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Understanding the Diversity and Community Structure of Bdelloid Rotifer Soil Communities.

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UNDERSTANDING THE DIVERSITY AND COMMUNITY STRUCTURE
OF BDELOID ROTIFER SOIL COMMUNITIES

by

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A thesis submitted to the
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The final copy of this thesis has been examined by the signatories,

and we find that both the content and the form meet

acceptable presentation standards of scholarly work

in the above mentioned discipline.
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Understanding the Diversity and Community Structure of Bdelloid Rotifer Soil Communities.

Thesis directed by Professor Steven K. Schmidt

Abstract

Rotifers, though very important to the microbial food-web and energy flux of a system, are still poorly understood in terms of their taxonomy and geographical distributions. Rotifers pose problems for taxonomists and evolutionary biologists due to the difficulties associated with isolation, identification and enumeration of organisms that have few distinguishing morphological characters. This lack of morphological information and their extreme abundance of micro-invertebrates make identification of rare, or even common cryptic, taxa a large and unwieldy task as only painstaking microscopy can be used to identify synapomorphies. To overcome these problems I made use of environmental DNA sequencing to perform large-scale surveys of bdelloid rotifer communities. The goal of this study was to describe the diversity and distribution of bdelloid rotifer communities as they relate to space, soil environment, and co-occurring bacterial communities. I found that bdelloid rotifers are significantly limited in their dispersal capabilities on the order of 100 meters, despite historically being viewed as having easy-to-disperse propagules. Although dispersal limitation is a significant contributor to the diversity structure of bdelloid communities, its influence constitutes much less of an effect than local biogeochemistry. In contrast, co-occurring bacterial communities are less affected by both
dispersal limitation and local habitat. However, both bdelloid rotifer and bacterial communities share patterns of diversity between three ecologically distinct sites within the Niwot Ridge LTER. Comparisons of the diversity and distribution of microscopic animal communities to bacterial communities have been anecdotal, and have not made direct comparisons among samples, as done here. Analyses comparing soil invertebrate communities to co-occurring microbial communities remain in their infancy, indicating that more inclusive concepts and theories are needed to explain observed patterns of distribution and diversity.
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Chapter 1

INTRODUCTION
The importance of mesofauna

Between the macro-scale of visible plants and animals, and the micro-scale of bacteria and eukaryotic microbes, exists a world of meso-organisms responsible for cycling large amounts of organic matter. These soil animals link the micro- and macro-ecology of ecosystems, feeding on bacteria and other single celled microbes, and are preyed upon by larger organisms (Arndt, 1993; Kutikova, 2003). Mesofaunal invertebrates are also important to the maintenance of soil structure, decomposition, nutrient cycling and plant community dynamics (Wall, 2004; Lavelle et al., 2006). A group that has been gaining recognition as a significant player in the mesofaunal community are bdelloid rotifers (Arndt, 1993; Bledzki and Ellison, 1998; Kutikova, 2003; Lapinski and Tunnaciffe, 2003), one of the most abundant non-nematode mesozoans soils (Sohlenius, 1979; Anderson et al., 1984; Petz, 1997; Devetter, 2010). We know very little about the diversity and distribution of bdelloid rotifers, despite their abundance within soil ($6.0 \times 10^3$ to $2.1 \times 10^6$ individuals/m$^2$ (Schmid-Araya, 1998; Devetter, 2007)).

The abundance and diversity of bdelloids are substantially underestimated in the literature and may rival that of nematodes (Devetter, 2010). In fact the majority of soil animal extraction methods are often just simple modifications of those used for nematodes, e.g. Bearmann and Tullgren funnel techniques. These techniques are biased against less-actively mobile soil fauna such as rotifers. Unlike tardigrades and nematodes, rotifers can adhere to soil particles and the funnel walls themselves, or they can die due to anoxic conditions within those funnels (Devetter, 2010). These technical issues combined with confounding morphology of many undescribed species (Behan-Pelletier
and Newton, 1999) have inspired my work to determine the diversity and distribution of bdelloidrotifer communities via environmental DNA sequencing strategies (Chapters 2-4).

In the remainder of this chapter I provide some basic background information about the Phylum Rotifera and its three free-living Classes the Siesonidea, Monogononta, and the Bdelloidea. Since most of the work outlined in this dissertation is focused on the Bdelloidea, I have provided more detailed information about this Class. What follows in the remaining chapters is an account of how I have made significant progress in ascertaining the diversity and distribution of mesofaunal organisms at multiple spatial scales. I start by comparing the basic phylogentic patterns of communities of eutardigrades and bdelloid rotifers (Chapter 2). I then discuss how I further refined my assessment of bdelloid soil community diversity by addressing the ease, or lack thereof, by which bdelloids can disperse about the globe; and the implications for other microscopic soil invertebrates (Chapter 3). Finally, I show for the first time, how biogeochemistry and bacterial diversity relate to bdelloid community diversity along an alpine altitudinal gradient (Chapter 4).

The Phylum Rotifera

The phylum Rotifera is comprised of approximately 2000 species of bilaterally symmetrical unsegmented animals that possess a pseudocoeolom. They can range in size from 10 to 3000 μm in length (Wallace and Snell, 1991). Rotifers have a ciliated head region, called the corona, which appears to have rotating wheels (due to the movement of the cilia) (Figure 1.1). This optical illusion is reflected in the Latin root word origins of their name: *rota* (wheel) and *ferre* (to bear), thus the literal translation is “wheel bearers”.
Figure 1.1: Image modified from Fontaneto et al. (2007b) with permission. (A) Rotaria neptunia, lateral view; (B) Rotariamacrura, ventral view; (C) Rotariatardigrada, dorsal view; (D) Rotariasordida, lateral view. Scale bar: 100 μm.
This iconic structure is used for both locomotion and feeding. Rotifers that rely on suspension feeding usually have larger amounts of coronal ciliation opposed to raptorial rotifers. This ciliation works by creating a current so that food particles will flow toward the mouth. When the food reaches the mouth it is ground by translucent jaws called trophi, which are within a structure called the mastax (pharynx). The trophi of raptorial feeders, however, are specially developed for catching nearby prey (Brusca and Brusca, 1990).

Rotifers contain a hydrostatic skeleton that, depending on the flexibility of the cuticle and the musculature used, the rotifer can increase the hydrostatic pressure of the pseudocoel within the body cavity. This increase in pressure can be used to protrude parts of the body (i.e. foot and corona). This allows them to appear vermiciform (worm-like) so that they may move more efficiently about the substrata and between the pore spaces of soil. While moving, some rotifers may use their pedal glands (ducts that open and release adhesive substances) on their foot for temporary attachment on various surfaces (Hyman, 1951; Wallace and Snell, 1991).

Phylogenetically, the Phylum Rotifera belong to a group of non-molting protostomes usually referred to as the Platyzoa, a microscopic collection of invertebrates within the Lophotrochozoa (Cavalier-Smith, 1998), of the Bilateria, Kingdom Animalia (Figure 1.2). The name Lophotrochozoa stems from the two major groups that compose it, the Lophophorata and the Trochozoa. The Trochozoa are represented by the Mollusca and the Annelida due to their similar trochophor larvae, while the Lophophorata are represented by the Bryozoa (moss animals) and Brachiopods, and are defined by their feeding structures that are appendages with hollow tentacles, the lophophore.
Figure 1.2: General phylogeny of Protostomes. The Rotifera is denoted with an asterisk.
The Rotifera are composed of three free-living classes: Siesonidea, Monogononta, and Bdelloidea (Wallace and Colburn, 1989; Wallace and Snell, 1991; Melone et al., 1998; Sørensen, 2002; Sørensen and Giribet, 2006). Recently it has become evident that Acanthocephela (vertebrate gut parasites) may be an in-group of the Rotifera, but there are differing views of which of these existing Classes of rotifers is most phylogenetically related to acanthocephelans (Garey et al., 1996; 1998; Zrzavy, 2001; Herlyn et al., 2003; Sørensen and Giribet, 2006). Next, I will briefly discuss the different free-living groups of the Rotifera, each displaying alternate modes of reproduction.

**Class Siesonidea**

The Siesonidea comprise only three known species from the genera Seison and Paraseison (Sørensen and Segers, 2005). Siesonids are currently known to exist only as epizoic symbionts that live on the gills of the marine leptostracan crustacean Nebalia. Seisonids can grow to a size of 2-3 mm, and have very reduced corona, which is not very efficient for food gathering. Seisonids also have both male and females that are similar in size and morphology (Ricci et al., 1993).

**Class Monogononta**

Monogononts are the largest group within the Rotifera containing approximately 1,500 described species of benthic, free-swimming, and sessile/colonial forms that exist in freshwater, brackish water, and marine habitats. Monogononts are dioecious with both sexes having one gonad, hence their name. However, the majority species have never had
a male observed, when males are observed they only last for a few days or weeks because their gut serves only as energy storage (i.e. they do not feed). Monogonont rotifers follow a complex reproductive cycle that is composed of a parthenogenetic amictic phase and a mictic phase that includes sexual reproduction via hyperdermic impregnation by ephemeral haploid males (Birky and Gilbert, 1971; Gilbert, 1974).

Monogononts are the most studied of the Rotifera, mainly due to their economic importance in aquaculture (Lubzens et al., 1989), ecotoxicology (Halbach et al., 1983) and wastewater treatment (Fiałkowska and Pajdak-Stós, 2008). Even more recently, there has been interest in the molecular biology of this group. The first rotifer mitochondrial genome was sequenced from the rotifer Brachionus plicatilis. The mitochondrial genome of this Monogonont rotifer was found to be composed of two separate circular molecules (Robeson, 2001; Suga et al., 2008). The structure of the mitochondrial genome of this rotifer goes against the standard dogma of animal mitochondrial genomes (Wolstenholme, 1992). That is, animal mitochondrial DNA is generally thought to have few or non-existent inter-genic spaces (i.e. very compact gene arrangement). Whereas the mitochondrial genome of B. plicatilis has very large non-coding segments of DNA on both molecules, flanked by repeat sequences (Robeson, 2001; Suga et al., 2008). This data will be crucial for comparative genomic studies that endeavor to elucidate the evolutionary relationships of the Rotifera, as DNA sequencing and assembly of the first bdelloid rotifer (Bdelloidea; discussed below) genome is currently underway.

Class Bdelloidea
Bdelloid rotifers are the focus of this dissertation so; I will go into the detail of this rotifer class more than the others. Bdelloids comprise roughly 400 described species of obligate parthenogens and have a very conserved vermiform body plan consisting of several semi-rigid lorica (shells) separated by annuli (rings). This allows them to shorten or lengthen by telescoping their body (Wallace and Snell, 1991; Segers, 2007). Bdelloids can propel themselves by using cilia to swim or by taking alternate steps with their head and foot along a substrate. The latter movement type is the basis for their Greek derived name which stems from the root-word βδελλα, (bdella) meaning leech or leech-like. Bdelloïdstypically range in size from 0.2 mm to 2 mm in length and are quite adept at surviving unfavorable environmental conditions such as ionizing radiation (Gladyshev and Meselson, 2008), extreme desiccation, acidic pH and cold temperatures (Gilbert, 1974; Ricci, 1987; Ricci et al., 1987; Ricci, 1998; Deneke, 2000).

Bdelloids often occur within sediments, plant debris, surfaces of aquatic plants, or within thin water-films on the surface of moss and lichen. When bdelloids are biologically active they are very important to the development of soil biocenoses (self-regulating ecological communities) and energy flux of systems (Kutikova, 2003). They can even play a large role in the carbon budget of soils (Sohlenius, 1979). Some bdelloid rotifers can be quite specific in what they consume, many limiting their diet to only one or a few species of algae (Starkweather, 1980).

Bdelloids have substantial ecological effects at both the micro and macro-scale, they aide in freeing up resources in microbial food webs by mechanically breaking-down leaf litter, and are both consumer and prey items of other micro-organisms (Kutikova, 2003). Bdelloids are also parasitized by a variety of fungi (Wilson and Sherman, 2010; Wilson,
In fact, predatory fungi related to the genus Lecophagus have been found in the talus soils of Colorado (Schmidt et al., 2012), a location in which I have discovered highly diverse communities of eutardigrades and bdelloid rotifers (Chapters 2 & 3). Finally, bdelloid rotifers are large contributors of nitrogen and phosphorus to pitcher plants and bogs, such that the nutrients supplied by insects and rainfall is minimal by comparison (Bledzki and Ellison, 1998; 2003). Based on these studies it becomes apparent that bdelloid rotifers effectively link nutrient flow from microbes to macro-fauna / flora and back again (Lavelle, 1996; 1996; van Hannen et al., 1999). These links need to be further studied and should be apart of ever expanding soil microbiome projects.

Bdelloids do have a central role in microbial food web interactions. The overwhelming importance of this interaction can best observed in waste-water treatment systems (Lapinski and Tunnaciffe, 2003; Fiałkowska and Pajdak-Stós, 2008). Bdelloid rotifers are present in these activated-sludge (AS) systems as top predators where they graze on suspended bacteria and algae. Rotifers are the most abundant within AS, comprising 97% of total metazoan biomass, of which bdelloids themselves make up 74% of the rotifer biomass (Lapinski and Tunnaciffe, 2003). At the current level of understanding the potential impact of bdelloids in natural systems can only be estimated, but when compared to the observation that bdelloids can filter large volumes within an hour. For example, single individual of Habrotrochathienemanni with a mean bod size of 76.2 ±19.6μ can filter ~9.6μL•hour⁻¹ of water (Devetter, 2009a). This suggests that bdelloids are likely to have large impacts within the soil microbial food web (Wallace and Starkweather, 1985; Devetter, 2009a). Thus, it is important to begin elucidating the diversity and distribution of bdelloids as they relate to their co-occurring microbial
communities as I discuss in Chapter 4.

Historical problems estimating soil bdelloid rotifer diversity and distribution

Bdelloid rotifers, like many micro and mesofaunal organisms, pose problems for taxonomists and evolutionary biologists due to the difficulties associated with isolation, identification and enumeration of such small organisms. Moreover, even when it is possible to successfully culture organisms, limited phenotypic differentiation among taxa and the possible effects cyclomorphosis (seasonal change in body shape (Fontaneto and Melone, 2003a; Gómez, 2005)) confound accurate taxonomy. This lack of taxonomic information and the extreme abundance of meiofaunal organisms makes identification of rare, or even common cryptic taxa, a large and unwieldy task (Blaxter et al., 2005; Fontaneto et al., 2007b). Finally, most studies regarding the diversity and distribution of rotifers are biased, as studies have largely have been centered in Europe with little representation from the rest of the world. This has been labeled as the ‘rotiferologist effect’ (Fontaneto et al., 2011a). That is, most research on rotifers is carried out near the residences of local rotifer biologists.

The above-mentioned issues reveal why bdelloids are still poorly understood in terms of their taxonomy and geographical distributions (Kutikova, 2003; Stead et al., 2005; Woodward et al., 2005; Fontaneto et al., 2007b). Rotifer species are thought to be ubiquitous due to their small size and abundance. A recent debate in the literature (as reviewed in (Green and Bohannan, 2006)), revolves around the Bass-Becking idea of ‘everything is everywhere and the environment selects’ (EisE), for organisms smaller than 2 mm in size (de Wit and Bouvier, 2006). Bdelloids, more than any other
microscopic invertebrate, would be the ideal candidate to support this conjecture, as they have extreme survival capabilities as mentioned earlier (Gilbert, 1974; Ricci, 1998; Gladyshev and Meselson, 2008). However, I show in Chapter 3, that most bdelloids do have limited patterns of distribution.

**Expanding our understanding of soil invertebrate communities**

To avoid the aforementioned issues of confounding morphological data, I initially developed a culture-independent molecular survey approach for the amplification and sequencing of eutardigrade and bdelloid rotifer communities directly from soil using the 18S rDNA marker gene (Chapter 2). I was able to determine that both eutardigrade and bdelloid communities showed geographic structure among clades. However, there was greater lack of phylogenetic resolution of bdelloid rotifer phylotypes compared to eutardigrade phylotypes, suggesting that there are a few truly dispersed bdelloid phylotypes. Furthermore, bdelloid sequences from Socompa Volcano (Costello et al., 2009) and Japan (Fukushima and Ibaraki), reside within a larger clade (Clade B, Figure 2.4) containing sequences from Calhoun Experimental Forest in South Carolina. This suggests that several groups of bdelloids may have wide ecological tolerance and can survive within quite different habitats.

Due to the lack of representative 18S rDNA bdelloid sequences (only two of four families are currently represented in all online databases), along with exceedingly short branchlengths and many polytomies within the 18S phylogeny makes the description of environmentally derived sequence data difficult to interpret. This was not an issue for eutardigrades (Chapter 2). For reasons that are not entirely clear, substantially more
progress has been made in generating large amounts of sequence data from a variety of
genes to elucidate the phylogeny and genetic diversity of the Tardigrada. Anecdotally,
tardigrade research does not suffer from the ‘rotiferologist effect’ that plagues rotifer
research in (Fontaneto et al., 2011a).

I set out to increase our understanding of bdelloid genetic diversity within the
United States, and other regions of the globe, by making use of the cytochrome oxidase
subunit 1 (\textit{cox1}) gene. Most of the morphologically known genera within the Bdelloidea
are represented by this marker gene due to recent barcoding initiatives (Birky, 2007). So,
I developed bdelloid rotifer specific primers for \textit{cox1} so that I can amplify and sequence
their DNA directly from soil. My environmental molecular survey approach has allowed
me to generate bdelloid community data from a variety of geographically distant
locations. With these data I was able to show that bdelloid rotifer communities are
spatially autocorrelated locally and highly diverse globally (Chapter 3).

After determining that bdelloid rotifers are spatially structured I set out to
determine how much of this spatial structuring between communities is affected by local
biogeochemistry and co-occurring bacterial communities. I chose three distinct habitats
(sub-alpine forest, dry meadow tundra, and talus) within the Niwot Ridge LTER (Chapter
4). This enabled me to determine that although geographic distance is a significant
contributor to the beta diversity structure of bdelloid communities, bdelloids are more
influenced by local environment. Most interestingly, I was able to show that bdelloid
rotifer and bacterial communities share patterns of \(\alpha\)- and \(\beta\)-diversity structure between
three ecologically distinct sites within the Niwot Ridge LTER (Chapter 4). Indicating
many, as yet known interactions between soil invertebrates and their co-occurring
microbial communities await study and should be made a part of future biological and environmental sequencing surveys.
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Chapter 2

ENVIRONMENTAL DNA SEQUENCING PRIMERS FOR EUTARDIGRADES AND BDELLOID ROTIFERS
Summary

The time it takes to isolate individuals from environmental samples and then extract DNA from each individual is one of the problems with generating molecular data from meiofauna such as eutardigrades and bdelloid rotifers. The lack of consistent morphological information and the extreme abundance of these classes makes morphological identification of rare, or even common cryptic taxa a large and unwieldy task. This limits the ability to perform large-scale surveys of the diversity of these organisms.

Here we demonstrate a culture-independent molecular survey approach that enables the generation of large amounts of eutardigrade and bdelloid rotifer sequence data directly from soil. Our PCR primers, specific to the 18S small-subunit rRNA gene, were developed for both eutardigrades and bdelloid rotifers.

The developed primers successfully amplified DNA of their target organism from various soil DNA extracts. This was confirmed by both the BLAST similarity searches and phylogenetic analyses. Tardigrades showed much better phylogenetic resolution than bdelloids. Both groups of organisms exhibited varying levels of endemism.

The development of clade-specific primers for characterizing eutardigrades and bdelloid rotifers from environmental samples should greatly increase our ability to characterize the composition of these taxa in environmental samples. Environmental sequencing as shown here differs from other molecular survey methods in that there is no need to pre-isolate the organisms of interest from soil in order to amplify their DNA. The DNA sequences obtained from methods that do not require culturing can be identified post-hoc and placed phylogenetically as additional closely related sequences are obtained
from morphologically identified conspecifics. Our non- cultured environmental sequence based approach will be able to provide a rapid and large-scale screening of the presence, absence and diversity of Bdelloidea and Eutardigrada in a variety of soils.
Introduction

Micro-invertebrates, though very important to the soil biocenose (self-regulating ecological communities) and energy flux of a system, are still poorly understood in terms of their taxonomy and geographical distributions (Kutikova, 2003; Stead et al., 2005; Woodward et al., 2005; Fontaneto et al., 2007a). Like many microfaunal organisms, Rotifera and Tardigrada pose problems for taxonomists and evolutionary biologists due to the difficulties associated with isolation, identification and enumeration of organisms that do not preserve any discernable morphological characters. Even when it is possible to successfully culture these organisms, limited phenotypic differentiation among taxa and cyclomorphosis (seasonal change in body shape; (Gómez, 2005)) confound accurate taxonomy. This lack of consistent morphological information and the extreme abundance of meiofaunal organisms makes identification of rare, or even common, cryptic taxa a large and unwieldy task (Blaxter et al., 2005; Fontaneto et al., 2007a) as only painstaking microscopy can be used to identify synapomorphies.

Environmental sequencing is valuable for performing large-scale surveys of the diversity of organisms that cannot be cultured or grown in the laboratory or when species are difficult to distinguish using phenotypic characters. These issues argue for culture independent molecular surveys of meiofaunal diversity in natural ecosystems. Microbiologists have faced many of the same problems and solved them by turning to conserved DNA sequences as a means of describing communities (Reysenbach et al., 1992; Pace, 1997). Instead of isolating and culturing individuals, communities are characterized by extracting all of the DNA in a particular sample (soil, water, air), amplifying a specific gene using PCR, cloning individual PCR products, and then
sequencing individual clones. This environmental DNA approach has revolutionized microbiology. For example, these techniques have been successfully used to provide new insights into fungi (Schadt et al., 2003; Schmidt et al., 2007), novel Chloroflexi (Costello and Schmidt, 2006), abundance and distribution of Psychrobacter and Exiguobacterium (Rodrigues et al., 2009) and have been used to provide information about the structure and function of alpine and arctic soil microbial communities (Nemergut et al., 2005).

Our survey focuses on the 18S rRNA gene, commonly used for phylogenetic inference of eukaryotes due to its highly conserved sequence and ability to resolve relatively deep nodes. This is the first description of the general utility of environmental DNA sequencing approaches for characterizing difficult to study ecological communities of eutardigrades and bdelloid rotifers.

Environmental sequencing as described here differs from other molecular survey methods (Blaxter et al., 2005; Sands et al., 2008a) in that there is no need to pre-isolate the bdelloid rotifers or eutardigrades of interest from soil (or other mediums) before amplifying their DNA. The successful development of clade-specific 18S SSU primers has shown to be effective when surveying the diversity of targeted groups of organisms. For example, clade specific 18S SSU primers have been used to describe soil metazoans (Waite et al., 2003; Wu et al., 2009) and reveal the hidden diversity and biogeographic endemism of kinetoplastids (flagellate protozoa) (Heyden and Cavalier-Smith, 2005).

The use of 18S rDNA allows for sequences to be combined into already existing 18S and 16S rDNA databases, including those being developed by microbial ecologists.
through their large scale molecular surveys as referred to above. Here we describe the utility of screening for bdelloid rotifer and eutardigrade diversity in two very distinct sample sites with targeted 18S primers: the high-elevation sites located within the Niwot Ridge Long Term Ecological Research (LTER) site in the Colorado Rockies, and the low-elevation sites located within the Calhoun Experimental Forest in South Carolina.
Results

We developed two forward primers for taxon specific amplification of eutardigrades and bdelloid rotifers. These primers were used in combination with a universal reverse 18S rDNA primer to specifically characterize the diversity of these two groups from several environments. PCR, BLAST and phylogenetic analysis confirmed that each set of primers amplifies the targeted groups with fidelity and specificity (Figures 2.1 & 2.2). We have observed many invertebrates within the soils prior to DNA extraction and amplification, including mites, nematodes, and insects; none of these were observed within the sequencing data produced using the specific primers in this study. Thus, our primers are shown to be specific to the targeted groups of organisms. The closest known sequences or clades to the environmental sequences are noted below. Note that we do not infer that the environmental sequences are of the same species or genera to those closest to them.
Figure 2.1: Gel image of PCR results for eutardigrade specific 18SrDNA primers. First four lanes are from replicate individuals from a single population eutardigrades. Lanes five through 7 are from heterotardigrades. Lanes eight through ten are nematodes.
Figure 2.2: Gel image of PCR results for bdelloid specific 18SrDNA primers. Lane 1 is the Hyper ladder 1 from Bioline USA Inc. MA, Brachionusplicatilisis the Monogonont in lane 2. Lanes three through six are from individual representatives of the following bdelloid rotifers: Philodina, Adineta, Macrotrachela, and Habrotrocha. The final two lanes are from unidentified bdelloids taken from a pond.
**Tardigrada**

Out of 1,814 nucleotide positions there were 900 variable sites, of which 677 were phylogenetically informative, comprising 68 unique phylotypes. Phylogenetic analysis clearly separates the two main groups of tardigrades: the Heterotardigrada and the Eutardigrada (Figure 2.3). Many of the environmental sequences from the high-elevation talus sites clustered into distinct clades, suggesting each clade may comprise a separate species. Eutardigrade sequences from soils near the Arikiree Glacier (AGL) grouped within the Macrobiotoidea and Hypsibiodea groups. Those within the Macrobiotidea are most closely related to *Richtersius coronifer*, a cosmopolitan species sampled from high elevation and arctic habitats (Ramlov and Westh, 2001; 2001). The AGL sequences that grouped within the Hypsibiodea are related to those of the englacial dominating Hypsibius genus. These Hypsibius sequences from the AGL site are nearby and grouped with the two talus sites (T1T2 & T3T6).

The Calhoun Hardwood site sequences cluster closest to *Isohypsibius papillifer* typically found in Europe, Asia, Australia, & South America (McInnes, 1994). The genus *Isohypsibius* is composed of species that are widespread and has been documented circumglobally as well (McInnes, 1994) (GBIF Data Portal [http://www.gbif.net]. 09-24-2009 Sweden, 17 records; National Museum of Natural History, 10 records; Australian Antarctic Data Centre, 3 records).

The Calhoun Grassland sequences cluster basally with the Arikiree and Talus sites within the Hypsibius group, noted as "Acutuncus/Hypsibius" in contrast to another group labeled "Acutuncus/Calohypsibius" in Figure 2.3, (see (Dastych, 1991; Sands et al.,...
2008b) for clarification about taxonomic identification issues with Hypsibius and Acutuncus).
Figure 2.3: Cladogram representations of phylogenetic trees obtained from TNT (Goloboff et al., 2008) and MrBayes (Altekar et al., 2004) on tardigrades. Bootstrap values below 50 and posterior probability values below 70 are not represented. All environmental sequences fall within the Eutardigrada.
Bdelloidea

Out of 1638 sites 896 were variable and 718 were phylogenetically informative. The environmentally obtained sequences totaled 54 unique phylotypes (49 from this study). Phylogenetic analysis clearly separates all of the main clades of rotifers: Seisonidea, Monogononta and Bdelloidea (Figure 2.4). All of the environmental sequences we sampled grouped within the Bdelloidea. We also discovered three relatively diverse clades. The first is dominated by Niwot Ridge sequences (Clade A). One of the clades within Clade A (Sub A) is mainly dominated by sequence types from the T1T2 site. The second clade (Clade B) is dominated by those sequences from the Calhoun sites. What is interesting here is that the most derived cluster within Clade B contains uncultured sequences from Japan (Ibaraki upland soils) along with sequences from a high elevation site in Socompa, South America (Sub B) (Costello et al., 2009).

The final main group of sequences, Clade C, contains sequences from several locales, but mostly those from the AGL site. Again, like in Sub B, we observe uncultured sequence data from Japan (Fukushima and Ibaraki) clustering with a sequence from Socompa.

The lack of 18S rDNA sequence information in online data bases (14 bdelloid sequences in GenBank(Benson et al., 2005) as of this writing), makes the identification of environmentally obtained sequences even more difficult.
Figure 2.4: Cladogram representations of phylogenetic trees obtained from TNT (Goloboff et al., 2008) and MrBayes (Altekar et al., 2004) on bdelloid rotifers. Bootstrap values below 50 and posterior probability values below 70 are not represented. All environmental sequences fall within the bdelloidea.
Discussion

The development of clade-specific primers that allows characterization of eutardigrade and bdelloid rotifer communities from environmental samples should greatly increase our ability to discern the community diversity of these taxa in environmental samples. Moreover, the rDNA sequence data can be directly stored (within software packages like ARB (Ludwig et al., 2004)) and compared with other surveys that attempt to characterize invertebrate community composition (Porazinska et al., 2009; Wu et al., 2009).

We anticipate that environmental DNA surveys using clade-specific primers, like those we have developed, will be used to complement more directed studies that cultivate individual micro-eukaryotes as a means of more fully describing the diversity of ecological communities. We have yet to assess whether isolation of individuals and environmental DNA surveys yield different estimates of community composition, as is the case for surveys of bacteria (but see (Porazinska et al., 2009; Wu et al., 2009)) and bdelloid rotifers (Kaya et al., 2009).

Environmental sequencing as shown here differs from other molecular survey methods (Blaxter et al., 2005; Sands et al., 2008a; Porazinska et al., 2009; Wu et al., 2009) in that there is no need to pre-isolate the organisms of interest from soil (or other media), in order to amplify their DNA. Here, we simply extract total cellular DNA from all organisms in the soil and use targeted primers for the group of interest. This allows for a single DNA extraction prep instead of DNA extraction prep for each targeted organism of interest.
Eutardigrada

Although there are too few data to make robust biological inferences, several results are noteworthy. We found sequences from the highest elevation site in Colorado (near the Arikiree glacier) that grouped together with R. coronifer, a cosmopolitan morpho-species known to exist in high mountain and arctic habitats which is also known to survive extreme desiccation and temperatures down to -196°C (Ramløv and Westh, 1992). Additionally, several sequences from the Calhoun hardwood forest were very similar to Isohypsibius papillifer, a widespread European species. Moreover, the genus Isohypsibius is ubiquitous, distributed from North America, Northern Europe, and Asia, all the way to Antarctica.

Interestingly, the sequences from the AGL site seem to have the most distant set of sequences compared to the other sites. One set of sequences is from within the Macrobiotidae, Richtersius group and the other from the Hypsibiodea, Hypsibius group. This is probably due to the longer duration of moist and wet soils that allows for a greater diversity of eutardigrade groups.

It is not too surprising that the majority of the Eutardigrade sequences amplified from the Talus and glacier sites are dominated by Hypsibius-related sequences. The Hypsibidae are known to dominate englacial habitats and are the dominate family of polar and cryoconitetardigrades. Hypsibius species are hydrophilic and are composed of bacteriophagous and/or algivorous feeding types. These biological factors aid in the colonization of nunatuks and glacial habitats (as reviewed in (Pugh and McInnes, 1998)).

However, several sequences from the Macrobiotidae were also found within the glacial habitat of the AGL site. Macrobiotidae are traditionally considered cosmopolitan
occurring in many habitats, including those that are periodically frozen (Pugh and McInnes, 1998). The AGL sequences cluster closest to the known sequences of Richtersius sp. (Figure 2.3). Richtersius have been the focus of many anhydrobiosis studies and have shown significant improvements in desiccation survival when many individuals aggregate together during anhydrobiosis (Ivarsson and Jönsson, 2004). This could lead to positive density dependence and even allow these animals to achieve greater monopolization [as reviewed in (Pilato and Binda, 2001)] to local habitats that encounter extreme desiccation events like the high elevation AGL and talus sites. However, aggregation can create problems with environmental sequencing strategies like the one proposed here. If aggregation in the wild occurs within other eutardigrade groups then environmental sequencing may lead to amplification of only those extremely high-abundant clusters of animals.

**Bdelloidea**

In contrast to the tardigrades, there was less agreement of support between the two different phylogenetic reconstruction methods of Bayesian and parsimony analysis for bdelloid rotifers. It was not possible to identify what bdelloids the environmental sequences were related to due to lack of abundant reference sequences. However, while it was possible to make some general statements about the bdelloid communities at the listed sample sites, the lack of resolution of 18S rDNA compared to 28S rDNA (Fontaneto et al., 2007b) makes it difficult to delineate the more recent clades of Bdelloidea (Figure 2.4). In fact, a similar level of poor resolution of bdelloids is also seen from phylogenies produced via cytochrome oxidase subunit 1 sequence data, wherein the early nodes are
mostly saturated with polytomies (Robeson & Birky unpublished). Better resolution of this group at the tips of the phylogeny is often seen regardless of the phylogenetic reconstruction method chosen.

It is interesting that sequences from Socompa (Costello et al., 2009) cluster with the Calhoun sequences as opposed to other high elevation sites like the dry Talus, in Niwot Ridge. Although Socompa is a very high elevation site (5824 m above sea level), it is most likely similar in its microhabitat to the Calhoun sites, where there is greater moisture compared to the dry Talus. The Socompa site is characterized as a fumarole environment (Costello et al., 2009). Typically fumaroles are areas where steam and volcanic gases vent out of the earth's crust due to the degassing of magma and/or geothermal heating of shallow ground water. This particular fumarole site is weakly active, creating an environment in which communities of mosses and liverworts are sustained by warm water vapor. The potentially similar microhabitats may be the reason for finding such similar sequence types in very different locales.

Bdelloid rotifers in particular show evidence for geographic structure among clades. Whether this apparent pattern reflects environmental filtering, priority effects (differences in arrival time that can have a lasting effect on differences in species dominance), or some other process remains to be seen. Nonetheless, the data presented here support the contention of (Fontaneto et al., 2008), in which instances of endemism are seen (Clade A & B), with a few phylogenetic clusters of widespread bdelloids sampled from very different locales (Clade C and Sub B). It may be that harsher conditions in which there are very ephemeral moments of soil moisture creates higher levels of endemism of bdelloids, whereas environments in which soil moisture is
sustained for longer periods of time allow for increased chances of long distance dispersal to suitable habitats and persistence. The location of the Socompa fumarole sites in the phylogeny (Figure 2.4) and its high similarity to sequences from Japan and within the Calhoun sites (Clade B & C) may be an indication of the latter point. One caveat here is that the 18SrDNA sequences are more conserved than their cytochrome oxidase subunit 1 counterparts (Fontaneto et al., 2007a; a; 2008) preserving more ancient than contemporary relatedness.
Conclusion

Large-scale surveys of rotifer and tardigrade diversity using traditional approaches make for a large and unwieldy set of tasks (i.e. difficulties associated with isolation, identification and enumeration of organisms that do not preserve any discernable morphological characters).

Environmental sequencing is valuable for performing large-scale surveys of the diversity of organisms that cannot be cultured or grown in the laboratory or in which species are difficult to distinguish using phenotypic characters. The DNA sequences obtained from non-cultured based methods can be identified post-hoc (placed phylogenetically) as closely related sequences are obtained from morphologically identified conspecifics. Our environmental sequence based approach, which does not require culturing or isolation of animals from soils, provides a rapid and large-scale screening for the presence, absence and diversity of Bdelloidea and Eutardigrada in a variety of soils.

We have shown that targeted amplification of eutardigrades and bdelloid rotifers are possible from a range of soil types. This sequence data can be used to quickly assess the peculiar biogeography (Pilato and Binda, 2001; Nelson, 2002) and genetic diversity of soil samples, more often informing us of dominate groups within each sample.

It should also be emphasized that environmental sequencing strategies like this are not intended to replace, but instead complement ongoing morphological work, explore the possible effects of heterogeneity within individuals, and the effect of this variation on phylogenetic analysis (Carranza et al., 1996). This highlights the need for morphological taxonomists and molecular ecologists to work together in order to make
environmental sequencing methods, like the one proposed here, more robust. In particular, studies such as these are most empowered by the cataloging of sequence data from vouchered specimens.
Methods

Soil DNA extraction

Soil samples (~5 g) were taken from all sites. Three sites from within the Niwot Ridge Long Term Ecological Research (LTER) area in the Front Range of the Colorado Rocky Mountains, United States of America (40° 03' N, 105° 35' W). These sites are: the Arikiree Glacier (AGL), Talus site 1 (T1T2), and Talus site 2 (T3T6) as described previously by (Freeman et al., 2009). Other soil samples were also obtained from the Calhoun Experimental Forest (managed by the US Department of Agriculture located in northwestern South Carolina in the Piedmont region, 34.5°N, 82°W), these sites are: Hardwood (H), Grassland (G), and Cultivated (C). Total cellular DNA was extracted from soil using the PowerSoil DNA Isolation Kit #12888 (Mo Bio Laboratories, Inc, Carlsbad, CA).

Primer development

Only forward 18S SSU primers were developed to target specific groups (bdelloids and eutardigrades). Primer development entailed downloading all available target sequences of interest along with their closest set of out-group taxa from GenBank(Benson et al., 2005) and aligned using Muscle (Edgar, 2004) and edited in ARB (Ludwig et al., 2004) to align conserved regions only. A region of bases unique to the target group that excluded as many matches as possible to the outgroup taxa were chosen for primer development. Bdel_2: 5'- CGG CTC ATT ACA TCA GCT ATA ACT T-3' was used for bdelloid rotifers, and Tard_1: 5'-TCT CAG TAC TTG CTT TAA CAA GGC-3' was used for eutardigrades. Amplicon products produced were ~1700 base pairs
in length. All eutardigrade and bdelloid rotifer environmental sequences had a sequence identity to those in GenBank ranging from 91 to 98% with a query coverage of 99 to 100% and 95-99% with a query coverage of 97-100% respectively.

Other 'universal' primers used in this study were taken or derived from [38-40] and are listed here as follows: 18S2a: 5'-GAT CCT TCC GCA GGT TCA CC-3'; 18S3: 5'-GAC TCA ACA CGG GAA ACC TCA CC-3'; 18S10: 5'-CTA AGG GCA TCA CAG ACC-3'

PCR

The reverse primer 18S2a was used in conjunction with either the Tard_1 or Bdel_2 primer in order to amplify the DNA of either eutardigrades or bdelloid rotifers directly from soil. The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of: 94°C for 30", 60°C for 30", 72°C for 2', with a final extension at 72°C for 10'. PCR reaction contained (all reagents from Invitrogen, Carlsbad, CA, USA) 1× PCR Buffer, 1.5 mM MgCl2, 0.2 μM dNTPs, 0.4 μM of each primer, Taq polymerase (0.5 units), template DNA: 2 μL.

Cloning & Sequencing

The final PCR product was purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) or the QIAquick Gel Extraction Kit 28704 (QIAGEN, Valencia, CA). Purified PCR product was then cloned using the Invitrogen TOPO TA Kit (with pCR2.1-TOPO vector) with One Shot TOP10 Chemically Competent E. coli (K4500-01). Pelleted cells were sent to Functional Biosciences, Inc (Madison, WI) for
sequencing. The 18S3 and 18S10 primers were only used at this step for internal sequencing along with M13 primers to generate robust sequence data for contig assembly.

**Sequence analysis**

Sequence data was assembled, vector and primer sequence removed, then edited by hand using Sequencher 4.7 (Gene Codes Cooperation, Ann Arbor MI). Sequences were chimera-checked using the Bellerophon server (Huber et al., 2004) and determined that no chimeras by sample site amplicons were detected. Usable data were then exported for BLAST (Benson et al., 2005) searches. All sequences produced and/or used in this study are listed by accession in Table 2.1.
<table>
<thead>
<tr>
<th>Environmentally obtained Bdelloids (this study)</th>
<th>GQ922286 - GQ922334</th>
</tr>
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<td><strong>Bdelloidea</strong></td>
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<tr>
<td><strong>Uncultured Bdelloidea</strong></td>
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<td><strong>Monogononta</strong></td>
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<td><strong>Seisonidea</strong></td>
<td>AF469411, DQ089737, DQ297761</td>
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<tr>
<td><strong>Gnathostomulida</strong></td>
<td>AY218111</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmentally obtained Eutardigrades (this study)</th>
<th>GQ922218 - GQ922285</th>
</tr>
</thead>
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<td><strong>Heterotardigrada</strong></td>
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<td><strong>Pycnogonida</strong></td>
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<tr>
<td><strong>Mollusca</strong></td>
<td>AF120503, X91977</td>
</tr>
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</table>

Table 2.1: List of Accession numbers by major groups. Sequences used as guides as well as those generated from this study.
Pre-aligned guide and outgroup sequences were downloaded from the SILVA database (Pruesse et al., 2007). The SILVA aligner was used to align the environmental 18SrDNA SSU sequence data according to secondary structure (Pruesse et al., 2007). The data was further edited by eye and exported from ARB (Ludwig et al., 2004) using an 'in-house' filter to remove highly ambiguous regions of the alignment. All terminal gaps in the alignment were converted to missing (i.e. as '?' characters) and gaps '-' counted as a 5th character state. TNT (Goloboff et al., 2008) and a multi-core version of MrBayes (Altekar et al., 2004) were used to confirm the phylogenetic placement of environmentally obtained sequences. Parsimony analysis was performed by generating 1000 bootstrap replicates. Before re-sampling, the trees were collapsed using TBR. Each bootstrap replicate was composed of twenty iterations of 'Wagner addition trees' (trees formed by sequentially adding the taxa at the best available position, using Fitch parsimony) followed by swapping with TBR, the single best tree was then used for random sector searches and trees saved. MrBayes was used to perform 5 and 8 million generations using the GTR + G + I model of evolution as specified by MultiPhyl Online on the bdelloid and eutardigrade data sets respectively (Keane et al., 2007).
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This work was previously published as:

References:


Chapter 3

SOIL ROTIFER COMMUNITIES ARE EXTREMELY DIVERSE GLOBALLY BUT SPATIALLY AUTOCORRELATED LOCALLY
Summary

Bdelloid rotifers are important contributors to biogeochemical cycling and trophic dynamics of both aquatic and terrestrial ecosystems, but little is known about their biogeographic distribution and community structure in terrestrial environments. This lack of knowledge stems from a lack of phylogenetic information and assumptions that microbial eukaryotes are globally distributed and have very limited diversity across vast geographic distances. However, these assumptions have been based more on assessments of their morphology than any measure of their true genetic diversity and biogeographic distribution. We developed specific primers for the cytochrome c oxidase subunit 1 (cox1) gene of bdelloid rotifers and amplified and cloned sequences using a nested sampling scheme that represented local (0–10 m) to global (up to 10,000 km) scales. Using phylogenetic community analyses (UniFrac) and geospatial statistics (semivariograms, mantel tests), we were able to reject the hypothesis that communities of rotifers are the same across even fairly small geographic distances. Bdelloid communities showed highly significant spatial structuring with spatial autocorrelation ranges of 54–133 m, but beyond that distance communities were extremely dissimilar. Furthermore, we show that these spatial patterns are driven not only by changes in relative abundance of phylotypes but also by absolute changes in phylotype occurrence (richness). There is almost no overlap in phylotype [or operational taxonomic unit (OTU)] occurrence between communities at distances beyond the autocorrelation range (~133 m). Such small species ranges, combined with their ubiquity in soils, make it increasingly clear that the biodiversity of bdelloid rotifers (and other less easily dispersed microbes) is much higher than previously thought.
Introduction

Microbiota have been described dogmatically as having similar global and local distributions, i.e., “everything is everywhere, and the environment selects,” as initially discussed by Beijerinck and Baas Becking (de Wit and Bouvier, 2006; O'Malley, 2007). This view is supported by the massive population sizes of microorganisms, by the general ease with which they can disperse (e.g., via wind), and by data based on the distribution of species defined by morphology. However, recent work has suggested significant dispersal limitation in some groups of microorganisms including bacteria and archaea (Cho and Tiedje, 2000; Whitaker et al., 2003) and microbial eukaryotes (Green et al., 1996; Fontaneto and Ricci, 2006).

As reviewed elsewhere (Whitaker et al., 2003; Martiny et al., 2006; Fontaneto et al., 2008), the case of whether or not small organisms have any discernable biogeography can be split into two camps: those that think “everything is everywhere” (EisE) (Figure 3.1A), and those that think all organisms have some level of biogeography, regardless of size (Figure 3.1B).
Figure 3.1: Hypothetical community versus biogeographic relationships as they relate to microorganisms. (A) Assuming EisE, community relatedness is not dependent upon distance; so all communities appear similar to each other. (B) Assuming a continuous distance–decay relationship of community dissimilarity over geographic distance. (C) Spatial autocorrelation up to the autocorrelation range (vertical dashed line), after which the limit of spatial dependence is reached (sill), and communities are just as likely to be as similar to or as different from communities before the range. (D) Spatial autocorrelation as in C but with a lag before the start of autocorrelation indicating we are sampling from within the same community.
Basically, the issue is rooted in whether historical or contemporary effects are responsible for present patterns of distribution (Martiny et al., 2006). For those on the side of EisE, the local environment plays a role larger than that of any historical biogeographical effects. The degree of similarity between any two communities should be independent of the geographical distance between them, given similar environments. Therefore, no matter the geographic scale sampled, all communities should maintain the same level of average, but low, dissimilarity (Figure 3.1A). Those who maintain historical effects trump local environment effects would expect that communities increasingly further from each other should become increasingly dissimilar (Figure 3.1B). Debates often revolve around which of these two community patterns is the rule.

However, it also is possible that both historical and environmental effects determine the biogeographic distribution of microbes (Martiny et al., 2006). If so, then one would expect local communities to show more similarity than that seen with geographically distant communities. In other words, local communities should show a high degree of autocorrelation, and distant communities should present a random array of pair-wise community relatedness. This latter case is depicted graphically in Figure 3.1C. However, to detect a pattern as shown in Figure 3.1C, a nested sampling strategy is essential to reveal the spatial structure of communities at scales ranging from local (centimeter to meter scales) to global. A fourth and final possibility is one that resembles the trend in Figure 3.1C with the addition of a flat lag at very small geographic distances (Figure 3.1D) before spatial autocorrelation is observed. This pattern reflects the case in which, at small spatial distances, communities would be identical. That is, what we think
are different communities are really independent spatial replicates from the same community at a local site (Mackas, 1984); this case is represented in Figure 3.1D.

One method to describe biogeographic patterns quantitatively at all scales is through the use of spatial autocorrelation statistics (Franklin and Mills, 2007). Spatial autocorrelation is defined as the level of dissimilarity in a variable as the distance of separation between sample locations increases. Thus, all the patterns depicted in Figure 3.1 can be described using spatial autocorrelation statistics. For example, Figure 3.1C, shows a hypothetical dataset that is spatially correlated only up to a certain autocorrelation range (denoted by the vertical dashed line). After this range any two communities are equally likely to be as similar to or as different from other pair-wise comparisons because the limit of spatial dependence has been surpassed.

Here we use spatial autocorrelation statistics to describe the biogeographic distributions of bdelloid rotifers, a ubiquitously distributed group of microbial eukaryotes that is a prey item for larger organisms and is an important predator of smaller microbial species in aquatic and terrestrial systems (Arndt, 1993; Pejler, 1995; Kutikova, 2003). Fortunately, despite the debates on issues of cryptic speciation within this group, exact species identification is not necessary to describe the spatial patterning and community diversity based on phylogenetic relatedness (Green et al., 1996; Ramette and Tiedje, 2007; Schmidt et al., 2011). We describe a culture-independent sequencing strategy that resulted in the generation of many long-read sequences directly from environmental samples and allowed us to elucidate the spatial scale at which microbial eukaryote communities are structured.
Results

*Amplification and Utility of the cox1.*

We developed primers specific to the cytochrome oxidase subunit 1 (*cox1*) gene of bdelloid rotifers. PCR, BLAST (Zhang et al., 2000), and phylogenetic analyses confirmed that the *cox1* primers amplify bdelloid rotifer DNA with specificity and fidelity (Figure 3.2). Our local-to-global scale sampling resulted in 1,024 sequences comprising 790 unique sequences (GenBank accession numbers HQ174968–HQ175991).
Figure 3.2: Gel image of amplification products obtained with bdelloid-specific *cox1* primers. Adineta, Habroctrocha, and Macrotrachela are from morphologically identified bdelloids. The negative control and the outgroup Monogonont rotifer Brachionus plicatilis produced no bands.
Spatial and Community Analysis.

Semivariogram plots (Franklin and Mills, 2007) of the decay in community similarity with increasing geographic distance (Figures 3.3 and 3.4) were best fit (best r² values) by the exponential autocorrelation model for both the weighted and unweighted UniFrac (Lozupone and Knight, 2005) metrics (using the phylogeny from Dataset S2). There was strong linear relationship [Mantel tests (Mantel, 1967) (P < 0.002)] in the data across geographic distances up to autocorrelation range (see below). This result was supported by more traditional operational taxonomic unit (OTU)-based methods, which, despite overestimation of differences between communities, showed the same spatial patterns over similar distances (Figures 3.5 and 3.6).

Overall, our sampling of bdelloid communities from local to global scales demonstrates that local communities show strong spatial structuring, whereas more distant communities are very different from one another, even in similar environments, thus best matching the theoretical model depicted in Figure 3.1C. This local effect was evident at distances up to 54 m (autocorrelation range) when relative abundance of sequences was taken into account (weighted UniFrac; Figure 3.3) and at distances up to 133 m when occurrence (richness) data were analyzed (unweighted UniFrac; Figure 3.4). Beyond the autocorrelation range, all communities averaged community dissimilarity of about 0.8 and 0.9 (Figure 3.3 and 3.4).
Figure 3.3: Weighted UniFrac (Lozupone and Knight, 2005) variogram (bdelloid abundance) plotted as the UniFrac metric (Community Dissimilarity) versus the log of geographic distance. Values close to 1 indicate completely different communities, and values close to 0 indicate identical communities. The dashed line indicates the autocorrelation range.
Figure 3.4: Unweighted UniFrac (Lozupone and Knight, 2005) variogram (bdelloid occurrence) plotted as the UniFrac metric (Community Dissimilarity) versus the log of geographic distance. Values close to 1 indicate completely different communities, and values close to 0 indicate identical communities. The dashed line indicates the autocorrelation range.
Figure 3.5: Operational taxonomic unit (OTU)-based variogram using the Bray–Curtis metric (bdelloid abundance) as implemented in QIIME (Caporaso et al., 2010) plotted as community dissimilarity versus the log of geographic distance. A value of 1 indicates completely different communities, and a value of 0 indicates identical communities. The vertical dashed line indicates the autocorrelation range. Note the overestimation of community dissimilarity after the autocorrelation range.
Figure 3.6: OTU-based variogram using the Sorensen metric (bdelloid occurrence) as implemented in QIIME (Caporaso et al., 2010) and plotted as community dissimilarity versus the log of geographic distance. A value of 1 indicates completely different communities, and a value of 0 indicates identical communities. The vertical dashed line indicates the autocorrelation range. Note the overestimation of community dissimilarity after the autocorrelation range.
This high level of dissimilarity suggests that unique clades of bdelloids exist at each location beyond the autocorrelation range, a conclusion independently supported by the decreasing probability of sampling specific OTUs at increasing distances (Figure 3.7). We further tested this idea by using the Net Relatedness Index (NRI) (Webb, 2000; Webb et al., 2002); the large positive NRI values obtained for geographically distant communities (Table 3.1) indicate that communities are “phylogenetically constrained,” i.e., each community is composed of unique clades compared with all others.

We failed to observe a noticeable lag at small spatial scales (Figure 3.1D), indicating that we may not have sampled at small enough spatial distances to resample the same community. This result is informative, because our closest samples were 0.16 m apart. To observe such disparate bdelloid clones and communities at such close distances reveal that these limnoterrestrial bdelloid communities are heterogeneous at small scales (De Meester et al., 2002), being composed of similar but not identical bdelloid clones.

To verify that we sampled communities to a level sufficiently deep to characterize phylogenetic differences adequately, we performed both rarefaction analyses (Figure 3.8) and jackknife analyses (randomly resampling sequences without replacement) (Figure 3.9 and 3.10). These analyses demonstrated that we sampled a majority of the phylotypes (48–100%) at most of our sites (Figure 3.8) and that, even if we jackknifed all our sites using the minimum sampling intensity for any site, we still recovered the same community patterns (compare Figures 3.3 and 3.4 and Figures 3.5 and 3.6).
Figure 3.7: The probability curve of sampling any one specific OTU (at the 97% sequence similarity cutoff) as geographic distance increases between any pair of samples. Any pair-wise comparison of samples that shared one or more OTUs was cataloged as “one,” and any pair-wise comparison of samples that shared no OTUs was cataloged as “zero.” The probability of sampling the same OTU drops to zero for almost all pair-wise comparisons beyond a geographic distance of about 100 m.
Table 3.1: Mean Phylogenetic Diversity (MPD) and Net Relatedness Index (NRI) for every geographic location in the present study. Higher values of NRI (low MPD) indicate phylogenetically constrained communities, and low NRI (high MPD) indicates more even communities (Webb, 2000; Webb et al., 2002). Community designations and geospatial coordinates also are listed along with the number of sequences obtained for each geographic location.

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<th>Latitude</th>
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Total | 1024 |
Figure 3.8: Rarefaction curves for four representative sample sites including the site that was least fully sampled (dry meadow). Three OTU sequence similarity cutoffs of 97, 95, and 93% are plotted. The percent of the estimated total OTUs (at the 97% similarity level) sampled at each site (Chao, 1984) is included at the bottom right in each graph. All other sites had similar curves and showed that we sampled between 64 and 100% of possible OTUs at those sites. These results indicate that we did not limit sampling to only the dominant bdelloid clones of a given community.
Figure 3.9: Jackknife analyses of phylogenetic data using both unweighted (UW) and weighted (W) UniFrac as implemented in QIIME (Caporaso et al., 2010). Differing jackknife subsampling depths are shown; that is, 4, 10, or 20 sequences were subsampled (without replacement) from each geographic location. Each level of sub-sampling was performed for 1,000 iterations, and the means of each and all pair-wise comparisons are plotted. Local spatial autocorrelation was captured at all levels of jackknifing, confirming that at each geographic location we sampled at sufficient depth to characterize all communities.
Figure 3.10: Jackknife analysis of OTU-based data using both Bray–Curtis and Sorensen metrics as implemented in QIIME (Caporaso et al., 2010). Differing jackknife subsampling depths are shown; that is 4, 10, or 20 sequences were subsampled (without replacement) from each geographic location. Each level of subsampling was performed for 1,000 iterations, and the means of each and all pair-wise comparisons are plotted. Local spatial autocorrelation was captured at all levels of jackknifing, confirming that at each geographic location we sampled at sufficient depth to characterize all communities.
Discussion

Based on most previous studies of the biogeography of small eukaryotes (Green et al., 1996; Finlay, 2002; Whitaker et al., 2003; Fenchel and Finlay, 2004; Darling et al., 2004; Foissner, 2006; Martiny et al., 2006; Taylor et al., 2006), our expectation was that bdelloid rotifers either would show no spatial structure (Figure 3.1) or would show increasingly different communities as the distance between pair-wise samples became greater (Figure 3.1B). Instead, we found that these communities showed the pattern depicted in Fig. 3.1C: Local communities show a high degree of spatial autocorrelation, whereas geographically more distant communities show a high level of community dissimilarity. Our analyses also produced estimates of spatial autocorrelation ranges for microbial eukaryotes; quantitative (weighted UniFrac) and qualitative (unweighted UniFrac) metrics indicate that this range is around 54–133 m. This unexpectedly small autocorrelation range highlights the importance of sampling at scales that pick up local, regional, and global biogeographic patterns. It is apparent that if we had not sampled intensively at local scales (0.2–100 m), we would have concluded that all communities at all scales are equally dissimilar.

Even more surprising than the small spatial autocorrelation ranges for bdelloid communities was the lack of similarity between communities at geographic distances beyond the autocorrelation range (Figures 3.3 and 3.4). This pattern indicates not that “everything is everywhere” but just the opposite: There is little overlap in community composition at distances greater than about 54–133 m. This unexpected pattern is driven not just by changes in abundance (weighted UniFrac; Figure 3.3) of the dominant clades,
as has been documented for the geographic distribution of soil bacterial communities
(King et al., 2010), but also by the presence of many novel phylogenotypes at each site so that
there is almost no overlap in OTUs (defined at the 97% level of similarity; Figures 3.5
and 3.6) at sites further apart than the autocorrelation range. This finding directly
contradicts the idea that rotifers (and other less easily dispersed microbial eukaryotes) are
cosmopolitan and supports the idea that there are vastly more “cryptic species”
(Fontaneto et al., 2008; Kaya et al., 2009) of bdelloid rotifers than previously was
thought.

Of the models depicted in Figure 3.1, our data are most similar to Figure 3.1C, but,
unexpectedly, the level of sequence dissimilarity between communities (at distances
beyond the autocorrelation range) was much greater than predicted or observed in
previous studies (Martiny et al., 2006; King et al., 2010). Therefore in Figure 3.11 we
expand upon the model shown in Figure 3.1C to contrast the extreme cases of EisE and
the very different findings of the current study. EisE would result in very low community
dissimilarity values (circles in Figure 3.11). In contrast, the pattern we observed is that
every community is mostly unique (triangles in Figure 3.11) at distances beyond the auto-
correlation range. Our phylogenetic (Figures 3.3 and 3.4) and OTU- based (Figures 3.5
and 3.6) analyses strongly support a global view of microbial distribution in which
communities are dominated by endemic species and share very few common clades
between sites (circles in Figure 3.11). Local endemcity is supported further by the largely
positive NRI (Webb, 2000; Webb et al., 2002) values (Table 3.1), which show that local
communities tend to be comprised of closely related lineages relative to the phylogenetic
diversity of rotifers across the planet. This world-view directly contradicts the idea of
EisE and suggests that the diversity of microbial eukaryotes such as rotifers may be vast beyond our imagining, especially given that endemic microbes may have species ranges of about 100 m (54–133 m).
Figure 3.11: Theoretical expectations for “everything is everywhere” (circular points) and “everywhere is different” (triangular points). One would expect very low community dissimilarity values if most bdelloid communities are composed predominately of ubiquitous bdelloid species that are not impeded in their dispersal (circles). An equal but opposite extreme is that all communities are highly unique and are composed predominately of unique endemic bdelloid rotifers (triangles), revealing differences in dispersal and or habitat.
Bdelloids are known to produce small, resistant resting stages (Ricci, 2001; Gladyshev and Meselson, 2008) that should disperse easily by wind. The fact that our results show that bdelloids are not widely distributed implies that other microbial eukaryotes with less resistant stages (Jenkins and Underwood, 1998; Foissner, 2006) should have even more geographically restricted distributions; unfortunately, few phylogenetically based studies have been done at the range of spatial scales needed to see the patterns observed in the present study (Green et al., 1996; Martiny et al., 2006). Importantly, the ability to form resistant stages probably evolved not for dispersal but to survive periods of unfavorable environmental conditions (i.e., dry and cold conditions) (Ricci, 2001), thereby maintaining unique local communities (De Meester et al., 2002). Resistant survival structures also result in large banks of propagules (Ricci, 2001), which can undergo rearrangement of nuclear genes during rehydration (Mark Welch et al., 2008), allowing long-term monopolization (De Meester et al., 2002) by local cox1/phylotypes. Monopolization could be especially important in intermittently wet soils (such as those studied here), because indigenous propagules would be numerically dominant in the soil on the rare occasions when abundant water becomes available. Thus, our observation of strong geographic differentiation of bdelloid communities presumably reflects local numerical abundance of locally adapted clones that persist over relatively long periods of time.

In community spatial studies it is important to distinguish between conclusions based on relative abundance (diversity) data and those based on occurrence (richness) data. In our analyses, relative abundance data (weighted UniFrac) gave a stronger spatial signal (Figures 3.3 and 3.5) than our analyses based on richness (unweighted UniFrac;
Figures 3.4 and 3.6). This finding could indicate that large-scale patterns of microbial diversity are driven mostly by changes in relative abundance, so that the same organisms occur everywhere, but the dominant organisms are different in different sites. However, our data do not support this model because, even though relative abundance does have a strong effect, we still see spatial patterning when only species richness is taken into account (Figures 3.4 and 3.6). As discussed above, there are novel clades or OTUs at every site we sampled. In fact, the probability of encountering the same OTU (defined at the 97% similarity level) drops from near 100% to zero as geographic distance increases beyond the autocorrelation range (Figure 3.7), and this finding is not attributable to under sampling. Under-sampling would account for the observed patterns only if dominant phylotypes at one site were rare at other sites (and vice versa) and therefore were missed because of under sampling. However, rarefaction curves show that we sampled many of our sites to near saturation, encountering 48–100% of the estimated number of phylotypes (Figure 3.8) (Chao, 1984), making it highly unlikely that changes in abundance alone explain the spatial patterns we observed.

Finally, it is important to note that the phylogenetic approach used in the present study (UniFrac) actually underestimates spatial structuring of communities compared with traditional OTU-based metrics. When we analyzed all our data using traditional OTU-based metrics, the spatial patterns were exaggerated compared with UniFrac (which preserves actual phylogenetic relatedness in the analyses). This difference is easily seen by comparing Figures 3.3 and 3.4 with the OTU-derived views of spatial structure (Figures 3.5 and 3.6). Collapsing the data into OTUs gives the false impression that almost all communities are 100% different (dissimilarity values of 1) at distances beyond
the auto-correlation range (Figures 3.5 and 3.6). This false impression occurs because collapsing data into OTUs makes all OTUs equally dissimilar, obscuring deeper levels of genetic relatedness (Martin, 2002; Lozupone et al., 2007). Thus, our phylogenetic analyses reveal that, although bdelloid communities are very different at large spatial scales, they still show deeper levels of phylogenetic relatedness than one would predict from OTU-based metrics. Nonetheless both OTU and phylogenetic metrics support our conclusion that bdelloid rotifer communities are highly spatially autocorrelated at local scales and are very different at larger spatial scales.
Methods

DNA Extraction and PCR.

Soil samples were collected from the sites listed in Table S1 and represented seasonally dry, high-elevation ecosystems across the western United States and similar sites at greater geographic distances (e.g., the high Andes). Samples consisted of 100-g soil cores of the top 4 cm of soil, which subsequently were homogenized; 10-g subsamples were used for DNA extraction. Total cellular DNA was extracted from soil using the UltraClean Mega Soil DNA Isolation Kit (#12900; Mo Bio Laboratories, Inc.). Bdelloid rotifer DNA was amplified from these soil DNA extractions by using a two-step PCR protocol to amplify coxl.

We chose the coxl gene because it provides some analytical advantages with regard to bdelloid rotifers (Birky, 2007), not only in what one would expect to find regarding branch length and tree topology (Birky, 1996; Barraclough et al., 2003) but also because (i) bar-coding initiatives have been accruing coxl data (Ratnasingham and Hebert, 2007) to which our sequence data can be compared; (ii) robust primers (Folmer et al., 1994) make it possible to amplify the coxl gene from almost any invertebrate, and these sequences then can be used to design taxon-specific primers, as in the present study; and (iii) coxl is an effectively haploid gene which eliminates the “Meselson effect” that can make it difficult or impossible to recover the correct phylogenetic tree using nuclear gene sequences (Mark Welch, 2000; Birky, 2007) (also see Chapter 1(Robeson et al., 2009) for a discussion of problems with elucidating bdelloid diversity with the 18S rDNA gene).
The first PCR made use of primers from Folmer et al. (1994). The second PCR made use of bdelloid-specific primers developed for this study: Bdell_CO1_FW: 5’-CGT ACW GAG TTA GGA ATR GTA-3’, and Bdell_CO1_Rev: 5’-CCA AAA TTW CGA TCT AAY A-3’. Touch-down PCR was used for both reactions and was set up as follows: 94 °C for 5 min, followed by eight cycles of (−1 °C annealing per cycle) 94 °C for 30 s, 55 °C for 30 s, and 62 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, and 62 °C for 1 min. The amplified template from the first primers (Folmer et al., 1994) was diluted 20-fold and used as a template for the second PCR using our bdelloid-specific primers.

A 50-µL PCR contained the following: 10× PCR buffer, 0.5 units Taq polymerase, 1.5 mM MgCl₂ (catalog nos. M0267L and B9021S; New England Biolabs), 0.2 μMdNTPs (dNTP stock was made as follows from individual 100-mM stocks: 12 μL A, 12 μL T, 8 μL G, 8 μL C, and 360 μL water; catalog no. 10297–018; Invitrogen), 0.4 μM of each primer (1–2 μL template DNA). We want to emphasize that the thermocycling protocol listed above in combination with the dNTP mix ratios produced the best-quality sequence for this very AT-rich region (Su et al., 1996).

**DNA Purification, Cloning, and Sequencing.**

The final PCR product was purified using the UltraCleanGelSpin DNA Extraction Kit (#12400; Mo Bio Laboratories, Inc.). Purified PCR product then was cloned using the Invitrogen TOPO TA Kit (with pCR4-TOPO vector) with One Shot TOP10 chemically competent Escherichia coli (K4575-01). Pelleted cells were sent to Functional Biosciences, Inc. for sequencing. Sequence data were assembled, the vector sequence
was removed, and data were edited by hand using Sequencher 4.7 (Gene Codes Corporation). Data then were exported for use in various phylogenetic and community-based analysis programs.

**Phylogenetic and OTU-Based Analyses.**

MUSCLE (Edgar, 2004) was used to generate two cox1 alignments (with and without outgroup taxa), which then were edited by hand to ensure sequences were aligned by codon. These alignment data then were analyzed in RAxML v.7.2.6 (Stamatakis et al., 2005). The best-scoring likelihood tree, from 100 full-alignment inferences using the GTR + I + Γ model as chosen by MultiPhyl Online (Keane et al., 2007) and partitioned by codon position, was retained. Once placement of sequence data was confirmed with outgroup taxa using Dendroscope (Huson et al., 2007), the phylogeny containing only bdelloids was submitted as a midpoint-rooted tree to several phylogenetic community-comparison programs such as UniFrac (Lozupone and Knight, 2005), Phylocom (Webb, 2000; Webb et al., 2002), and mothur (Schloss et al., 2009). UniFrac and OTU-based metrics like Sorensen and Bray-Curtis were implemented via QIIME (Caporaso et al., 2010) to determine the relatedness of bdelloid communities. For OTU-based metrics, a 97% sequence similarity cutoff was implemented, based on the results of Birky et al. (2010). Phylocom was used to generate Mean Phylogenetic Diversity (MPD) and NRI values. Mothur (Schloss et al., 2009) was used to generate a distance matrix for Mantel tests (Mantel, 1967) along with rarefaction and Chao1 (Chao et al., 2005) analysis.
Spatial Analyses.

To determine which model of biogeographical distribution applies to bdelloid rotifers in soil, we determined rotifer sequence diversity at scales ranging from 0.16 m to 9,100 km. Each 10-g soil sample was assumed a priori to represent a community. UniFrac (Lozupone and Knight, 2005) was used to generate community distance matrices taking into account sequence abundance (weighted; quantitative diversity measure taking into account relative abundance) or only occurrence (unweighted; qualitative diversity measure). Data were plotted against the log of geographic distance (Figures 3.3 and 3.4). Spatial autocorrelation models were fit in Kaliedograph (Synergy Software) using the Levenberg–Marquardt algorithm (Levenberg, 1944; 1944; Marquardt, 1963; 1963), and the best-fit model (out of nugget, spherical, Gaussian, and exponential) (Bailey and Gatrell, 1995) was chosen based on the highest r² value. Significance of spatial autocorrelation was tested using the Mantel test (Mantel, 1967) to the maximum distance of spatial autocorrelation as given by the spatial model. General patterns of spatial autocorrelation were confirmed and validated via Jackknife analyses at varying sampling depths for all beta-diversity metrics (Lozupone et al., 2010).
ACKNOWLEDGMENTS

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Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for


Chapter 4

BROAD PATTERNS OF DIVERSITY ARE SHARED BETWEEN SOIL ROTIFER AND BACTERIAL COMMUNITIES
Summary

Bdelloid rotifers and other mesofauna are integral to biogeochemical cycling and trophic dynamics of soil systems. Despite the potential for rotifers to exert top-down control on bacterially mediate biogeochemical cycling, there is no previous work on the relationship between rotifers and bacteria in soils. We sampled bacterial and bdelloid communities within three distinct ecosystems (conifer forest, dry meadow tundra, and talus) within the Colorado Rockies Niwot Ridge LTER study area. We sequenced amplicon libraries of bacterial 16S rRNA gene and compared the richness and evenness of the resulting operational taxonomic units to clone library data of bdelloid communities and to biogeochemical data. Procrustes analysis demonstrated that bdelloid and bacterial communities had similar patterns of community dissimilarity. Highest levels of richness and diversity for both bacterial and bdelloid rotifer communities occur within the dry meadow tundra. Environmental variables explained more of the observed variation for bdelloid rotifer communities than it did for bacterial communities. Our results indicate that mesofaunalike bdelloidssshare patterns of diversity with their co-occurring bacterial communities.
Introduction

Connecting the macro-scale of visible plants and animals, and the micro-scale of bacteria and eukaryotic microbes, are a world of meso-organisms responsible for cycling large amounts of organic matter. These soil animals link the micro- and macro-ecology of ecosystems, feeding on bacteria and other single celled microbes, and are preyed upon by larger organisms (Arndt, 1993; Kutikova, 2003). Mesofaunal invertebrates are also important to the maintenance of soil structure, decomposition, nutrient cycling and plant community dynamics (Wall, 2004; Lavelle et al., 2006). A group that has been gaining recognition as a significant player in the mesofaunal community are bdelloid rotifers (Arndt, 1993; Bledzki and Ellison, 1998; Kutikova, 2003; Lapinski and Tunnaccliffe, 2003), one of the most abundant non-nematode mesozoans in soil communities (Sohlenius, 1979; Anderson et al., 1984; Petz, 1997; Devetter, 2010). Despite the abundance of bdelloids within soil ($\sim 2.1 \times 10^6$ individuals/m$^2$ (Schmid-Araya, 1998; Devetter, 2007)), the extent to which biotic and abiotic factors translate directly to soil bdelloid community diversity is largely incomplete (Devetter, 2009b).

A primary reason why such analyses are rarely performed on bdelloids is that live specimens are generally required for identification (Fontaneto and Melone, 2003b) and they are composed of notoriously cryptic species (Fontaneto et al., 2009). These issues confound the already existing problems associated with isolation and enumeration of bdelloids (Devetter, 2010). As a result, the assessment of informative associations between bdelloid community diversity and their environment, including co-occurring bacterial communities, are very elusive and often intractable to study. However, recent advances in targeted amplification and sequencing of DNA from bdelloid rotifer communities directly
from soil has made the analysis of bdelloid communities increasingly tenable (Robeson et al., 2009; 2011).

There has been increasing interest in recent years dedicated to the study of high-elevation ecosystems due to their sensitivity to human induced climate change. These climate effects can alter the microbial and biogeochemical cycles of these systems, such as the Niwot Ridge Long Term Ecological Research station (Colorado, USA) (Nemergut et al., 2005). Much of these effects have been underestimated since the discovery of active microbial communities in ‘barren’ soils and under deep snowpack (Lipson et al., 2002; Schadt et al., 2003; Costello and Schmidt, 2006; Freeman et al., 2009). However, recent research into the diversity of mesofauna, such as bdelloid rotifers (Robeson et al., 2011) and eutardigrades (Robeson et al., 2009) suggests more complex microbial food-web interactions may be occurring within the Niwot Ridge LTER than previously thought.

The Niwot Ridge LTER is a complex system in which the topography, wind exposure, and snow-pack duration are largely responsible for driving differences in soil development, biogeochemistry, nutrient transport, and plant community structure throughout its range (Seastedt et al., 2004). These processes can result in substantially different habitats over a short geographic distances, that allow for comparative studies of co-occurring mesofaunal and bacterial community diversity. Here we describe how patterns of mesofaunal and bacterial diversity shift among three distinct ecosystems located within the Niwot Ridge LTER: a sub-alpine forest floor (~<3400 m.a.s.l.), an alpinedry meadow (~3550 m.a.s.l.), and high-elevation talus sediment (~3750 m.a.s.l.) (Nemergut et al. 2005).
Results

The bacterial data generated in this study (243,399 sequences) were merged to those of (King et al., 2010) (16,894 sequences) for a total of 260,293 sequences. These data collapsed to 4,194 OTUs at the 94% sequence similarity level (Family-to-Genus). Newly generated *cox1* sequences were merged with existing sequence data from the dry meadow tundra and sub-alpine forest from Robeson et al.(2011) (Chapter 3) for a total of 1,354 sequences that collapsed to 289 OTUs at the 97% sequence similarity level (Species).

Across the alpine landscape of Niwot Ridge, bdelloid rotifer and bacterial communities showed very similar spatial patterns and non-random association with one-another. PCoA plots showed clear separation by ecosystem type for both rotifers and bacteria even at low levels of sub-sampling via jackknife analysis (Figures 4.1 & 4.2). In addition, Procrustes analyses show strong associations between the bacterial and rotifer communities by ecosystem type (Figure 4.3). Other non-phylogenetic based beta-diversity metrics (Bray-Curtis and Sorensen-Dice) also revealed significant non-random association of bdelloid rotifer communities to their respective bacterial communities (Figure 4.3 & 4.4).

Pairwise T-tests (Table 4.1) of sub-samples between the three sites revealed that the Dry Meadow and Spruce Fir forest were significantly different in pH and Dissolved C:N ratios. The pH of the Talus and Spruce Fir forest were not significantly different from one another. Other than sharing similar pH with the Spruce Fir Forest, the Talus was significantly different from both the Dry Meadow and Spruce Fir forest for almost all biogeochemical parameters (Table 4.1 & 4.2).
Figure 4.1: PCoA plots of bacterial and bdelloid rotifer communities via weighted-normalized and unweighted UniFrac; Spruce-Fir Forest (blue), Dry Meadow (red), Talus (orange). The sizes of the ellipsoids represent the standard deviation about the mean for each sample. All other richness and quantitative beta diversity measures show the similar partitioning by site. Bacterial plots were constructed by jackknife sampling 41 sequences from the data set 1000 times, at 94% similarity. Bdelloid plots constructed by jackknife sampling 15 sequences from the data set 1000 times, at 97% similarity.
Figure 4.2: PCoA plots of bacterial and bdelloid rotifer communities via Bray-Curtis and Sorensen-Dice; Spruce-Fir Forest (blue), Dry Meadow (red), Talus (orange). The sizes of the ellipsoids represent the standard deviation about the mean for each sample. Bacterial plots were constructed by jackknife sampling 41 sequences from the data set 1000 times, at 94% similarity. Bdelloid plots constructed by jackknife sampling 15 sequences from the data set 1000 times, at 97% similarity.
Figure 4.3: Procrustes analysis with 1000 randomized iterations of bdelloid and bacterial communities for both normalized weighted UniFrac (left; $P < 0.001$, Count better $= 0$, $M^2 = 0.248$) and unweighted UniFrac (right; $P < 0.001$, Count better $= 0$, $M^2 = 0.128$) and Forest (blue), DryMeadow (red), Talus (orange). Both analyses reveal significant association of bdelloid communities to their bacterial community counterparts by site. Lines connect bdelloid and bacteria data from the same sample. Spheres connected to the black-end of the lines are from bdelloid communities, the sphere connecting to the red end of the lines are from bacterial communities.
**Figure 4.4:** Procrustes analysis with 1000 randomized iterations of bdelloid and bacterial communities for both Bray-Curtis (left; $p < 0.001$, Count better = 0, $M^2 = 0.102$) and Sorensen-Dice (right; $P < 0.001$, Count better = 0, $M^2 = 0.083$). Forest (blue), DryMeadow (red), Talus (orange). Both analyses reveal significant association of bdelloid communities to their bacterial community counterparts by site. Lines connect bdelloid and bacteria data from the same sample. Spheres connected to the black-end of the lines are from bdelloid communities, the sphere connecting to the red end of the lines are from bacterial communities.
Table 4.1: Pairwise t-test results of biogeochemical values by site, Dry Meadow (DM), Spruce Fir forest (SF), Talus (TAL). Significant differences (P < 0.05) are noted with asterisks. Measured variables are: Total dissolved N (TDN), Microbial N (MN), Dissolved Organic C (DOC), Microbial C (MC), mass of dried soil (Dry Mass), Soil water present at time of sampling (Soil Water), Microbial C:N ratio (MBC_N), Dissolved Organic C:N ratio (DOC_N), and pH.

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Table 4.2: See following page for description.
Table 4.2: Geospatial Coordinates and measured biogeochemical factors from the 25 samples used in this study. Measured variables are: Total dissolved N (TDN), Microbial N (MN), Dissolved Organic C (DOC), Microbial C (MC), mass of dried soil (DryMass), Soil water present at time of sampling (SoilWater), Microbial C:N ratio (MBC_N), Dissolved Organic C:N ratio (DOC_N), and pH.

In order to parse out the contribution of environmental and spatial variables between ecosystem types, we performed Mantel tests and generalized linear models (Dixon, 2009; R Development Core Team, 2011) that control for these environmental influences. All Mantel $P$-values for bdelloids were $\leq 0.001$ indicating significant contribution of geospatial distance regardless of the contribution of all environmental parameters (Table 4.2). Conversely, bacterial communities were not significantly structured by geo-spatial distance when all environmental variables were taken into account ($P$-values 0.221 and 0.097 for unweighted and weighted UniFrac respectively). Thus, the significant explanatory power of geospatial distance for bdelloid community beta-diversity is due either to dispersal effects or unmeasured, spatially distributed, biogeochemical factors affecting bdelloid but not bacterial communities.

Environment was a strong predictor of bdelloid beta-diversity between ecosystem types but less so for bacterial beta-diversity variance (Figure 4.5, Table 4.3). The explanatory power of environment on both bdelloid and bacterial beta-diversity increased when relative abundance was considered (Figure 4.5, Table 4.3).
A) Unweighted UniFrac beta-diversity of bdelloid communities as explained by all significant variables.

Call:
\( \text{glm(formula = rot\_dist\_u \sim bact\_dist\_u + GeoDist + TDN + DOC + MC + MBC\_N + pH)} \)

Deviance Residuals:

<table>
<thead>
<tr>
<th></th>
<th>Min</th>
<th>1Q</th>
<th>Median</th>
<th>3Q</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.34405</td>
<td>-0.03324</td>
<td>0.01433</td>
<td>0.04878</td>
<td>0.19819</td>
</tr>
</tbody>
</table>

Coefficients:

|                  | Estimate | Std. Error | t value | Pr(>|t|) |
|------------------|----------|------------|---------|---------|
| (Intercept)      | 4.21E-01 | 7.72E-02   | 5.452   | 1.06E-07 *** |
| bact\_dist\_u    | 2.68E-01 | 1.00E-01   | 2.68    | 0.00779 ** |
| GeoDist          | 1.30E-05 | 1.59E-06   | 8.146   | 1.11E-14 *** |
| TDN              | 2.33E-03 | 4.32E-04   | 5.4     | 1.38E-07 *** |
| DOC              | -2.17E-04| 4.38E-05   | -4.949  | 1.26E-06 *** |
| MC               | 2.22E-05 | 9.73E-06   | 2.283   | 0.02312 *   |
| MBC\_N           | 4.33E-04 | 2.21E-04   | 1.956   | 0.05138 .   |
| pH               | 7.03E-02 | 9.26E-03   | 7.597   | 4.14E-13 *** |

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 1

(Dispersion parameter for gaussian family taken to be 0.007182627)

Null deviance: 3.7987 on 299 degrees of freedom
Residual deviance: 2.0973 on 292 degrees of freedom
AIC: -619.57

Number of Fisher Scoring iterations: 2

Table 4.3 (A): Raw output from Generalized Linear Models (GLM). Each table displays only those variables that significantly contribute to the β-diversity structure of bdelloid and bacterial communities. Variables input into the GLM are: Total dissolved N (TDN), Microbial N (MN), Dissolved Organic C (DOC), Microbial C (MC), mass of dried soil (DryMass), Soil water present at time of sampling (SoilWater), Microbial C:N ratio (MBC\_N), Dissolved Organic C:N ratio (DOC\_N), pH, and one of the following: weighted / unweighted UniFrac distances for bdelloid and bacterial communities. Example: Table A reveals how the β-diversity of, bdelloid communities are structured by contributing significant variables, the co-occurring bacterial communities (unweighted UniFrac) and environment. Table continues on following pages.
B) Unweighted UniFrac beta-diversity of bacterial communities as explained by all significant variables.

Call:
```
glm(formula = bact_dist_u ~ rot_dist_u + DOC + MC + DOC_N + pH)
```

Deviance Residuals:
```
                      Min           1Q          Median           3Q          Max
-0.156166       -0.034456       0.004088       0.038408       0.104211
```

Coefficients:
```
                     Estimate     Std. Error   t value  Pr(>|t|)  
(Intercept)          7.07E-01     2.03E-02    34.793    < 2.00E-16 ***
rot_dist_u           7.29E-02     2.92E-02    2.497     0.01308   *
DOC                  5.81E-05     1.92E-05    3.031     0.00265   **
MC                   -1.85E-05    5.24E-06    -3.52     0.0005    ***
DOC_N                4.60E-03     1.46E-03    3.148     0.00182   **
pH                   1.55E-02     5.61E-03    2.757     0.0062    **
```

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.002333944)

Null deviance: 0.81663  on 299 degrees of freedom
Residual deviance: 0.68618  on 294 degrees of freedom
AIC: -958.76

Number of Fisher Scoring iterations: 2

Table 4.3 continued (B): This table reveals how the β-diversity of bacterial communities are structured by contributing significant variables, the co-occurring bdelloid communities (unweighted UniFrac) and environment.
Table 4.3 continued (C): This table reveals how the β-diversity of bdelloid communities is structured by contributing significant variables, the co-occurring bacterial communities (normalized weighted UniFrac) and environment.
D) Normalized Weighted UniFrac beta-diversity of bacterial communities as explained by all significant variables.

Call:

glm(formula = bact_dist_w ~ rot_dist_w + TDN + DOC + MC + MBC_N + DOC_N)

Deviance Residuals:

  Min  1Q Median  3Q Max
-0.094641 -0.02747 0.004153 0.027034 0.097333

Coefficients:

                     Estimate Std. Error   t value Pr(>|t|)
(Intercept)    2.69E-01  8.35E-03 32.22400  < 2.00E-16 ***
rot_dist_w     1.53E-01  2.11E-02  7.21910  4.53E-12 ***
TDN           -3.64E-04  2.00E-04 -1.82111  0.069697 .
DOC           7.36E-05  2.07E-05  3.55828  0.000436 ***
MC            -1.62E-05  4.04E-06 -4.00909  7.74E-05 ***
MBC_N         -3.61E-04  1.11E-04 -3.24809  0.001298 **
DOC_N         2.38E-03  1.25E-03  1.90234  0.058192 .

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for gaussian family taken to be 0.001295957)

Null deviance: 0.51916  on 299 degrees of freedom
Residual deviance: 0.37972  on 293 degrees of freedom
AIC: -1134.3

Number of Fisher Scoring iterations: 2

Table 4.3 continued (D): This table reveals how the β-diversity of bacterial communities are structured by contributing significant variables, the co-occurring bdelloid communities (normalized weighted UniFrac) and environment.
In addition to the beta-diversity metrics discussed above, bdelloid and bacterial communities have similar patterns of alpha-diversity across this alpine landscape. The dry meadow tundra contained the greatest amounts of phylodiversity and OTU richness of bdelloid rotifers and bacterial communities, followed by the Spruce Fir Forest site, and the least amount of diversity found at the Talus (Table 4.4). Phylodiversity (Faith, 1992) levels are significantly different across sites for both bacteria and bdelloids (all P-vals < 0.001). Representative upper level taxonomic designations for each sample are shown in Figures 4.6 & 4.7.
Figure 4.5: Variance partitioning of factors contributing to patterns of beta diversity for bdelloids and bacteria based on richness (unweighted UniFrac) and abundance (normalized weighted UniFrac) of taxa. Synergistic Effects are the combined influences of all other variables shown. Space denotes the variance explained by geographic distance. Environment indicates the variance explained by the measured environmental variables in Table 1. Community indicates the amount of variance explained by the other community being compared (e.g. how much variance does bdelloid $\beta$-diversity explain the $\beta$-diversity structure of co-occurring bacteria). Bdelloids are more affected by space and environment than are bacteria. However, the influence of space and environment increases for both bdelloids and bacteria when abundance is taken into account.
<table>
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<tr>
<th>Sample</th>
<th>MPD unweighted</th>
<th>MPD weighted</th>
<th>Faith's PD - Jackknifed</th>
<th>Mean PD</th>
<th>MPD unweighted</th>
<th>MPD weighted</th>
<th>Faith's PD - Jackknifed</th>
<th>Mean PD</th>
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<td>0.016</td>
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<td></td>
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<td>0.002</td>
<td>6.670</td>
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<td>0.087</td>
<td>0.068</td>
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<td>7.082</td>
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<td>2.534</td>
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</table>

Table 4.4: See following page for description.
Table 4.4: P-values for richness (unweighted) and abundance-based (weighted) measures of Mean Phylogenetic Diversity (MPD; (Webb et al., 2006)) and overall Phylodiversity (Faith, 1992) for bacteria and bdelloids. Jackknifed PD values are means of 1000 jackknifes from re-sampling 41 and 15 sequences for both bacteria and bdelloids respectively.
Figure 4.6: Taxonomy summary of bacterial OTUs to known bacterial sequences as defined by reference based taxonomy via QIIME(Caporaso et al., 2010)(Caporaso et al., 2010). Columns 1-9 are forest samples, columns 10-17 are Dry Meadow samples, and columns 18-25 are talus. Note the substantial increase in unique OTUs in the talus compared to the Dry Meadow and Forest. This bar graph represents broad level bacterial taxonomy.
Figure 4.7: Taxonomy summary of bdelloid OTUs to known bdelloid sequences as defined by BLAST (90% confidence and e-value of $10^{-10}$) using reference sequences and taxonomy from GenBank (Benson et al., 2005) and processed via QIIME (Caporaso et al., 2010). Columns 1-9 are forest samples, columns 10-17 are Dry Meadow samples, and columns 18-25 are Talus.
Discussion

Recent studies investigating the effects of environment on the biodiversity of bacterial communities are often compared to macroflora and fauna (Bryant et al., 2008; Fierer et al., 2011). However, microscopic animals like bdelloid rotifers are left out of these comparative analysis even though they are thought to be highly integral to microbial food web interactions (Arndt, 1993; Pejler, 1995; Kutikova, 2003). We hypothesized that if microscopic animals are fundamentally linked to the microbial food web, then they should show similar patterns of diversity with co-occurring bacterial communities across three different ecosystems within the Niwot Ridge LTER (West et al., 1999): sub-alpine coniferous forest, dry meadow tundra, and talus. We chose bdelloid rotifers as our representative mesofaunal group due to their ubiquitous presence along the Niwot Ridge landscape (Robeson et al., 2009; 2011).

The development of bdelloid-specific primers (Robeson et al., 2009; 2011) has made it possible to directly investigate whether or not patterns of diversity are commensurate between bdelloid and co-occurring bacterial communities. Our environmental sequencing survey revealed that bdelloid communities have a significant non-random association with their respective bacterial communities (Figure 4.3 & 4.4). That is, the patterns of phylodiversity and community phylogenetic divergence between the forest, tundra and talus ecosystems are similar for both bdelloids and bacteria. This correspondence in bdelloid and bacterial distributions suggests that the known differences in biotic and abiotic factors across these three distinct ecosystems (Seastedt et al., 2004; Nemergut et al., 2005) are driving the assembly of bacterial and mesofaunal communities through both predator-prey interactions and shared environmental tolerances; as
suggested by the co-clustering of bacterial and bdelloid communities by ecosystem within the PCoA space (Figure 4.3 & 4.4).

A variety of geospatial and soil factors (Table 4.2) where analyzed in order to determine their contribution to the patterns observed in Figures 4.3 & 4.4. Geographic distance between sites is often a contributing factor to observable patterns and differences between communities, but understanding its contribution is often confounded by other spatially autocorrelated environmental factors (Bell, 2010). We performed Mantel tests and combined linear models (Dixon, 2009; R Development Core Team, 2011) in order control for potential co-correlating environmental influences between ecosystems. All Mantel $P$-values for bdelloid communities were $\leq 0.001$, indicating significant contribution of geospatial distance between bdelloid rotifer communities, as initially discovered in my previous work (Robeson et al., 2011). Conversely, bacterial communities were not significantly structured by geospatial distance between ecosystems, when all environmental variables were taken into account ($P$-values for unweighted and weighted UniFrac are 0.221 and 0.097 respectively). This strongly shows that substantial differences between mesofauna and microbes exist due to differences in dispersal capacity (Wilkinson et al., 2011) or responses to unmeasured but spatially autocorrelated environmental factors between ecosystems (King et al., 2010).

Although geospatial distance is a significant contributing factor to the structuring of bdelloid rotifer communities between the ecosystems studied here, its affect is substantially less than locally measured environmental factors according to variance partitioning (Figure 4.5). This result indicates that rotifers may be occupying habitats more selectively (Fontaneto and Ricci, 2006) than bacterial communities at the spatial
scale measured here. For example, our results suggest that the diversity of many bdelloids may be strongly dependent on survival and dispersal associated with desiccation events (Ricci et al., 2007; Wilson and Sherman, 2010) (i.e. variation in the persistence of a water-film). The persistence of water within soil does indeed vary between these three distinct ecosystems, as evidenced by differences in precipitation and snow-pack duration (Seastedt et al., 2004; Nemergut et al., 2005). For example, bdelloid sequences that are most closely related to the genus *Rotaria* were not observed to any major degree within our analysis (Figure 4.7). The limited detection of *Rotaria* fits with their known biology. *Rotaria* typically live in less ephemeral water film environments, compared to the three study sites discussed here, and do not survive desiccation events as well as other bdelloid groups (Ricci, 1998; Mark Welch et al., 2009), resulting in exclusion from these sites.

Previous studies of bdelloid ecology have found differing levels of ecological tolerance depending on the environment studied. Fontaneto et al. (2011b) found weak habitat selection (wide ecological tolerance) in bdelloids when diverse lichen environments where compared. However, in other environments, bdelloids were shown to be highly selective (low ecological tolerance) between differing environments and correlated with factors such as the size of soil-pore space (Ricci et al., 1987; Fontaneto and Melone, 2003b; Ejsmont-Karabin, 2004; Fontaneto et al., 2006; 2007a). The latter case agrees with what we find here across these three distinct alpine ecosystems. That is, bdelloids are most strongly influenced by effects of local environment and co-occurring bacterial communities (Figure 4.5). One noteworthy observation is that fungi of the genus *Lecophagus* (McInnes, 2003), a known predator of rotifers, have been detected in the talus soils of the Niwot Ridge LTER (Schmidt et al., 2012). This indicates that
bdelloid community structure is also influenced by biotic interactions with organisms not included in this study.

Although co-occurring bacteria significantly explain a portion of bdelloid β-diversity and vice versa, much of the structure of bacterial β-diversity remains largely unexplained (Figure 4.5). Of particular note are the substantial differences on the effects of measured soil factors (i.e. local environment) on explaining the β-diversity structure of bacterial communities. However, one caveat should be noted, the taxonomic breadth of our bacterial analysis was much larger than that of bdelloids. Thus, determining the environmental parameters which structure the community composition of an entire domain of life will be much more difficult to elucidate than those which affect only a class (Bdelloidea), since different clades within the bacterial domain may be affected differently by the same environmental parameter. Further analysis is required to reveal such fine scale associations.

However, the edaphic factors measured in this study may not be the primary factors (or combination of factors) that contribute to the co-clustering patterns of bacteria and bdelloid communities in Figure 4.3. This lack of equivalent explanatory power of the measured environmental factors on both bacterial and bdelloid communities (Figure 4.5) highlight that the mechanisms shaping bacterial and mesofaunal community assembly are still poorly understood and difficult to quantify, at least for the sampling scheme used in this study. This difficulty results from our current inability to measure all known environmental and biotic factors at relevant microscopic spatial scales and interactions that can influence community composition and diversity. However, past research and
knowledge of the Niwot Ridge LTER ecosystems chosen for this study may provide insight into the current study as well as future avenues of study.

Each of these ecosystems is broadly defined by elevation, snowpack duration, and intensity of solar radiation (see Methods section for detailed site descriptions). The substantial differences in local climate contributes to the biodiversity of these ecosystems, with most obvious effects on plant diversity and plant coverage (Seastedt et al., 2004; 2004; McCain and Grynes, 2010). The complex seasonal dynamics between plants and microbes have only recently been explored within Niwot Ridge (Nemergut et al., 2005). For example, it was recently found that some bacterial clades are indeed spatially autocorrelated with plant abundance (King et al., 2010), indicating tightly linked plant-microbe associations (Lipson et al., 2002; Nemergut et al., 2005). Similarly, other studies have also shown highly linked associations of soil invertebrates to the root systems of plants (Ricci, 1987; Wolters, 2000; Ayres et al., 2009; Pereira et al., 2012), including other locations within Colorado Rockies (Ayres et al., 2009). These patterns suggest that much of the unexplained variance observed in Figure 4.5 may be attributable to plant-microbe and/or plant-mesofaunal interactions. Although such plant diversity and distribution data was not collected as part of my study, some general inferences can be made based on what is historically known about the three ecosystems in question, and how each may partially explain the diversity patterns observed.

The overall low plant establishment and growth of plants in the talus system may explain the low diversity of bacterial and bdelloid communities, compared to the alpine tundra and sub-alpine forest. For example, plant growth within the talus is severely limited due to high winds and deep snow pack that result in a short growing season
(Caine, 1995). The continual cryogenic disturbance of the sand and gravel dominated talus contributes to the lack cohesive soil structure observed in typical vegetated soils and may limit heterotrophic microbial diversity across the talus landscape (Ley and Schmidt, 2002). Additionally, this lack of structure leads to reduced biomass (~2 % total organic carbon) within the talus that can limit the size of microbial communities (King et al., 2008), preventing the build up of multiple niches that allow for more diverse microbial communities [as reviewed in Ley & Schmidt (2002)]. Finally, carbon input into the system is limited to eolian deposition and CO$_2$ fixation by autotrophic bacteria (Freeman et al., 2009).

Unlike the talus soils, plants that produce dense and shallow root systems dominate the dry meadow tundra soils. This enables more efficient nutrient uptake as the root system stays within the upper warm microenvironment of the soil. The slowly decaying humus in this zone allows plants to live in a consistent and highly organic rich environment due to the strong cation binding capacity of organic molecules within the dry meadow tundra system (Monson et al., 2001). Thus, a more stable cohesive soil structure rich in organic compounds, leads to consistent biogeochemistry and plant diversity that maintains a stable and diverse microbial community. This can allow for a variety of consistent and available niche-space that enables the maintenance of complex and diverse trophic interactions (Pejler, 1995; Schmid-Araya and Schmid, 2000; Ley and Schmidt, 2002). The above plant-microbe interactions and dynamics taking place within the tundra system (Nemer gut et al., 2005) may be a contributing factor explaining why I have observed the highest levels of bacterial and bdelloid communities compared to the talus and forest (discussed below) soils.
The forest study system in question is largely homogeneous, consisting of mostly Engleman spruce (*Picea engelmannii*). The pH of the forest system is significantly lower that that of the tundra (Tables 4.1 & 4.2). Both the lack of plant diversity (Grüter et al., 2006) as well as the low pH (Fierer and Jackson, 2006) is typically associated with lower bacterial diversity. The reduced diversity of available prey items for mesofauna like bdelloid rotifers can affect the diversity of rotifers themselves (Pejler, 1995; Schmid-Araya and Schmid, 2000) as many are known to be specific in their prey-item preferences (Pourriot, 1979; Kutikova, 2003). Finally, the reduction in bacterial diversity can also be exacerbated by competition with fungi which typically fare better in aerobic low-pH environments that are rich in tree litter derived polymers compared to bacteria (Bååth and Anderson, 2003; Högberg et al., 2007). The combination of lower pH compared to the tundra, yet higher plant abundance and more stable soil structure in relation to the talus may explain why the diversity of bacterial and bdelloid communities of the forest system lie in between that of the tundra (highest diversity) and the talus (lowest diversity). My data support similar findings from a prior study by Neufeld (2005), in which the bacterial diversity of a boreal forest was substantially less that that of arctic tundra. Whether this diversity patterns is a general trend between coniferous forest systems and tundra remains to be explored.

The above highlights that plant diversity may be a contributing factor for the diversity of soil bdelloids in these environments, along with their co-occurring bacterial communities. Much remains to be explored regarding the interplay among environment, space, and trophic level interactions between microscopic animals and their co-occurring bacterial communities. In this study, we have used a multivariate approach to analyze the
relative contributions of factors that shape the community structure and diversity of both bdelloids and bacteria located between three different ecosystems of the Niwot Ridge LTER (West et al., 1999). Although both bdelloid and bacterial communities showed similar patterns of diversity and community divergence by ecosystem, the environmental factors contributing to these patterns are still inconclusive and merit further study. I propose that plant diversity, though not directly accounted for in this study, may have large influence on the composition of both bacterial and mesofaunal community diversity. Any future studies attempting to elucidate the drivers of community assembly and diversity should include plant diversity as a component.

Conclusion

Little has been done to compare mesofaunal to bacterial communities between differing ecosystem types. Comparisons of the diversity and distribution of mesofaunal communities to bacterial communities have been anecdotal, and have not made direct comparisons among samples, as we have done here. We show that broad patterns of diversity across discrete ecosystems are similar for bdelloid rotifer and bacterial communities. Modern high-throughput sequencing and informatics approaches are just now making it possible to perform fine-grained inter-taxonomic comparisons via co-occurrence analyses (Barberan et al., 2011). I propose that future analyses incorporate a sampling regime in which samples are taken at finer altitudinal, spatial, and temporal sampling scales (Gonzalez et al., 2011) that also take into account plant diversity. This will aid in the direct observations of community “turn-over” through space and time over elevational and environmental gradients. Analyses comparing microbial communities to
meso- and macro-organism communities remain in their infancy, indicating that more inclusive concepts and theories are needed to explain patterns not only between macro-, meso- and micro-organisms (Fierer et al., 2011), but between various study systems as well.
Methods

Sampling sites:

We sampled three ecosystems within the Niwot Ridge LTER site: a Spruce Fir Forest, Dry Meadow, and Talus. Sample geospatial locations are listed in Table 4.2.

Sub-Alpine Forest: Located at 3,030 meters above sea level, the forest is dominated by several species of conifer: lodgepole pine (*Pinuscontorta*), subalpine fir (*Abieslasiocarpa*), and Engelmann spruce (*Piceaengelmannii*) and encounters minimal wind velocities within the canopy (Turnipseed et al., 2003). Annual precipitation averages at about 800 mm (65% as snow) with a mean annual temperature of 1.5° C (Monson et al., 2005). For the current study, we chose a sparsely vegetated understory; the soil was covered with mostly pine needle-litter with few saplings.

Alpine Tundra Dry Meadow: Located at 3,577 meters above sea level, this alpine tundra meadow, is located within the Green Lakes Valley (GLV) watershed Colorado (USA) along an exposed windblown gradient (Fisk et al., 1998). The Dry Meadow encounters highly ephemeral snow-pack resulting in a very limited and dry growing season (Taylor and Seastedt, 1994; Walker et al., 1994; Fisk and Schmidt, 1995). This results in a plant community dominated by the tussock forming sedge *Kobresiamyosuroides* as well as *Selaginelladensa*, and *Acomastylisrossii* (Fisk et al., 1998).

Talus: Located at 3,734 - 3,887 meters above sea level, the mountain talus slopes have extreme climatic conditions: with mean annual temperatures below zero, intense solar
radiation, deep snow pack, and strong prevailing winds. These environmental components severely limit the presence of vegetation (Caine, 1995). The talus is predominantly composed of boulders, in between which sand and clay can accumulate (Williams et al., 1997). It is thought that both aeolian deposition of organic carbon, and in situ CO₂ fixation by soil cyanobacteria and algae (Ley et al., 2004), contributes carbon and energy to the talus ecosystem (Litaor, 1987). Microbial life can remain biologically active during the later stages of winter as the snow-pack acts as an insulator maintaining the availability of free water. However, the summer months are potentially even more harsh for microbial and mesofaunal life, as the talus environment becomes very dry (Ley et al., 2004).

**DNA Extraction and Soil Measurements:**

Using the scheme of Weintraub et al. (2007), we measured soil nutrient (DOC, DON, DIN) and microbial biomass. We measured soil pH by making a soil paste with 2-3 times the water holding capacity of each soil using deionized water. Soil DNA extraction, PCR, cloning, and DNA sequencing of bdelloid rotifer communities were performed as in Robeson et al. (2011) with modification of using DyNAzyme II DNA polymerase and its associated 10x buffer (F-501L, F-511; Thermo Scientific).

**Data preparation for Bdelloid rotifers:**

We generated alignments of the cytochrome oxidase subunit 1 gene (cox1) using MUSCLE (Edgar, 2004) followed by hand editing to ensure that sequences were aligned by codon. This alignment was then analyzed in RAxML v.7.2.6 (Stamatakis, 2006) with
outgroup taxa. Outgroup taxa were pruned from the tree. The resulting bdelloid-only phylogeny was used for downstream phylogenetic community analyses.

OTU picking, and various community analysis metrics were performed using QIIME (Caporaso et al., 2010). An OTU similarity cut-off at 97% was used as, this the typical variation for within species differences for bdelloids (Birky et al., 2010).

**Data preparation for Bacteria:**

The same DNA samples used to amplify bdelloid *cox1* were used to amplify the v4-v6 region of bacterial small ribosomal subunit gene as previously described. Amplicon libraries were sequenced on a 454 GSFLX using Titanium chemistry and the resulting reads processed through a set of quality filters that ensure a per-base accuracy of better than 99.75% in retained sequences (Huse et al., 2007). OTU picking, taxonomy assignment and various community analysis metrics were performed using QIIME (Caporaso et al., 2010).

**Reference database construction:** In order to robustly assign OTU taxonomy to our sequence data, an ‘in-house’ reference database was constructed. This was performed as follows: reference OTU assignment of the Silva 108 database (Pruesse et al., 2007) along with long-read sequences from the Niwot Ridge LTER Alpine Microbial Observatory (amo.colorado.edu) database were searched against the Greengenes (DeSantis et al., 2006) database at 94% sequence similarity. Any sequences that failed to be assigned to the Greengenes database where placed into *de novo* OTUs using the UCLUST (Edgar, 2010) option within QIIME (Caporaso et al., 2010) at 94% sequence similarity. These *de novo* OTUs were then aligned to the Greengenes
alignment. A final bacterial reference phylogeny including the reference assigned data and *de novo* OTUs was constructed via FastTree2 (Price et al., 2010; 2010) and rooted with Archaea.

*Reference bacterial OTU-picking:* The 454 GSFLX bacterial data were combined with that of King et al. (2010). The two data sets were merged by converting each representative sequence to its nearest taxonomic OTU-match using the constructed reference database referred to above, via the ‘pick_reference_otus_through_otu_table.py’ command in QIIME (Caporaso et al., 2010). This was required because the data from King et al. (2010) were produced via the 27F and 338R primers, (see Fierer et al. (2008) for more details), which are located upstream of that of the v4-v6 region obtained from the 454 GSFLX data (Huse et al., 2007). OTUs that could not be assigned to the reference database were excluded from downstream analyses.

The resulting reference OTU and taxonomy assignments were produced using the 94% sequence similarity threshold. This level of OTU similarity approximates that of Family-to-Genus levels of taxonomy for Bacteria (Konstantinidis and Tiedje, 2007) and has been implemented in the past to increase the robustness of identified OTUs for microbial communities (Barberan et al., 2011). This threshold also allows us to circumvent potential taxonomic miss-classifications resulting from merging data-sets derived from different sequencing primers and any potential sequencing anomalies (Barberan et al., 2011). Once OTU-tables where constructed for each of the two datasets, they were merged for subsequent analyses via tools within QIIME (Caporaso et al., 2010).
Data analyses

QIIME (Caporaso et al., 2010) was used to generate both traditional OTU-based (Bray-Curtis, Sorensen-dice) and phylogenetic (weighted normalized UniFrac, unweighted UniFrac) beta-diversity metrics for both bdelloid and bacterial communities. Phylodiversity and Mean Phylogenetic Distance (MPD) estimates of phylogenetic over- and under-dispersion were calculated using the Picante R-package (Kembel et al., 2010) and QIIME (Caporaso et al., 2010).

Data were visualized via Principle Co-ordinate Analysis (PCoA) plots, and then subjected to Procrustes analysis via QIIME (Caporaso et al., 2010). Procrustes analysis (Caporaso et al., 2011), takes as input two PCoA matrices (in the current case each matrix, is composed of the mean values of 1000 jackknife matrices), that are compared to one-another using a rotational-fit algorithm that minimizes the sum-of-squared distances between the two matrices. The best observation that minimizes this distance is reported as the goodness-of-fit statistic $M^2$. The number of times that the randomized data was as good or better (smaller sum-of-squared distances) than the original data was recorded and used as a measure of procrustes significance.

Variance Partitioning:

Mantel tests, using VEGAN (Dixon, 2009), where used to test for the contribution of spatial effects while controlling for significantly different environmental parameters between sites. Generalized linear models (GLM) were used to determine which measured environmental and geo-spatial parameters were significantly contributing to observed $\beta$-
diversity patterns. For each of the four possible phylo-beta-diversity distance matrices a combined generalized linear model was created using AIC to select the most informative of the potential explanatory variables assuming a Gaussian relationship with the phylo-beta-diversity matrix in question (R Development Core Team, 2011). Variance partitioning was achieved by using the same AIC process to fit a model excluding one of the categories of explanatory variables, calculating the residuals, and then regressing the residuals against the remaining variable using a generalized linear model with a Gaussian relationship because biogeochemistry is represented by multiple variables, a second round of AIC model selection was performed to identify the biogeochemical variables most contributing to the biogeochemistry-only variance in the residuals from the partial model including only the spatial and co-occurring β-diversity as explanatory variables.
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Chapter 5

CONCLUSIONS
Review & Synthesis

My research has focused on the ability to detect and describe microscopic animal communities at local and global scales (Chapters 2-4) using bdelloid rotifers as my subject organism. In Chapter 2, I alluded to the difficulties of studying soil invertebrates like bdelloids due to limited taxonomic knowledge and non-distinguishable morphological traits. I further suggested that molecular characterization of communities via environmental sequencing is currently the best available means to describe bdelloid rotifer communities. This, was supported by the fact that molecular data are able to capture two to three times as much diversity as current methods based on morphology (Kaya et al., 2009). This highlights how morphological comparisons of bdelloids are often unreliable. Initially, I started my work using the 18SrDNA small sub-unit (SSU) in an attempt to gather information about the diversity and distribution of these lesser-known organisms. However, the abysmal representation of bdelloids within databases such as GenBank (Benson et al., 2005) and SILVA (Pruesse et al., 2007) can be problematic for microbial ecologists if their goal is to elucidate the full spectrum of microscopic eukaryotes within a study system. For example, SILVA is often used to help identify anonymous eukaryotic DNA sequences resulting from high-throughput environmental sequencing surveys. Other than the obvious ‘putative’ taxonomic identification applied to these anonymous sequence data, researchers often would like to know if their sequences are known from other studies and locations. As of this writing my geo-referenced bdelloidSSU data (Chapter 2) accounts for 85% of the representative bdelloid sequences within the SILVA database and increases the confidence by which anonymous sequences are correctly assigned to Bdelloidea.
My SSU data showed strong support for biogeographically disparate bdelloids and eutardigrades despite using such a conserved gene. Whether this apparent pattern reflects environmental filtering, priority effects (differences in arrival time that can have a lasting effect on differences in species dominance), or some other process remains to be seen. These initial estimates of the distribution and diversity of bdelloids and eutardigrades revealed that there are instances of endemism as defined by phylogenetic clusters being different at each locality (Chapter 2).

The representation of known SSU bdelloid sequence data that correspond to known morphotypes is excessively limited. Although, I was able to find biogeographical differences using SSU sequence data, the limited phylogenetic information resulting from my initial survey didn’t allow me to determine reliable family-to-species-level classification of these bdelloids. This prompted a more refined approach using a different phylogenetic marker gene that resides within the mitochondrial genome, cytochrome oxidase subunit 1 (cox1). I chose the cox1 gene because it provides some analytical advantages with regard to bdelloid rotifers (Birky, 2007), not only in what one would expect to find regarding branch length and tree topology (Birky, 1996; Barraclough et al., 2003) but also because (i) bar-coding initiatives have been accruing cox1 data (Ratnasingham and Hebert, 2007) to which our sequence data can be compared; (ii) robust primers (Folmer et al., 1994) make it possible to amplify the cox1 gene from almost any invertebrate, and these sequences then can be used to design taxon-specific primers, as in the present study; and (iii) cox1 is an effectively haploid gene which eliminates the “Meselson effect” that can make it difficult or impossible to recover the
correct phylogenetic tree using nuclear gene sequences (Mark Welch, 2000; Birky, 2007). This latter point may be a contributing factor to the poor internal resolution of my SSU bdelloid rotifer phylogenies (Chapter 2), although this has only been shown with several representative nuclear protein coding genes and not rDNA.

I used my cox1 primers to generate a high number of bdelloid rotifer sequences directly from soil (Chapter 3). As of this writing and using this approach, I have single-handedly doubled the amount of bdelloid cox1 data residing within GenBank (Benson et al., 2005). Although the ability to tell community samples apart using cox1 is more robust than that of 18S SSU sequence data, deeper phylogenetic relationships within the bdelloidea were shown to be inconclusive, as seen by the lack of phylogenetic support of monophyletic families or genera as defined by morphology (Birky et al., 2010). That is, using environmentally derived anonymous cox1 sequence alone is insufficient to identify which bdelloids are present, as morphologically defined taxonomy at the family or genus level is not monophyletic. However, phylogenetic β-diversity metrics are quite robust against potential issues of poor phylogenetic resolution resulting from errors in topological resolution and differences in tree reconstruction methods (Lozupone et al., 2007). As a result, detailed knowledge about presence of particular “species” per se is not required to make general assessments about community diversity and relatedness. Indeed, cox1 community sequence data was quite robust for my community-based analyses of bdelloid rotifer communities and my results were also supported by traditional OTU-based β-diversity metrics (Chapter 3). For the first time, I have provided an explicit assessment of the spatial structure of bdelloid rotifer communities using both phylogenetic and OTU-based β-diversity metrics. Soil bdelloid rotifer communities are
spatially autocorrelated from 54-133 meters. This is surprising, as one would expect ubiquitous distribution of all or most bdelloid species given their ability to survive a wide range of harsh conditions (Ricci and Perletti, 2006; Gladyshev and Meselson, 2008) that allows bdelloids to tolerate to a variety of habitat types (Fontaneto et al., 2011b). This raises questions about what this means for soil invertebrates and other microorganisms that also have highly resistant life stages: Are all resistant life stages meant for the production of propagules for dispersal only, or do these dormant stages to help prevent local extinction to maintain a foot-hold in favored habitats via Monopolization (De Meester et al., 2002)? Perhaps cryptobiosis is a means of spatiotemporal escape from being preyed upon by other organisms like predatory fungi (Wilson, 2011) and dispersal is an occasional secondary benefit to seed new habitats? I would like to pursue these questions in the future.

To further understand how bdelloid rotifer communities are structured due to factors other than geospatial distance (Chapter 3), I analyzed how local biogeochemical factors and co-occurring bacterial communities contribute to the diversity patterns of bdelloid rotifer communities (Chapter 4). Recent studies investigating the effects of environment on the biodiversity of bacterial communities are often compared to macroflora and fauna (Bryant et al., 2008; Fierer et al., 2011). However, mesofauna like bdelloid rotifers are left out of these comparative analysis even though they are thought to be highly integral to microbial food web interactions (Arndt, 1993; Pejler, 1995; Kutikova, 2003). I hypothesized that if mesofauna are fundamentally linked to the microbial food web (i.e. mesofaunal diversity is limited by their own prey-item preferences and trophic interactions with bacteria (Pejler, 1995; Schmid-Araya and
Schmid, 2000; Kutikova, 2003), then they should show similar patterns of diversity with co-occurring bacterial communities across three different ecosystems within the Niwot Ridge LTER (West et al., 1999): sub-alpine coniferous forest, dry meadow tundra, and talus. Indeed, I was able to show that bdelloid rotifer and bacterial communities do share patterns of α- and β-diversity structure between these three ecologically distinct sites (Chapter 4). Local environmental factors played a large role in the diversity and phylogenetic divergence of bdelloid rotifer communities between ecosystems. However, the environmental factors measured in this study explained very little for the observed differences bacterial community diversity between ecosystems. Although both bdelloid and bacterial communities showed similar patterns of diversity and community divergence by ecosystem, the environmental factors contributing to these patterns are still inconclusive and merit further study. I proposed that plant diversity, though not directly accounted for in this study, may have large influence on the composition of both bacterial and mesofaunal community diversity. Any future studies attempting to elucidate the drivers of community assembly and diversity should include plant diversity as a component.

**Current Issues and Future Directions:**

*a) Rotifer phylogeny.*

SSU rDNA sequences are known to be problematic for resolving many regions of the metazoan tree and is often combined with 28S large-subunit (LSU)rDNA sequences to increase the resolution of the Lophotrochozoan phylogeny (Passamanbeck and
Halanych, 2006). In fact, many problematic groups are often removed to create stable phylogenies (i.e. gastrotrichs, gnathostomulids, rotifers, acocelomorphs, bryozoans or chaetognaths) for the metazoan tree when using SSU rDNA sequences alone (as reviewed in Paps et al. (2009)). In general, the current limitations of SSU rDNA sequence data for metazoan phylogenetics (Abouheif et al., 1998) have not been overcome or replaced by other marker genes. However, it has been proposed that the E23 helix of the SSU be used for further refinement of rotifer phylogenetic relationships (Miquelis et al., 2000). However, no one has continued with this work and generated additional sequence data from known cultured representatives. Until taxonomists and molecular ecologists work together in order to develop a database of sequence data linked to known vouchered specimens, the ability to resolve rotifer phylogeny will not be possible; and the utility of E23 or any other molecular marker will remain untested. This is of particular interest to me, and I hope to collaborate and receive future training in the taxonomic identification of bdelloid rotifers to aide in this regard.

b) Are soil invertebrates incorrectly assigned to the ‘rare-biosphere’?

Recently, lichen associated eukaryotic communities have been surveyed via pyrosequencing (Bates et al., 2012). Micro-invertebrate taxa like the Tardigrada and Rotifera are known to be quite abundant in lichen habitats (Stubbs, 1989). However, the aforementioned study reveals that these two groups eachonly compose 0.1 % of lichen-associated eukaryotes (i.e., only several sequences out of thousands). This brings into question weather or not these low numbers would incorrectly place these taxa as members of the ‘rare-biosphere’, in the absence of other observational data. This suggests
that current ‘universal’ primers are unable to cope with amplifying the full diversity of eukaryotic organisms, which can alter our interpretation of the diversity of these habitats. For example, refinement of PCR primers for the 16S SSU have enabled better detection of the Verrucomicrobia in soils, revealing abundance levels much higher than previous primer sets had suggested (Bergmann et al., 2011). Similarly, current metazoan ‘universal’ primers have known biases against the detection of some groups of soil invertebrates like rotifers, meanwhile preferentially amplifying other groups like arthropods (Hamilton et al., 2009; Wu et al., 2009). In fact Wu et al. (2009) revealed discrepancies of the type and abundance of soil invertebrates found when comparing environmental molecular data to direct observational data based on morphology.

These metazoan primer biases call into question general claims about the inverse relationships of soil invertebrate diversity compared to above ground plant diversity as observed by Wu et al. (2011). Especially, since the PCR primers used do have biases that result in low representation of particular taxa (rotifers) that are known by direct observation to be in higher abundance. Alternatively, organisms that were not directly observed in samples can appear to dominate DNA sequence libraries. However, as reviewed by Wu et al. (2009), this is may be no worse than comparing whole-organism isolation methods to one another, as some methods (e.g. Baermann and Tullgren funnels) are better at isolating actively mobile soil invertebrates and underestimate the diversity of less mobile taxa due to this differential extraction efficiency.

PCR primer bias and organism extraction efficiency is a generally known issue across many environmental surveys and each is difficult to overcome. Clearly, further analysis and development of better molecular and whole-organism extractions tools are
required to properly determine the diversity and distribution of soil invertebrates as they relate to co-occurring micro- and macro-organisms. Recently, Devetter (2010) developed a highly robust soil rotifer extraction method to isolate live rotifers from soil. I am very interested in using this approach in further studies of high-elevation soil rotifers and comparing them to my environmental DNA sequencing approach. This is of particular interest as I would very much like to explore the diversity of predatory fungi on bdelloid rotifers in the Talus, and being able to extract live organisms would increase our assessment of ‘who’ is there and what these fungi (e.g. Lecophagus; (Schmidt et al., 2012)) are feeding upon. I am also interested in future work that focus on developing more robust eukaryotic ‘universal’ or metazoan specific primers (or a primer cocktail) if studies of eukaryotic diversity are to continue via culture-independent methods. Again, microscopic animals may be mistakenly classified via high-throughput sequencing analyses as part of the ‘rare metazoan biosphere’, when in fact they are not rare at all.

c) Are we missing out on taxonomic expertise?

If the interests I mentioned immediately above are to be of benefit; the scientific community needs to maintain the training of soil invertebrate taxonomists. The number and quality of trained soil invertebrate taxonomists is dwindling and will make future biodiversity and ecosystem function research involving these taxa more difficult to interpret (Behan-Pelletier and Newton, 1999). As pointed out by the example above, we have no way of knowing how accurate environmental DNA sequencing surveys are if they can not be validated by trained taxonomists. To reiterate, most of these trained taxonomists reside in Europe, which partly explains the “rotiferologist effect” (Fontaneto
et al., 2011a). I hope to collaborate more in the future with bdelloid taxonomists so that I can be trained to identify bdelloids based on morphology. This will allow me to engage in studies that try to elucidate how and why certain bdelloids reside in the habitats they do, based on ecologically important traits that help them to survive in those habitats. Further attention must be given to microscopic soil invertebrates as they inherently link the micro and macro food web interactions.

d) Understanding community assembly and trophic interactions.

Based on the topics I discussed in subsections a-c above, it becomes obvious how lack of phylogenetic, molecular, and simple taxonomic expertise can impede interdisciplinary research that attempts to elucidate the interactions of soil invertebrate taxa and their co-occurring microbial communities; and how these affect our understanding of community assembly and trophic interactions. Comparisons of the diversity and distribution of microscopic animal communities to bacterial communities have been anecdotal, and have not made direct comparisons among samples, as I have done (Chapter 4). Modern high-throughput sequencing and informatics approaches are just now making it possible to perform fine-grained inter-taxonomic comparisons via methods like co-occurrence analysis (Barberan et al., 2011). I propose that future analyses incorporate a sampling regime in which samples are taken at finer altitudinal, spatial, and temporal sampling scales (Gonzalez et al., 2011). This will aid in the direct observations of community assembly, persistence, and “turn-over” through space and time over altitudinal and environmental gradients. Analyses comparing micro-, meso-, and macro-organism communities remain in their infancy, indicating that more inclusive
concepts and theories are needed to explain disparities not only between micro- through macro-organism scales (Fierer et al., 2011), but between various study systems as well.
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