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Soil Characteristics and Bacterial Communities from the Microhabitats of a Widespread Salamander Compared to Two Microendemic Species

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Microhabitats require robust characterization to assess species and habitat conservation needs effectively. Because climate and other abiotic environmental data are typically measured at a resolution of 1 km^2 or greater, quantifying microhabitat heterogeneity is especially relevant for species limited to small geographic scales (<1 km^2). However, species with small home ranges can have expansive species distributions. For example, *Plethodon cinereus* is geographically widespread (>1 million km^2), whereas microendemics such as *P. hubrichti* and *P. sherando* have relatively small distributions (<100 km^2), yet all three species have small home ranges tightly linked to microhabitats. Although studies have examined mechanisms that maintain these distributions such as the effects of interspecific competition and thermal and hydric preferences, the role of soil characteristics on salamander habitat selection in terrestrial environments is unclear. Soil has many functional traits that can influence its floral, faunal, and microbial inhabitants. Here, we quantified soil properties (density, porosity, particle size, pH, and organic matter) as well as bacterial community composition within the range of the two microendemic species of salamanders and compared those to the same microhabitat characteristics in nearby populations of *P. cinereus*. Our results suggest that soil texture and bacterial communities vary across the range of both widespread and microendemic species. However, soil properties did not significantly predict the differences in bacterial composition among sites. The pH was also lower at microendemic sites compared to adjacent *P. cinereus* sites. Because salamanders are critical components of forest floor food webs and contribute to a large proportion of vertebrate biomass, our findings highlight the importance of soil structure and composition as these factors may play a role in determining salamander's activity, behavior, and physiological processes and should be considered in ecological studies of plethodontids.

CONSERVATION efforts are centered around understanding the requirements and limitations that shape species distributions, which are dictated by biotic and abiotic factors including intra- and interspecific competition, habitat and resource availability, and climate, among many others (Ashcroft et al., 2011; Leach et al., 2016). Thus, factors limiting species distributions are location-dependent due to high environmental variability (Zhang et al., 2021). While macro-scale (>1 km^2) habitat is often assessed through satellite or aerial imagery (Balmford et al., 2003; Mikula et al., 2021; McNeil et al., 2023), this scope is well beyond the geographic scale of biologically relevant processes such as reproduction and foraging (<1 m^2; Suggitt et al., 2011; Farallo and Miles, 2016; Farallo et al., 2018). Therefore, the scale of data that are readily available lacks the necessary detail to capture what many organisms experience.

Lungless salamanders (family Plethodontidae) are one such group of organisms with individuals that exist at small spatial scales. These salamanders are lungless and rely on cutaneous respiration, which is influenced by both temperature and moisture conditions (Feder, 1983). Very little is known about plethodontid salamander activity and behaviors within the soil due to the challenging nature of monitoring organisms underground (Sterrett et al., 2022). Furthermore, large portions of populations of *Plethodon* remain underground even when surface activity is high (Test and Bingham, 1948; Fraser, 1976; Marsh and Goicochea, 2003). Salamanders are in direct contact with soil both below ground and at the surface when hiding under cover objects. Soil will therefore influence salamander physiology including the temperatures they experience and their ability to retain moisture, both factors that influence cutaneous respiration. Soil properties will also influence how far salamanders are able to retreat underground, as soil density limits their burrowing ability and that of heterospecifics that make burrows used by salamanders (Stovold et al., 2004). Both salamander physiology and soil properties will presumably influence where a species can occur and whether they are able to persist in a location.

Soil is vital for sustaining ecosystems by enabling the cycling of nutrients; it is the basis of terrestrial primary production, plays a regulatory role in climate and flood control, and can even filter out toxic components (Coleman et al., 2018). An important factor in soil structure is texture, quantified by the clay-silt-sand-gravel complex, or the percentages of each type of particle. Clay particles are the smallest (<0.0039 mm), while gravel particles are the largest (>2 mm; Wentworth, 1922; Rai et al., 2017). Soil texture and porosity, the percentage of void space in soil, both affect soil water holding capacity, which is critical for salamander respiration (Riddell et al., 2017, 2018; Brischke and Wegener, 2019). Bulk density is another factor contributing to soil structure, calculated as the ratio of weight to volume of a soil sample. Soil texture, porosity, and bulk density may also affect the ability of salamanders to burrow underground (Heatwole, 1960), as well as other organisms that create burrows that can be utilized by salamanders especially in substrates that

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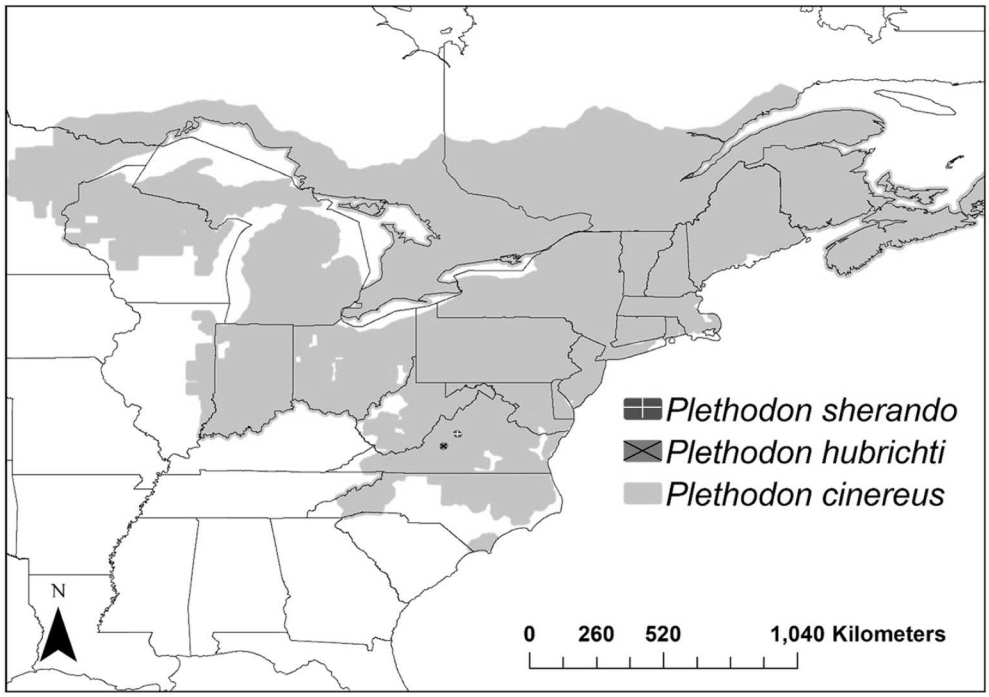


Fig. 1. A map of the range (based on IUCN Red List range maps) of our three focal salamander species including the widespread *Plethodon cinereus* and two microendemic species, *P. hubrichti* and *P. sherando*. We sampled soil at four sites, one within each of the two microendemic ranges and one within 10 km of each of the two microendemic ranges, where only *P. cinereus* can be found.

would be impermeable to smaller plethodontids (Caceres-Charneco and Ransom, 2010; Ransom, 2012). Less porous, denser soil is tightly packed and more difficult to burrow in, while more porous, less dense soils could be too loose to create structured burrows (Langmaack et al., 1999; Stovold et al., 2004). Soil organic matter (SOM) is made up of decomposed tissues of animals, plants, and microbes. Thus, it provides essential nutrients to the soil ecosystem and can be used as an indicator for estimating soil health (Sullivan et al., 2019). Decomposed leaf litter, which also provides moist refugia to salamanders and houses much of the prey items they consume for several days after rain events (Jaeger et al., 2016), is a major component of SOM. Salamanders can also impact leaf litter decomposition, which contributes to SOM, primarily through consumption of invertebrate detritivores (Best and Welsh, 2014; Hickerson et al., 2017). Although studies indicate that *P. cinereus* is resistant to low pH (Wyman and Hawksley-Lescault, 1987; Bondi et al., 2016), we do not know how acidity of soil affects other species of *Plethodon*, including the microendemics. If these factors do not affect salamanders directly, they certainly affect the plants and microbes surrounding the salamanders (Kaiser et al., 2016; Zhou et al., 2024), thereby indicating heterogeneity in nutrient availability (Shen et al., 2021; Jahn et al., 2025) in the ecosystem, which can affect the habitat and prey available to the salamanders.

Distributions of plethodontid species vary considerably. Many closely related species have extremely limited distributions, sometimes under 100 km^2. The Peaks of Otter Salamander, *Plethodon hubrichti*, only occurs within a 19 km stretch of the Blue Ridge Mountains in Virginia but is locally abundant (Taub, 1961; Farallo and Miles, 2016). Similarly, the Big Levels Salamander, *P. sherando*, is located in a very limited range of less than 80 km^2, but is also plentiful where it is found (Bayer et al., 2012). In contrast, the Eastern Red-backed Salamander, *P. cinereus*, is one of the most abundant and widespread salamanders in the eastern United States (Petranka, 1998), found over nearly 1 million km^2, including within parts of and outside the ranges of both *P. hubrichti* and *P. sherando* (Fig. 1). Given the importance of these salamanders to forest ecosystems through a top-down regulation of forest floor food webs (Walton, 2005, 2013; Walton and Steckler, 2005; Walton et al., 2006; Hickerson et al., 2012; Paluh et al., 2015), understanding what limits their ranges is critical for conserving these species in the face of global climate change.

Morphological differences can have an impact on a species' ability to traverse their environment. For example, rocky substrates are associated with longer limbs in Australian skinks (Goodman et al., 2008). Morphologically, the microendemic species are distinct from the widespread *P. cinereus*, which may offer advantages in certain microhabitats. *Plethodon hubrichti* is more elongated than *P. cinereus*, with longer limbs and a larger head (Arif et al., 2007). In the salamander genus *Ambystoma*, longer snout–vent length was not related to the ability to burrow; however, ambystomatids are known to actively burrow, whereas plethodontids will typically utilize pre-established burrows and underground crevices with some capacity to enlarge them (Heatwole, 1960; Semlitsch, 1983). Compared to *P. cinereus*, *P. sherando* has a larger head and longer limbs, but a shorter body (Highton, 2004). Although vertebra number is similar between our three focal species (19–21), there is variation and increased vertebra number is associated with fossoriality (Jockusch, 1997; Fisher-Reid and Wiens, 2015). Differences in morphology could result in microhabitat selection, especially if soil structure differs geographically.

Microbial communities are also part of the soil components that are important in salamander biology, especially for their health and survival. The soil acts as an environmental reservoir, promoting the assembly of skin symbionts (Loudon et al., 2014a). For example, controlled experiments have demonstrated that soil bioaugmentation with specific bacteria with anti-fungal activity can protect salamanders from infection with *Batrachochytrium dendrobatidis*, a major pathogen threatening amphibians globally (Muletz et al., 2012; Loudon et al., 2014b). In addition, studies have shown

that the type of vegetation influences the abundance of certain bacterial taxa, such as those in the family Chlamydiaceae. Taxa from the family Chlamydiaceae include potential salamander pathogens and were found to be more prevalent on the skin of salamanders inhabiting invasive *Eucalyptus* environments when compared to native *Quercus* habitat (Hernández-Gómez et al., 2020), highlighting a connection to habitat and microbial communities that impact salamanders. However, soil microbial diversity remains relatively underexplored, and the few available studies mentioned above highlight essential interactions between soil microbial communities and amphibian health.

This project aimed to provide exploratory data characterizing the soil within the ranges of the two microendemic species, *P. hubrichti* and *P. sherando*, and make comparisons to soil in adjacent locations, where the widespread *P. cinereus* occurs in allopatry. We measured abiotic aspects of soil structure including soil texture, bulk density, porosity, and pH. We also quantified the bacterial soil microbiota at each site. Our hypotheses are rooted in morphological differences between the species in conjunction with observational data of their microhabitats, specifically belowground refuges. We predicted that both abiotic and biotic soil properties in the ranges of *P. hubrichti* and *P. sherando*, and *P. cinereus* would be different. Specifically, we hypothesized that soil within the range of both microendemics will have lower bulk density, higher porosity, less organic matter, and larger soil particles, as the microendemics' morphological differences (e.g., larger heads and longer limbs) may be advantageous moving underground with those soil conditions. We also hypothesized that microendemic sites will have a higher pH than the *P. cinereus* sites, as *P. cinereus* is shown to be tolerant to acidic soils. Finally, we hypothesized that microbial communities would be more diverse within the *P. cinereus* sites compared to the sites of both *P. hubrichti* and *P. sherando*, as limited microbial diversity in the range of the microendemics could cause adaptation preventing dispersal outside their current distribution.

MATERIALS AND METHODS

Field sites and sample collection.—We utilized four sampling sites for our study. Two of the sites were chosen within the range of microendemics *Plethodon hubrichti* (37.51211, –79.52304) and *Plethodon sherando* (37.92070, –79.06765) located in George Washington and Jefferson National Forests. The remaining two sites were paired with the microendemic sites but outside of their range, yet still within 10 km. The widespread *Plethodon cinereus* is present at both of these sites while neither microendemic species is present, as we expect that either *P. cinereus* is able to outcompete the microendemics at these locations or aspects of the environment are not suitable to the microendemic species. However, the factors that shape the microendemic species distributions are not clear, as numerous studies have suggested different mechanisms maintaining the range of high elevation microendemic species of *Plethodon*, including climate, microhabitat use, morphology, and competition (Arif et al., 2007; Grant et al., 2018; Amburgey et al., 2020; Waldron et al., 2024). At each site, an approximately 30 cm^3 volume portion of soil was collected from a randomly selected location. This single sample from each site was used to provide approximations of large rocks within the soil. Within each site, 12 random plots were selected. In each plot, two soil samples were taken from the surface, including one soil core (15.24 cm tall cylinder with a radius of 2.54 cm; AMS Inc.) that provided an exact volume (308.89 cm^3), and one medium-sized bucket (1.9 L). We used soil cores to find bulk density and porosity, and the bucket samples to find pH, SOM, and soil texture. Additionally, a small scoop (~10–15 g) of soil was also collected from the 12 random plots at each site in a 532 mL Whirl-Pak for analyses of bacterial communities. Soil sampling locations did not directly correlate with the capture of a salamander, but based on previous work, salamander density is high, and they are found throughout each of the sites. All equipment used to sample soil for this collection was sanitized between sites with 70% ethanol. These bacterial samples were transported in a cooler box to our lab at the University of Scranton where they were stored in a freezer before being sent (packed in dry ice) to the University of Florida for analysis.

Soil processing and data collection

Sample preparation.—Samples that required oven-drying (for bulk density, porosity, and soil texture) were transferred to aluminum pans and dried at 105°C for 24 hours before use. Samples that required air-drying (for pH and SOM) were transferred to aluminum pans and dried at room temperature (20°C) for >4 days or until constant weight was achieved. Mass was recorded to the nearest 0.01 g using a digital analytical balance.

Bulk density and porosity.—Bulk density was found by the oven-dried weight of each sample (from a soil core) divided by the volume of the soil core (Eq. 1).

$$\text{Bulk Density} = \text{Mass}_{\text{Dry Soil}}/\text{Volume}_{\text{Soil Core}} \quad \text{Eq. 1}$$

Porosity was determined by using the displacement of soil solids in 50 mL pycnometers. We used the samples from the same soil cores used to calculate bulk density and then sieved the samples through a #10 sieve (2 mm) to remove gravel and larger rocks. We calibrated each pycnometer for volume by weighing the mass of the pycnometer and the mass of the pycnometer filled with water; the difference is the true volume of each pycnometer. Then, we transferred 10 g of oven-dried soil to the pycnometer and weighed it again. We added water to fill approximately half the pycnometer and then placed the pycnometers in a heated sand bath for 10 minutes to allow air bubbles to escape. We then filled the pycnometer completely with water, replaced the top pin of each pycnometer, and recorded the final mass measurement. We calculated porosity by Eq. 2–5.

$$\text{Volume}_{\text{Water Displaced by Soil}} = (\text{Mass}_{\text{Pycnometer}} + \text{Mass}_{\text{Soil}} + \text{Mass}_{\text{Water}}) - (\text{Mass}_{\text{Pycnometer+Soil}}) \quad \text{Eq. 2}$$

$$\text{Volume}_{\text{Soil}} = \text{Volume}_{\text{Pycnometer}} - \text{Volume}_{\text{Water Displaced by Soil}} \quad \text{Eq. 3}$$

$$\text{Particle Density} = \text{Mass}_{\text{Soil}}/\text{Volume}_{\text{Soil}} \quad \text{Eq. 4}$$

$$\text{Porosity} = 1 - (\text{Bulk Density}/\text{Particle Density}) \quad \text{Eq. 5}$$

pH.—To measure soil pH, we created a soil slurry. We mixed neutral-pH water with each soil sample at a 1:1.5 soil to water

mass ratio. We used approximately 10 g of soil from each air-dried sample and 15 mL of water. We mixed the sample intermittently for 30 minutes and immersed a pH probe (ETI 8100 Plus pH Meter) in the water that pooled above the soil until it had a stable pH reading (Gavlak et al., 2005).

Soil organic matter.—Soil organic matter was found by the loss by ignition method (Sullivan et al., 2019). Each sample was processed in a ceramic crucible. First, we recorded the empty crucible mass, then added 10 g air-dried soil to the crucible. The crucible was then placed in a drying oven set to 105°C for 2 hours to remove any excess water. We re-weighed the mass of the crucible and the dried soil, then placed the crucible in a muffle furnace. The samples baked in the furnace for two hours at 360°C, burning off any organic matter in the sample. Afterward, we made the final mass measurement of the crucible with the soil without organic matter. The soil organic matter is calculated by the percentage of mass lost after ignition (Eq. 6).

$$\%\,\mathrm{SOM} = (\mathrm{Mass}_{\text{before ignition}} - \mathrm{Mass}_{\text{after ignition}})/\mathrm{Mass}_{\text{before ignition}} \quad \text{Eq. 6}$$

Soil texture.—Two methods were used to determine soil texture. First, the sieve method was used to find the size distribution of soil particles larger than 63 microns. We used the remaining soil from the 1.9 L containers (12 from each site) and oven-dried them before processing. We also processed the 30 cm^3 samples (one from each site); if there were rocks too large to fit in the soil sieves, we weighed them separately. Although we feel the texture analyses from the 30 cm^3 samples are important, given we only processed a single sample per site, our assessment is anecdotal. We weighed each soil sieve (Frey Scientific Fieldmaster Steel Sieves) and stacked them with the largest opening sieves at the top and the smallest at the bottom. Then, we added the dried soil to them, placed the sieves in a sieve shaker (Gilson Company, Inc. Sieve Shaker SS-15D), and allowed it to shake each sample for 10 minutes. Afterward, we weighed each sieve. Percent retained by each sieve and percent passing (or percent finer) were calculated according to Eq. 7–10.

$$\mathrm{Soil}_{\text{Retained}} = \mathrm{Mass}_{\text{Sieve + Soil Retained}} - \mathrm{Mass}_{\text{Sieve}} \quad \text{Eq. 7}$$

$$\mathrm{Percent}_{\text{Retained}} = \mathrm{Soil}_{\text{Retained}}/\text{Total Dry Mass of Sample} \quad \text{Eq. 8}$$

$$\mathrm{Cumulative}_{\text{Retained}} = \text{Previous Cumulative}_{\text{Retained}} + \text{Current Percent Retained} \quad \text{Eq. 9}$$

$$\text{Percent Finer} = 100 - \mathrm{Cumulative}_{\text{Retained}} \quad \text{Eq. 10}$$

The size distribution of particles smaller than 63 microns were determined by the fractionation or hydrometer method (Gavlak et al., 2005). We used ASTM 151H hydrometers, which we calibrated a zero-correction factor (Z_C) and meniscus correction (C_M) for each use (Bowles, 1992). The soil that was collected by the pan in the sieve portion of analysis was saved to use in the hydrometer portion. Each sample soaked for 24 hours in 125 mL of 5% by weight sodium hexametaphosphate (SHMP), a particle dispersing agent. Then, we added the soil-SHMP mixture to a soil dispersion cup, washed down the edges with DI water, and blended the solution with a soil dispersion mixer (Hamilton Beach) for 2 minutes. Then, we added the soil-SHMP mixture to a 1 L sedimentation cylinder and filled it with DI water just below the 1 L mark. We placed a stopper on the top of the cylinder and inverted the cylinder 30 times. Immediately after inversions, we started a stopwatch and washed down the sides of the cylinder to bring the solution to the 1 L mark. Readings were made with the hydrometer at 2, 4, 8, 16, and 30 minutes and 1, 2, 4, 8, and 24 hours and recorded as R_A. We also recorded the temperature of the soil solution for the temperature correction factor (Bowles, 1992). We made calculations of percent finer according to Eq. 11–12 and particle diameter (D) according to Eq. 13 and constants L and K (Bowles, 1992), which allowed us to determine the percentage retained of particle sizes under 63 microns that was then combined with the particle size data from the sieve measurements.

$$\text{Corrected Hydrometer Reading}\,(R_C) = R_A - Z_C + C_T + C_M \quad \text{Eq. 11}$$

$$\%\ \text{Finer} = R_C/\mathrm{Mass}_{\text{Dry Sample}} * 100 \quad \text{Eq. 12}$$

$$D = K\sqrt{(L/t)} \quad \text{Eq. 13}$$

Microbial community processing.—After collection, an aliquot of soil from each site was frozen and sent to the University of Florida for bacterial community characterization. Soil was homogenized in a laminar flow hood using sterilized scoopulas and subsampled to obtain five replicates per site. Total genomic DNA (gDNA) was extracted using a DNeasy PowerSoil Pro kit (QIAGEN, Germantown, MD, USA) following the manufacturer's protocol with an initial input of 0.25 g of soil. UltraPure™ DNase/RNase-Free distilled water (Invitrogen, MA, USA) and ZymoBIOMICS™ Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA) were also extracted and included in sequencing as controls. We eluted DNA in 50 µL for downstream analyses, kept at –20°C. Post extraction, we quantified each DNA sample using a Qubit 4 fluorometer (Invitrogen, MA, USA) and, when necessary, normalized using 10 mM Tris-HCl pH 8.0–8.5 prior to amplification. Full-length 16S rRNA gene regions were then PCR amplified using barcoded primers following the 'Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell® Library Preparation and Sequencing' protocol (Pacific Biosciences, Menlo Park, CA, USA) for 27 cycles. Amplification products were visualized via electrophoresis on a 1.2% agarose gel, pooled, and purified using AMPure PB beads (Pacific Biosciences, Menlo Park, CA, USA) before library construction. SMRTbell adapters were then ligated onto purified PCR products using SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA). Libraries were sequenced on a PacBio Sequel IIe system at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR).

We processed sequence data in R v4.1.1 (R Core Team, 2024), first filtering reads less than 1,000 base pairs (bp) and greater than 1,600 bp. Filtered reads were further processed using the DADA2 pipeline (Callahan et al., 2016), with default settings for PacBio reads (v1.20.0, following tutorial https://benjjneb.github.io/LRASManuscript/LRASms_fecal.html), providing a table of amplicon sequence variants (ASVs). Taxonomy was assigned using the Silva database v138.1 (Quast et al., 2013; accessed 17 May 2022). Sequence and taxonomy data were combined using the package *phyloseq* (McMurdie and Holmes, 2013) under which we conducted microbial community analyses. To determine the presence of bacterial contaminants, we ran our data through the *decontam* package (Davis et al., 2018) using laboratory controls (e.g., PCR water control) as 'controls' with all others assigned as 'true samples.' We found no instances of contamination present in the dataset. Mock community standard results matched manufacturer estimates.

Statistical Analysis

Abiotic soil characteristics.—Statistical analyses of the abiotic soil characteristics were performed in R v4.4.2 (R Core Team, 2024). We specifically made comparisons between the microendemics and the respective adjacent *P. cinereus* sites (e.g., *P. hubrichti* and *P. cinereus* near *P. hubrichti*). For bulk density, porosity, pH, and SOM, we tested the assumption of normality with the Shapiro-Wilkes test with the *shapiro.test* function and equal variance by the Bartlett test with the *bartlett.test* function. If assumptions were met, we used the function *t.test* to conduct a t-test to find if there were any differences between sites. If assumptions were not met, we used the function *wilcox.test* to conduct a non-parametric Wilcoxon rank-sum test. Soil texture data were separated into categories based on the Wentworth particle size scale (Wentworth, 1922). Specifically, we used the categories of gravel (>2 mm), sand (2 mm > 0.0625 mm), silt (0.0625 mm > 0.0078 mm), and very fine silt/clay (<0.0078 mm). We removed seven samples with noted measurement errors during the sieve data collection (e.g., total mass retained deviated more than 5% from 100%). Each site had a minimum of ten samples even after removal. It should be noted that based on our hydrometer measurements, our particle size delimitation is 0.007 mm, which means our categories are approximating the size class at the boundaries of silt and clay (see Wentworth, 1922 for detailed description of size classes). We used the *clr* function in the *compositions* package (Boogaart et al., 2024) to perform a center-log transformation to account for the lack of independence of the data, as the percentage of the four categories sum to 100%. The transformed data were analyzed using the function *adonis2* from the *vegan* package (Oksanen et al., 2025) to perform a permutational analyses of variance (PERMANOVA) comparing soil texture between the microendemics and the respective adjacent *P. cinereus* sites (e.g., *P. hubrichti* and *P. cinereus* near *P. hubrichti*). If differences were detected, we performed separate Wilcoxon rank-sum tests for each particle size category. The *P*-values were adjusted for multiple comparisons with a Benjamini and Hochberg correction (Benjamini and Hochberg, 1995) using the function *p.adjust*.

Biotic soil characteristics.—To test differences in bacterial community composition between paired sites, we used package *phyloseq* to calculate three diversity metrics based on the ASVs at their highest level of taxonomic resolution (i.e., richness, Shannon diversity index, and Bray-Curtis distances). We compared average values of ASV richness and Shannon diversity among paired sites using ANOVAs. We also tested the association of bacterial composition (Bray-Curtis distances) with each site using PERMANOVA. In addition, we visualized shared ASVs among sites and salamander species using R package *ggVennDiagram* (Gao et al., 2021). We constructed relative abundance plots at the phylum level after filtering for low abundance reads comprising less than 0.02% of the total ASVs using R package *bbplot*. To explain the variance, we integrated abiotic and biotic soil characteristics and performed a distance-based redundancy analysis (dbRDA) using Bray-Curtis distances and bulk density, porosity, pH, percent soil organic matter, and soil texture.

RESULTS

Abiotic soil characteristics.—We found significantly lower bulk density of the soil at the *P. sherando* sites (0.880 g/cm^3) compared to *P. cinereus* near *P. sherando* sites (0.979 g/cm^3; $t_{22} = -2.55$, $P = 0.018$; Fig. 2A). The *P. hubrichti* site (0.733 g/cm^3) did not have a significantly different bulk density than its respective *P. cinereus* site (0.811 g/cm^3). We found no differences in the porosity of the soil between the *P. hubrichti* site (63.9%) and the *P. cinereus* near *P. hubrichti* site (63.2%; $t_{22} = 0.317$, $P = 0.754$; Fig. 2B) and no differences in porosity between the *P. sherando* site (50.1%) and *P. cinereus* near *P. sherando* site (53.7%; $t_{44} = -1.802$, $P = 0.085$; Fig. 2B). We found significantly lower pH of the soil at the *P. hubrichti* site (4.90) compared to the *P. cinereus* near *P. hubrichti* site (5.69; $W = 33.5$, $P = 0.028$; Fig. 2C) and at *P. sherando* site (4.37) compared to *P. cinereus* near *P. sherando* site (4.84; $W = 0.5$, $P < 0.001$; Fig. 2C). We found no differences in the soil organic matter of the soil at the *P. hubrichti* site (9.48%) and the *P. cinereus* near *P. hubrichti* site (8.45%; $W = 100$, $P = 0.112$; see Fig. 2D), and no differences in soil organic matter at the *P. sherando* site (7.85%) and *P. cinereus* near *P. sherando* site (7.43%; $W = 84$, $P = 0.507$; see Fig. 2D).

We found significant differences in the soil texture between the *P. hubrichti* site and the *P. cinereus* near *P. hubrichti* site (PERMANOVA: $F_{1,19} = 6.85$, $P = 0.001$). There was no significant difference in the composition of gravel ($P = 0.126$) or silt ($P = 0.126$), but significantly less sand ($P = 0.003$) and more clay ($P = 0.002$) at the *P. hubrichti* site (Fig. 3). We also found significant differences in the soil texture between the *P. sherando* site and the *P. cinereus* near *P. sherando* site (PERMANOVA: $F_{1,20} = 6.85$, $P = 0.005$). There was no significant difference in the composition of gravel ($P = 0.809$) or very fine silt/clay ($P = 0.467$), but significantly less sand ($P < 0.001$) and more silt ($P = 0.012$) at the *P. sherando* site (Fig. 3). We did not statistically compare the large soil samples (~30 cm^3), as we collected one sample per site. However, it should be noted that only the microendemic sites contained large rocks (>100 mm). The sample from the *P. hubrichti* site was 27.1% rock by mass and *P. sherando* 19.8% rock by mass, with the remaining soil including gravel, sand, silt, and clay. The two samples from *P. cinereus* sites contained 0% rock.

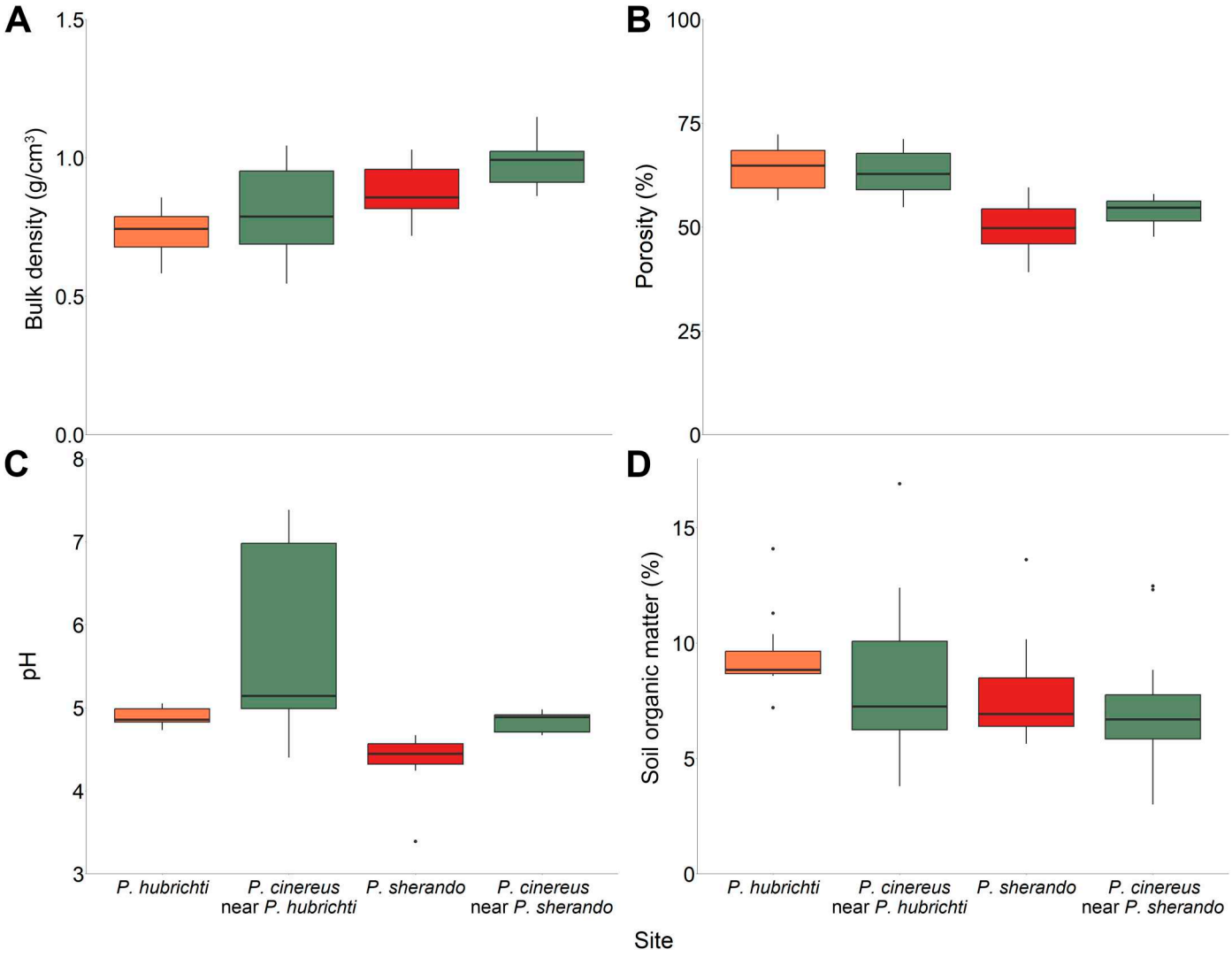


Fig. 2. Box plots of bulk density (A), porosity (B), pH (C), and soil organic matter (D) of soil within the geographic ranges of *P. hubrichti* and *P. sherando* and 10 km outside each range, where *P. cinereus* occurs. Samples collected from George Washington and Jefferson National Forests during the summer of 2021. The line within the box represents the median, while the box boundaries indicate the 25th and 75th percentiles. The whiskers extend to the smallest and largest values within 1.5 * interquartile range from the quartiles. Points beyond the whiskers represent outliers.

Microbial community composition.—Soil microbial communities were mostly comprised of bacteria in the phylum Proteobacteria, with 37% of total sequences followed by Planctomycetota (23%), Acidobacteriota (16%), Verrucomicrobiota (7%), and Actinobacteriota (5%). We found that both observed and Shannon diversity estimates varied across sites (Fig. 4A), but did not differ between microendemic and widespread species habitat (Kruskal-Wallis: observed—$X^2 = 1.74$, $P = 0.62$, df = 3; Shannon—$X^2 = 1.37$, $P = 0.71$, df = 3). However, sites harbored distinct bacterial communities when comparing diversity between samples (PERMANOVA: $F_{3,16} = 2.95$, $P = 0.001$; pairwise PERMANOVA: $P < 0.05$ for all comparisons; Fig. 4B). Bacterial communities associated with microendemic salamander habitats differed from nearby habitat in the widespread salamander, *P. cinereus*, range (PERMANOVA: $F_{1,18} = 1.44$, $P = 0.008$; Fig. 4B). Microendemic sites only shared 1% of total amplicon sequence variants (ASVs) compared to the 38 (2%) and 116 (5%) of ASVs shared between *P. hubrichti* and *P. sherando* with their widespread range adjacent sites, respectively (Fig. 4C). Sequence variants specific to one habitat comprised 86% of all sequences, leaving few shared ASVs between sites, even when comparing similarities across all sites (1%; Fig. 4C). Bacterial phyla show differences in relative abundance among sites, especially for the most abundant taxa, Proteobacteria, Planctomycetota, and Acidobacteriota (Fig. 4D). Soil abiotic factors potentially driving the terrestrial microbial community showed bulk density contributing more to *P. sherando* associated sites and porosity, pH, and percent soil organic matter having a greater influence on *P. hubrichti* associated sites (Fig. 5); however, these predictors alone did not explain a significant portion of the community variability (ANOVA: $F_{5,14} = 1.04$, $P = 0.074$).

DISCUSSION

Our analysis of abiotic and biotic soil characteristics points to several potential factors that may influence the distributions of the two microendemic species, *P. hubrichti* and *P. sherando*. We found significantly lower bulk density at the *P. sherando* site compared to the adjacent *P. cinereus* site, which supported our hypothesis; however, it is unclear if

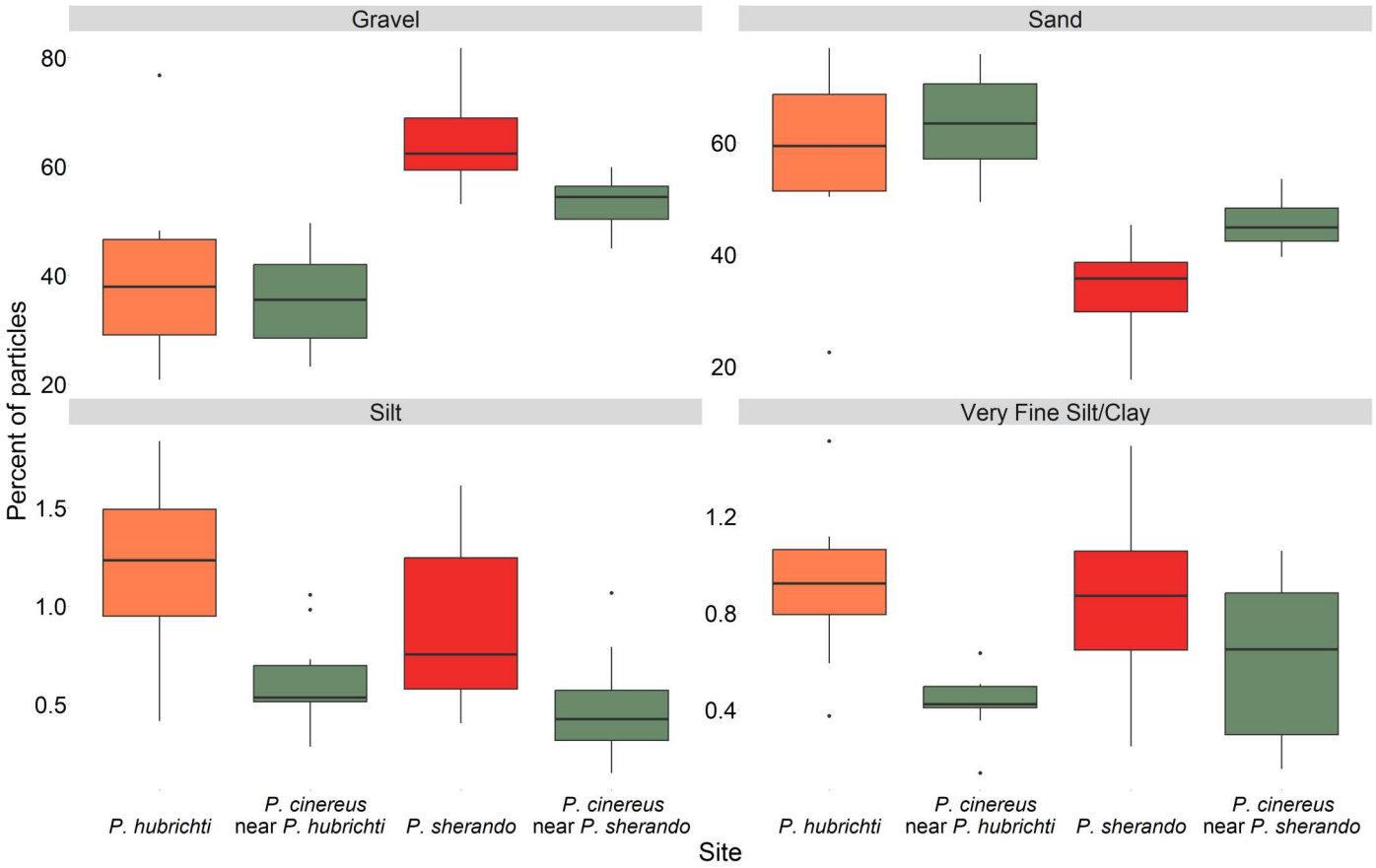


Fig. 3. Box plots of gravel, sand, silt, and very fine silt/clay from our soil texture measurements. Gravel and sand were determined using a sieve method, while silt and very fine silt/clay were determined using a hydrometer method. Samples were collected from George Washington and Jefferson National Forests during the summer of 2021. The line within the box represents the median, while the box boundaries indicate the 25th and 75th percentiles. The whiskers extend to the smallest and largest values within 1.5 * interquartile range from the quartiles. Points beyond the whiskers represent outliers.

this is an ecologically significant difference. For example, bulk density can impact soil moisture retention, with higher bulk densities resulting in lower soil moisture content as the compaction can prevent water infiltration into the soil (Nemček-Korenkova and Urík, 2012; Ilek et al., 2017). Our values of bulk density were all low and typical of a temperate forest, so the difference would likely have a limited impact on soil moisture. However, given the lack of available data on how bulk density directly affects salamanders and their ability to use the soil, more research is needed to determine if this degree of difference would impact belowground movement between the species. The porosity and soil organic matter of the soil within the microendemic ranges did not differ from the *P. cinereus* sites outside each of the microendemics respective range, which did not support our hypothesis. Therefore, it is not likely that porosity or soil organic matter are directly impacting the distribution of *P. hubrichti* and *P. sherando*.

Soil at both of the microendemic sites had a significantly lower proportion of sand compared to the adjacent *P. cinereus* sites. Soils with a higher percentage of sand particles do not retain as much water as soils with higher silt and clay percentages (Rai et al., 2017). Terrestrial salamanders need moist conditions to respire by cutaneous respiration, but not so wet as to prevent gas exchange (Welsh and Droege, 2001). These results coincide with previous microhabitat work on these microendemics (Farallo and Miles, 2016), with lower soil moisture found in the range of both *P. hubrichti* and *P. sherando*. Although no statistical differences were found in the soil moisture at salamander capture points compared to random absence points, both microendemics trended toward selecting higher soil moisture locations compared to lower soil moisture absence points (Farallo and Miles, 2016). Again, although we did not detect a statistical difference in gravel, both microendemic sites trended toward more gravel, with 65% gravel for *P. sherando* compared to 53% gravel in the adjacent *P. cinereus* site, and 40% gravel at the *P. hubrichti* site compared to 36% at the adjacent *P. cinereus* site. Additional evidence indicating larger particle size of substrates in the microendemics was seen in our 30 cm^3 soil samples. We only found large rocks in the samples from the microendemic ranges with none found in the samples from *P. cinereus* sites. The microendemic salamanders may select for microhabitats within the cavities created by these rocks within the soil, outcompeting *P. cinereus* where this habitat type occurs, while the presence of these rocks may also contribute to soil acidity and microbial community composition (Agnelli et al., 2001; Hong et al., 2021; Huang et al., 2023). This characteristic of the habitat in microendemic ranges, including talus slopes and otherwise rocky habitat, has been mentioned anecdotally in the literature before

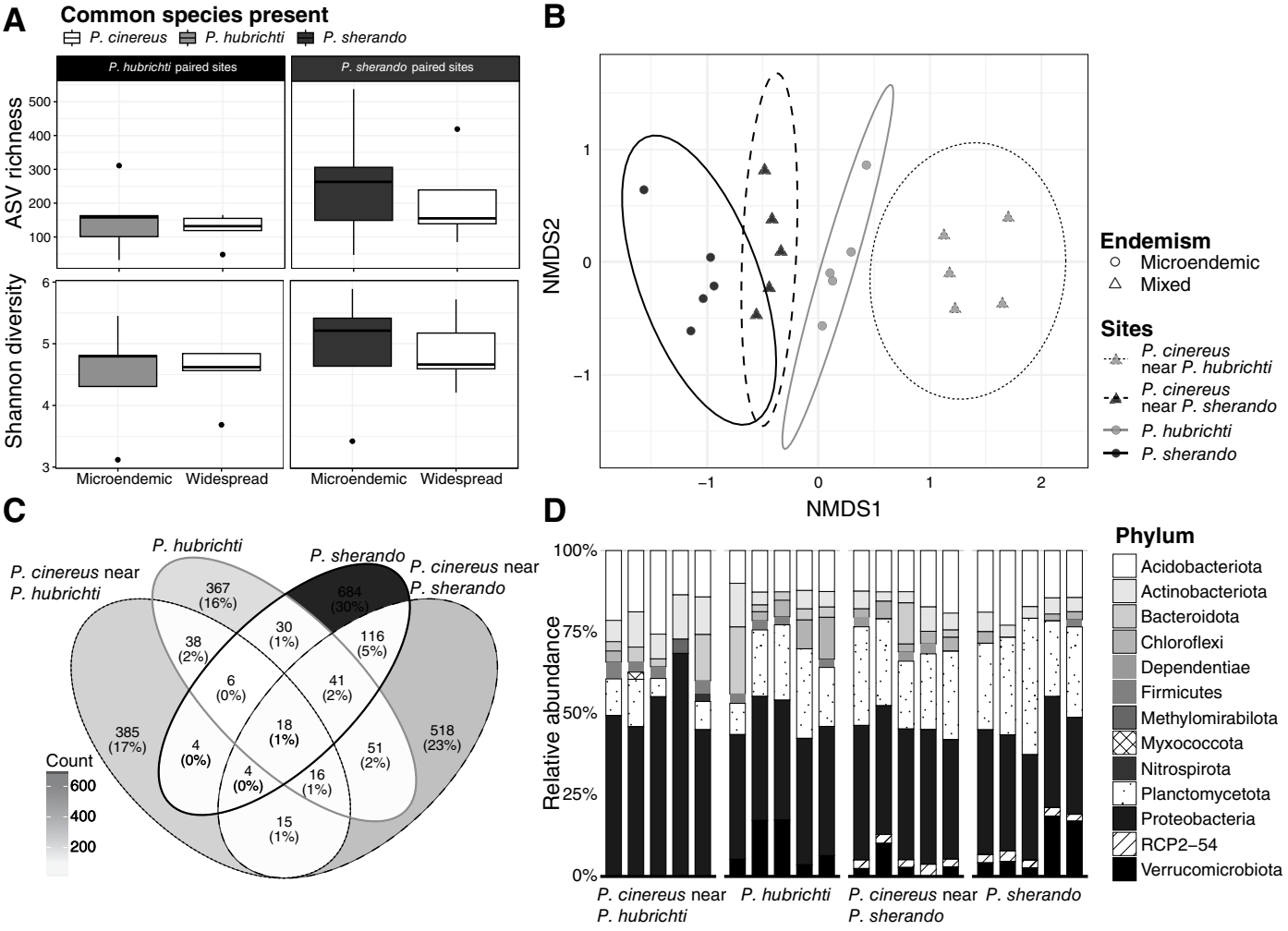


Fig. 4. Soil bacterial diversity across the four study sites. (A) Box plots of alpha diversity in terms of the number of amplicon sequence variants (ASVs) and Shannon diversity index. (B) NMDS ordination plot using Bray-Curtis distances shows significant differences among the centroids of each site cluster. (C) Venn diagram indicates the differences in the number of shared ASVs across sites. (D) Bar plot shows the relative abundance of ASVs across bacterial phyla. Each bar represents one soil sample.

(Highton, 2004; Farallo and Miles, 2016). As has been suggested previously (Farallo and Miles, 2016) morphological difference in head shape and limb length may impact how microendemics are able to navigate these larger rocks and resulting crevices below ground. Unfortunately, there are limited options for quantifying these types of habitat differences. Collecting multiple samples that are large enough to allow for quantifying the presence of large rocks could have a negative impact on the habitat of these species with very small ranges. Furthermore, transporting samples of this large size would be extremely challenging. The best option may require techniques able to monitor underground conditions without disturbing the surface, such as ground penetrating radar (Jin and Duan, 2020).

In contrast to our expectation, soil pH was more acidic in the microendemic ranges and microbial communities also differed. The soil pH in all sites was acidic, with averages ranging from 4.37 to 5.69. This is likely due to historic acidic rain of pH 4.3 (USDA, 2004). While acidic soil can be tolerated by *P. cinereus* (pH < 3.4), it is known to cause disrupted embryonic development and lower growth rates (Bondi et al., 2016), and *P. cinereus* are typically found less frequently when soil pH is lower than 3.7 (Wyman and Hawksley-Lescault, 1987). Other plethodontid salamander species have also shown preference for more basic substrate (Mushinsky, 1975). Despite their restricted range, our data indicate that *P. sherando* and *P. hubrichti* may be able to tolerate more acidic soils than *P. cinereus*. Historic acid deposition and regional geology could have influenced the evolution of the microendemic species such that they are now better adapted to acidic conditions, therefore outcompeting *P. cinereus* in regions of lower pH.

In addition to abiotic differences, we also found significantly different bacterial composition, which can link belowground and aboveground community assembly processes (Crowther et al., 2019). Microbial communities can be sensitive to relatively small changes in abiotic soil characteristics (Kuramae et al., 2012). Our findings suggest significantly different soil bacterial composition at each site, even though alpha diversity metrics were similar. Decoupling of alpha and beta diversity is expected, as the latter can reflect large-scale dynamic changes governed by bacterial dispersal (Walters and Martiny, 2020). However, we expected to find similar bacterial communities inhabiting the sites dominated by *P. cinereus*. In

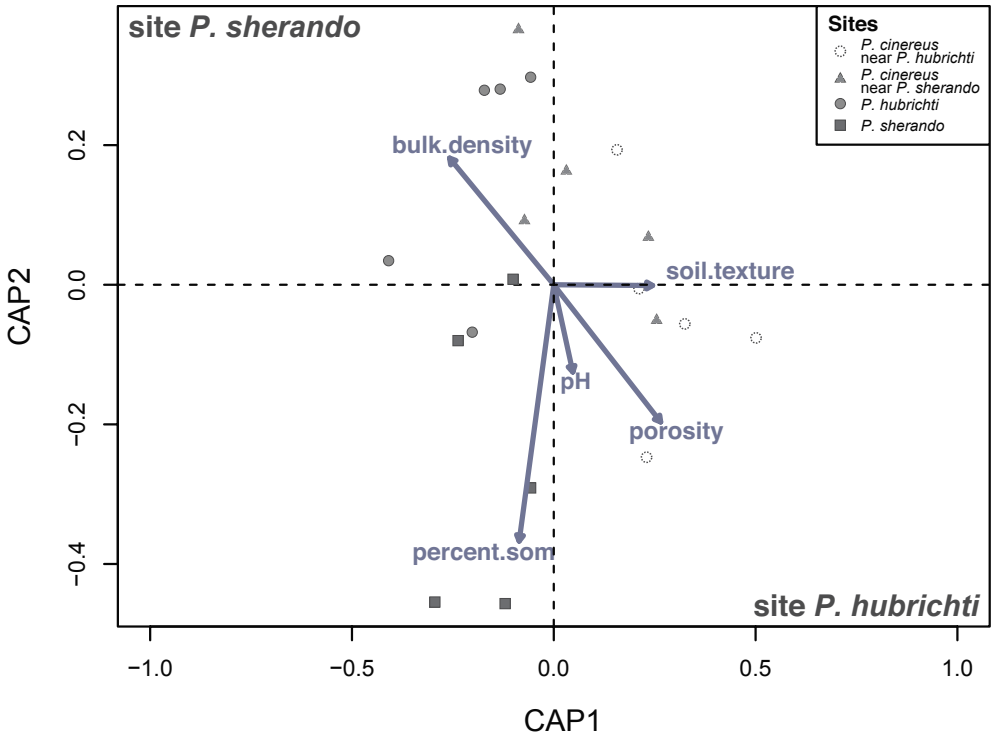


Fig. 5. Distance-based redundancy analysis testing environmental predictors of soil bacterial communities. Although the direction of the predictor arrows and microendemic sites are at opposite sides of the ordination space, the permutation test was not significant ($F = 1.02$, df = 5, $P = 0.247$). This result indicated that the environmental predictors had a negligible relationship with bacterial community composition.

contrast, soil bacteria from neighboring sites were more similar to each other. Most of the differences were driven by bacteria in phyla Proteobacteria, Verrucomicrobiota, Planctomycetota, and Bacteroidota. These patterns became more evident when we compared shared versus unique ASVs across sites, as the overlap in ASV occurrence was extremely low. Bacterial communities in plethodontids form an integral part of their defenses against diseases (Muletz et al., 2012; Loudon et al., 2014a; Walker et al., 2020). Thus, understanding the factors that shape soil microbial communities can help us predict differences in microbial recruitment that could lead to increased susceptibility to pathogens.

An important aspect of studying soil microhabitats is the variability through geographic regions. Throughout all our sample sites and with all variables, high standard deviations were common. It is also worth noting that our soil samples were not tested for the correlation with the presence of any salamanders—disparities in the soil characteristics that we described in this project could be increased (or reduced) if the samples are specific to salamander habitat preference at a smaller spatial scale. Further research should also investigate exactly how the microendemic salamander species interact with their soil habitat with experiments that test salamander fitness and performance under different soil conditions.

The goal of this study was to explore potential mechanisms for the restricted distribution of mountaintop endemic salamanders. Despite being an ever-present component of the life of plethodontid salamanders, there are still large gaps in our knowledge of how soil directly and indirectly impacts aspects of their ecology. The data collected during this project help narrow the focus to key components of soil that may have more substantial impacts on these salamanders. Furthermore, the data can be used to recreate soil conditions for use in controlled experiments. Salamanders can be maintained under these established soil conditions in a laboratory setting to determine if the soil impacts their fitness or behaviors. Overall, this project is an initial step for better understanding the role of soils in maintaining salamander distributions and hopefully inspires additional studies on the topic.

DATA ACCESSIBILITY

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AI STATEMENT

The authors declare that no AI-assisted technologies were used in the design and generation of this article and its figures.

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Janice Healy: Investigation, Writing—review & editing; Sarah McGrath: Formal analysis, Visualization, Investigation, Writing—review & editing; Ana Longo: Formal analysis, Visualization, Writing—review & editing; Vincent Farallo: Conceptualization, Project administration, Formal analysis, Visualization, Writing—review & editing.

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