Assessing Subterranean Arthropod Diversity through COI Barcoding in Two Ecoregions of Southwestern Virginia, USA.

Garrett Harrison

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Paul Marek, Chair

Alejandro Del Pozo-Valdivia

Kaloyan Ivanov

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Academic Abstract

Subterranean arthropod communities are important components of North American ecosystems, contributing numerous ecosystem services and essential food-web functions. Despite this, fundamental information about species diversity in these communities remains unknown, and their taxonomic composition and ecological diversity have scarcely been assessed. Subterranean pitfall traps are a commonly used method for sampling endogean and hypogean soil habitats in Europe but have never been widely implemented in North America. Here, I employed this method to sample subterranean arthropod communities in the Ridge and Valley and Blue Ridge ecoregions of Virginia, USA in the winter and spring. In total, 2,260 arthropod specimens were collected constituting 319 distinct species. I extracted and purified DNA and amplified the mitochondrial gene: cytochrome C oxidase subunit I (COI) from each recovered morphospecies and derived a unique COI barcode for each species sequenced. Objective sequence clustering was used to establish molecular operational taxonomic units (mOTUs) for downstream diversity analyses and establishment of dynamic identification resources. Total species richness and average species richness per site were assessed and compared for both regions and seasons. The Shannon-Wiener diversity index, Hutcheson's t-test, and effective numbers of species (ENS) were employed to compare regional subterranean arthropod diversity. The richness, Shannon-Wiener, and ENS comparisons indicated that both the Ridge and Valley and Blue Ridge regions of Virginia, USA encompass highly diverse subterranean arthropod communities with those of the Ridge and Valley being significantly more diverse than those of the Blue Ridge.

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General Audience Abstract

Arthropods are invertebrate animals with hard exoskeletons, segmented bodies, and jointed paired appendages, and include insects, arachnids, crustaceans, and myriapods. Arthropods make up the majority of animal species on the planet and are important parts of ecosystems, making it important for researchers to study them. While we know a fair amount about North American arthropods that live above-ground, relatively little is known about those that live deep within the soil. Subterranean pitfall traps are a common method used for collecting arthropods from subterranean habitats in various regions of the world but have rarely been used in the USA. As a result, much of the life beneath our feet may be unknown. I used these traps to collect subterranean arthropods in the Ridge and Valley and Blue Ridge regions of the Appalachian Mountains. Approximately 2,260 individual arthropods were collected, belonging to 319 different species. I used DNA sequencing to establish a unique fingerprint-like "barcode" for each species. These barcodes serve as helpful identification resources and will help name new species in the future. They also allowed me to measure the number of species (diversity) of subterranean arthropods collected from each region and compare the two to determine which region is more diverse. I used common statistical metrics of diversity including species richness (number of species) and the Shannon-Wiener diversity index to compare regional subterranean arthropod diversity. My results show that both the Ridge and Valley and Blue Ridge regions of Virginia, USA are home to highly diverse subterranean arthropod communities and those of the Ridge and Valley are significantly more diverse than those of the Blue Ridge.

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1. Introduction

The need for understanding global biodiversity has never been more urgent. Anthropogenic habitat degradation and climate change have been implicated as the main drivers of the sixth great mass extinction event in geological history (Bellard et al. 2012). Biodiversity is at the greatest risk in highly diverse regions known as biodiversity hotspots (Myers et al. 2000; Hamilton et al. 2022). The Appalachian Mountain region of North America constitutes one such biodiversity hotspot (Stein 2000). Low-dispersal arthropods and other invertebrates are at the greatest risk of extinction due to greater rates of endemism and physiological constraints on dispersal (Canepuccia et al. 2009).

Arthropods constitute the most species-rich animal group, with recent estimations projecting approximately 7.0 million species. Of those, 5.5 million belong to the class Insecta (Santos et al. 2021). It has been estimated that 1,128,168 species of arthropods have been described by taxonomists, making up only 16.17% of the species thought to exist (Catalog of Life 2022). This displays how arthropods constitute a woefully understudied pool of biodiversity globally, one which is at high risk of species loss and anonymous extinction. A recent review of the literature assessing global declines in insect diversity, abundance, and biomass has stated that of the insect species currently described, twice as many show long-term population declines as those showing increases in abundance (Sánchez-Bayo et al. 2021). It is highly unlikely that the majority of these declining population trends are associated with natural species turnover, making assessing and understanding insect diversity now of paramount importance in the face of widespread decline.

Significant shifts in the ranges of several mobile arthropod groups such as butterflies have been implicated as impacts of anthropogenic climate change (Hickling et al. 2006). Between 1977 and 2003, a northward range shift of up to 200 km was observed in 38 species of butterflies as responses to average habitat temperature increases (Parmesan et al. 1999; Parmesan 2003). Subterranean and soil-dwelling arthropods are less mobile than their winged-lepidopteran counterparts, thus less capable of dispersal. Soil-dwelling arthropods are those that live within the soil strata beneath the leaf litter. They are highly adapted for the specific soil conditions present within their habitats, making them especially susceptible to habitat degradation (Menta and Remelli 2020). Their subterranean soil substrate may buffer immediate temperature increases, but this is poorly understood. Soil moisture and temperature rank highly amongst the abiotic factors that serve as major determinants of soil-dwelling arthropod fitness (Barnett and Johnson 2013). Global increases in soil aridity due to projected shifts in temperature and precipitation will impact the mobility and survival of subterranean arthropods, driving local and global extinctions (Sánchez-Bayo et al. 2021).

Previous reviews of literature have asserted that climate related declines in temperate soil-dwelling arthropods would produce significant bi-directional cascading effects through associated food-webs. Soil-dwelling arthropods provide essential contributions to food-web structure predominantly through plant/microbial interactions and as food sources for surface-dwelling organisms (Lang et al. 2014). They also contribute significantly to several important soil processes such as biogeochemical cycling, moisture retention, organic matter fragmentation and breakdown (Menta and Remelli 2020). These arthropod communities are significant and impactful components of their ecosystems, making describing, understanding, and conserving their diversity important for both taxonomic and conservation purposes. Assessing the

composition and diversity of these subterranean communities may provide insight into how they stand to be impacted by further anthropogenic habitat disruption.

Taxonomists, and funding for taxonomic work, are both in decline despite this work being essential for all avenues of biological research (Wheeler 2004; Godfray 2007). Taxonomy as a field is experiencing a molecular renaissance in which rapidly developing molecular techniques are hastening the pace at which taxonomists are able to discover, describe, and identify new species as well as characterize biodiversity (de Kerdrel et al. 2020; Meierotto et al. 2019). Molecular approaches to these traditionally taxonomic questions have been referred to as DNA taxonomy (Blaxter 2004). DNA taxonomy offers a promise to speed the description of planetary biodiversity but may not be a panacea to solve all challenges of the impending biodiversity crisis. It has been argued that the rise of popular DNA taxonomic techniques should not fully displace the necessary morphological perspectives that have underlined our taxonomic understanding of the natural world for centuries but should instead be integrated with these traditional perspectives to enhance them (Will et al. 2005; Rubinoff et al. 2006). DNA barcoding is one such molecular application that has enhanced the capacity for taxonomists to describe biodiversity since becoming common in the mid-2000s.

DNA barcoding is described as the sequencing of variable genetic regions between diverse taxa to establish a unique finger-print-like sequence for each taxon referred to as a barcode (Burns et al. 2007). For the purposes of this paper, this technique will be referred to as both DNA barcoding and just barcoding. Applications of barcoding include but are not limited to species delimitation, identification, characterization, and discovery. Barcoding has also been widely applied in the fields of conservation and forensics. This technique has become increasingly common with the number of papers employing and/or discussing barcoding

exceeding 3,500 as of 2018 (DeSalle and Goldstein 2019).

The mitochondrial cytochrome C oxidase subunit I (COI) gene region has become the standard gene used for DNA barcoding in animals (DeSalle and Goldstein 2019). This is especially true for invertebrates, in which the COI primer sites are highly conserved making comparative species delimitation feasible within diverse communities (Burns et al. 2007). Mitochondrial genes are haploid, maternally inherited, and have small effective population sizes (Brower 1994). In arthropods, they have been shown to evolve up to six times faster than nuclear protein-coding genes (Lin and Danforth 2012). These characteristics of mitochondrial genes and COI specifically, make it an ideal gene for barcoding in arthropods (Folmer et al. 1994; Hebert et al. 2003a; Jinbo et al. 2011). COI barcoding, when incorporated with traditional morphological taxonomy, may help to combat some of the challenges that currently face taxonomy as a field (Blaxter 2004). Essential processes such as species description, identification, and the assessment of biodiversity currently suffer from the taxonomic impediment in which immense amounts of time, labor, and specialized expertise still are direly required (Hebert et al. 2003a; Ball and Armstrong 2011; Meierotto et al. 2019).

Morphological identification can act as a major bottleneck in the assessment of biodiversity as it is time and resource intensive. Species delimitation via barcode clustering can be used to partially overcome this bottleneck by producing molecular operational taxonomic units (mOTUs) which serve as species proxies that allow diversity analyses to be conducted without complete morphological identification (Blaxter 2004). The use of mOTUs drastically increases the pace at which biodiversity assessments can be conducted (Bukowski et al. 2022). Barcode clustering relies upon divergence thresholds to molecularly delimit species. Clustering

algorithms employ barcode gaps for this. A barcode gap is defined as a divergence value, often expressed as a percentage, that differentiates interspecies divergence from intraspecific variation. Effective barcode gaps can only be established for taxa in which there is minimal overlap between interspecies divergence and intraspecific variation (Meyer and Paulay 2005).

Appropriate COI barcode gaps have been established for some groups of arthropods, but several major groups remain poorly understood (Myriapoda, Acari, several groups of Hexapoda and Insecta, etc). Previous studies have indicated that the optimal COI barcode gap for the phylum Arthropoda is 2–3% for objective clustering (Smith et al. 2005). It is rare for intraspecific variation at COI to exceed 2%, but exceptions do occur (Hebert et al. 2003a; Hebert et al. 2003b). COI divergence is an imperfect marker for establishing barcode gaps in some closely related species due to low interspecies divergence (Ratnasingham and Hebert 2013). In some closely related butterflies, it has been shown that interspecies divergence at COI can be as small as a 1 – 3 nucleotide difference (Burns et al. 2007). Due to this, erroneous clustering and splitting of closely related species can occur when using COI barcodes for species delimitation (Burns et al. 2007; Ranasinghe et al. 2022). This further illustrates how barcoding, and DNA taxonomy more generally, is not a panacea for all challenges associated with the biodiversity crisis.

The taxonomic potential of COI barcoding cannot be reached without the saturation of molecular reference databases with expertly identified voucher records representing diverse taxa. Over two million animal COI records have been uploaded to the National Center for Biotechnology Information (NCBI) and/or the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2013). This material is freely available and easily searchable using Basic Local Alignment Search Tools (BLAST) (Altschul et al. 1990). The need for this work is especially urgent in understudied and underrepresented groups such as subterranean arthropods.

Subterranean arthropod communities in North America have rarely been sampled outside of studies targeting superficial soil habitats (≤ 10 cm below-surface) (Bredeson and Lundgren 2015), cave habitats (Elliott 2000; Reeves 2002; Christman et al. 2005; Wynne and Voyles 2013; Peck and Wynne 2013; among others) and/or various taxonomically narrow groups such as ants (Hymenoptera: Formicidae) (Thompson 1980; Lubertazzi and Tschinkel 2003; Foard 2015; among others), beetles (Coleoptera) (Faille 2019; Harden et al. 2019; among others) and various others. Callaham et al. (2003) represents one of the rare instances of taxonomically indiscriminate soil arthropod sampling in North America beyond 10 cm below-surface. However, characterizing the taxonomic composition of the communities sampled was not the aim and thus little taxonomic information was published (Callaham et al. 2003).

Due to this lack of study, we know little to nothing about the diversity and taxonomic composition of the arthropod communities that occupy the shallow subterranean habitats (SSH) of North America, and especially those within the Appalachian biodiversity hotspot. The SSH can be defined as a collection of various habitats that extend up to 10 meters below soil surface, including lava-tubes, aquifers, and soil habitats containing interstitial spaces and crevices formed by pockets of eroding rock (Novak 2014). Specifically, soil SSHs can be considered ecotones as they serve as a sort of transition zone between the adjacent epigean habitats and superficial hypogean habitats (Prous 2004; Novak 2014). Epigean is a term often used to describe above ground, or surface-dwelling taxa/habitats. Hypogean is often used to describe taxa/habitats associated with the rocky substrate that lays deep below the soil surface, including portions of cave systems distant from the mouth or surface-opening (Prous 2004). Establishing concrete distinctions between different subterranean habitats is difficult as they exist more as a gradient than as distinct zones, further contributing to the challenges of characterizing their diversity. For

our purposes, SSH will henceforth be used to refer to soil habitats existing at least 10 cm below surface, but not extending into the bedrock or any cave system considered hypogean.

This study sought to sample the arthropod communities within the SSHs of the Ridge and Valley and Blue Ridge ecoregions of southwestern Virginia, USA to broadly characterize and compare their alpha diversities using common diversity metrics (observed species richness, Shannon-Wiener index, Hill numbers, rarefaction/extrapolation). These ecoregions were selected as they both were known to contain relatively shallow underlying rock substrates of differing type and were accessible for sampling (U.S. Geologic Survey 2021). We also sought to generate COI barcodes for the species sampled to contribute to the representation of subterranean arthropod genetic data in NCBI and BOLD. These habitats were previously untouched and the potential for the discovery and description of new arthropod species is high as a result.

1.1. Objectives

The objectives of this study included the assessment and comparison of subterranean arthropod diversity within SSHs sampled within the Ridge and Valley (RV) and Blue Ridge (BR) ecoregions of Virginia. Morphospecies were sorted and imaged, DNA was extracted, and the COI gene region amplified and sequenced for the generation of DNA barcodes. These barcodes were then used as a basis for generating molecular operational taxonomic units (mOTUs) and producing diversity metrics for comparative analyses of regional biodiversity.

1.2. Hypothesis and Rationale

We hypothesized that subterranean diversity would be higher in the Ridge and Valley region than in the Blue Ridge region. The rationale for this hypothesis is derived from the difference in underlying rock substrate found in the two regions. The Ridge and Valley region of Virginia is characterized by a sedimentary rock substrate, potentially indicating a greater abundance of eroded rock creating an increased number of interstitial spaces and microhabitats for endogean and hypogean arthropods when compared to the Blue Ridge region which is characterized by a harder, metamorphic rock substrate (U.S. Geologic Survey 2021).

2. MATERIALS AND METHODS

2.1. Sampling and Site Selection

Subterranean pitfall traps (HOLEs) were constructed according to the design of López and Oromí (2010). This design was selected due to the semi-permanent nature of the installation. The outer shell and main body of the trap is constructed of perforated polyvinyl chloride (PVC) piping and can remain in place while the trap receptacle itself is removable (Figs. 1 and 2). This allows for multiple sampling trips without repetitive removal and installation of the entire trap apparatus. This produces less disturbance to the subterranean habitat between sampling trips, reducing the impact of trap installation on the communities being sampled. The trap receptacle is composed of a thin plastic cup with a smaller polypropylene and polyethylene plastic bait chamber bolted within the center. Small rubber gaskets, stainless steel washers, and small bolt-nut combinations were used to stabilize the bait chamber within the center of the trapping receptacle. This bait chamber is capped with a perforated lid to allow the bait odors to disperse. This trap design allows for sampling within a range of 10–67 centimeters below the soil surface. The perforations in the outer shelling allow specimens to enter the trap and begin 10 cm below the soil surface to decrease the likelihood of epigean species burrowing into the sampling range.

Traps of this design were utilized to sample the subterranean arthropod communities of the Ridge and Valley and the Blue Ridge ecoregions of Virginia (Omernik 1995) (Fig. 1). The trapping receptacles were baited with Limburger cheese (Wisconsin Cheese Mart- Madison, Wisconsin) and filled with propylene glycol for specimen preservation. Limburger cheese was selected as bait due to its strong odor and propensity for attracting arthropods. Propylene glycol was selected due to its DNA preservative and thermal buffering qualities (López and Oromí

2010). Each trap was loaded with bait and preservative and allowed to collect specimens for a two-week period before the receptacles and specimens were collected. Two collections were conducted. The first was on December 28th, 2021, and the second on June 1st, 2022, constituting four total weeks of collection time.

Figure 1. Subterranean pitfall trap design adapted from López and Oromí (2010).

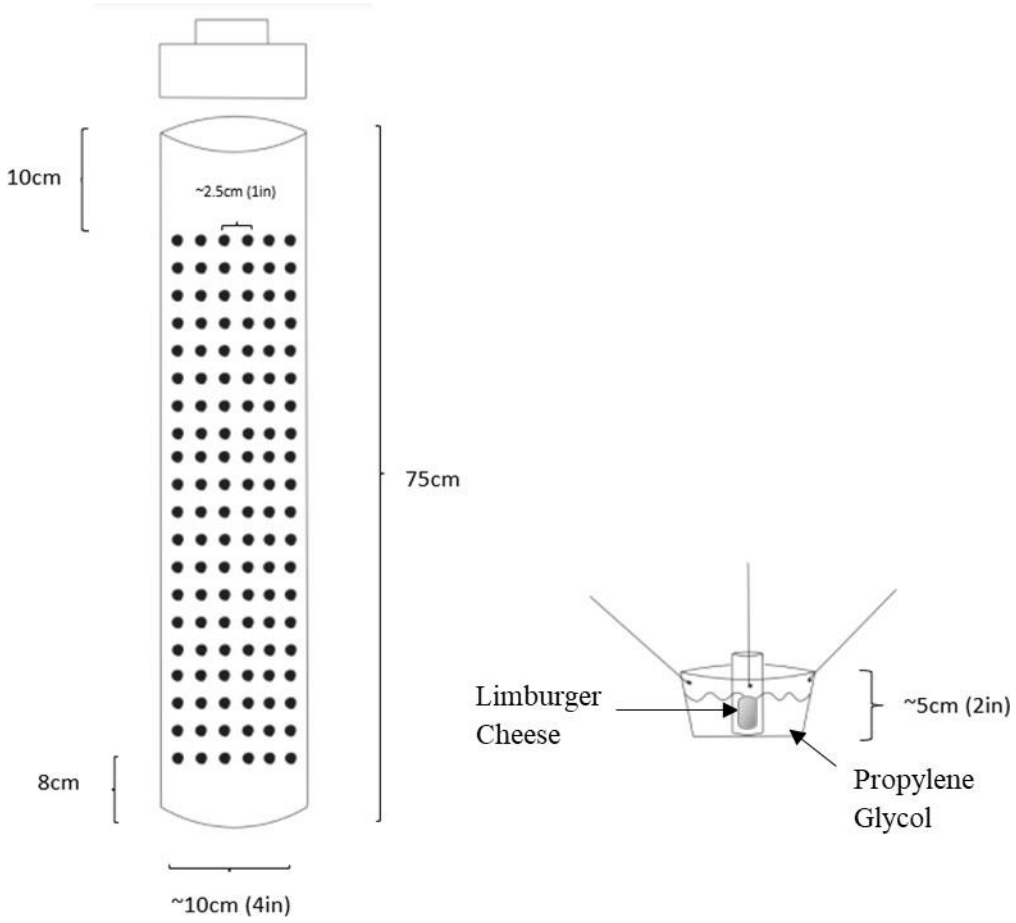
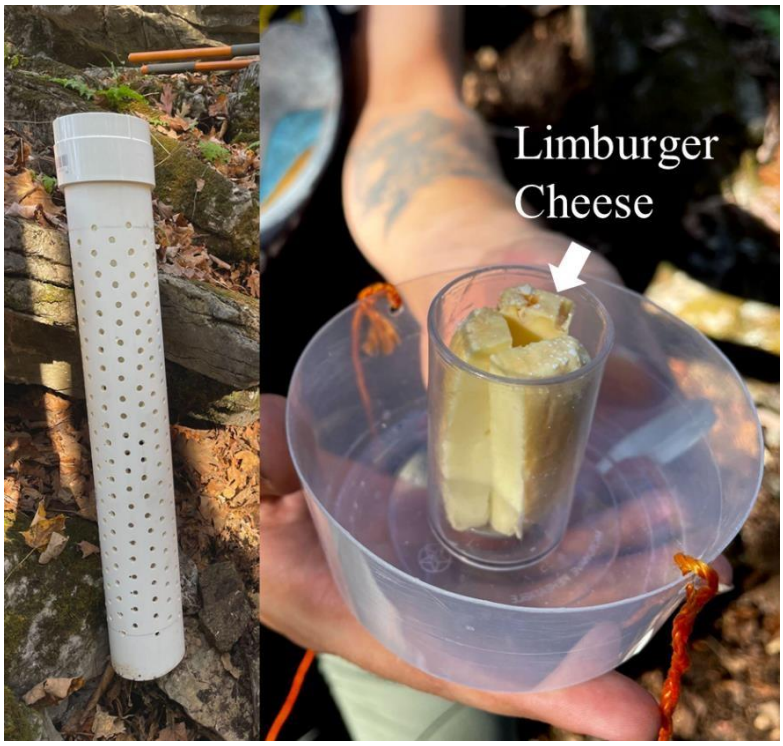


Figure 2. Subterranean pitfall trap used for this study with close-up of trapping receptacle shown on right.

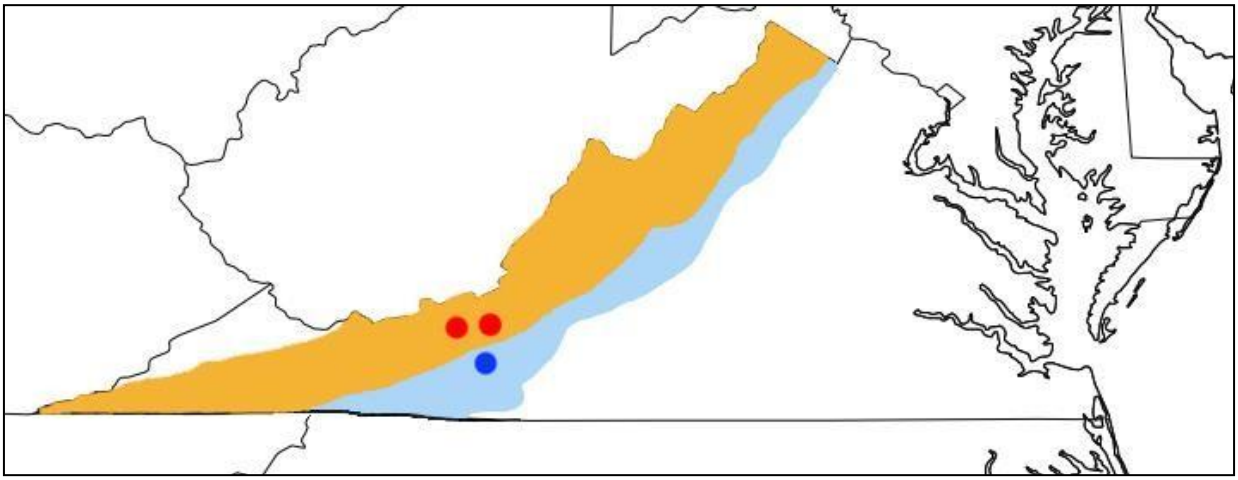


A total of 20 subterranean pitfall traps were installed within three different localities in southwestern Virginia (Fig. 2) (Table 1). Each trap site was wooded, and traps were installed where evidence of frequent human disturbance was absent. These sites and their respective traps are referred to as HOLEs for this project. HOLE numbers 1–5 were installed on Virginia Tech Foundation-owned land near the university's dolomite quarry. HOLE numbers 6 –10 were installed on Virginia Tech-owned land near Blacksburg, Virginia. These 10 traps were all located within the Ridge and Valley ecoregion of Virginia with the rock substrate being heavily composed of sedimentary rock (U.S. Geologic Survey 2021). Dolomite (commonly known as "Hokie stone" amongst the Virginia Tech community) was the rock type most frequently encountered while installing traps 1–10. HOLE numbers 11–20 were installed on private land near Floyd, Virginia owned by a local naturalist and artist, Ruth Neumann. This land is located within the Blue Ridge ecoregion of Virginia with the rock substrate being heavily composed of

metamorphic rock (U.S. Geologic Survey 2021). Quartzite was the rock type most frequently encountered while installing trap numbers 11–20.

Both the RV and BR regions were dominated by oak-hickory hardwood deciduous forests with oak-pine sparsely scattered throughout (Virginia Department of Forestry, https://dof.virginia.gov/forest-markets-sustainability/learn-about-forest-markets-sustainability/virginias-forest-composition). All sites were located within mixed hardwood forests where oaks were most abundant with occasional pine, beech, and maples scattered throughout. All sites were selected as they offered ideal elevational slopes with underlying rocky substrates that aligned with the conditions described by López and Oromí (2010). The latitude, longitude, and elevation for each site is reported below (Table 1).

Figure 3. Virginia map displaying trap (HOLE) sites and RV and BR.



Red points - RV sites (HOLEs 01-10), Blue points - BR sites (HOLEs 11-20), Orange – RV ecoregion, Light blue – BR ecoregion

Table 1. Locality coordinates and elevation for trap sites (HOLEs).

Trap #	Lat	Long	Elevation (m)	County	State	GPS Satellite #	GPS Accuracy (m)
HOLE-2021-001	37.2231	-80.383	631	Montgomery	VA	15	3
HOLE-2021-002	37.2229	-80.38	610	Montgomery	VA	5	6
HOLE-2021-003	37.2228	-80.382	639	Montgomery	VA	12	5
HOLE-2021-004	37.2225	-80.387	667	Montgomery	VA	9	9
HOLE-2021-005	37.2232	-80.384	619	Montgomery	VA	15	2
HOLE-2021-006	37.2124	-80.609	560	Montgomery	VA	10	3
HOLE-2021-007	37.2127	-80.609	558	Montgomery	VA	4	34
HOLE-2021-008	37.2132	-80.605	600	Montgomery	VA	16	3
HOLE-2021-009	37.2133	-80.605	592	Montgomery	VA	13	3
HOLE-2021-010	37.2119	-80.609	559	Montgomery	VA	14	4
HOLE-2021-011	36.9656	-80.419	751	Floyd	VA	13	6
HOLE-2021-012	36.9663	-80.418	773	Floyd	VA	15	3

HOLE-2021-013	36.9685	-80.417	771	Floyd	VA	13	9
HOLE-2021-014	36.9684	-80.418	778	Floyd	VA	12	3
HOLE-2021-015	36.9673	-80.417	780	Floyd	VA	14	3
HOLE-2021-016	36.967	-80.417	783	Floyd	VA	15	5
HOLE-2021-017	36.9668	-80.417	781	Floyd	VA	16	3
HOLE-2021-018	36.9657	-80.419	776	Floyd	VA	13	3
HOLE-2021-019	36.9651	-80.419	790	Floyd	VA	13	3
HOLE-2021-020	36.9639	-80.418	772	Floyd	VA	11	3

GPS – Global Positioning System (handheld Garmin eTrex 10)

2.2. Sorting/Designating Morphospecies

Specimens were first removed from the propylene glycol filled trap receptacles using forceps, then sorted by order for each HOLE and stored in 8.0 milliliter (mL) Sarstedt vials with 100% ethanol (EtOH). Morphospecies were then designated for each site from each order vial. Morphospecies represent a form of taxonomic operational unit and are designated by examination of easily observable morphological characters (Derraik et al. 2010). Because morphospecies are determined solely by morphology, different life stages of holometabolous

insects are typically designated as separate morphospecies. Morphospecies were designated by examining specimens using a Leica M125 stereomicroscope (Leica, Wetzlar, Germany). Each morphospecies was photographed in EtOH at 7 –12 focal planes with a Canon EOS 6D SLR camera and Canon MP-E 65mm macro lens mounted on a Visionary Digital Passport portable imaging system (Canon, Tokyo, Japan; Visionary Digital, Balaclava, Australia). The software Helicon Focus was used to integrate the focal stacks into single, high resolution JPG image (HeliconSoft, Kharkiv, Ukraine). Each morphospecies from the second collection trip was imaged from dorsal, ventral, and both lateral perspectives, while those from the first collection trip were imaged from the dorsal view only.

Morphospecies from different sampling sites thought to be the same based on morphological similarity were treated as distinct and unique in order to capture potential cryptic species from one site to another. Each morphospecies was morphologically identified to at least the family level using various morphological identification resources (Stehr 1991; Goulet and Huber 1993; Arnett and Thomas 2000; Arnett et al. 2002; Government of Canada 2002; Triplehorn et al. 2005; Whitfield et al. 2014). Exceptions included immature dipteran, coleopteran, and hemipteran specimens for which morphological identification resources do not exist or were not accessible. These juvenile morphospecies were left at the order level morphologically.

2.3. Generating, Identifying, and Clustering Barcodes

DNA was extracted from each morphospecies using a DNeasy (Qiagen) extraction kit. The extraction protocol was modified to be less destructive, keeping specimens largely intact for morphological identification, potential species description, and future deposition in the Virginia Tech Insect Collection (https://collection.ento.vt.edu). Rather than homogenizing the specimen by grinding body parts in buffer, a single puncture was made in the cuticle with a flame - sterilized pin (see above) and the specimen transferred to the DNeasy minicolumn along with the buffer solution. This puncturing allows the lysis buffer to bypass the cuticle and access the softer tissues within without grinding the specimen completely. The specimen is then recovered from the minicolumn following the final buffer wash and stored in 100% ethanol. The COI mitochondrial gene region was amplified utilizing polymerase chain reaction (PCR) employing the primers LCO 1490 and HCO 2198 (Folmer et al. 1994). LCO 1490 and HCO 2198 were selected as primers for PCR as they have been shown to be ideal for the amplification of the COI gene region in arthropods (Folmer et al. 1994; Elbrecht et al. 2019).

The PCR protocol was conducted according to Means et al (2021). Cleaning, quantification, normalization, and sequencing of amplicons was conducted by the University of Arizona Genetics Core on an Applied Biosystems ABI 3730 capillary sequencer. The resulting chromatograms were concatenated into high quality consensus sequences in the program Mesquite (Version 3.61) via base calling, trimming, and quality assessment using the sequence analysis module Chromaseq (Version 1.52) as well as the software PHRED and PHRAP (Ewing et al. 1998; Maddison and Maddison 2019; Maddison and Maddison 2021). This was carried out according to the methods outlined by Vasquez-Valverde and Marek (2022), producing a consensus COI sequence approximately 500–600 base-pairs in length for each morphospecies.

For the chromatograms that did not contig in Mesquite, additional assembly attempts were made using the program Geneious Prime and various assembler algorithms (Version 2022.1.1, Build 2022-03-15). No additional contigs were retrievable. To generate the completed DNA barcodes, the consensus sequences were cleaned of stop codons within Mesquite using the sequence processing tools Reading Frame and Codon Position and aligned within Geneious Prime using the program MAFFT (Katoh and Standley 2013) (Version 7.490). Stop codons were removed by deletion of erroneous single nucleotides detected by Mesquite, followed by designation of the proper reading frame orientation after each deletion.

The barcodes were matched with existing BOLD records using GBIF's sequence-id engine (https://www.gbif.org/tools/sequence-id). An additional local BLAST analysis was conducted by downloading all arthropod COI sequence data uploaded to NCBI as of 7-March-2023 and utilizing the custom batch BLAST feature in Geneious Prime. BLAST hits with a percent identity match of ≥97% were accepted as molecular identifications up to the species level. Hits with a percent identity match below 97%, but ≥95% were accepted up to the genus level (Srivathsan et al. 2022). Searches with no matches at or above these thresholds were left with solely morphological identifications—predominantly at the family level. Sequences were then clustered at 3% dissimilarity (or 97% similarity by nucleotide identity) using objective clustering in order to establish molecular operational taxonomic units (mOTUs) for diversity analyses (Meier et al. 2006; https://github.com/asrivathsan/obj_cluster).The divergence threshold of 3% was employed in accordance with the original methods for objective clustering as well as similar insect diversity studies employing objective clustering (Hebert et al. 2003a; Smith et al. 2005; Meier et al. 2006; Srivathsan et al. 2022).

2.4. Comparing Taxonomic Composition

Taxonomic composition was assessed for RV and BR by observed richness and abundance. Percent observed richness was calculated by class for both regions and is shown in Figures 4 and 5. Abundance was compared by order and limited to the ten most abundant orders for each region (Figure 6; Table 2). These comparisons were conducted to broadly characterize the taxonomic composition of the subterranean arthropod communities sampled as they were entirely unknown previously.

2.5. Assessing Diversity

Observed species richness was compared seasonally and regionally using Wilcoxon signed-rank tests conducted in IBM SPSS (Version 29.0.1.0) (Table 4) (Wilcoxon 1945) (IBM Corp). Wilcoxon signed-rank tests are non-parametric tests for comparing matched-pair data (Wilcoxon 1945). This analysis tests whether the probability distribution of one sample of data is equal to that of another sample (Woolson 2008). This allows us to use Wilcoxon analyses to test for significant differences between our observed richness data from multiple perspectives (Table 4). As our observation data is not normally distributed, a non-parametric (distribution-free) analysis was necessary. Our data included small counts (< 5) for some sites in the winter, making a test robust to small sample sizes necessary as well. The Wilcoxon signed-rank test was selected as it meets these necessary parameters and provides powerful hypothesis testing while remaining computationally simple (Woolson 2008). The null hypothesis for each of our Wilcoxon signed-rank tests is there is no significant difference between the observed richness of each matched-data-pair within the samples being compared. The alternative hypothesis for each test is observed richness is higher for RV and for spring than for BR and winter.

Regional diversity with respect to richness, abundance, and evenness was assessed using the Shannon-Wiener diversity index calculated in Microsoft Excel (Shannon 1948) (Microsoft Corporation, https://office.microsoft.com/excel) (Fig. 3). Hutcheson's t-test was used to test for statistically significant differences between the Shannon indices (Hutcheson, 1970). The null hypothesis is there are no significant differences between the RV and BR Shannon indices. The alternative hypothesis is the Shannon index for RV is significantly higher than that of BR. By nature, Shannon indices are non-linear and thus difficult to compare beyond "statistically different" or "relatively high/low". This fails to account for the magnitude of a community's diversity. In other words, we can use Shannon indices to determine that one community is more diverse than another, but we cannot assess how much more diverse in an intuitive way. This lack of comparability arises when using several common diversity indices including Shannon-Wiener (Staff and Chao 2009; Chao et al. 2014).

The Shannon indices of RV and BR were converted to effective numbers of species (ENS or Hill numbers) to compare their magnitudes and determine the fractional difference between the two (Table 5). ENS is a metric used to extrapolate Shannon indices into equivalent linear values, thus making comparisons easier to understand (Chao et al. 2014). The Shannon index accounts for species evenness resulting from unequal abundance data, making it useful for assessing real data collected from assemblages in nature, which rarely display equal abundances across all species. ENS, sometimes referred to as "true diversity", is an extrapolation of the Shannon index for a given community and represents a hypothetical community with the same Shannon index, but with equal species abundances. ENS essentially takes a Shannon value and tells us how many equally abundant species a hypothetical community would need to constitute

the same Shannon value. This serves as a linear representation of a community's true diversity, allowing for intuitive comparisons that account for magnitude.

Additional regional comparisons of alpha diversity were conducted using integrated sample-size based rarefaction/extrapolation curves generated in the program iNEXT Online (Hsieh et al. 2016). Rarefaction is a statistical method for comparing alpha diversities while accounting for differences in sampling error and discrepancies in observed species occurrences between assemblages (Sanders 1968; Willis 2019). It involves the down-sampling of larger assemblages until they match the number of observations or observed individuals as the smallest assemblage (Chao et al. 2014; Budka et al. 2019). This adjusts the diversity estimates for each of the larger assemblages to balance them with the smallest, producing fair estimates adjusted for discrepancies in sampling (Hsieh et al. 2016). Unobserved and rare species are often the most informative when estimating an assemblage's true diversity (Willis 2019). Extrapolation was employed to estimate the expected number of species within each assemblage while accounting for the species our sampling failed to detect. Extrapolation was carried out to twice the number of individuals observed during sampling which is consistent with the methods employed by Chao et al. (2014). The rarefaction/extrapolation curves were generated for Hill's number: $q = 1$, representing the use of the Shannon indices and ENS values for RV and BR. Rarefaction/extrapolation was conducted employing a 95% confidence interval with 1,000 bootstrap replicates (Fig. 8). A sample completeness curve was generated in iNEXT under the same parameters ($q = 1$; 95% confidence; 1,000 replicates) to ensure our sampling was sufficient to draw meaningful diversity estimates (Fig. 9).

3. RESULTS

3.1. Collection and Barcoding

The two sampling periods generated 2,260 arthropod specimens that constituted 344 morphospecies after sorting and designation. Of those, 320 successfully amplified with COI primers with 276 viable, concatenated sequences being generated from the successful amplicons. Objective clustering revealed 16 molecular clusters that displayed <3% sequence dissimilarity within each cluster. These clusters represented 2 –4 morphospecies each, collapsing the 276 sequenced morphospecies to a total 255 molecularly distinct species. Morphospecies that did not sequence were treated as morphologically distinct, bringing the total number of species sampled between all traps to 319 after clustering. High quality COI barcodes were generated for the 276 sequenced morphospecies.

3.2. Taxonomic Composition

Figure 4. Observed species richness by class for Ridge and Valley.

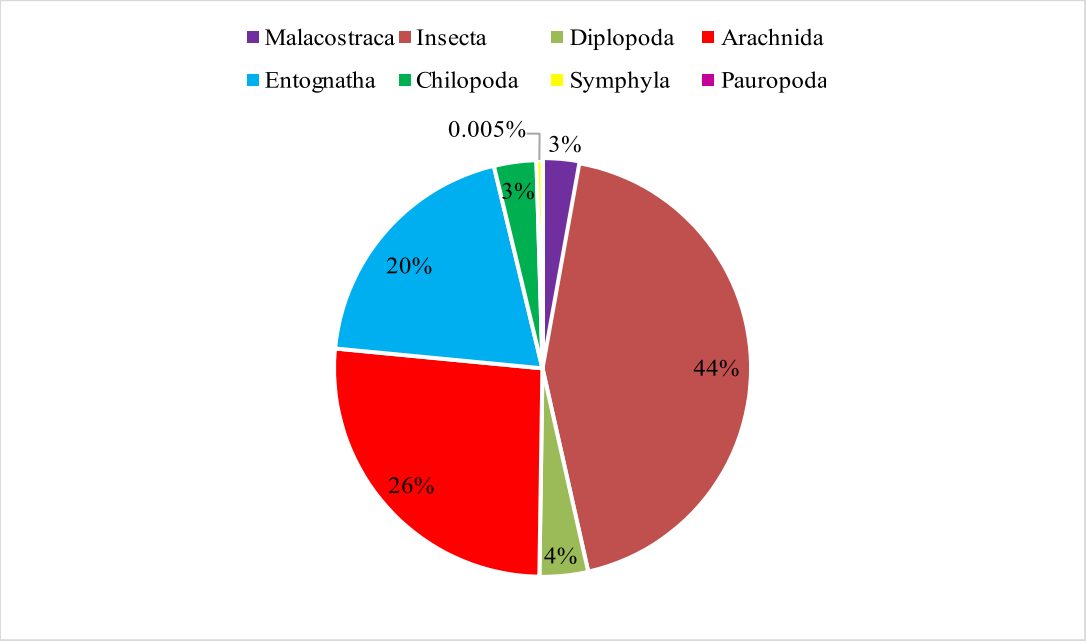


Figure 5. Observed species richness by class for Blue Ridge.

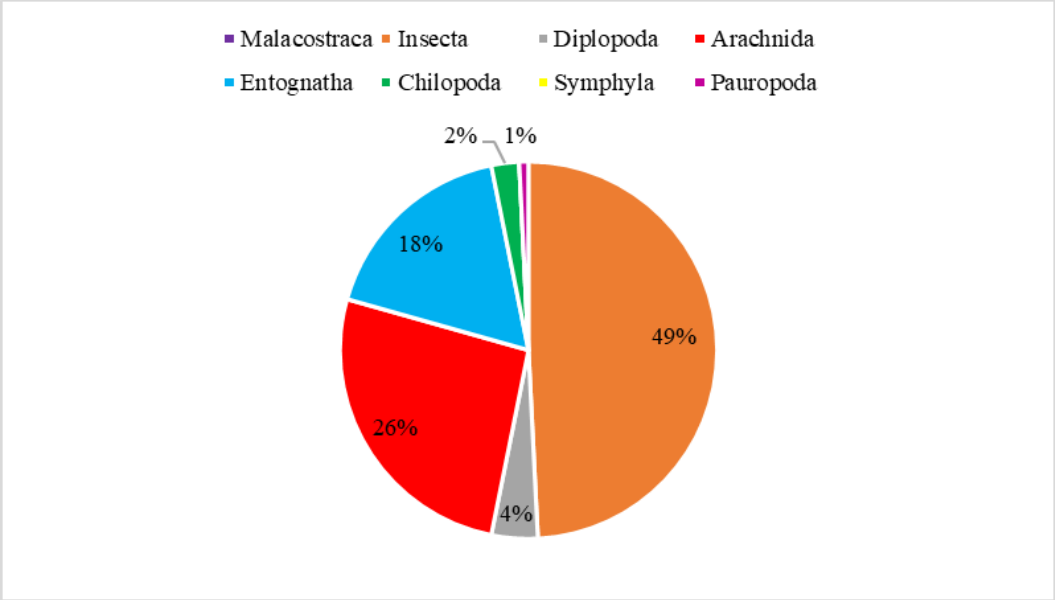
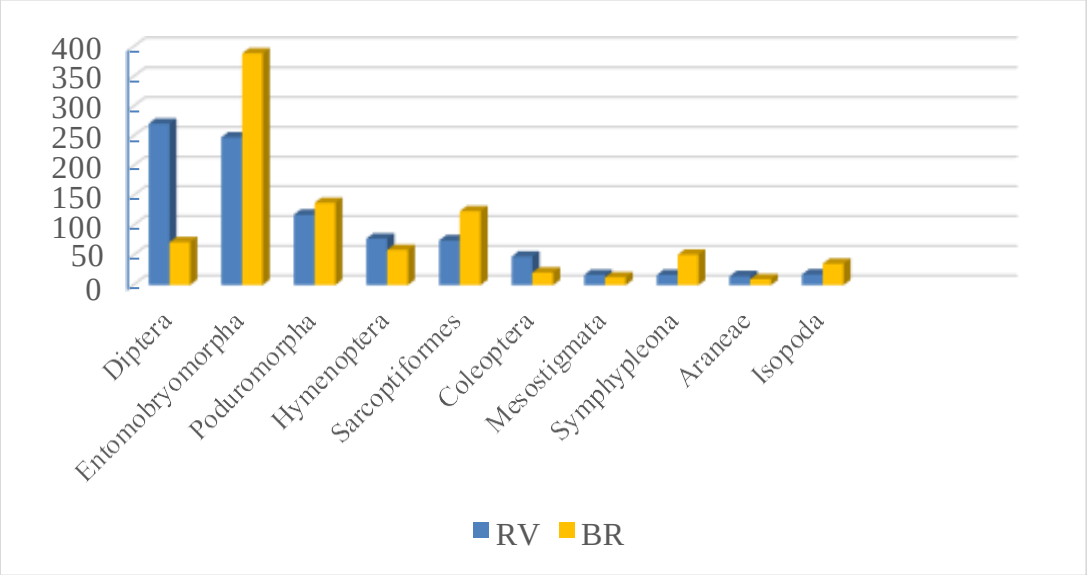


Figure 6. Ten most abundant orders for Ridge and Valley (RV) and Blue Ridge (BR).



Observed species richness by class was similar for both regions (Figs. 4 and 5). The top three classes for richness in both regions were Insecta (≥44%), Arachnida (26%), and Entognatha (≥18). Insecta was largely represented by Diptera (~41%) and Coleoptera (~33%). Arachnida was largely represented by subclass Acari (mites) (~69%). Entognatha was predominantly represented by Collembola (~98%). The ten most abundant orders for both regions are reported in Table 2 and shown in Figure 6. It is interesting to note that the five most abundant orders were the same for RV and BR with only their respective rankings varying between regions (Table 2). Three orders of subclass Collembola were within the top eight in abundance for both regions: Entomobryomorpha, Poduromorpha, and Symphypleona. A large portion of the Dipteran specimens collected from RV were early instar larvae from the family Phoridae. The order Hymenoptera was predominantly represented by ants (Hymenoptera: Formicidae).

Table 2. Ten most abundant orders for RV and for BR.

Rank	RV	Count	BR	Count
1	Diptera	271	Entomobryomorpha	389
2	Entomobryomorpha	248	Poduromorpha	138
3	Poduromorpha	118	Sarcoptiformes	124
4	Hymenoptera	78	Diptera	72
5	Sarcoptiformes	75	Hymenoptera	59
6	Coleoptera	48	Symphypleona	51
7	Mesostigmata	17	Orthoptera	36
8	Symphypleona	17	Coleoptera	21
9	Araneae	15	Mesostigmata	13
10	Isopoda	18	Pseudoscorpiones	10

3.3. Observed Species Richness

Table 3. Observed species richness totals for each HOLE split by season and region.

Region	**Traps**	**Winter Richness**	**Spring Richness**	**Total Richness**
RV	HOLE-001	19	19	38
RV	HOLE-002	11	17	28
RV	HOLE-003	2	8	10
RV	HOLE-004	2	13	15
RV	HOLE-005	3	18	21
RV	HOLE-006	13	14	27
RV	HOLE-007	0	21	21
RV	HOLE-008	4	11	15
RV	HOLE-009	6	13	19

RV	HOLE-010	4	17	21
BR	HOLE-011	4	1	5
BR	HOLE-012	6	7	13
BR	HOLE-013	2	9	11
BR	HOLE-014	0	2	2
BR	HOLE-015	1	10	11
BR	HOLE-016	2	10	12
BR	HOLE-017	1	5	6
BR	HOLE-018	8	19	27
BR	HOLE-019	1	20	21
BR	HOLE-020	3	14	17
RV Totals		**64**	**151**	**215***
BR Totals		**28**	**97**	**125***

RV - Ridge and Valley, BR - Blue Ridge, * = Repeat species allowed within each region to calculate average observed richness per site. Regional total richness values with no duplicates reported in Table 5.

Six Wilcoxon signed-rank tests were performed to compare observed species richness per site for:

a. Total Winter vs Total Spring.

b. RV Winter vs RV Spring.

c. BR Winter vs BR Spring.

d. RV Spring vs BR Spring.

e. RV Winter vs BR Winter.

f. RV Total vs BR Total

Average observed richness per site (mean) and results of the Wilcoxon signed-rank tests are reported in Table 4. For tests **a** and **f** the critical p-value is 0.05. The critical p-values for tests **b**, **c**, **d**, and **e** were adjusted to 0.0125 via the Bonferroni correction (critical p-value/n, where n = 4) in order to account for four comparisons (Bonferroni 1935).

Table 4. Results for Wilcoxon signed-rank tests **a**—**f** comparing seasonal and total species richness* within and between regions.

	Variables	AR/S	Test Stat	Standardized Test Stat	Standard Error	p value
a	Total Winter	4.6	186	3.666	24.824	<0.001***
	Total Spring	12.4				
b	RV Winter	6.4	0	-2.67	8.426	0.008***
	RV Spring	15.1				
c	BR Winter	2.8	3	-2.499	9.804	0.012***
	BR Spring	9.7				
d	RV Spring	15.1	10.5	-1.734	9.804	0.083
	BR Spring	9.7				
e	RV Winter	6.4	6.5	-1.901	8.419	0.057
	BR Winter	2.8				
f	RV Total	21.4	8	-1.993	9.785	0.046**
	BR Total	12.5				

AR/S - Average observed Richness per Site, * - Observed species richness, ** - $p < 0.05$, *** - $p < 0.0125$

Observed species richness was assessed with respect to region and season. Richness was assessed predominantly as average observed richness per site (AR/S) (Table 4). The average observed richness per site for the RV samples was significantly higher than that of the BR samples (Table 4 - f). The total observed species richness was also higher for the RV (Table 5). Both the total observed richness and average observed richness per site were significantly higher

in the spring for both regions (Table 4 - a, b, and c). When comparing regions, there were no significant differences in average observed richness per site for spring or winter (Table 4 - d and e).

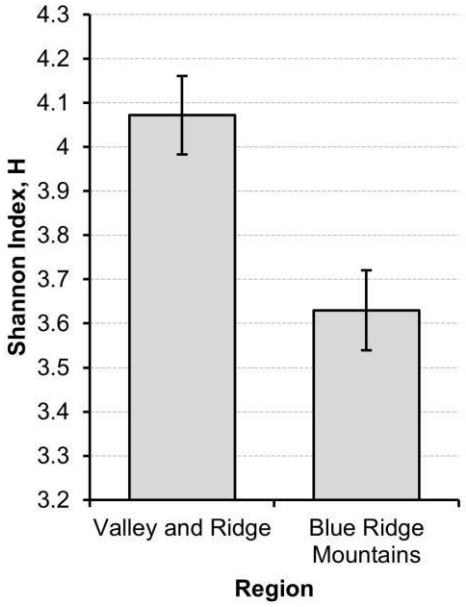
3.4. Shannon Diversity Indices and Effective Numbers of Species

Table 5. Total specimen count, species richness, Shannon index value, and ENS for each region.

Region	RV	BR
Total specimens	1224	1036
Total Richness	197	122
Shannon Index (H)	4.072	3.629
ENS	58.673	37.691

ENS - Effective Number of Species

Figure 7. Shannon indices for RV and BR.

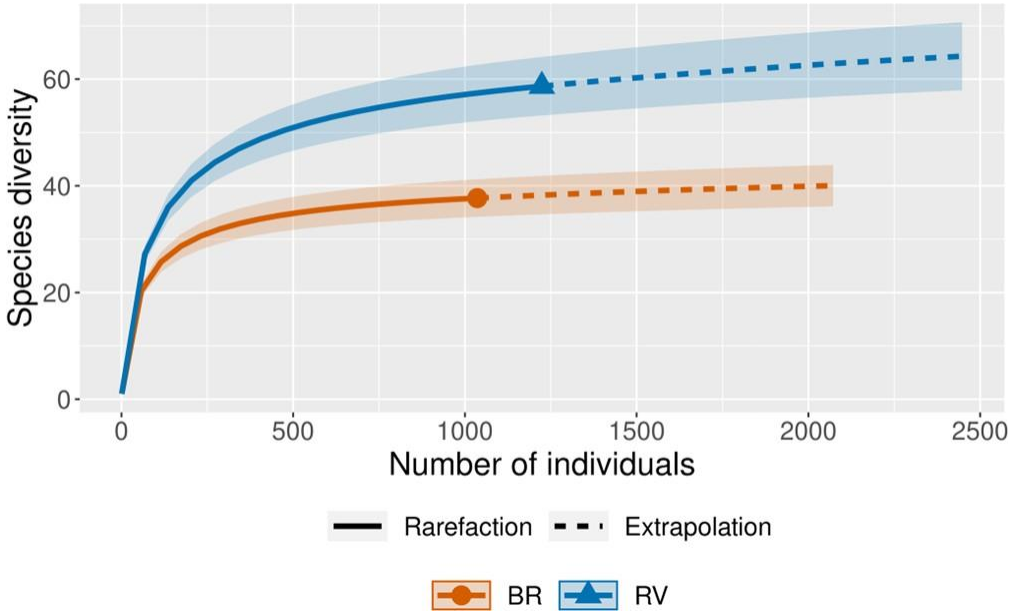


Error bars – 95% confidence interval for Hutcheson's t-test

The Shannon index for the RV was significantly higher than that of the BR as indicated by Hutcheson's t-test ($p < 0.001$) (Fig. 7) (Hutcheson 1970). Both indices are relatively high, indicating that the subterranean arthropod communities of RV and BR are highly diverse. The Shannon indices were converted to ENS to compare their magnitudes (Table 5). Dividing the smaller ENS value (BR) by the larger (RV) yields a fractional drop in diversity value which serves as a direct, intuitive, comparison of true diversity. The fractional drop in diversity of 0.6424 indicates that the sampled subterranean communities of BR constituted only 64.24% of the diversity found in those of RV. This indicates a difference in diversity of approximately 35.76% by ENS. For the sake of comparison, the difference between Shannon indices is 0.443. This illustrates the usefulness of ENS as a measure of diversity as the direct Shannon comparison is uninformative and does far less to convey the magnitude of the difference between regional diversities.

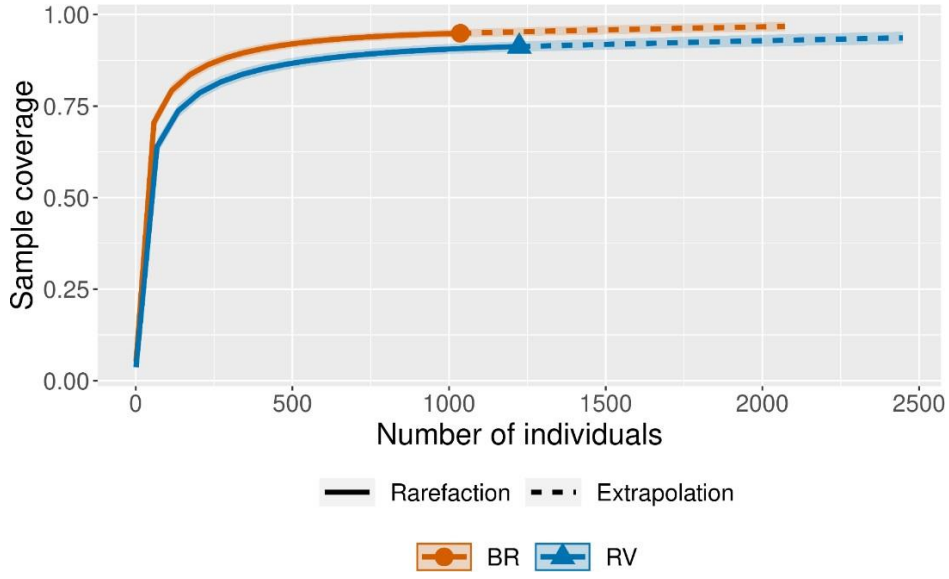
3.5. Rarefaction, Extrapolation, and Sample Completeness

Figure 8. Sample-size rarefaction/extrapolation for RV and BR (q = 1).



Y-axis – ENS values (or Hill numbers)

Figure 9. Sample completeness curves for Ridge and Valley and Blue Ridge (q = 1).



The confidence intervals for the rarefaction/extrapolation curves did not overlap indicating that the RV samples were significantly more diverse than the BR samples by alpha diversity within a 95% confidence interval with 1,000 bootstrap replicates (Fig. 8). The rarefaction and extrapolation portions of the BR curve reached a slower rate of increase with increased sampling than those of the RV curve. This indicates a higher sample completeness was achieved for the BR samples. This is consistent with the sample completeness curves which display higher coverage for BR as the number of sampled individuals increases (Fig. 9). The rarefied portions of the sample completeness curves for both the RV and BR approached asymptote, indicating sufficient sampling for statistical comparison with low rates of increase in coverage as sampled individuals increased throughout the extrapolated portions of the curves.

4. DISCUSSION

This study represents the first taxonomically indiscriminate assessment of subterranean arthropod diversity conducted in North America beyond 10 cm below soil-surface. It is also the first in Appalachia to employ subterranean pitfall traps adapted from the method described by López and Oromí (2010). 2,260 individual arthropod specimens were collected representing 8 classes, 33 orders, and 319 species. 255 high quality COI barcodes were generated and published to BOLD, many of which for species previously missing from both NCBI and BOLD. Several morphospecies exhibited hypogean morphology (depigmentation and reduction of eyes and/or limbs), suggesting that they may be obligate subterranean species. These included several species of ant (Hymenoptera: Formicidae), beetle (Coleoptera), fly (Diptera: Cecidomyiidae), springtail (Collembola), dipluran (Diplura: Japygidae), spider (Arachnida: Araneae) and mite (Arachnida: Mesostigmata). However, most of the individuals collected displayed morphology associated with epigean origin. This is consistent with the findings of similar subterranean studies conducted outside the United States and indicates that our sampling was likely confined to the SHH which is characterized as being dominated by epigean taxa (Mammola et al. 2017). This dominance of epigean taxa most likely contributed to the significant decreases in both richness and abundance observed in winter (Table 4 - a). It has been shown that the richness and abundance of epigean arthropods in subterranean systems, especially insects, are significantly impacted by seasonality as surface temperature impacts superficial SHH temperature to some extent. This is not the case for hypogean taxa as climatic stability tends to increase as depth from surface increases (Mammola et al. 2017).

The primary taxonomic compositions of our samples were comparable to those of similar shallow subterranean studies in the Canary Islands (Pipan et al. 2011; Mammola et al. 2017),

mainland Spain (Gilgado et al. 2014), Bulgaria (Langourov et al. 2014), and France (Juberthie 2000) with arthropods and annelids being most abundant. Our findings differ in detection of mollusks and crustaceans (Isopoda) as we collected only one mollusk individual and few isopods while both were common in the European studies cited above. For insect abundance by order, our results align well with those of Mammola et al. (2017) in that Diptera, Hymenoptera, and Coleoptera were among the most abundant for both regions (Table 2). Our findings are consistent with those of Moldenke and Lattin (1990) in that the families Phoridae, Cecidomyiidae, and Sciaridae were among the most common Dipterans encountered. Dipteran abundance was highly variable between regions due in part to large numbers of early instar larvae from a single family (Diptera: Phoridae) found in two individual traps in the RV. The same is true for Hymenoptera as ant specimens (Hymenoptera: Formicidae) dominated specific traps in both regions. These patterns are consistent with the findings of Mammola et al. (2017).

For observed beetle (Coleoptera) richness by family, our findings are consistent with those of Moldovan (2005) where ground beetles (Coleoptera: Carabidae) (~14.3%) were less common than round fungus beetles (Coleoptera: Leiodidae) (~18.4%). This is the reverse of the findings of Mammola et al. (2017). Carabids were largely observed as larvae with only a single mature specimen being collected. This may be a result of bait-bias. Rove beetles (Coleoptera: Staphylinidae) (~24.5%) were the most species rich group of beetles observed which differs from the studies cited above. Minimal overlap in species occurrence was observed between regions as only a single species (*Cicurina pallida*, Keyserling 1887) was recorded from both RV and BR. This lack of overlap in species observed between North American subterranean sites is consistent with the findings of Lamoncha (1994) who recorded minimal overlap in oribatid mites between nearby subterranean sites in North and South Carolina. For Hemipterans, our findings are

consistent with those of Langourov et al. (2014) in that members of the order Hemiptera were rare with only five collected individuals. This could also be due to bait-bias as phytophagous Hemipterans may be less attracted to Limburger cheese which showed a propensity for attracting carnivores and/or detritivores.

The Shannon indices, ENS results, and Wilcoxon comparison for total regional observed richness support our hypothesis that the arthropod communities sampled from the SSHs of RV are more diverse than those of BR (Table 4 - f, Table 5, Fig. 7). The Shannon indices and ENS results indicate that both sampled communities are highly diverse (Table 5, Fig. 7). This is consistent with the findings of several studies characterizing subterranean arthropod diversity both broadly and within particular groups including beetles, spiders, and mites (Lamoncha 1994; Christman et al. 2005; Mammola et al. 2017; Ledesma et al. 2019). The extrapolation portion of the rarefaction/extrapolation curve for RV displayed a steeper slope than the curve for BR, indicating that further sampling may be warranted to capture additional rare species undetected by this study (Fig. 8). This is consistent with the sample completeness curve for RV which displayed lower sample coverage than the BR curve (Fig. 9). The findings of this study support the notion that the subterranean arthropod communities within the SHHs of Appalachia, and potentially North America more generally, are hyper-diverse and warrant further study.

4.1. Future Directions

The 255 mOTUs established by this study serve as powerful identification resources that contain morphological and molecular information for the subterranean arthropod species they represent. These resources serve as beneficial tools for future research in this system by

streamlining taxonomic identification and directly combatting the lack of subterranean arthropod representation in NCBI and BOLD. Future steps for the molecular data generated from this study include the establishment of precise barcode gaps for the species sequenced as the majority are underrepresented within the literature. The objective clustering analyses indicated that the barcode gaps for most of our sequenced species are somewhere between 2.2 and 3% at COI. The assignment of Barcode Index Numbers (BINs) for each mOTU in BOLD will allow for Automatic Barcode Gap Analyses (ABGA), enabling us to pinpoint these gaps more precisely (Puillandre et al. 2012; Ratnasingham and Hebert 2013).

Additional future steps include the establishment of long-term, continuous sampling initiatives with adapted subterranean pitfall traps similar to a design employed in several European studies (Rendoš et al. 2012; Baquero et al. 2017; Haľková et al. 2020). This design features multiple collection receptacles that rest at regular depth intervals spanning the length of the entire trap apparatus. It also features temperature and moisture recording equipment. This design allows for the collection of more ecologically precise data including the depth each specimen entered the trap at. This would allow for the observation of differences in species occurrence and morphology between the stratifications in the trap. It would also allow for the assessment of two significant abiotic factors, temperature and moisture, allowing for more thorough characterization of North American subterranean habitats and their diversity.

5. Annotated Literature Review

Elbrecht V, Braukmann TWA, Ivanova NV, Prosser SWJ, Hajibabaei M, Wright M, Zakharov EV, Hebert PDN, Steinke D (2019) Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ 7: e7745. https://doi.org/10.7717/peerj.7745

Here the performance of 36 primer sets were tested to assess amplification bias and suitability for use in the metabarcoding of terrestrial arthropods for community composition analyses. 21 of these sets were used to retrieve barcodes from field specimens as well as specimens from a mock community. The resulting taxon recovery percentages revealed that primer sets that lacked degeneracy such as LCOI490 and HCO2198, were less suitable for the metabarcoding of terrestrial arthropod communities. The authors recommended the set BF3+BR2 as they provide maximal taxonomic resolution and are not impacted by primer slippage. The study also compared the use of primer combinations and concluded that the use of multiple primer sets was unnecessary for metabarcoding terrestrial arthropod communities.

de Kerdrel GA, Andersen JC, Kennedy SR, Gillespie R, Krehenwinkel H (2020) Rapid and cost-effective generation of single specimen multilocus barcoding data from whole arthropod communities by multiple levels of multiplexing. Scientific Reports 10: 78. https://doi.org/10.1038/s41598-019-54927-z

Here the authors developed a more efficient and affordable method for conducting large-scale analysis of arthropod communities by adapting the pooled-sequencing approach commonly used within Illumina metabarcoding. By utilizing pre-sorted specimen pools for extraction and amplification, multiplexing PCR of different amplicons, and two levels of indexing during library preparation the authors were able to achieve up to a 100-fold reduction in workload and

cost. The authors' method also improves on traditional pooled barcoding approaches by allowing the linking of sequences to physical specimens as well as the assessment of multiple loci within one amplification.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proceedings. Biological Sciences 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

This paper describes the taxonomic impediment that characterizes the slow pace of morphological species identification. It argues that traditional taxonomic approaches which center solely around morphological assessment are not sufficient tools for the study and understanding of global biodiversity as the time, labor, and expertise required for this type of work is both immense and in decline due to dwindling experts and funding. The authors argue that a new protocol for the assessment of diversity and identification of species is necessary, and that the establishment and use of COI barcodes and profiles should be the backbone upon which this protocol is built. The advantages of such a system would be objective criteria for species discrimination, quantifiable intra-species diversity parameters, and the possibility of identification of all life stages. They also argue for the establishment of accessible, web -based databases for use in this barcode system and assert that within 20 years of publishing this paper it would be possible to establish barcodes for all known animal species either directly or through the barcoding of related taxa.

López H, Oromí P (2010) A type of trap for sampling the mesovoid shallow substratum (MSS) fauna. Speleobiology Notes 2: 7–11.

This paper describes a method for sampling hypogean fauna that dwell within the Mesovoid Shallow Substratum (MSS) habitats. The authors describe and diagram a permanent pitf all trap of their design that has been utilized to sample subterranean fauna in the Canary Islands. The trap is predominantly constructed using PVC pipe and a baited receptacle filled with propylene glycol for specimen trapping and initial DNA preservation.

Oromí P, Martín AL, Medina AL, Izquierdo I (1990) The Evolution of the Hypogean Fauna in the Canary Islands. In: Fourth International Congress of Systematic and Evolutionary Biology. University of Marylands. Volume I

This paper describes the subterranean fauna found in cave and MSS habitats of the Canary Islands and organizes each species into one of four distinct groups. Group 1 includes species most likely introduced by humans while group 2 describes eyeless species that also occur outside of the Canary Islands. Group 3 encompasses endemic species but with epigean, eyed sister species also present. Group 4 describes endemic species lacking any epigean sister species on the Island. This paper also briefly discusses the authors' hypotheses and supporting evidence surrounding the claim that many of the hypogean species found in the Canary Islands are products of allopatric speciation.

Ratnasingham S, Hebert PDN (2013) A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. PLoS ONE 8(8): e66213.

Many studies conducted with morphologically distinct taxonomic groups utilize operational taxonomic units (OTU) in order to distinguish species boundaries. This approach has traditionally relied upon morphological sorting with no clear universal standard, contributing to a number of challenges regarding replication and standardization when utilizing OTUs. The

increasing prominence and accessibility of molecular approaches to species level identification offers a new approach to distinguishing species among dubious, taxonomically diverse specimen pools. The use of the Barcode Index Number (BIN) system addresses the challenges associated with the use of OTUs by standardizing the parameters for species distinguishment, as well as other various benefits over the OTU system. It has been shown that within the animal kingdom, the divergence in COI sequences between members of the same species rarely exceeds 2% and is almost always higher than 2% among individuals of different species. By barcodin g specimens and assessing the percent variation between them, functional species units can be established without utilizing morphological identification methods.

Srivathsan A, Hartop E, Puniamoorthy J, Lee WT, Kutty SN, Kurina O, Meier R (2019) Rapid, large-scale species discovery in hyperdiverse taxa using 1D MinION sequencing. BMC biology 17: 96. https://doi.org/10.1186/s12915 -019-0706-9

This paper describes a more affordable, accessible, and portable method for largescale specimen sequencing utilizing MinION sequencing. This method was employed to sequence 7059 specimens collected in one Malaise trap in Kibale National Park in Uganda. Of those specimens, over 650 species of phorid flies were discovered. The authors argue that MinION sequencing is more affordable than current leading next-generation sequencing methods and would allow for accurate DNA sequencing to occur in places that lack expensive next-generation facilities. An accuracy comparison was conducted between the barcodes generated using MinION and barcodes generated from the same extraction plates using Illumina to assess the reliability of MinION sequencing. The comparison showed that MinION sequencing, following the authors' methods, was highly accurate (>99.9% in all samples). The authors argue that their results support the concept that MinION sequencing is suitable for rapid, large-scale species discovery,

and that it would drastically speed up the processes of species discovery and description particularly in specimen- and species-rich taxon.

Zizka VMA, Elbrecht V, Macher J-N, Leese F (2019) Assessing the influence of sample tagging and library preparation on DNA metabarcoding. Molecular Ecology Resources 19: 893–899. https://doi.org/10.1111/1755-0998.13018

In this paper, the authors compared three metabarcoding methods to assess the taxa detection efficiency and consistency of fusion primers within each. Commercially manufactured Illumina kits, one-step, and two-step PCR protocols were used to sequence and detect taxa within five mock communities with similar taxa composition. The authors reported that in terms of taxonomic discrimination, the Illumina kit produced the most efficient and consistent results but was vastly more expensive than the other two methods. The two-step protocol produced results only marginally less consistent than the Illumina kit products and for a fraction of the price. The one-step protocol was the most affordable method but produced the least reliable results. The authors concluded that all three methods were suitable for community diversity assessments, but that the degree of accuracy necessary for, and financial capacity of each study should drive which method is employed.

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