Biology and biodiversity of tardigrades in the world and in Sweden

Current status and future visions

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Abstract

Tardigrades are small water-dwelling invertebrates that can live almost anywhere in the world. Even though they are well-known our knowledge about them is still scarce. The aim of this study was therefore to explore our current knowledge about tardigrades by: (1) explore their global phylogeny and biogeography based on bioinformatics (2) screen for tardigrades in select locations of northern Sweden and compare with other Swedish locations, and (3) identify at least one tardigrade from northern Sweden and explore the published biomarkers for further identification. The bulk of this thesis was based on evaluation of the Silva database for analyzing SSU (small subunit) and LSU (large subunit) tardigrade sequences and create phylogenetic trees. Some initial lab work was performed using samples of moss and lichen from Piteå, Vindeln and Öland. Results show that only few countries have been explored with regard to tardigrades, and in Sweden more research have been performed in the south compared to the north. The phylogenetic trees give a rough overview of tardigrade relatedness but many of the sequences need to be improved and more sequence work from additional environments is needed. In the lab tardigrades were only found from the Piteå samples, and one of those was identified as Macrobiotus hufelandi, for which a new biomarker was created. Overall, tardigrade research need to continue and expand to other regions in order to understand how these organisms differ between different environments, and more work is needed to ensure higher quality of sequences added to databases.
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1. Introduction

Tardigrades (also known as water bears or moss piglets, in Swedish “björndjur”) are small water-dwelling invertebrates (Bordenstein 2016). The animals vary in length from 0.05 to 1.2 mm and have a “plump” bilaterally symmetrical body with four pairs of legs that end in claws (Miller 2011). They can be found almost anywhere in the world; from mountaintops to the deep sea and from tropical forests to the Antarctic (Bordenstein 2016). Over 1100 species have been described so far (Bartels et al. 2016), most of these have been found in freshwater or semiaquatic environments (Zhang 2011; Degma, et al. 2015). They can generally be found on mosses and lichens, but also for example in sand, soil, sediments and leaf litter (Brusca and Brusca 2003; Ruppert, et al. 2004).

Tardigrades are mostly known for their impressive ability to withstand harsh environments and undergo cryptobiosis. They can therefore survive extreme conditions that would prove fatal for most other organisms. They can withstand temperatures as low as −272 °C and up to around 150 °C. They can also survive pressures that are around six times greater than what is found in the deepest parts of the ocean, and X-ray radiation 1000 times the level it takes to kill a human (Brusca and Brusca 2003; Nelson 2002; Zhang 2011). They can even survive the vacuum of lower Earth orbit (Jönsson et al. 2008). If there is no food and water available, tardigrades can dry themselves out until they only consist of 3% water or less, and stay in this state for more than 30 years. When living conditions improve the tardigrades can then return back to their normal active state (Dean 2015).

1.1 Anatomy

The body of a tardigrade consists of a head, three body segments and four pairs of legs. (Ciobanu et al. 2014; Kaczmarek et al. 2014). The legs lack joints and the feet can have four to eight claws. The cuticle around the body consists of chitin and protein. Tardigrades are eutelic in nature, which means that all grown adults of the same species have the same number of cells (Devasurmutt and Arpitha 2016). Normally, tardigrades have two eyespots on their head and one or more pairs of sensory whiskers on the head and on other parts as well (Greven 2007). The body contains a haemocoel, which is an open circulatory system where blood flows freely (Perry et al. 2015). Tardigrades do not have a respiratory system, so gas exchange occurs all over the body. The biggest part of its inside is made up of an intestine where most of the digestion takes place (Kaczmarek et al. 2014; Perry et al. 2015). A mass of nerve cells in the head makes up the majority of the nervous system. These go from the head to a double ventral nerve chord in the bottom of the body cavity, and then branches out to all the legs (Zantke et al. 2007).

1.2 Habitat & distribution

Tardigrades are found in both aquatic and terrestrial habitats all over the world, and they can in both cases be found on for example bryophytes, lichens and in leaf litter (Utsugi et al. 1997). So far, the tropics seem to be an unfavorable habitat (Mathews 1938), perhaps because those locations are warm and wet at the same time, which may also encourage the growth of other (possibly competing) microbes such as bacteria and fungi (Glime 2014).

The majority of all tardigrade species are limnoterrestrial (Garey et al 2008), meaning that they live in the film of water that can be found on bryophytes, lichens, algae and other plants (Crum 1976). The assemblages of tardigrade species seem to be very similar no matter the location in the world. Many studies have tried to show some kind of species preference for bryophytes, but most have failed (Kathman & Cross 1991; Miller & Heatwolde 1995; Meyer &
Hinton 2007). Some studies have managed to indicate that mosses and lichens could be preferred over other kinds of substrate. One problem when trying to study tardigrade habitats is that substrate records are often inconsistent or missing from many collections and scientific reports (Glime 2014).

Plenty of researchers have been able to show a relationship between the altitude and the distribution of tardigrades (Nelson, 1975; Ramazzotti & Maucchi 1983; Dastych 1988; Beasley 1988), which suggest that species numbers increase with altitude. Not all studies have come to these conclusions though (Meininger and Spatt 1988). Guil et al. (2009) theorize that the altitudinal differences in species composition could be explained by differences in soil, climate, vegetation, and litter type. Other factors that also could influence species assemblages of tardigrades are competition and food relations. Milnesium tardigradum for example can be found with two Hypsibius species that it can consume (Wright 1991).

Forests have a more temperate climate than do polar regions. Trees help to reduce water loss and offer bryophytes protection from the sun. In 2003, Jönsson examined mosses in southern Swedish forests and found 16 different species of tardigrades. Five of those had not been described for that region earlier. He found that the pine forest had more species than the clearcut areas, and that of all the tardigrades recorded, the species Macrobiotus hufelandi was by far the most common.

1.3 Life cycle

Reproductive mechanisms vary among tardigrades from different habitats (Bertolani 2001). Those that live on bryophytes or in freshwater are often parthenogenetic or sometimes hermaphrodites (that self-fertilize). However, tardigrades living in marine environments have so far been found to have separate sexes (Glime 2014).

Dispersal by wind with mosses is known to happen amongst tardigrades and might be one of their primary ways of dispersal (Pilato 1979). Since many tardigrades which live on bryophytes are parthenogenetic, this allows them to reproduce even if it is only one individual (or egg) that reaches a new location. Depending on the species there are two ways that tardigrade eggs are deposited: some just lay eggs freely exposed on the substrate, while others deposit them in the exuvia (outer skin) after molting (Mach 2010). Eggs that are not in a molt often have decorative structures (Mach 2010). Kinchin (1994) suggest that these structures have various functions such as anchorage to the substrate, defense, water storage and gas exchange. The eggs can be few or as many as 40 and the number can differ even within the same species (Altiero et al. 2006). The amount of eggs does not only depend on species however, but also on the nutritional value of the mother (Mach 2010).

Not much is known about egg development in tardigrades. Eggs generally develop inside the exuvia until the tardigrade is fully formed, a process that takes several weeks (Mach 2010). Young tardigrades look like adults but are smaller and require many molts as they grow. Growth occurs by enlargement of cells instead of addition of cells. The process of molting usually takes 5-10 days and happens 4-12 times during a tardigrades active life, which is about 3-30 months long (Walz 1982; Nelson 2002).

Tardigrades that live on moss include both tardigrades that actually consume the moss and tardigrades with other feeding strategies, such as carnivory. The tardigrade has a special pair of stylets and a muscular pharynx that produce suction into the gut. This allows it to suck fluids from the interior of plants or from other small organisms (Glime 2014). According to Schill et al. (2011) bryophyte food webs provides a rich food supply for both carnivorous and herbivorous species, as they include nematodes, rotifers, plant cells, algae, yeast, bacteria and the bryophyte itself. Tardigrades could also eat other smaller tardigrades. Larger species like Macrobiotus and Milnesium have been shown to consume smaller members such as
Diphascon and Hypsibius (Nelson 2002). Tardigrades are not only important consumers but they are in many cases the top carnivore in their micro-ecosystem (Sánchez-Moreno et al. 2008). However organisms that consume tardigrades exist as well. Snails (Fox 1966) and some fungi such as Ballocephala pedicellata (Pohlad & Bernard 1978) have been known to predate on tardigrades.

1.4 History

Two of the earliest texts that describe tardigrades are written in German. The earliest report is from a German pastor and zoologist named Johann Conrad Eichhorn, who apparently had found a tardigrade already in 1767 (Fig. 1) but failed to let the public know about his discovery. He described the tardigrade as follows; "In contrast to other insects with their nice and artistic shells and movements there was nothing which might have made it appear attractive to the eye of the observer". He also reported that the tardigrade had ten legs instead of eight; a statement which was corrected in a later publication (Mach 2010).

Johann August Ephraim Goeze was the first person to actually publish his discovery of the tardigrade, which he did in 1773 (Fig. 1). Goeze (who also was a German zoologist) seemed to be much more interested in the little animals and called them “kleiner Wasserbär”, meaning “little water bear” in German. According to him the animals looked and moved like miniature bears (Mach 2010). Water bears did not get their proper name “tardigrada” (meaning slow walker) until 1776 by the Italian biologist Lazzaro Spallanzani (Devasurmutt and Arpitha 2016). Richters (1904) was the first to report tardigrades in Sweden, which he did in the beginning of the 20th century. He had found 12 different species in moss from the southern part of Sweden.

1.5 Phylogeny & taxonomy

A large number of both morphological and molecular studies have been carried out in order to understand how tardigrades are related to other microorganisms. Two more credible theories have been noted. One is that tardigrades nearest relatives are Arthropoda (insects, spiders and crustaceans) and Onychophora (velvet worms); a result of morphological studies (Devasurmutt and Arpitha 2016). The other theory is that tardigrades nearest relatives are nematodes, a theory that have gotten support in some molecular analysis. This idea has in more recent times been rejected based on EST (Expressed Sequence Tag) analysis (Telford et al. 2008), which leaves us with three different tardigrade relationships within the arthropod group:
1) The lobopodia hypothesis: Tardigrades are a sister group to Onychophora plus Arthropoda.
2) The tactopoda hypothesis: Onychophora are a sister group to tardigrades plus Arthropoda.
3) Onychophora are a sister group to tardigrades (Budd 2001).

Analysis have shown that Panarthropoda is a monophyletic group and that tardigrades are a sister group to Lobopodia, which consists of both Onychophora and Arthropoda (Cooper 1964).

Marcus (1929) was the man who established the major taxa within tardigrades and split the group into two classes; Heterotardigrada and Eutardigrada (Fig. 2). Heterotardigrada (meaning “other” tardigrades) have armor with cuticular dorsal plates. Their other major characteristics include cephalic appendages, cuticular extensions, claws and the pattern of dorsal cuticular plates. Eutardigrada (meaning “true” tardigrades) are naked and lack any dorsal plates. They also have claws, a buccopharyngeal apparatus and a cuticle structure that can be smooth, granulated or have tubercles (Romano 2003).

![Fig. 2. The current phylogenetic topology of Tardigrada (Marley et al. 2011).](image)

The taxonomy we have today of tardigrades come from a number of scientific studies, but primarily from Thulin, Marcus, Ramazzotti and Maucci who have all contributed by writing books and monographs about the subject from the middle to the end of the 20th century. The Eutardigrade genus Macrobiotus was described in 1834 and the Heterotardigrade genus Echiniscus in 1840. A third class of tardigrades called Mesotardigrada (meso meaning “middle”) was established by Rahm in 1937 because of a tardigrade found near a hot spring in Nagasaki, Japan that got the name Thermozodium esakii. However, ever since then neither the tardigrade nor the locality have survived and no other Mesotardigrade have been found. Most scientists today therefore agree that the class should be removed (Romano 2003).
1.6 Tardigrade fossils

The fact that tardigrades are so small and mostly consist of soft membrane makes their fossilization hard to find and thus rare. The only fossils that have been found today are from mid-Cambrian deposits in Siberia and a few individuals enclosed in Cretaceous amber (Grimaldi & Engel 2005).

The fossilized Siberian tardigrades are different from their modern counterparts in many ways (Fig. 3): They have six legs instead of eight, their head morphology is very simple and they lack posterior head appendages. But one thing they have in common with today’s tardigrades is their columnar cuticle construction (Budd 2001). Scientists have argued that these tardigrades could be a stem group to tardigrades living today (Grimaldi & Engel 2005).

![Fig. 3. Siberian tardigrade fossil from the middle Cambrian](http://ksuweb.kennesaw.edu/~jdirnber/InvertZoo/LecTardOny/tardigrade.html)

The few tardigrades that have been found in Cretaceous amber are from two North American locations; New Jersey and western Canada. *Milnesium swolenskyi* from New Jersey is the older specimen. Its claws and mouthparts are no different from the *Milnesium Tardigradum* alive today. One of the other fossilized tardigrades has been given their own genus and family; *Beorn leggi*. In spite of this, it still look very similar to many living tardigrades of the family Hypsibiidae (Cooper 1964; Grimaldi & Engel 2005).

1.7 Tardigrade-Bacteria relationship

Understanding how much and in what ways organisms interact with their environment is very important in evolutionary ecology (Vecchi et al. 2016). Most organisms are in some way interacting with bacteria, ranging from pathogenesis (the bacteria causing disease to the host) to symbiotic relationships (McFall-Ngai et al. 2013). A few animal taxa have obtained a lot of scientific attention regarding this subject, but currently minimal research has been done on the bacteria-tardigrade relationships (Vecchi et al. 2016). Several marine arthrotardigrades have been shown to carry bacteria in special cephalic vesicles. Kristensen (1984) hypothesized that these tardigrades can use substances that are secreted by bacteria as a secondary energy source when food is not available. Eutardigrades can also have bacteria in their bodies; bacteria have been found in the gut of *Ramazzottius varieornatus* (Kinchin 1994), where there was no sign that they might have been digested. This leads to the conclusion that these bacteria could possibly be gut symbionts. Some few studies have suggested that tardigrades may spread phytopathogenic bacteria (Vecchi et al. 2016). However, none of these sporadic observations provide convincing evidence of the role of microbes for tardigrades. Thus, further studies are needed about the role of microbes for tardigrades.
1.8 Aim of the study

Although tardigrades are very well known organisms, our knowledge about them is still far from well explored. For example, today about 1100 tardigrade species have been described but it has been estimated that the total global number of species is 2654 (meaning we have achieved a completeness of 44.5%), or it could even be as high as 5407 (so only about 22% completeness) (Bartels et al. 2016). Out of these 1100+ species only 101 have been found in Sweden at some selected locations, in particular in southern Sweden (Guidetti et al. 2015). Tardigrades represent a special branch in the evolutionary tree and have many interesting traits, such as their tolerance against extreme conditions – which has expanded our knowledge about the potential of life on both Earth as well as in space. Furthermore, if only a fraction of all tardigrades found on Earth has been described, it is fundamental to explore them further. This will not only expand our understanding of their biodiversity and biogeography, but may also expand our knowledge about the tolerance of other extremophilic organisms on Earth.

It is generally assumed that the microbes are crucial to all complex life forms – but when did these come into play in the evolution of eukaryotes and are there any differences between complex versus simpler life forms? Tardigrades are relatively primitive organisms, but they do have a gut. Very few studies however have stated that tardigrades possess microbes. Dedicated investigations and experiments have not been performed to explore the tardigrade-microbe relationship further. Thus, basic studies on this are needed.

Therefore, this thesis has three aims:

1) To explore the global phylogeny and biogeography of tardigrades, based on some applied bioinformatics, such as evaluation of gene sequences retrieved from public databases and evaluation of the suitability of so far published biomarkers (for PCR (Polymerase Chain Reaction) and FISH (Fluorescence In Situ Hybridization)) for molecular identification.

2) To screen for tardigrades in select locations in northern Sweden and compare the results with other observations on other locations in Sweden. For this, we intend to screen for tardigrades in different moss and lichen samples from Västerbotten (Vindeln) and Norrbotten (Piteå), as well as explore some of their biology with some basic methods (classic methods such as morphology, and where possible also their associated microflora).

3) Identify at least one of the tardigrades from the northern Swedish samples and explore the suitability of so far published biomarkers for further detailed identification and further analyses.
2. Material and Methods

2.1 Bioinformatics

2.1.1 SILVA Database

The Silva database (www.arb-silva.de) is an online recourse for quality checked and aligned ribosomal RNA sequence data. The database is regularly updated and consists of datasets of aligned small (16S/18S, SSU) and large (23S/28S, LSU) subunit ribosomal rRNA sequences from Bacteria, Archaea and Eukarya. In this study both small (SSU) and large (LSU) subunit ribosomal RNA sequence data for tardigrades were downloaded from the Silva database (version 128). The number of tardigrade SSU sequences at the time of this study was 1291, while the number of LSU sequences was 230. The total number of SSU Parc sequences for all organisms were 5 616 941, while the total number of LSU Parc sequences were 735 238. This means that tardigrades only make up 0.023% of all SSU sequences, and 0.031% of all LSU sequences.

After downloading the metadata for all SSU and LSU tardigrade sequences, the published articles were evaluated to add, where possible, extra information that was not present in the metadata table; in particular the sample location for the tardigrades in the studies and what tardigrade species that had been used.

2.1.2 Sequence alignment

Two LSU datasets were downloaded from the Silva database 128; the “LSU Ref” and “LSU Parc” datasets. The difference between the two is that the Ref dataset includes high quality aligned 23S/28S sequences with a minimum length of 1900 bases, while the Parc dataset includes all aligned, quality checked rRNA sequences longer than 300 bases. The LSU Ref dataset was opened using the ARB software (http://www.arb-home.de/); a program with various tools for sequence database handling and analysis. The dataset included in total 230 tardigrade sequences. Eighteen of these were suggested by the Silva database as “the best” and thus included in the reference phylogenetic tree. Of these sequences, 17 belonged to the class Eutardigrada while one sequence belonged to the class Heterotardigrada (the genus Echiniscus). In total 10 outgroup organisms were chosen; Tenthredo koehleri, Semibalanus balanoides, Lorryia sp., Sacculinidae sp., 2 Saccharomyces cerevisiae, Kudoa hexapunctata, Kudoa septempunctata, Podocoryna carnea and Symplectoseyphus corvatus (with the accession numbers: gaww01000169, eu370440, kp276404, ay859599, jsaco1000031, cp006392, ab902957, ab731755, gbeh01172497 and kt757144 respectively) which would be used later for the final tree. The two LSU datasets were merged and the new total number of sequences became 154 527 (154 297 from before + 230 tardigrades). After loading the new dataset in ARB, each genus was evaluated for sequence alignment, starting with the 18 sequences included in the tree, and then aligning the rest of the sequences. Most genera contained several sequences, but 22 of them only contained one sequence representative and could not be as thoroughly aligned.

After alignment of all LSU sequences, the same procedure was done with the SSU sequences. The two SSU datasets were downloaded from the Silva database 128; the “SSU Ref” dataset and the “SSU Ref NR 99” dataset. The Ref dataset includes high quality aligned 16S/18S sequences with a minimum length of 1200 bases for bacteria and eukarya, while the Ref NR 99 dataset have applied a 99% criterion to remove redundant sequences. The SSU Ref dataset was opened using ARB and included in total 645 151 sequences where 1291 belonged to tardigrades. 124 of these were suggested by Silva as “the best” and thus included in the reference phylogenetic tree. Of these sequences, 95 belonged to the class Eutardigrada while
29 belonged to the class Heterotardigrada. The same outgroups that were used for the LSU dataset were selected, apart from one: *Sacculinidae* sp. which was not included as it was not present in the SSU database, instead 262 rotifer sequences were downloaded from the Silva database 128 to be used as an additional outgroup. The tardigrade sequences from the SSU Parc and rotifer sequences were merged into one dataset. The new total number of sequences became 1553. After loading the new dataset each genus was evaluated in more detail for sequence alignment, starting with the 124 that were included in the original reference tree. After aligning all genera with at least two sequences, the remaining 7 “lonely” genera (with only 1 sequence each) were aligned as good as possible. When this was done 572 sequences had been aligned.

The remaining sequences (719) were unspecific since they had not been affiliated to any genus by their authors. Some reasons for this could be that the authors either had no time or possibility to identify the sequences, or that some of these sequences represent novel taxa. A temporary phylogenetic reference tree was therefore created using the parsimony model as a base. All sequences were added successively to the temporary reference tree; both tardigrades, rotifers and the unknown sequences. The reconstruction of this temporary phylogenetic tree made it possible to see which organisms these unknown sequences were most closely related to, and by doing that those sequences could be better aligned compared to their most closely related genus.

### 2.1.3 Phylogenetic tree construction

After alignment of all LSU and SSU sequences, the final phylogenetic trees could be created in ARB. For LSU, the 18 sequences from the reference tree were chosen as a base, and for this reason the parsimony model was used. All aligned LSU sequences were successively added to the tree. For SSU all tardigrades over 1700 bases and rotifers (from the outgroup) over 1800 bases were used as a base. These were 70 sequences in total. The parsimony model was used for SSU as well, partly because it had been used previously when constructing the temporary reference tree (for alignment of the unspecific sequences), and partly because it had a better structure compared to the other two models; Jukes-Cantor and Maximum Likelihood. Just like with LSU the aligned SSU sequences were added successively to the tree. When the trees were created the sequences were added into genus-groups for easier overview and analysis.

### 2.1.4 Biomarker evaluation

From the SSU and LSU metadata tables every published article was surveyed to find out which PCR primers (or other type of biomarkers like for FISH, fluorescence in situ hybridization) were used in the different studies. These primers were recorded together with their sequence and reference.

The online Nucleotide BLAST tool ([Basic Local Alignment Search Tool](https://blast.ncbi.nlm.nih.gov/Blast.cgi)) was used to perform “probematch” for the PCR primers – in order to find out additional information: highest similarity percentage to an organism, total number of sequences, “best fitting” organism, how many different organisms that fit and if any non-ribosomal genes fit. FISH probes were surveyed from the probe database: [http://131.130.66.201/probebase%5Fold/default.asp?mode=search](http://131.130.66.201/probebase%5Fold/default.asp?mode=search). No specific FISH probes were available for tardigrades, thus, searches were done for the domain level Eukarya.

A probematch was also performed online from the Silva database ([http://www.arb-silva.de/search/testprobe/](http://www.arb-silva.de/search/testprobe/)) to find out additional information about the FISH probes; number of matched and mismatched sequences, how big proportion (in %) of sequences that fit
with archaea, bacteria and eukarya, and how big portion (in %) that fit with tardigrades. (Even though probematch can also be performed from the Silva database with PCR primers, this was not done in this study as most PCR primers found in the articles either did not have a clear reverse primer stated so they could not be searched, or they contained “nucleotide ambiguities” that the program could not handle).

The difference between the NBCI BLAST tool and the Silva database is that they contain different databases; NCBI contains all kinds of gene sequences while the Silva database is more curated and contains only ribosomal gene sequences of high quality.

2.1.5 FISH probe design and FISH probe match

A FISH probe was designed using the SSU and LSU datasets for the genus Macrobiotus in ARB. Macrobiotus was chosen as that was the tardigrade that had been identified from the petri dish (P2) in the lab exercise.

The LSU dataset contained 30 sequences of Macrobiotus belonging to different species. The best sequences over 2000 bases were chosen. These were only four and had the accession numbers: FJ435751, FJ435756, FJ435754 and FJ435755. After doing a FISH probe design two biomarkers of good quality were chosen; one at E. coli position 1372 and another at position 548.

The SSU dataset contained 130 Macrobiotus sequences. The sequences of better quality and over 1500 bases were chosen. These were in total seven sequences with the accession numbers: FJ435740, FJ435739, JX296284, JX296290, JX296279, JX296327 and JX296321. After doing a FISH probe design one good biomarker was chosen that had the E. coli position 846.

2.1.6 Survey of northern Swedish tardigrades

Guidetti et al. (2015) had previously made a report where they had compiled all studies about tardigrades found in Sweden and made a check-list of all recorded species and their locality. As the aim with this study was mainly to look at tardigrades from the northern part of Sweden, only the entries from Lappland, Norrbotten and Västerbotten were extracted from Guidetti et al. (2015) excel table of species. With this information, a map over northern Sweden was created where the sample sites for tardigrade collection (from the studies) was marked.

Information was gathered about common moss, lichen, and tardigrade species found in northern Sweden, and information compendia were made for easier analysis later in the lab.

2.2 Laboratory Study

2.2.1 Sample collection and experimental setup

Moss samples were collected from a small pine forest in Svensbyn village (coordinates: 65.332848, 21.250348) outside Piteå the 4th of November 2016. The samples were kept in closed plastic bags and stored in a refrigerator at the university until the time of the experiment (approximately 4 weeks later). Moss and lichen samples were also collected by Natuschka Lee (EMT, Umeå University) from the Krycklan research site in Vindeln (coordinates: 64.235268, 19.570069) on the 23rd of September 2015 and left to dry in open
plastic bags in the lab. Two other dried samples of moss from Öland collected by Ingemar Jönsson at Högskolan in Kristianstad in August 2009 and March 2010 were also used.

Lab work started in the beginning of December 2016. A small amount of moss and lichen from all samples were put in separate petri dishes and got 10-20 ml of tap water (as some samples were very small, those got a smaller amount of water). In total there were 20 specimens; 13 from Vindeln, 5 from Svensbyn and 2 from Öland. A lamp was set up close to the petri dishes to allow them constant light (later a periodic light was introduced with a timer). Every day, 10 ml more water was added to the samples (depending on how much had evaporated since the day before). After a few days some of the sphagnum samples had started to mold. These were put aside and new samples were made from the same material.

2.2.2 Petri dishes

The moss and lichen species were identified using the information booklets made previously for this thesis (not shown in the thesis but instead in the DVD medium of this thesis) and the field guide-books “Mossor” by Holmåsen and Hallingbäck (1985) and “Lavar” by Holmåsen and Moberg (1990). The samples were looked with a light microscope after eight days had passed since the initial setup. The presence and abundance of various microorganisms (such as ciliates, flagellates, nematodes, rotifers and tardigrades) were recorded. Microorganisms were identified using “Guide to Identification of Fresh Water Microorganisms” (https://www.msnucleus.org/watersheds/mission/plankton.pdf) and “Free-Living Freshwater Protozoa” by Patterson and Hedley (2009). The dishes were surveyed again after 14 and 29 days had passed and the change in microorganisms was recorded. The tardigrades that could be found were extracted with a Pasteur pipette from the petri dishes and placed in smaller glass dishes together with some tap water and water, microorganisms and a smaller piece of moss from the dishes they came from. In total there were 5 glass dishes with tardigrades which all came from Piteå. After one day, 10 days, and 14 days, the glass dishes were checked and the amount of tardigrades counted. The petri dishes were also checked after one day and 14 days and any new tardigrades found were moved to the glass dishes.

At the end of the experiment one tardigrade was extracted from one glass dish (Code: P2) and put on a microscope slide for identification under a light microscope. The premade information compendium about tardigrades found in northern Sweden was used for identification. The tardigrade was identified as Macrobiotus hufelandi.

2.2.3 Agar plates

Preparation of microbiological media: Agar was made by mixing one tenth (5 g/L) of the standard bacteriological medium nutrient broth, agar (15 g/L) and water and placed in an autoclave for sterilization. Still warm, the liquid was poured onto petri dishes and left for cooling and setting.

Inoculation of agar plates: After the agar plates had solidified, they were marked with a pen into 2 sections; a wet and a dry. A bit of dry moss from all samples was dabbed onto one half of the dish, while a bit of wet moss was dabbed onto the other half. Two dishes were made as control; one which was exposed to air (by removing the lid) for 15 seconds, and another which obtained a hand print (by Niki Andersson). The lid was placed back on all dishes and they were left for bacteria to grow. Twenty-four hours later the dishes were observed with a light microscope and the appearance of the bacteria was recorded. After 48 and 96 hours the agar plates were checked again and the change of the bacterial growth was recorded. A lot of
mold had started to develop on all agar plates after 16 days except for those from Öland, so pictures were taken of the plates before the mold had overtaken the whole surface.

**Picking of bacterial isolates:** Using a sterile inoculation loop heated under a flame, selected parts of the bacteria colonies were scraped off some of the agar plates and spread onto new agar plates. In total 24 bacterial colonies were taken; both plates from Öland got 4 samples each as those were the only plates without mold. The rest of the selected plates got either 2 or just one sample taken where there was the least amount of mold. Twenty-four, 48, and 240 hours after scraping the new agar plates were checked and the appearance of the bacteria recorded.

**Harvest of bacteria for FISH analysis:** Small Eppendorf tubes were filled with 0.5 ml of a 50% solution of EtOH and 1xPBS according to [http://www.microbial-systems-ecology.de/pdf_files/Fixation_for_fish_2march2013.pdf](http://www.microbial-systems-ecology.de/pdf_files/Fixation_for_fish_2march2013.pdf) (protocol II). The bacteria on the new agar plates were scraped off using a sterile inoculation loop and a small amount from all 24 samples were put into the Eppendorf tubes. After this they were stored at -20 °C.

**Determination of Gram stain characteristics:** The gram property of the isolates was evaluated with the quick test KOH [http://wiki.bugwood.org/KOH_test](http://wiki.bugwood.org/KOH_test); A “string test” was performed using a sterile inoculation loop and it was recorded which bacteria were gram-positive (did not develop a string) and which were gram-negative (did develop a string).

**FISH:** Isolates were tentatively identified with fluorescence in situ hybridization (FISH). Different probes were selected based on the database for FISH gene probes: [http://131.130.66.201/probebase%5Fold/](http://131.130.66.201/probebase%5Fold/). For G+ bacteria, the probes HGC69A (for Actinobacteria) and LGC354 (for Firmicutes) were selected. For G- bacteria the probes EUB338 (I) (for Bacteria) and Bet 42a/Gam42a (for beta and gamma-Proteobacteria) were selected. For the G- bacteria red color got associated to Bacteria while green color got associated to G-Proteobacteria. For the G+ bacteria red color got associated to Actinobacteria while blue got associated to Firmicutes. Out of the 24 total samples only four were considered to be G+ and the remaining 20 G-. FISH was performed based on the Protocol for standard FISH and DOPE-FISH for prokaryotes: [http://www.microbial-systems-ecology.de/pdf_files/StandardFISH_DOPEFISH_9may2013.pdf](http://www.microbial-systems-ecology.de/pdf_files/StandardFISH_DOPEFISH_9may2013.pdf).

**Microscopy:** The finished microscope slides were looked at through a Zeiss fluorescence microscope and the appearance of the bacteria (their structure and color) was recorded.
3. Results

3.1 Bioinformatics

3.1.1 World map & northern Sweden map

The world map (Fig. 4) shows the locations for tardigrade collection from the studies included in the Silva database version 128. Tardigrades were collected from 32 different countries in total. For 24 of those countries the collected tardigrade species were specified while for 8 countries they were not. Most species were collected from Greenland (16 different species) while most studies were carried out using Antarctic tardigrades (8 studies). Four studies investigated tardigrades in Sweden and five different species were collected there. Eight regions only had one single species collected (Faroe Islands, Iceland, Alaska, Argentina, Greece, Kenya, Seychelles and England) (See Appendix 1 for list of species and locations).

Fig. 4. World map with marked countries where tardigrades were collected from the studies in the Silva version 128 database. Countries in red are those where tardigrade species have been specified while countries in blue are collected unspecific tardigrades.

The full map of Sweden from the Guidetti et al. (2015) report (Fig. 5) shows how many species of tardigrades that have been found in the different provinces in the country. The created map of northern Sweden (Fig. 6) shows the exact sites for tardigrade collection in the provinces Norrbotten, Västerbotten and Lappland, together with two of the collection sites used in this study (third one being Öland which is not shown on the map). Lappland has the highest amount of collection sites while Västerbotten has the lowest. Information regarding the precise description of the substrate (such as moss and lichen species for example) could not be recorded from the studies as they often lacked this information.
Fig. 5. Number of tardigrade species collected in the different provinces of Sweden from report by Guidetti et al. (2015). Bl = Blekinge; Bo = Bohuslän; Da = Dalarna; Dl = Dalsland; Go = Gotland; Gä = Gästrikland; Ha = Halland; Hd = Härjedalen; Hä = Hälsingland; Jä = Jämtland; La = Lapland; Me = Medelpad; No = Norrbotten; Nä = Närke; Sk = Skåne; Sm = Småland; Sö = Södermanland; Up = Uppland; Vä = Västerbotten; Vl = Värmland; Vg = Västergötland; Vm = Västmanland; Ån = Ångermanland; Öl = Öland; Ös = Östergötland.
3.1.2 Phylogenetic trees

Although the Silva database is known for containing ribosomal gene sequences of better quality than the NCBI database as well as for having performed a basic alignment, their alignments are still far from perfect. Prior to the phylogenetic tree reconstructions, a great effort was spent on manually checking up the alignments and introducing minor improvements. Due to the limited time of the thesis, only basic changes could be made – nevertheless, this still improved the quality of the sequences to some extent, so that a better reconstruction of the trees could be made for this thesis.

The SSU phylogenetic tree include in total 1291 sequences (shown in Appendix 2). It contains 49 different tardigrade genera where 14 of those are specific to SSU (they do not exist in the LSU tree). More than half of all the sequences (719) are “unspecific”, meaning they are not affiliated to any genus but are instead named “Tardigrada sp.” “Tardigrada environmental sample”, “Uncultured eukaryote” etc. The genera including most sequences are Macrobiotus (130 sequences), Echiniscus (76 sequences), Diphascon (41 sequences), Hypsibius (37 sequences), Ramazzottius (35 sequences) and Milnesium (33 sequences). The outgroup (Fig. 7) contain not only the pre-chosen outgroup organisms but, oddly enough, tardigrades as well; a few sequences of Hypsibius dujardini.

The LSU phylogenetic tree (Fig. 8) include in total 230 tardigrade sequences. It contains 39 genera, including 4 that do not appear to exist in the SSU tree based on current analyses. All sequences in the LSU tree are affiliated to a genus; there are no unspecific sequences included. The genera including most sequences are Echiniscus (38 sequences), Hypsibius (37 sequences), Macrobiotus (30 sequences) and Echiniscoides (29 sequences). The outgroup (Fig. 9) is much more “contaminated” with tardigrade sequences than in the SSU tree.
outgroup, including many *Ramazzottius*, *Hypsibius*, *Diphascon* sequences, and one *Glabratella* sequence.

Fig. 7. Subset of SSU phylogenetic tree. Outgroup highlighted in red.

Fig. 8. LSU phylogenetic tree created in ARB using parsimony.
3.1.3 PCR BLAST Probematch & FISH SILVA Probematch

PCR primers used in the tardigrade studies from Silva version 128 together with the BLAST probematch can be found in the file “Biomarker_PCR_BLASTprobematch.xlsx”. FISH primers used for Eukarya are found in Appendix 3 together with Silva probematch. Most of the PCR primers were of decent quality, but they also match a wide variety of organisms and not just tardigrades. Some of the sequences could not be used in BLAST probematch as the authors had not stated enough information for a search to be performed. The FISH primers were far fewer than the PCR primers, and after performing Silva probematch only 18 out of 29 probes matched with tardigrades.

3.2 Laboratory Study

3.2.1 Petri dishes

The type and amount of microorganisms found in the petri dishes with moss and lichen samples from Vindeln, Piteå and Öland (Fig. 10) after 192, 336 and 696 hours are shown in Appendix 4. Moss and lichen species are also stated. The following organisms were found: flagellates (F), Aspidisca ciliates (CA), Glaucoma ciliates (CG), Coleps ciliates (CC), Loxophyllum ciliates (CL), flatworms (P), nematodes (N), rotifers (R) and tardigrades (T). The amount of different organisms “few” (f) or “many” (m) was recorded except for tardigrades where the exact number was stated. The dishes containing tardigrades are marked with “X”.

Fig. 9. Subset of LSU phylogenetic tree. Outgroup highlighted in red.

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The extracted tardigrades in the new dishes were checked and counted at various times and the numbers were recorded (Table 1). Twenty-two tardigrades were found when extracting the first time, while 19 were found at the end of the experiment.

Table 1. Number of tardigrades found in the glass dish after 24, 240 and 336 hours and number of tardigrades added from the original petri dishes after 24 and 336 hours. Samples came from the following moss species: P1 & P2 = Pleurozium schreberi, P3 = Hylocomium splendens, P4 = Dicranum montanum, P5 = Syntrichia ruralis.

<table>
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<th>24h: added</th>
<th>240h: found</th>
<th>336h: found</th>
<th>336h: added</th>
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<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
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<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>1 (+1 shell)</td>
<td>1 (+1 shell)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>4</td>
<td>0</td>
<td></td>
<td>2 (+1 shell)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2 Macrobiotus hufelandi

The tardigrade examined under light microscope at the end of the experiment was tentatively identified based on basic morphological criteria as Macrobiotus hufelandi (Fig. 11). The genus Macrobiotus was established in 1834 by C.A.S. Schultze when he described the first species of the genus; M. hufelandi (Mach 2010). It was not only the first Macrobiotus, but also the first species of tardigrade to be described in the literature (Bertolani and Rebecchi 1993).
The most important characteristic of *Macrobiotus* tardigrades are their claws which have a special kind of branching (Fig. 12) but the pharyngeal apparatus is important for identification as well (Mach 2010). *Macrobiotus* are fairly large, have eye spots (Bertolani and Rebecchi 1993), and move more quickly than many other tardigrades. Their color is usually transparent or different shades of brown, but its stomach contents can also be green. Around 100 *Macrobiotus* species have been described so far, and they have been collected all over the world (Mach 2010).

### 3.2.3 Agar plates

The development of the bacteria (and mold) on the agar plates that had been dabbed with dry and wet moss and lichen was recorded after 24, 48, 96 and 384 hours (Fig. 13). General characteristics such as color, consistency and spread were stated (Appendix 5).
Selected bacteria colonies were extracted from the agar plates and spread onto new clean agar plates. Development of bacteria (and mold) on the new places was recorded after 24, 48 and 240 hours (Appendix 6) (Fig.14). The bacteria colonies were tested for G+/G- and later examined under a fluorescence microscope with the structure and color of the bacteria recorded. An example of the typical appearance of G- bacteria can be seen in Fig. 15.
Fig. 14. Appearance of the new agar plates (used for FISH) after 24, 48 and 240 hours.

Fig. 15. Photo of G- bacteria (sample 1, FISH probe: EUB338 (I)) taken through fluorescence microscope after performing FISH. (Magnification: 400x)

3.2.5 *Macrobiotus* FISH probe design and FISH probe match
The results of the FISH probe design for LSU and SSU are shown in Appendix 7 and Appendix 8. The FISH probe match tables for LSU (biomarker 1372 and biomarker 548) are located in the files “FISHprobematch_LSU_Macrobiotus_1372.pdf” and “FISHprobematch_LSU_Macrobiotus_548.pdf”, while the FISH probe match table for SSU (biomarker 846) are located in the file “FISHprobematch_SSU_Macrobiotus_846.pdf”. A small overview of the biomarkers can be seen in Table 2.
Table 2. LSU and SSU biomarkers designed in ARB for the genus *Macrobiotus* together with *E. coli* position and probe sequence.

<table>
<thead>
<tr>
<th>Type</th>
<th><em>E. coli</em> position</th>
<th>Probe sequence (5'-3')</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>LSU</td>
<td>548</td>
<td>GCACUAGCCCAACGCAAA</td>
</tr>
<tr>
<td>SSU</td>
<td>846</td>
<td>CGGAAAGCUGCCCCGUCG</td>
</tr>
</tbody>
</table>
4. Discussion

4.1 World map & northern Swedish map

The world map (Fig. 4) clearly shows that there needs to be a continuation of tardigrade research in more regions. While many studies have focused on tardigrades from various parts in Europe and America, other parts of the world, such as the majority of Asia and Australia, have obtained much less attention. Many of the studies from the Silva database 128 have solely focused on tardigrades found in arctic regions, but as tardigrades are found everywhere in the world perhaps more effort should be placed on studying tardigrades from warmer regions as well. Doing that would contribute to our understanding of how tardigrades differ in different environments, both when it comes to their biology but also to their tolerance spectra. It should also be noted that many of the studies were only performed in the lab (that is; the researchers never ventured out to collect any tardigrades themselves, but only used material already available from various collections). The countries with “unspecific” tardigrades also make analysis of the map more complicated, as it is not certain that some of these even are tardigrades. They could have been misidentified. If this is the case maybe tardigrades have not been collected from 32 different countries but in fact in less than that.

The northern Swedish map gives an overview exactly where tardigrades have been collected from Norrbotten, Västerbotten and Lappland, but there is still a lot of information missing. Even though the number of tardigrade species has been recorded for the whole provinces by Guidetti et al. (2015), it has not been stated exactly where in the province these tardigrades were found. In many cases the articles also do not mention what substrate was collected, and if it is mentioned it is often on a very basic level such as only “moss” or “lichen”. Overall northern Sweden has also obtained much less attention compared to southern Sweden when it comes to tardigrade studies. For the tardigrades found in Norrbotten, Västerbotten and Lappland only six articles have been written, and the most recent of these was published in 1997 by Sohlenius et al.

4.2 Phylogenetic trees

The SSU phylogenetic tree is overall very large and does not provide a very good overview of tardigrade genus relatedness, partly because of the many unspecific sequences that are scattered throughout the tree, and partly because some genera contain huge amounts of sequences (like the genus *Macrobiotus*), whereas other tardigrade genus are only represented by one sequence, often of a lower quality. As all sequences are of varying length and quality the bigger genera end up having sequences in many places throughout the tree. LSU have a much neater phylogenetic tree (since the LSU database does not have as many sequences) but some sequences do still have odd placements. *Echiniscus, Diphascon* and *Hypsibius* for example have more than one placement.

Comparing the SSU and LSU trees we do see that some genera have similar positions in both. *Echiniscus* are closely related to a lot of other “-chiniscus” genera in both trees. For a lot of other genera the positions differ however. There are several possible reasons for the strange placement of some tardigrades including those in the outgroups: incorrect species identification by the original authors, bad quality of the sequences, too short sequences, sequence alignment not done properly, or a combination of these factors. As mentioned in the result part, although the alignment was improved to some extent further work is most likely needed for more detailed improvements, in addition it will also be necessary to improve the quality of many of the so far published gene sequences.
4.3 Development of microbial cultures on petri dishes

Since the start of the experiment the microorganisms in the petri dishes developed according to the same principle; all dishes started with none, or very few and very small microorganisms, and as time passed acquired more, bigger and a greater variety of different kinds of organisms. However there were still differences between the different cultures on the petri dishes. Those with moss from Öland had the least amount of microorganisms. The mosses and lichens from Vindeln had varying amounts; some nothing and some (like the Cladonia stellaris) developed a fair amount. The dishes from Piteå, however, were the ones with most organisms and most varying kinds of organisms throughout the whole experiment, and it was only in those dishes that tardigrades were found. A reason for this difference in amount of organisms is probably due to the freshness of the collected samples: the Öland samples had been collected in 2009-2010 while the Vindeln samples had been collected in 2015. The mosses and lichens from those locations had been dry for some time before the start of the experiment. The moss from Piteå on the other hand was collected only a month before the start and was kept in closed plastic bags that prevented the samples from drying. Even after only eight days the Piteå samples had plenty of life, while the samples from Vindeln and Öland did not. Most likely, the dried samples would have needed a longer time to acquire a sufficient amount of microorganisms for tardigrades to appear.

4.4 Development of microbial cultures on agar plates

Overall the majority of the cultures on the agar plates had a very similar pattern of development (Fig. 13). The dry and wet parts developed differently however. The wet parts started off getting a turbid whitish color during the first days, which later turned into a more brown hue. After a few days had passed white mold started to sprout which quickly spread over the wet area and soon covered half of the plate. A few days later, black and green mold started to appear in various patches across the surface. The dry parts had a slower development. The first days there were only small colorless colonies. After some time these colonies grew larger and got a brownish hue. Later the dry parts also started to develop white mold and after some additional days also green and black mold. Some agar plates developed cultures in different colors such as yellow or pink by the end of the experiment, but most did not. The control plate with the hand-print developed several bacteria colonies (and later also mold), but at a slower rate than the mosses and lichens. The other control plate that had gotten air (had the lid off) for 15 seconds stayed clear throughout the whole experiment, indicating the air in the lab was very clean. The plates that stood out mostly from the rest were those that had gotten dabbed with moss from Öland: those developed a different kind of white branching bacteria. The reason for this could be that while both Piteå and Vindeln are somewhat located fairly close to each other Öland is an island in southern Sweden that is well separated from the mainland.

4.5 Bacteria from agar plates

Looking at the bacteria from the agar plates after FISH in a fluorescence microscope some general conclusions can be made (see Appendix 6): red rods of various sizes could be found in almost all G- samples (Fig. 15). This color indicates the presence of bacteria so they should (preferably) be present in all samples. Almost all G- samples also had a bit of green color, indicating there were some Proteobacteria in most samples as well. One G- sample stood out, however; sample 21 (from Vindeln) had weak greenish rods but no red rods at all. Perhaps the reason for the lack of red is because these were Archaeabacteria instead of the assumed “ordinary” Bacteria? The G+ samples looked very similar; they all had no red color and just a hint of blue. Maybe this points to there being a misidentification of G+ bacteria and they were in fact G- bacteria, or maybe the reason for the weak color could be due to there being
too little biomass in the samples, or that it these samples were not a pure colony of one species (could contain a mixture of G+ and G- species).

4.6 Macrobiothus biomarker

Macrobiothus hufelandi is a common tardigrade species, both in Sweden and in the rest of the world, but even though the Silva SSU dataset included 130 Macrobiothus sequences and the LSU dataset included 30 Macrobiothus sequences, none of these had been collected in Sweden. Furthermore, specific Macrobiothus biomarkers are not available according to our survey, thus there was a need for a Macrobiothus biomarker to be made for Swedish tardigrades.

4.7 Conclusions

More tardigrade studies are needed from not yet explored parts of the world, for example from warmer regions. While some countries in Europe have obtained somewhat more attention in terms of tardigrades, there are several other countries where only one or few species have been studied. When it comes to Sweden, it is evident that more studies have been performed in the south compared to the north. Öland has for example been a very popular island for tardigrade research and collection, while the regions in the north have obtained less attention during the past 20 years.

Reconstruction of phylogenetic trees of two biomarkers (SSU and LSU) showed clearly that the quality of the gene sequences of tardigrades are far from perfect. Thus, there is a need for a better quality, identification and alignment of the so far published gene sequences in the public databases, and of course of new added sequences as well. Better biomarkers are needed for tardigrades to improve molecular identification. In this study, we designed one for the Macrobiothus genus.

Although a rather simple procedure with mixed quality of samples (with regard to sampling occasion, age of sample etc.) was employed to explore microbes associated with tardigrades, this work shows that different types of microbes can be found in different moss and lichen samples; however, we are far from understanding their role for tardigrades. Thus, more research is needed regarding the Bactecia-Tardigrada relationship.
5. References


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