Comparison of Skin Microbial Communities Between Striped and Unstriped Morphs of Plethodon cinereus in Massachusetts

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Abstract

The skin-microbial community on amphibians plays a large role in maintaining the general health of the organism. One of the most common patterns found in amphibian skin microbiomes pertains to the antifungal aspect of these communities: beneficial microbes protect amphibians against harmful pathogenic fungi. The eastern red-backed salamander is one of the few amphibian species with well-documented skin microbes that produce antifungal compounds. These compounds are potentially lethal to chytrid fungus, one of the deadliest amphibian pathogens. The eastern red-backed salamander also has two primary color morphs that coexist over a large geographic range. Physiological differences between morphs have been well-documented across the range. However, morph differences in skin microbial community composition and relative abundance have not been explicitly looked at. In my study, I extracted whole-community DNA from skin swabs for six striped morph salamanders and four unstriped morphs. DNA was sequenced for 16S rRNA gene using Illumina MiSeq. Although I experienced a high rate of sequencing failure (60%), the results for one striped morph and three unstriped morphs are suggestive of different levels of microbial diversity between morphs. Unstriped morphs of *P. cinereus* had similar levels of both OTU abundance and diversity, while the striped morph had greater abundance of OTU’s, and significantly greater alpha diversity compared to the unstriped morph. Additional samples will be needed to confirm this pattern more broadly.

Introduction

The cutaneous microbial community on amphibian hosts is hypothesized to provide a variety of benefits to their hosts, including defensive functions against pathogens (Loudon et al. 2014). These defensive functions include manipulating host behavior, conferring disease resistance, activating the host’s immune system, maintaining host metabolism, and synthesizing vitamins (Loudon et al. 2014). A fluctuating microbial community structure on an amphibian host may result in fluctuating defensive functions as the microbiota produce antifungal metabolites (Loudon et al. 2014). The stability of an amphibian’s cutaneous microbiota is likely dependent on the bacterial reservoirs which surround it, such as those microbiota found in soil and water (Loudon et al. 2014). While all amphibian species harbor these symbiotic microbes, very few comprehensive studies have been carried out which assess whether different
amphibians harbor distinct microbial symbionts (McKenzie et al. 2012). Nor have many studies been designed to distinguish or examine within-host differences in microbial communities, such as those which might be hypothesized between different morphs of the same species (McKenzie et al. 2012).

One example of the role of skin microbes in defense against amphibian pathogens has been well-documented. Chytridiomycosis, one of the most virulent amphibian diseases, is caused by two species of chytrid fungi: Batrachochytrium dendrobatidi (or Bd, primarily infects frogs), and the more recently discovered B. salamandrivorans (or Bsal, primarily infects salamanders; Berger et al. 2016). In the late 1970s and early 1980s, Bd caused some of the most dramatic population declines of naïve amphibian species, particularly of tropical frogs, in the remote mountain regions of Australia and Central and South America (Berger et al. 2016).

Chytridiomycosis is a superficial skin infection which causes the skin to become keratinized, interfering with cutaneous gas exchange, and ultimately leads to suffocation (Berger et al. 2016). Chytrid fungi affect an array of amphibians including lungless salamanders, newts, frogs, and toads. (Berger et al. 2016). Recent studies have found that the microbial taxa which live on the skin of eastern red-backed salamanders may provide resistance to Bd and Bsal, as these microbial taxa are known to have both antibacterial and antifungal properties that are lethal to chytrid fungus in high enough concentrations (Lauer et al. 2007; Brucker et al. 2008; Loudon et al. 2014).

Figure 1. The two color morphs of the eastern red-backed salamander (Plethodon cinereus). Striped (or red) morph on the left, and unstriped (or lead) morph on the right. Photo by M.C. Fisher-Reid.

The eastern red-backed salamander (Plethodon cinereus; Fig. 1) is found in mature deciduous, northern conifer, and mixed deciduous-conifer forests throughout northeastern North America (Petranka 1998). These forest types often have broad, full canopies that allow for adequate shade and moisture. Plethodon cinereus is sensitive to both overall precipitation and temperature as they are lungless ectotherms, and all respiration is conducted through their skin. Cutaneous respiration requires moist, cool environments (Petranka 1998). Deep soils with scattered logs or rocks provide optimal habitats while areas of highly acidic, or shallow rocky soils result in the absence of this species (Petranka 1998). Plethodon cinereus is the focal species
of the Salamander Population and Adaptation Research Collaboration Network (SPARCnet; Fig. 2), which consists of over 20 research sites collecting mark-recapture data throughout the range of *P. cinereus* (Fig. 2). One of those sites is located on the campus of Bridgewater State University (BSU) in the Great Hill forest and is managed by Dr. Fisher-Reid.

**Figure 2.** SPARCnet research sites (red and yellow bubbles) across the range of *Plethodon cinereus* (in blue). Map made by SPARCnet members H. Coovert and J. Fleming and used with their permission.

There are two common color morphs for this species: striped and unstriped (Fig. 1). Previous work on these color morphs has suggested a suite of physiological differences. For example, the unstriped color morph may have a higher mortality rate in cooler temperatures, compared to the striped morph (Gibbs and Karraker 2006) and retreats underground earlier in the year (Anthony et al. 2008). Striped and unstriped morphs of *P. cinereus* have in some cases been found in different thermal microhabitats and physiological studies have shown a significant difference in basal metabolic rate of the two morphs at 15ºC (Moreno 1989; Petruzzi et al. 2006). Throughout their range, striped morphs of are generally found at a higher frequency than unstriped morphs: the most common population ratio is 70% striped to 30% unstriped (Petranka 1998).

Previous studies have looked at *P. cinereus* cutaneous microbiota (e.g., Loudon et al. 2014). Evidence from these studies suggests that alpha diversity of cutaneous microbes stays the same over time when salamanders are housed with forest-collected soil containing bacterial
reservoirs (Loudon et al. 2014). Alpha diversity of skin microbes decreased in the presence of sterile media, meaning that bacterial reservoirs, like those found in the forest soil, are needed to maintain diverse and complex populations of microbiota on *P. cinereus* (Loudon et al. 2014). Common cutaneous bacteria in *P. cinereus* have also been studied to determine if it could inhibit pathogenic fungi (Lauer et al. 2007). Lauer et al. (2007) found that antifungal skin bacteria may form mutualistic relationships with amphibian species which help protect against pathogenic fungi. Additionally, Brucker et al. (2008) was also able to find antifungal metabolites from *Janthinobacterium lividum* on the skin of *P. cinereus* at concentrations lethal to *Bd*.

In this study I compared core microbial communities between the two color morphs of *P. cinereus* (Fig. 1) and determine if there is a significant difference among them. I collected skin swab samples from 10 salamanders from one SPARCnet plot. To my knowledge, a direct comparison of microbial communities between the color morphs has not been conducted. Given the physiological differences between color morphs I hypothesized that the two morphs of *P. cinereus* may have different microbial communities, either in overall composition, relative abundance of taxa, or both.

**Methods**

**SPARCnet Plot Design**

The BSU SPARCnet site consists of eight separate study plots (Fig. 3). Each plot site consists of 50 cover boards, which are set up in five rows of ten, making a total of 400 boards across eight plots. These sites are used in the participation of a mark recapture survey for SPARCnet, which is a group with sites across New England that track the abundance rates of *P. cinereus*. Permission was granted to collect individuals from under these boards to use in this study. All individuals found were brough back to the lab for processing, and immediately returned once processing was completed.

**Skin Swab Sample Collection**

Skin swab samples were collected from 10 individuals found in Plot II in the Great Hill forest on the campus of Bridgewater State University, Bridgewater, MA (Fig. 3). All individuals were collected and swabbed on October 9th, 2020. Four of the individuals swabbed were of the unstriped morph, and six were of the striped morph. Salamanders were caught by hand then transported to the lab in a chilled container. Samples were collected in the lab. To remove dirt and environmental bacteria, *P. cinereus* were placed into sterile petri dishes and rinsed twice by pouring sterile water over top (i.e., previously autoclaved RODI water) prior to swabbing. This ensured the samples collected would reflect skin-associated microorganisms, rather than transient microbes from the external environment. Following rinsing, individuals were swabbed with Qiagen OmniSwabs using 20 strokes (10 ventral and 10 dorsal) while applying minimal
pressure to the skin. Vinyl gloves were worn during the rinsing and swabbing process, to prevent contamination of the sample with my own microbiota, and gloves were changed in between each individual salamander. Immediately after collection, swabs were stored in 1.5mL microcentrifuge tubes and placed at -20ºC. All salamanders were returned to their original location of capture after swabbing.

**DNA Extraction & Sequencing**

DNA was extracted from the swabs using a Qiagen PowerSoil Pro Kit following the kit’s quick start protocol. DNA was quantified in each sample by using a NanoDrop in the Department of Biological Sciences (Table 1). After quantification, all extracted DNA was sent out for Illumina 16S rRNA sequencing at Wright Labs LLC (Huntingdon, PA) to identify and quantify the bacterial species in each sample.

**Data Analysis**

Once sequencing was completed, an analysis of the skin microbiome data was conducted using the web-based bioinformatics pipeline Nephele (Weber et al. 2018). A rarefaction curve showing species accumulation as a function of sequencing depth, an operational taxonomic unit (OTU; bacterial species) heatmap and estimates of alpha diversity graphs was generated. The following settings were used in Nephele: 16S FASTQ paired end job, GreenGenes taxonomic database with sequences clustered at 99% identity, and QIIME 1 pipeline (Caporaso et al. 2010).
Figure 3. SPARCnet plots around Bridgewater State University (BSU) in Bridgewater, MA. Map made in QGIS by M.C. Fisher-Reid.

Results

Despite adequate amounts of genetic material from DNA extraction (Table 1), out of the ten individual samples that were sequenced, only three samples from the unstriped morph and one sample from the striped morph were successfully sequenced. The successful samples include L1-L3 and R6 (Fig. 4). With only four samples being successful there was a failure rate of 60%.

While I do not have the statistical power to make conclusions based on only four samples, the results depicted in Figures 4–6 are promising for future work testing my hypothesis. The rarefaction curve (Fig. 4) shows that the species richness of microbial taxa for the three unstriped salamanders is similar and that the species richness for the striped morph was higher than for the unstriped morphs. The alpha diversity chart (Fig. 5), which incorporates both species richness and relative abundance, also suggests a difference in between the unstriped and striped morphs of salamanders. The single striped morph individual had a greater alpha diversity value compared to the three unstriped morph individuals (Fig. 5). A variety of class abundance was found among the individuals who were successfully analyzed, where an overview is provided in the form of a heat map (Fig. 6). From this we recognize the top five classes which account for 70-80% of OUT’s in abundance out of the overall 45 classes identified (Fig. 6). These five classes include acidobacteria, betaproteobacteria, gammaproteobacteria, actinobacteria, and alphaproteobacteria (Fig 6). Due to the small sample size and lack of statistical power of the
results, more data is needed to make any conclusion on whether or not this is statistically significant.

**Table 1**: DNA was quantified using a Nanodrop. L1-L4 are indicative of the unstriped salamander samples, while R1-R6 are indicative of the striped salamander samples. Bolded samples were sequenced successfully. The volume used was 48µL for all concentrations.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (ng/ µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>42.7</td>
</tr>
<tr>
<td>L2</td>
<td>6.2</td>
</tr>
<tr>
<td>L3</td>
<td>5.3</td>
</tr>
<tr>
<td>L4</td>
<td>17.0</td>
</tr>
<tr>
<td>R1</td>
<td>91.6</td>
</tr>
<tr>
<td>R2</td>
<td>50.0</td>
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<td>R3</td>
<td>13.7</td>
</tr>
<tr>
<td>R4</td>
<td>28.4</td>
</tr>
<tr>
<td>R5</td>
<td>0.2</td>
</tr>
<tr>
<td>R6</td>
<td>40.7</td>
</tr>
</tbody>
</table>
Figure 4. Rarefaction curve showing the accumulation of OTUs as a function of sequencing depth (total number of reads). The single striped salamander (red line) had a more diverse skin microbial community than the three unstriped (black lines) salamanders, who all had similar levels of diversity and sequencing reads.
Figure 5. Alpha diversity by color morph using Shannon Diversity index. The alpha diversity for the single striped salamander (red) was greater than the alpha diversity for all three unstriped (lead; green box) salamanders.
Figure 6. Heatmap of microbial classes found on salamander skin using 16S rRNA Illumina sequencing. Size of the bar is proportional to the frequency of that class in the sample.
Discussion

The microbiota on a host species perform an array of functions important to host health, such as the development and activity of the immune system, disease resistance, metabolism, and vitamin production (Loudon et al. 2013). Microbial communities of a host’s skin play such a role that it can be considered a continuation of the immune system (Bletz et al. 2018). It has been found that soil reservoirs strongly impact the community of a host's cutaneous microbial diversity resulting in changes in community microbial structures when *P. cinereus* is removed from its current environment and placed into captivity (Loudon et al. 2013). The samples in this study were from individuals taken from their native habitats and thus, should be a true reflection of the microbial communities present in this population.

The top five classes of microorganisms for all four samples, in sequential order from lowest to highest, are: **acidobacteria, betaproteobacteria, gammaproteobacteria, actinobacteria, and alphaproteobacteria** (Fig. 6). This similarity between bacterial classes present is a good indicator that these main bacterial groups may be shared throughout all individuals of *P. cinereus* in this habitat. *Acidobacteria* are most commonly recognized as a bacterial species abundant in soils, especially those low in organic carbon environments (Kielak et al. 2016). Betaproteobacteria play a role in the nitrogen fixation for plants, converting ammonium to nitrate (LumenCandela 2021). Betaproteobacteria are also commonly found in terrestrial ecosystems, mainly soil and wastewater (LumenCandela 2021). The presence of these two soil microbial groups on the skin surface of salamanders is likely due to only partial effectiveness of the washing step. The remaining three groups, *gammaproteobacteria, actinobacteria, and alphaproteobacteria*, all likely represent symbionts of *P. cinereus*. All three classes have been previously documented in amphibian gut microbiota as well as having cutaneous antifungal activity in this species (Lauer et al. 2007; Bletz et al. 2016). A byproduct of bacterial interference competition from these three classes can produce antibiotic resistance, which may help in protection against fungal infections such as *Bd* (Bletz et al. 2018). When *Bsal*, a pathogenic cousin of *Bd*, was seen in cases of fire salamanders it was noted that *Bsal* inhibiting bacteria were able to elicit protection and slow disease progression (Bletz et al. 2018). However, the bacterium which increased in abundance after infection overlapped with bacteria involved in septicemic events (Bletz et al. 2018).

I created an appendix of the top ten microbial species for each sample. This was used to provide insight to the functional role of the most abundant microbial species, define how microbial species differed in between morphs, and as a baseline for comparison to other research. The appendix details the known functional roles of different microbial species found. It was found that eight of the 16 microbial species listed in the appendix were likely transient environmental bacteria (50%; Appendix 1). Additional precautions should be taken in the future to reduce the abundance of these species so that swabbing may reflect the true cutaneous microbial communities of *P. cinereus*. 
The striped morph (R6) had 15 of the 16 listed species present, missing only *Granulicella*, where *Granulicella* was present in all three of the unstriped morphs (Appendix 1). *Granulicella* is responsible for metal ion homeostasis, particularly Mn, and is often found in areas with decaying urban wood, making this a likely transient environmental species (Costa et al. 2020). The striped morph also had three species specific to this individual, indicating an overall higher degree of species richness (Appendix 1). These three species include *Noyosphingobium*, *Leifsonia* sp. 24, *Chryseobacterium*. *Leifsonia* sp. 24 is associated with the breakdown of cellulose and is likely a transient environmental species (Rastogi & Banerjee 2019), whereas *Noyosphingobium* and *Chryseobacterium* are previously documented mutualistic species of *P. cinereus* (Sohn et al. 2004 & Lauer et al. 2007). *Noyosphingobium* is a bacterium which is capable of breaking down high-molecular-mass polycyclic aromatic hydrocarbons, an often-associated carcinogen of combustion (Sohn et al. 2004). *Chryseobacterium* is a strong antifungal bacterium which may have a mutualistic relationship with *P. cinereus* in the protection of *Bd* and other pathogenic fungi (Lauer et al. 2007).

Due to sample size we were not able to define core communities, however we did have a top 10% of communities found to be present throughout all samples collected. Overlap was found between the samples taken here and research done by Louden et al. (2014). The organisms found in this study are comparable to what has been found in other surveys done of microbial communities on *P. cinereus*. Louden et al. (2014) suggests that core microbial communities may not be derived from environmental factors, but rather are selected for by host factors such as peptide secretions and maintained by host-associated microbial communities. However, diverse bacterial reservoirs, such as soils and vernal waters, house transient bacterial species which may compete with core microbes in an attempt to colonize the individual (Louden et al. 2014).

Louden et al. (2014) found that dominant core OTU phyla consisted of *Verrucomicrobia*, particularly the class *Opitutae*. In Louden et al.’s (2014) study on sterile media *Verrucomicrobia* contributed up to 92.5% of the bacterial community on *P. cinereus*, whereas this was only seen at a mean rate of 5.1% for our unstriped morph, and 5.2% for our striped morph. While *Verrucomicrobia* was still seen, it was found at a much lower rate than what was expected, if we are to use previous research as a baseline for microbial community abundance on *P. cinereus*. This may be due in part to our sample species not being housed in sterile environments, but rather taken from the field, and as such may have an overwhelming community of transient environmental species which are residual. Additionally, the samples from Louden et al. (2014) which were used for comparison came from Virginia, where the samples in this study originate from Massachusetts, so geographical variation may play a role. *Opitutae* was also found within the top 45% of species relative frequency, among all sampled individuals. This tells us that our results are similar to previous research.

Other previously documented core communities include families such as *Pseudomonadaceae*, *Staphylococcaceae*, and *Comamonadaceae* (Louden et al. 2014). Both *Staphylococcaceae* and *Comamonadaceae* are found in our samples, however *Pseudomonadaceae* was not (Appendix 1). These families are associated with antimicrobial
activity, where the largest antimicrobial contributor to individuals in our samples was in the phylum of Actinobacteria (Louden et al. 2014). The average antimicrobial Actinobacteria for the unstriped morph was at 30.2% and for the striped morph was 22.6%. As there was only one individual successfully for the striped morph, there is no real average for the data.

The high failure rate found in sequencing left only four samples to analyze. This rate of failure may have been due to my sampling method, specifically my choice of swab and stroke number. In the future, a full 30 strokes should be taken, and HydraFlock swabs may be used to better capture microbial DNA. When tested against four other competing swabs, HydraFlock was shown to exhibit both the highest water absorption and protein absorption (Harry et al. 2013). When tested among other brands it was also shown that HydraFlock had the highest percent recovery of viable bacterial species (Harry et al. 2013).

The next steps of action would be to continue sequencing genetic material from individuals from all eight SPARCnet plots around Bridgewater State University (BSU) in Bridgewater, MA. This would be done by sampling the microbial communities of ten individuals (five striped and five unstriped) from each plot following the guidelines used in this study. In future sampling HydraFlock swabs should be used to better capture microbial DNA, along with a full 30 strokes. Furthermore, swabbing samples should be stored at -80°C, instead of the -20°C used in this study, until DNA extraction in order to improve the integrity of genetic materials. This addition in sampling size would help to give a more resolute image of the overall results as well as the statistical power to make any claims in defining differences in microbial species between morphs.
References:


https://courses.lumenlearning.com/boundless-microbiology/chapter/proteobacteria/.
Appendix 1

The appendix is arranged in alphabetical order by genus and includes which sample the microbial species was found on and what percentage of abundance the microbial species was found at. Using the species taxonomic level, a brief description is provided for the functioning and purpose of each microbe listed. This appendix is limited to the top 16 microbial species with the highest rates of abundance, which ensured that the top ten species for each sample were included.

Figure A1.1: An overview of all microbial species listed in Appendix 1. The overall abundance rates for what is shown is as follows: L1-35.392% L2-20.269% L3-50.286%% R6-41.206%. The total number of microbial species for each sample is as follows: L1-10, L2-11, L3-11, R6-15.
Figure A1.2: Actinospica (L3-0.955%, R6-3.426%) – A part of the new order of Catenulisporales in the phylum Actinobacteria which has antimicrobial activity against gram-positive bacteria (Iorio et al. 2012). This was initially thought of as a soil microbe (Iorio et al. 2012).

Figure A1.3: Acidothermus (L1-3.973%, L2-0.178%, L3-12.957%, R6-0.417%) – This is likely a transient environmental bacterium which is found on wood and in dirt/mud (Baker et al. 1994).
Acidothermus is thermophilic and is found to secrete thermostable cellulose-degrading enzymes which may be associated with disease suppression (Backer et al. 1994).

Figure A1.4: Bryocella (L1-1.099%, L2-0.155%, L3-2.105%, R6-2.338%) – This is a type of Actinobacteria. A slow growing species found in coniferous soils which produces beta-glucosidase, an enzyme involved in the last step of cellulose-degradation (lladό et al. 2015).

Figure A1.5: Burkholderia-Paraburkholderia (L1-6.428%, L2-9.058%, L3-5.014%, R6-1.939%) – This is likely a transient environmental bacterium found in dirt (Cao et al. 2021). The main functions of this species are β-oxidation and FAS II (Cao et al. 2021). Paraburkholderia is often studied for its carbon use efficiency (Cyle et al. 2020).
Figure A1.6: *Chryseobacterium* (R6-5.835%) – Strong antifungal bacteria which may have a mutualistic relationship with *P. cinereus* in the protection of *Bd* and other pathogenic fungi (Lauer et al. 2007).

Figure A1.7: *Comamonadaceae* (L1-4.267%, L2-3.664%, L3-3.498%, R6-2.886%) – A study done on Panamanian golden frogs found that *Comamonadaceae* was found on individuals that cleared *Bd* infections but not those that died (Becker et al. 2015). This would indicate a
symbiotic relationship between Comamonadaceae and amphibians where Comamonadaceae is utilized for its antifungal metabolites.

Figure A1.8: Dyella (Xanthomonadacea) (L1-0.495%, L2-1.427%, L3-4.973%, R6-2.614%) – The genus Dyella is found in forest soils and are yellow-colored rods that are urease-negative but are catalase and oxidative-positive (Weon et al. 2009).

Figure A1.9: Granulicella (L1-5.093%, L2-2.266%, L3-1.648%) – Strains of Granulicella have mechanisms to deal with stress as well as metal ion homeostasis, particularly Mn, allowing its species to be better adapt in environments with decaying wood-rich Mn (Costa et al. 2020).
Figure A1.10: Leifsonia sp. D24 (R6-3.995%) – Leifsonia sp. is found in fruit wastes and synthesizes cellulose (Rastogi & Banerjee 2019).

Figure A1.11: Mycobacterium (L1-3.093%, L2-0.381%, L3-5.065%, R6-1.335%) – This genus is known to cause infections in both amphibians and mammals, where the epidemiology of disease in different in mammals than it is in amphibians (Miller & Fowler 2012). The pathogenicity of the 130 species varies significantly (Miller & Fowler 2012).
Figure A1.12: Novosphingobium (R6-3.484%) – a bacterium which is capable of breaking down high-molecular-mass polycyclic aromatic hydrocarbons, an often-associated carcinogen of combustion (Sohn et al. 2004).

Figure A1.13: Obesumbacterium proteus (L1-1.020%, L2-2.046%, L3-2.800%, R6-2.145%) – a species from the Enterobacteria family related to E. coli, which produces similar periplasmic phytase activity (AppA), an enzyme which improves the availability of dietary phosphorus (Zinin et al. 2004).
Figure A1.14: *Opitutae vadinHA64* (L1-3.498%, L2-0.791%, R6-1.125%) – a freshwater bacterial community found to be more prevalent in the winters (Zwirglmaier et al. 2015).

Figure A1.15: *Rhizobiales* (L2-0.113%, R6-6.446%) – This is an order of gram-negative *Alphaproteobacteria* which has symbiotic relationships with leguminous plants fixing nitrogen and is also often found to be pathogenic to animals and plants (Carvalho et al 2010). *Rhizobiales* as *Alphaproteobacteria* have been detected in lichens using fluorescence in situ hybridization and confocal laser microscopy (Erlacher et al 2015).
Figure A1.16: Streptomyces (L2-0.190%, L3-4.754%, R6-1.427%) — This is a rich source of antibiotics as well as antiparasitics and they are able to synthesize a large array of secondary bioactive metabolites (Ikeda et al. 2003). Because of these antibacterial metabolites, they may form a mutualistic or symbiotic relationship with _P. cinereus_.

Figure A1.17: YNPFFP1 (L1-6.426%, L3-6.477%, R6-2.211%) — This is likely a transient environmental bacterium found in thermal soil (Singleton et al. 2003).
Appendix References


