					Murrayon cf. dianeae	GG		*		FJ435737	-	-
						GG		*		-	FJ435801	-
						GG		*				FJ435762
					Murrayon pullari	J10		*		GQ849026	-	-
						Gu05		*		-	AY598772	-
						J10		*		-	-	GQ849050
HYPSIBIOIDEA Pilato, 1969												
Hypsibiidae Pilato, 1969												
					Hypsibius sp.	Sb		*		EU266939	-	-
						Sb		*		EF632429	-	-

						Sb		*		EF632428	-	-
						Sb		*		EF632427	-	-
						Sb		*		EF632425	-	-
						Sb		*		EF632424	-	-
						UP				Z93337	-	-
						UP				-	EF632522	-
						UP				-	EF632521	-
						UP				-	EF632520	-
						UP				-	EF632519	-
						UP				-	EF632518	-
					Hypsibius convergens	GG		*		FJ435725	-	-
						GG		*		FJ435726	-	-
						GG		*		-	FJ435798	-
						GG		*		-	-	FJ435770
						GG		*		-	-	FJ435771
						GG		*		-	-	FJ435772
					Hypsibiyus cf. convergens	K		*		AM500647	-	-
						K		*		AM500650	-	-
					Hypsibius dujardini	N				Nichols *et al.*, 2006	-	-
						UP				-	GU339057	-
					Hypsibius klebelsbergi	K		*		AM500648	-	-
					Hypsibius scabropygus	K		*		AM500649	-	-
					Thulinius stephaniae	Ga99	*	*		AF056023	-	-
						UP				GQ925701	-	-
						UP				GQ925700	-	-
						UP				GQ925699	-	-
						UP				GQ925698		-
						M07		*		-	EF620417	-
						M07		*		-	-	EF620407
					Eremobiotus alicatai	GG		*		FJ435722	-	-
						GG		*		FJ435723	-	-
						GG		*		-	FJ435796	-
						GG		*		-	-	FJ435766
						GG		*		-	-	FJ435767
					Acutuncus antarcticus	Sa		*		EU266943	-	-

						Sa		*		EU266944	-	-
						Sb		*		EF632426	-	-
						Sb		*		EF632430	-	-
						Sb		*		EF632431	-	-
						Sb		*		EF632432	-	-
					Halobiotus crispae	Mo07		*		EF620402	-	-
						Mo07		*		EF620401	-	-
						Mo07		*		-	EF620414	-
						Mo07		*		-	EF620413	-
						Mo07		*		-	EF620412	-
						Mo07		*		-	-	EF620408
						Mo07		*		-	-	EF620409
						Mo07		*		-	-	EF620411
					Halobiotus stenostomus	Mo07 & JK		*		AY582121	-	-
					Diphascon sp.	Sa		*		EU266950	-	-
						Sa		*		EU266951	-	-
						Sa		*		EU266952	-	-
						Sa		*		EU266953	-	-
						Sa		*		EU266954	-	-
						Sa		*		EU266955	-	-
						Sb		*		EF632443	-	-
						Sb		*		EF632444	-	-
						Sb		*		EF632445	-	-
						Sb		*		EF632446	-	-
						Sb		*		EF632447	-	-
						Sb		*		EF632448	-	-
						Sb		*		EF632449	-	-
						Sb		*		EF632450	-	-
						Sb		*		EF632451	-	-
						UP				-	EF632537	-
						UP				-	EF632536	-
						UP				-	EF632535	-
						UP				-	EF632534	-
						UP				-	EF632533	-
						UP				-	EF632532	-
						UP				-	EF632531	-

						UP				-	EF632530	-
					Diphascon maucci	Sa		*		EU266945	-	-
					Diphascon pingue	GG		*		FJ435734	-	-
						GG		*		FJ435735	-	-
						GG		*		FJ435736	-	-
						GG		*		-	FJ435794	-
						GG		*		-	FJ435795	-
						GG		*		-	FJ435793	-
						GG		*		-	-	FJ435777
						GG		*		-	-	FJ435778
						GG		*		-	-	FJ435776
					Diphascon puniceum	Sa		*		EU266946	-	-
						Sa				EU266947	-	-
						Sa		*		EU266948	-	-
						Sa		*		EU266949	-	-
					Hebesuncus sp.	Sa		*		EU266957	-	-
						Sa		*		EU266958	-	-
					Hebesuncus conjungens	K		*		AM500646	-	-
					Hebesuncus ryani	Sa		*		EU266956	-	-
					Astatumen trinacriae	GG		*		FJ435731	-	-
						GG		*		FJ435732	-	-
						GG		*		FJ435733	-	-
						GG		*		-	FJ435790	-
						GG		*		-	FJ435791	-
						GG		*		-	FJ435792	-
						GG		*		-	-	FJ435773
						GG		*		-	-	FJ435774
						GG		*		-	-	FJ435775
Calohypsibiidae Pilato, 1969												
					Calohypsibius sp.	Sb		*		EU266940	-	-
						Sb		*		EU266941	-	-
						Sb		*		EU266942	-	-
					Calohypsibius schusteri	N				Nichols *et al.*, 2006	-	-
Ramazzottiidae Marley *et al.* 2011												

					Ramazzottius oberhaeuseri	GG		*		FJ435727	-	-
						GG		*		FJ435728	-	-
						JK		*		AY582122	-	-
						Sb		*		EF632498	-	-
						Sb		*		EF632499	-	-
						Sb		*		EF632500	-	-
						Sb		*		EF632501	-	-
						Sb		*		EF632502	-	-
						Sb		*		EF632503	-	-
						Sb		*		EF632504	-	-
						Sb		*		EF632505	-	-
						Sb		*		EF632506	-	-
						Sb		*		EF632507	-	-
						Sb		*		EF632508	-	-
						Sb		*		EF632509	-	-
						Sb		*		EF632510	-	-
						Sb		*		EF632511	-	-
						Sb		*		EF632512	-	-
						Sb		*		EF632513	-	-
						Sb		*		EF632514	-	-
						Sb		*		EF632515	-	-
						Sb		*		EF632516	-	-
						Sb		*		EF632517	-	-
						GG		*		-	FJ435800	-
						GG		*		-	FJ435799	-
						Mo07		*		-	EF620418	-
						GG		*		-	-	FJ435769
						GG		*		-	-	FJ435768
						Mo07		*		-	-	EF620410
					Ramazzottius sp.	Sa		*		EU266959	-	-
ISOHYPSIBIOIDEA Marley *et al.*, 2011												
		Isohypsibiidae Marley *et al*,. 2011										
					Isohypsibius sp	GG		*		FJ435724	-	-
						GG		*		FJ435729	-	-
						GG				FJ435730	-	-
						GG		*		-	FJ435797	-

						GG		*		-	-	FJ435765
						GG		*		-	-	FJ435764
						GG		*		-	-	FJ435763
						D		*		-	-	DQ077800
					Isohypsibius asper	Sb		*		EF632452	-	-
						Sb		*		EF632467	-	-
						Sb		*		EF632468	-	-
						UP				-	EF632552	-
						UP				-	EF632538	-
					Isohypsibius cambrensis	K		*		AM500652	-	-
					Isohypsibius granulifer	Mo07		*		EF620403	-	-
						K		*		AM500651	-	-
						Mo07				-	EF620415	-
						Mo07		*		-	-	EF620405
					Isohypsibius papillifer	Sb		*		EU266925	-	-
					Isohypsibius prosostomus	Mo07		*		EF620404	-	-
						Mo07		*		-	EF620416	-
						Mo07		*		-	-	EF620406
EOHYPSIBIOIDEA Bertolani & Kristensen, 1987 Eohypsibiidae Bertolani & Kristensen, 1987												
					Bertolanius nebulosus	J10		*		GQ849023	-	-
						J10		*		-	-	GQ849046
					Bertolanius volubilis	Gu05		*		-	AY598769	-
						Gu05		*		-	AY598770	-
APOCHELA Schuster *et al.*, 1980 Milnesiidae Ramazzotti, 1962												
					Milnesium antarcticum	Sa		*		EU266923	-	-
					Milnesium sp.	Sa		*		EU266922	-	-
						Sa		*		EU266924	-	-
						Sb		*		EF632492	-	-
						Sb		*		EF632493	-	-
						Sb		*		EF632494	-	-
						Sb		*		EF632495	-	-
						Sb		*		EF632496	-	-

						Sb		*		EF632497	-	-
						UP				-	EF632553	-
						Ma04		*		-	-	AY210826
					Milnesium cf. tardigradum	A	*	*		U49909	-	-
						GG		*		FJ435749	-	-
						GG		*		FJ435750	-	-
						JK		*		AY582120	-	-
						UP		*		GQ925697	-	-
						UP		*		GQ925696	-	-
						UP		*		GQ925695	-	-
						UP		*		GQ925694	-	-
						UP		*		GQ925693	-	-
						UP		*		GQ925692	-	-
						UP				GQ925691	-	-
						UP				GQ925690	-	-
						UP				GQ925689	-	-
						UP		*		GQ925688	-	-
						UP		*		GQ925687	-	-
						UP		*		GQ925686	-	-
						UP		*		GQ925685	-	-
						UP				GQ925684	-	-
						UP		*		GQ925683	-	-
						UP				GQ925682	-	-
						UP				-	EU244603	-
						UP				-	EU244604	-
						GG		*		-	FJ435810	-
						GG		*		-	-	FJ435779
						GG		*		-	-	FJ435780
						J10		*		-	-	GQ849045
HETEROTARDIGRADA Marcus, 1927 ECHINISCOIDEA Marcus, 1927 Archechiniscidae Binda, 1978												
					Archechiniscus sp.	J10		*		-	-	GQ849031
Halechiniscidae Thulin, 1928												
					Dipodarctus sp.	J10		*		-	-	GQ849032

					Florarctus sp.	J10 J10 J10		* *	* *	GQ849017 - -	- - -	- GQ849034 GQ849033
					Halechiniscus perfectus	J10 J10		*		GQ849018 -	- -	- GQ849035
					Halechiniscus remanei	JK		*		AY582118	-	-
					Orzeliscus sp.	J10 J10		*		GQ849019 -	- -	- GQ849036
					Raiarctus colurus	J10 J10		* *		GQ849020 -	- -	- GQ849037
					Styraconyx sp.	J10		*		-	-	GQ849038
					Tetrakentron synaptae	J10		*		-	-	GQ849039
					Tanarctus dendriticus	J10		*		-	-	GQ849040
			Stygarctidae Schulz, 1951									
					Stygarctus sp.	J10		*		-	-	GQ849041
			Batillipedidae Ramazzotti, 1962									
					Batillipes mirus	N J10 J10		 * *	 * *	Nichols *et al.,* 2006 GQ849016 -	- - -	- - GQ849027
					Batillipes pennaki	J10		*		-	-	GQ849028
					Batillipes similis	J10		*		-	-	GQ849029
					Batillipes tubernatis	J10		*		-	-	GQ849030
	ECHINISCOIDEA Richters, 1926											
			Echiniscoididae Kristensen & Hallas, 1980									
					Echiniscoides sigismundi	J10 Sa Sa J11 J10		* * * 	* * *	GQ849021 EU266961 EU266960 - -	- - - HM193403 -	- - - - GQ849042
			Echiniscidae Thulin, 1928									
					Bryodelphax sp.	Sb		*		EF632433	-	-

						Sb		*		EF632434	-	-
						Sb		*		EF632435	-	-
						Sa		*		EU266963	-	-
					Bryodelphax parvulus	J11			*	HM193371	-	-
						J11			*	-	HM193405	-
						J11			*	-	-	HM193387
					Echiniscus sp.	Sa		*		EU266964	-	-
						Sa		*		EU266970	-	-
						Sa		*		EU266971	-	-
						Sa		*		EU266972	-	-
						Sa		*		EU266973	-	-
						Sa		*		EU266974	-	-
						Sa		*		EU266975	-	-
						Sa		*		EU266976	-	-
						Sa		*		EU266977	-	-
						Sa		*		EF632453	-	-
						Sa		*		EF632454	-	-
						Sa		*		EF632455	-	-
						Sa		*		EF632456	-	-
						Sa		*		EF632457	-	-
						Sa		*		EF632458	-	-
						Sa		*		EF632459	-	-
						Sa		*		EF632460	-	-
						Sa		*		EF632461	-	-
						Sa		*		EF632462	-	-
						Sa		*		EF632463	-	-
						Sa		*		EF632464	-	-
						Sa		*		EF632465	-	-
						Sa		*		EF632466	-	-
						UP				-	EF632551	-
						UP				-	EF632550	-
						UP				-	EF632549	-
						UP				-	EF632548	-
						UP				-	EF632547	-
						UP				-	EF632546	-
						UP				-	EF632545	-
						UP				-	EF632544	-

						UP				-	EF632543	-
						UP				-	EF632542	-
						UP				-	EF632541	-
						UP				-	EF632540	-
						UP				-	EF632539	-
					Echiniscus blumi	J11			*	HM193375	-	-
					Greenland	J07		*	*	-	EF620382	-
						J11			*	-	-	HM193391
					Echiniscus blumi Chile	J11			*	HM193374	-	-
						J11			*	-	HM193407	-
						J11			*	-	-	HM193390
					Echiniscus bigranulatus	J11			*	HM193373	-	-
						J11			*	-	HM193406	-
						J11			*	-	-	HM193389
					Echiniscus canadensis	GG		*		FJ435714	-	-
						GG		*		FJ435713	-	-
						GG		*		FJ435715	-	-
						GG		*		-	FJ435814	-
						GG		*		-	-	FJ435784
						GG		*		-	-	FJ435785
						GG		*		-	-	FJ435786
					Echiniscus granulatus	SS		*		DQ839606	-	-
						UP				-	EU244600	-
					Echiniscus jenningsi	Sa		*		EU266969	-	-
					Echiniscus merokensis	GG		*		FJ435719	-	-
					merokensis	GG		*		-	FJ435813	-
						GG		*		-	-	FJ435787
					Echinicus spiniger	J11			*	HM193376	-	-
						J11			*	-	HM193408	-
						J11			*	-	-	HM193392
					Echiniscus testudo	SS		*		DQ839607	-	-
						J10		*	*	GQ849022	-	-
						UP				-	EU244601	-
						J07		*		-	EF620367	-
						J07		*		-	EF620368	-
						J07		*		-	EF620369	-
						J07		*		-	EF620370	-

						J07		*		-	EF620371	-
						J07		*		-	EF620372	-
						J07		*		-	EF620373	-
						J07		*		-	EF620374	-
						J07		*		-	EF620375	-
						J07		*		-	EF620376	-
						J07		*		-	EF620377	-
						J07		*	*	-	EF620378	-
						J07		*		-	EF620379	-
						J07		*		-	EF620380	-
						J07		*		-	EF620381	-
						J10			*	-	-	GQ849043
					Echiniscus trisetosus	GG		*		FJ435716	-	-
						GG		*		FJ435718	-	-
						GG		*		FJ435717	-	-
						GG		*		-	FJ435815	-
						GG		*		-	FJ435817	-
						GG		*		-	FJ435816	-
						GG		*		-	-	FJ435781
						GG		*		-	-	FJ435783
						GG		*		-	-	FJ435782
					Echiniscus viridissimus	Ga99	*	*	*	AF056024	-	-
						J11			*	-	HM193409	-
						J11			*	-	-	HM193393
					Cornechiniscus lobatus	Gu09		*		EU038079	-	-
						Gu09		*		EU038077	-	-
						J11			*	HM193372	-	-
						UP				-	EU244602	-
						J11			*	-	-	HM193388
					Pseudechiniscus facettalis	GG		*		FJ435720	-	-
						GG		*		FJ435721	-	-
						J11			*	HM193382	-	-
						GG		*		-	FJ435811	-
						GG		*		-	FJ435812	-
						J11			*	-	HM193415	-
						GG		*		-	-	FJ435788
						GG		*		-	-	FJ435789

						J11			*	-	-	HM193399
					Pseudechiniscus islandicus Faroe Island	JK		*	*	AY582119	-	-
						J11			*	-	HM193416	-
						J10		*	*	-	-	GQ849044
					Pseudechiniscus islandicus Iceland	J11			*	HM193383	-	-
						J11			*	-	HM193417	-
						J11			*	-	-	HM193400
					Pseudechiniscus sp.	Sa		*		EU266965	-	-
						Sa		*		EU266966	-	-
					Pseudoechiniscus novaezelandiae	J11			*	HM193384	-	-
						J11			*	-	HM193418	-
						J11			*	-	-	HM193401
					Testechiniscus spitsbergensis	Sa		*		EU266967	-	-
						J11		*		EU266968	-	-
						J11			*	HM193385	-	-
						Sa			*	-	HM193419	-
						J11			*	-	-	HM193402
					Mopechiniscus granulosus	J11			*	HM193379	-	-
						J11			*	-	HM193412	-
						J11			*	-	-	HM193396
					Antechiniscus lateromamillatus	J11			*	HM193370	-	-
						J11			*	-	HM193404	-
						J11			*	-	-	HM193386
					Proechiniscus hanneae	J11			*	HM193381	-	-
						J11			*	-	HM193414	-
						J11			*	-	-	HM193398
					Parechiniscus chitonides	J11			*	HM193380	-	-
						J11			*	-	HM193413	-
						J11			*	-	-	HM193397
					Hypechiniscus exarmatus	J11			*	HM193377	-	-
						J11			*	-	HM193410	-
						J11			*	-	-	HM193394
					Hypechiniscus gladiator	J11			*	HM193378	-	-
						J11			*	-	HM193411	-
						J11			*	-	-	HM193395
Oreellidae Puglia, 1959												

					Oreella mollis	Sa		*	*	EU266962	-	-
OUTGROUP						Ref. No.				18S	COI	28S
Artemia salina (brine shrimp)						Ne Mu	*			X01723 -	- DQ426858	- -
Placopecten magellanicus (Mollusca)						Ri	*			X53899	-	-
Priapulus caudatus (Priapulida)						Wi Co Pe Ma04	*	 * *		X80234 AF025927	- - DQ087502 -	- - - AY210840
Tenebrio molitor (darkling beetle)						He	*			X07801	-	-
Meloe proscaraboeus (European oil beetle)						Ch	*			X77786	-	-
Okanagana utahensis (cicada)						Ca	*			U06478	-	-
Panulirus argus (Caribbean spiny lobster)						UP	*			U19182	-	-
Diplodasys meloriae (Gastrotricha)						To To To				JF357640 - -	- JF432031 -	- - JF357680
Pycnophyes sp. (Kinorhyncha)						Ma06 Ma06				AY859598 -	- -	- AY859597

Reference Legend: J07 – Jorgensen *et al.*, 2007; Gu05 – Guidetti *et al.*, 2005; Mo07 – Mojberg *et al.*, 2007; Gi96 – Giribet *et al.*, 1996; JK – Jorgensen & Kristensen, 2004; Ga99 - Garey *et al.*, 1999; A – Aguinaldo *et al.*, 1997; Ma04 – Mallatt *et al.*, 2004; K – Kiehl *et al.*, 2007; SS – Schill & Steinbruck, 2007; D – De Laet, 2005; GA96 – Garey *et al.*, 1996; Sa – Sands *et al.*, 2008a; Sb – Sands *et al.*, 2008b; J10 – Jorgensen *et al.*, 2010; Gu09 – Guidetti *et al.*, 2009; N – Nichols *et al.*, 2006; GG - Guil & Giribet, 2012; J11 – Jorgensen *et al.*, 2011; UP – Unpublished; Ne – Nelles *et al.*, 1984; Mu – Munoz *et al.*, 2008; Ri – Rice, 1990; Wi – Winnepenninckx *et al.*, 1995; Co – Cohen *et al.*, 1998; Pe – Peterson & Butterfield, 2005; He – Hendriks *et al.*, 1988; Ch – Chalwatzis *et al.*, 1995; Ca – Camphell *et al.*, 1994; To – Todaro *et al.*, 2011; Ma06 – Mallatt & Giribet, 2006