University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
December 2018

Phylogeography and Microhabitat of Weller's Salamander, *Plethodon welleri*

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Abstract

Plethodon welleri is a species of endangered terrestrial salamander found in western North Carolina, eastern Tennessee, and southwestern Virginia, primarily in areas of high-elevation spruce-fir forest. These forests harbor many endemic plant and animal species yet are highly threatened by climate change and invasive species. Endemic species such as *P. welleri* may be especially threatened by climate change as their preferred environments get pushed into higher elevations by warming temperatures— isolating populations on mountaintop "islands." Analyzing genetic data for *P. welleri* allows us to ascertain the extent of genetic diversity and population connectivity/divergence among mountaintop "island" populations, providing valuable information to conservation planners. Using over 60 previously collected samples of this species, plus several my own samples collected over the last 2 years, I generated and analyzed genetic data from *P. welleri* populations on Unaka and Grandfather mountains in North Carolina as well as Whitetop Mountain and Mount Rogers in Virginia. I examined a 1,100bp fragment of the mitochondrial DNA ND2-NADH locus for each of these samples using distance-based and phylogeographic analyses to determine their degree of genetic differentiation. I found that these populations were each genetically distinct, with North Carolina and Tennessee populations sharing a more recent common ancestor than those from Virginia as predicted by geographic distances alone. I further found that Unaka mountain harbors a considerable amount of genetic diversity relative to the other populations. Finally, I describe microhabitat characteristics of locations were *P. welleri* were found on Unaka Mountain. These data suggest a trend of *P. welleri* preference for high-elevation areas of *Picea rubens* forest corresponding to an increasing pH gradient with increasing elevation on Unaka Mountain.

1. Introduction

The Southern Appalachian Mountains are home to a wide variety of endemic species, including the highest diversity of salamanders in the world[14]. Many of these salamander species are relatively isolated on high-elevation mountaintops, separated from one another by low-elevation regions which are inhospitable to the cold-adapted species that dwell higher up[3]. Over the last several Pleistocene glacial cycles, populations that are now present on separate mountaintops were likely contiguous with one another during cooler interglacial periods but have now been pushed towards higher elevations during warmer interglacial periods and out of contact. As these populations become reproductively isolated from one another, the process of speciation might be initiated, and similar mechanisms have been proposed as having contributed to the region's vast biodiversity[6,15].

One such species, *Plethodon welleri*, the Weller's Salamander (Fig. 1), is found primarily in high-elevation spruce-fir forest in western North Carolina, eastern Tennessee, and southwestern Virginia (Fig. 2[11,14]). These habitats are isolated relics of southern boreal forest and are considered to be the second-most endangered ecoregion in the United States[3,12]. Since the last glacial maximum, warming climates have gradually pushed these habitats into higher elevations in this region, a trend which is likely to continue with climate change. Owing to such threats and to its limited range, *Plethodon welleri* is listed as a North Carolina Species of Special Concern[10].

As terrestrial salamanders typical occur in narrow microhabitats and rely heavily on objects for protection from predation, even slight perturbations to their preferred conditions might drastically impact survival of resident salamander species[5]. For example, increases in temperature and anthropogenic pollution (which alters soil pH) might decrease habitability of certain areas by *P. welleri*[17]. This means that extra attention must be given to characterizing the microhabitats of these species, which may be threatened by both climate change and pollution such as acid rain.

Additionally, climate change is predicted to induce high rates of extinction in biodiversity hotspots such as the Southern Appalachians, implying a significant threat to the continued survival of *Plethodon welleri* as well as other salamander species in this ecoregion[9]. Due to their sensitivity to habitat perturbations, salamanders are excellent indicators of forest habitat health[16]. Therefore, drops in their populations and diversity may also signify problems that could impact other species, including humans. Conservation efforts focused on salamanders may not only benefit these remarkable creatures, but also help to provide a habitable world for other organisms.

By developing a better understanding of the past range of *Plethodon welleri* before it was pushed up into its current elevation range, it might be possible to predict future range shifts and thus to formulate a conservation plan for this species. In order to do so, this study examines genetic distinction between several populations of *Plethodon welleri* to determine how closely related they are to one another. In particular, I examine three disjunct populations: one from Grandfather Mountain in North Carolina, one from Unaka Mountain on the North Carolina-Tennessee border, and one from Grayson Highlands (Mt. Rodgers and Whitetop) in Virginia. I predicted that the populations that are geographically closer together will be more closely related genetically owing to the population genetic pattern of isolation-by-distance, supporting the idea that they were contiguous more recently in their evolutionary history, but will exhibit varying levels of genetic diversity due to their idiosyncratic evolutionary histories[6].

Additionally, I examine characteristics of the microhabitats in which *Plethodon welleri* is found in order to better understand which conditions this species requires for survival. I predicted that *P. welleri* would be found primarily in areas of spruce-fir forest above 1350m with neutral pH soil.

Figure 1. *Plethodon welleri* from Rock Creek, Unicoi County, Tennessee. Photo by Dr. R. Graham Reynolds.

2. Methods

2.1 Phylogenetics

I analyzed genetic data from *Plethodon welleri* tissue samples collected over the past three years from several sample sites within the Southern Appalachian Mountains, primarily from Grandfather Mountain, Unaka Mountain, and Grayson Highlands (Table 1). All of these tissue samples were collected in the form of excised tail tips and were stored in 95% ethanol at -80 C. I extracted total genomic DNA from them using the Wizard SV Genomic DNA Purification System, then tested the extracted DNA for purity and concentration using a Qubit 3.0 Fluorometer. I diluted this extracted DNA with water and stored it at -20 C until further processing.

Following extraction, I performed polymerase chain reaction (PCR) amplification of mitochondrial ND2-NADH fragments using the ND2-L4437-F2 forward primer (5'-ATTCCCTTCACTCGCCCAAGC-3') and the KND2-R2 reverse primer (5'-AAAGTGTTTGAGTTGCATTCA-3')[5]. PCR was carried out in a 25-µL reaction mixture containing 3 µL DNA template, 10.4 µL molecular biology-grade water, 2.5 µL 25mM $MgCl_2$, 5 µL 5X Colorless GoTaq Flexi Buffer, 1.5 µL DNTPs, 1.25 µL ND2-L4437-F2, 1.25 µL KND2-R2, and 0.125 µL GoTaq Flexi DNA Polymerase. PCR was performed according to the following program: 95.0°C for 2.5 minutes, 35 cycles of 95.0°C for 35 seconds, 59.0°C for 35 seconds, and 72.0°C for 2.5 minutes, followed by 72.0°C for 2.5 minutes and incubated at 4.0°C until removal from PCR machine and storage in -20°C. Following PCR, I examined the obtained products using gel electrophoresis and sent successful amplicons to the North Carolina State University Genomics Core Lab in Raleigh, NC for purification and sequencing.

Following sequencing, I aligned the samples using GENEIOUS® 10.2.6 (Biomatters, Auckland, NZ) and exported the alignment as a Nexus file. I then used two methods to infer phylogenetic relationships among the different *Plethodon welleri* populations. First, I used the RaxML plugin in GENEIOUS to infer a maximum-likelihood phylogenetic tree of the data. Next, I used the program Beauti v1.10.2 to create an xml input file for the BEAST program[13]. I added a standard mtDNA rate of molecular evolution of 1.3% pairwise divergence per million years and a Yule speciation prior, an uncorrelated lognormal relaxed molecular clock model, and a GTR+G substitution model. I loaded the xml file into BEAST v1.10.2 and ran the analysis for 100 million generations taking advantage of the enhanced computational time offered by the BEAGLE v3.0.1 library and GPU computing[1,8,13].

2.2 Microhabitat Sampling

My research partner Robert Chambliss and I established 25 10m x 10m plots along a transect on Unaka Mountain on the North Carolina-Tennessee border between May and August of 2016. Within each of these plots, we searched beneath every cover object of reasonable size for a salamander and noted presence or absence of *P. welleri* under each. We also recorded several microhabitat characteristics of each cover object and plot. These included elevation, soil pH, and tree species present. We then mapped these data using R v.3.5.1 in RStudio in order to visually assess correlation between *P. welleri presence* and microhabitat.

Table 1. Genetic samples used in this study.

Specimen	**Date**	**Locality**
Unaka_1	6/3/2016	Unaka Mtn
Unaka_2	6/3/2016	Unaka Mtn
Unaka_3	6/3/2016	Unaka Mtn
Unaka_4	6/3/2016	Unaka Mtn
Unaka_5	6/3/2016	Unaka Mtn
GF_Mt_2	8/5/2016	Grandfather Mtn
GF_Mt_3	8/6/2016	Grandfather Mtn
GF_Mt_4	8/7/2016	Grandfather Mtn
GF_Mt_5	8/8/2016	Grandfather Mtn
GF_Mt_7	8/10/2016	Grandfather Mtn
GF_Mt_8	8/11/2016	Grandfather Mtn
GF_Mt_9	8/12/2016	Grandfather Mtn
GF_Mt_10	8/13/2016	Grandfather Mtn
GF_Mt_13	8/16/2016	Grandfather Mtn
GF_Mt_16	8/19/2016	Grandfather Mtn
GF_Mt_17	8/20/2016	Grandfather Mtn
GF_Mt_18	8/21/2016	Grandfather Mtn
GF_Mt_19	8/22/2016	Grandfather Mtn
GF_Mt_20	8/23/2016	Grandfather Mtn
Unaka_6	2016 (date not specified)	Unaka Mtn
Unaka_7	2016 (date not specified)	Unaka Mtn
Unaka_8	2016 (date not specified)	Unaka Mtn
Unaka_10	2016 (date not specified)	Unaka Mtn
Unaka_11	2016 (date not specified)	Unaka Mtn
Unaka_13	2016 (date not specified)	Unaka Mtn
Unaka_14	2016 (date not specified)	Unaka Mtn
GF_Mt_21	2016 (date not specified)	Grandfather Mtn
GF_Mt_22	2016 (date not specified)	Grandfather Mtn
GF_Mt_25	2016 (date not specified)	Grandfather Mtn
GF_Mt_26	2016 (date not specified)	Grandfather Mtn
Mt_Rogers_1	10/2/2016	Mt. Rogers, VA
Mt_Rogers_2	10/2/2016	Mt. Rogers, VA
Mt_Rogers_3	10/2/2016	Mt. Rogers, VA

Mt_Rogers_4	10/2/2016	Mt. Rogers, VA
Mt_Rogers_5	10/2/2016	Mt. Rogers, VA
Unaka_15	2016 (date not specified)	Unaka Mtn
Unaka_16	2016 (date not specified)	Unaka Mtn
Unaka_17	2016 (date not specified)	Unaka Mtn
Unaka_19	2016 (date not specified)	Unaka Mtn
Whitetop_1	9/30/2016	Whitetop Mtn, VA
Whitetop_2	9/30/2016	Whitetop Mtn, VA
Whitetop_3	9/30/2016	Whitetop Mtn, VA
Whitetop_4	9/30/2016	Whitetop Mtn, VA
Whitetop_5	9/30/2016	Whitetop Mtn, VA
Whitetop_6	10/1/2016	Whitetop Mtn, VA
Whitetop_7	10/1/2016	Whitetop Mtn, VA
Whitetop_8	10/1/2016	Whitetop Mtn, VA
Whitetop_9	10/1/2016	Whitetop Mtn, VA
Whitetop_10	10/1/2016	Whitetop Mtn, VA
Whitetop_11	10/1/2016	Whitetop Mtn, VA
Whitetop_12	10/1/2016	Whitetop Mtn, VA
Stamping_Grd_1	2016 (date not specified)	Stamping Ground Ridge, Unaka Mtn
Rock_Crk_1	3/29/2018	Rock Creek, Erwin
Rock_Crk_2	3/29/2018	Rock Creek, Erwin
Rock_Crk_3	3/29/2018	Rock Creek, Erwin
Rock_Crk_4	3/29/2018	Rock Creek, Erwin
Unaka_20	date not specified	Unaka Mtn
Unaka_21	date not specified	Unaka Mtn
Unaka_22	date not specified	Unaka Mtn
Unaka_23	date not specified	Unaka Mtn
Unaka_24	date not specified	Unaka Mtn
Unaka_25	date not specified	Unaka Mtn
Unaka_26	Nov 2017 (date not specified)	Unaka Mtn
Unaka_27	Nov 2017 (date not specified)	Unaka Mtn
Unaka_28	Nov 2017 (date not specified)	Unaka Mtn
HWY_197_1	3/29/2018	HWY 197 App. Trail
HWY_197_2	3/29/2018	HWY 197 App. Trail
HWY_197_3	3/29/2018	HWY 197 App. Trail
HWY_197_4	3/29/2018	HWY 197 App. Trail
HWY_197_5	3/29/2018	HWY 197 App. Trail
HWY_197_6	3/29/2018	HWY 197 App. Trail
HWY_197_7	3/29/2018	HWY 197 App. Trail

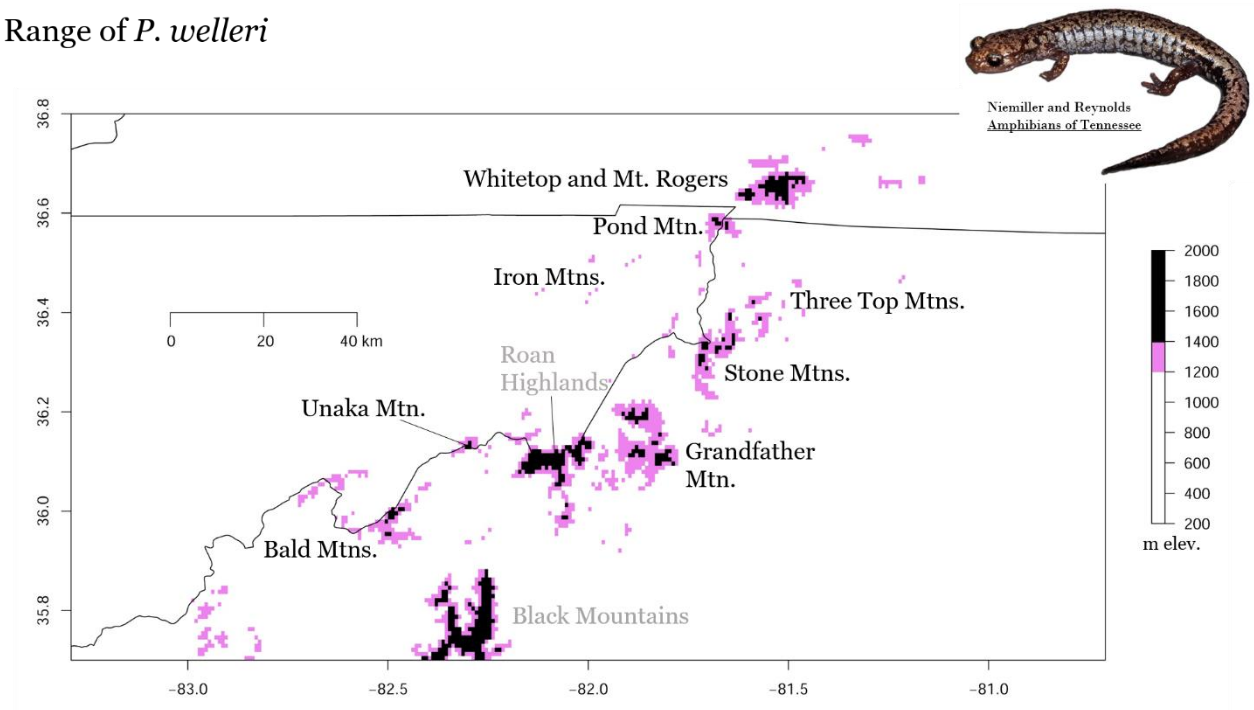

Figure 2. Entire range of *P. welleri*. Black areas are suitable high-elevation sites for the species, while purple represents potentially suitable elevational habitat.

Confirmed records are indicated by black text labels, while suitable habitat with no records are indicated by gray text (e.g., Roan Highlands and Black Mountains). Inset illustration of *P. welleri* is from Niemiller and Reynolds (2011) used with permission. Elevation data are BioClim layer from WorldClim and rendered in R 3.5.1.

3. Results

3.1 Phylogenetics

I found that the three populations of *P. welleri* that I examined in this study were genetically isolated, as indicated by both Bayesian (Fig. 3) and maximum-likelihood (Fig. 4) analyses. Within each of these populations, there is a small amount of genetic variation, indicated by shared haplotypes at the ND2 locus between multiple individuals. However, the three populations differ significantly from one another and those at Grandfather Mountain and Unaka Mountain are more closely related to one another than to that of the Grayson Highlands. I found a crown age of 0.9 million years for the three *P. welleri* populations and a divergence time of 0.59 million years for the Grandfather/Unaka lineages (Fig. 3).

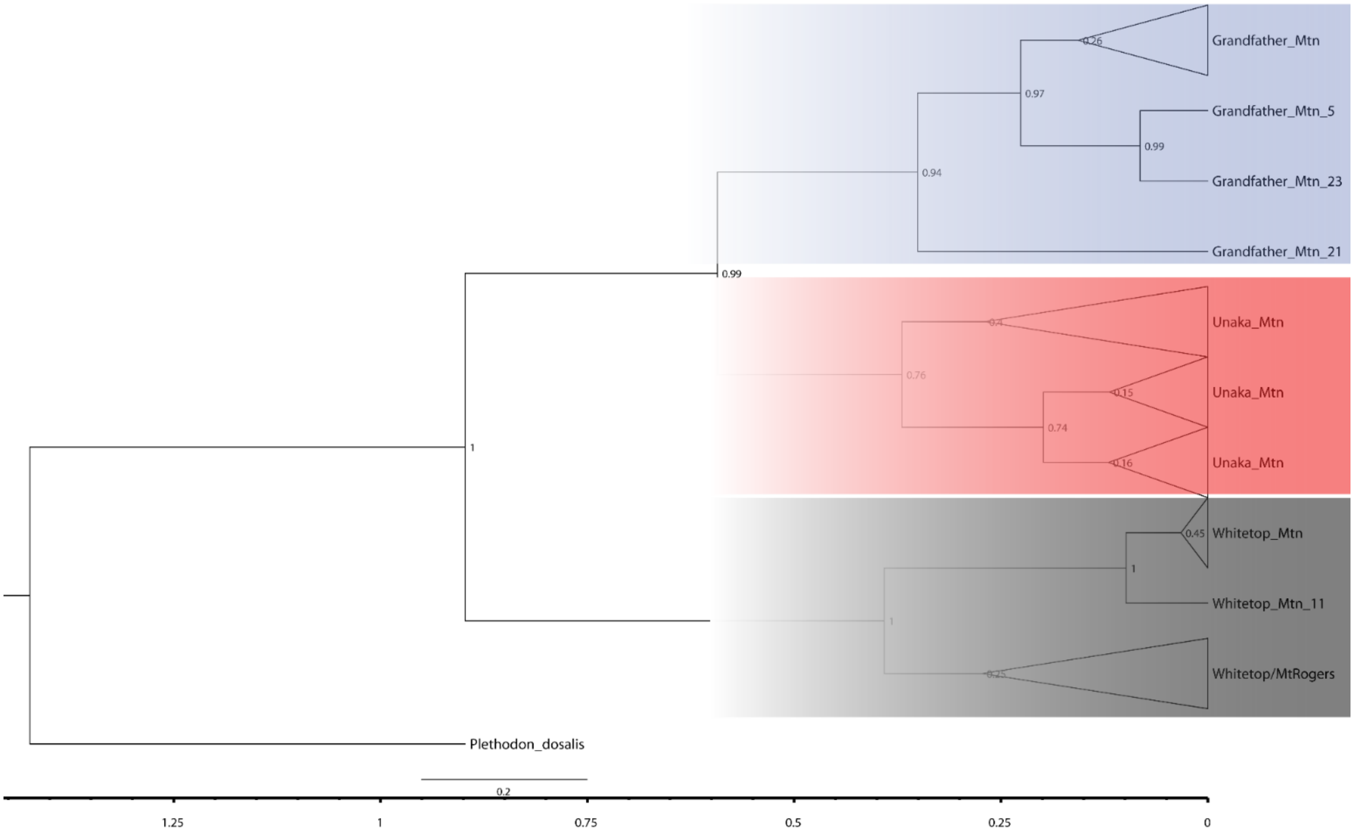

Figure 3. Molecular-clock calibrated phylogeny of 32 *P. welleri* mtDNA sequences from three geographic regions.

Sequences are sourced from the ND2-NADH locus of *Plethodon welleri* individuals from three populations, with *Plethodon dorsalis* as an outgroup. Numbers at nodes are posterior probabilities, and some clades are collapsed to improve visibility.

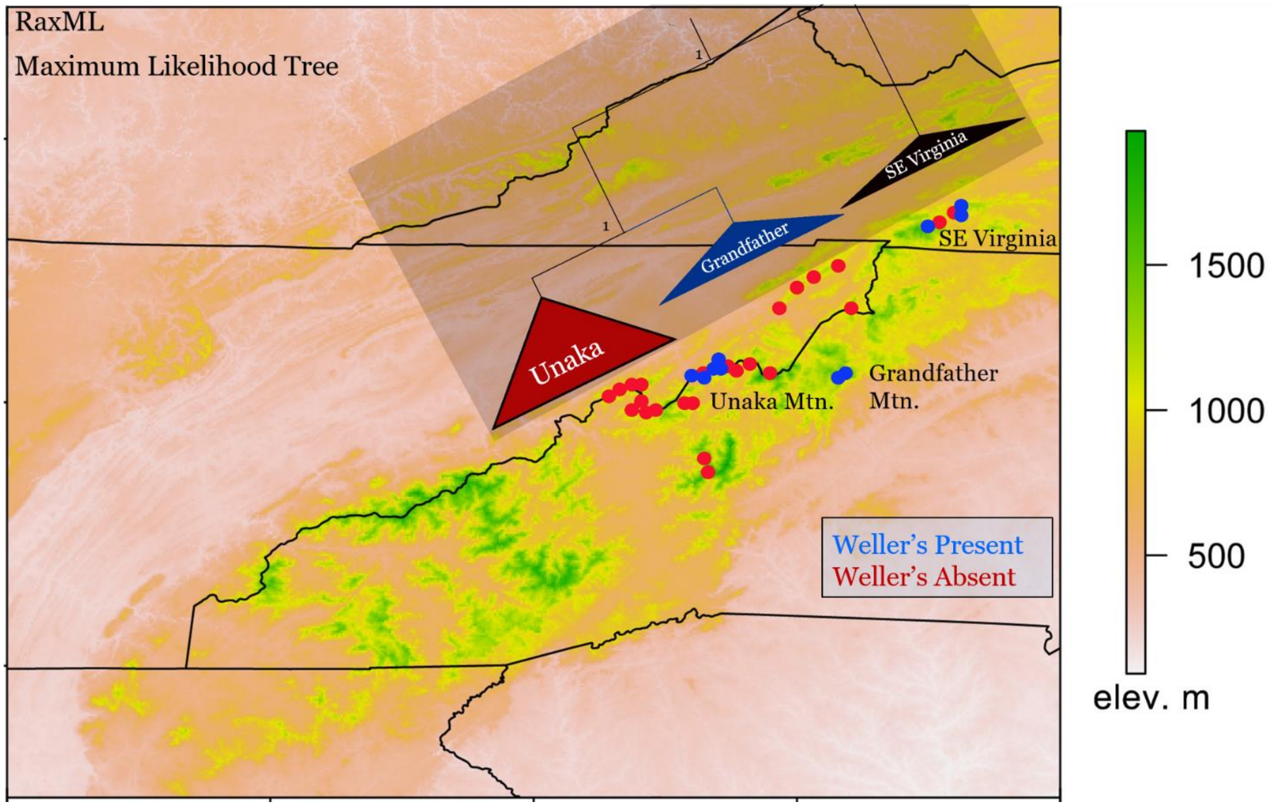

Figure 4. Maximum-likelihood phylogeny of 32 *P. welleri* mtDNA sequences from three geographic regions mapped to sampling localities (after Chambliss 2016).

Numbers at nodes are bootstrap support values, and clades are collapsed to improve visibility. Blue circles are sampling sites where the species was detected in surveys during 2016–2018, red dots are where the species was not detected.

3.2 Microhabitat

We found several transitions between dominant tree species as we proceed up Unaka Mountain, as well as a general transition from nearly neutral to more acidic soil (Fig. 5). We found *P. welleri* individuals throughout the entire range, although we found a higher concentration of individuals at higher elevations in areas of *Picea rubens* and *Betula alleghaniensis* forest than in other forest types (Fig. 5).

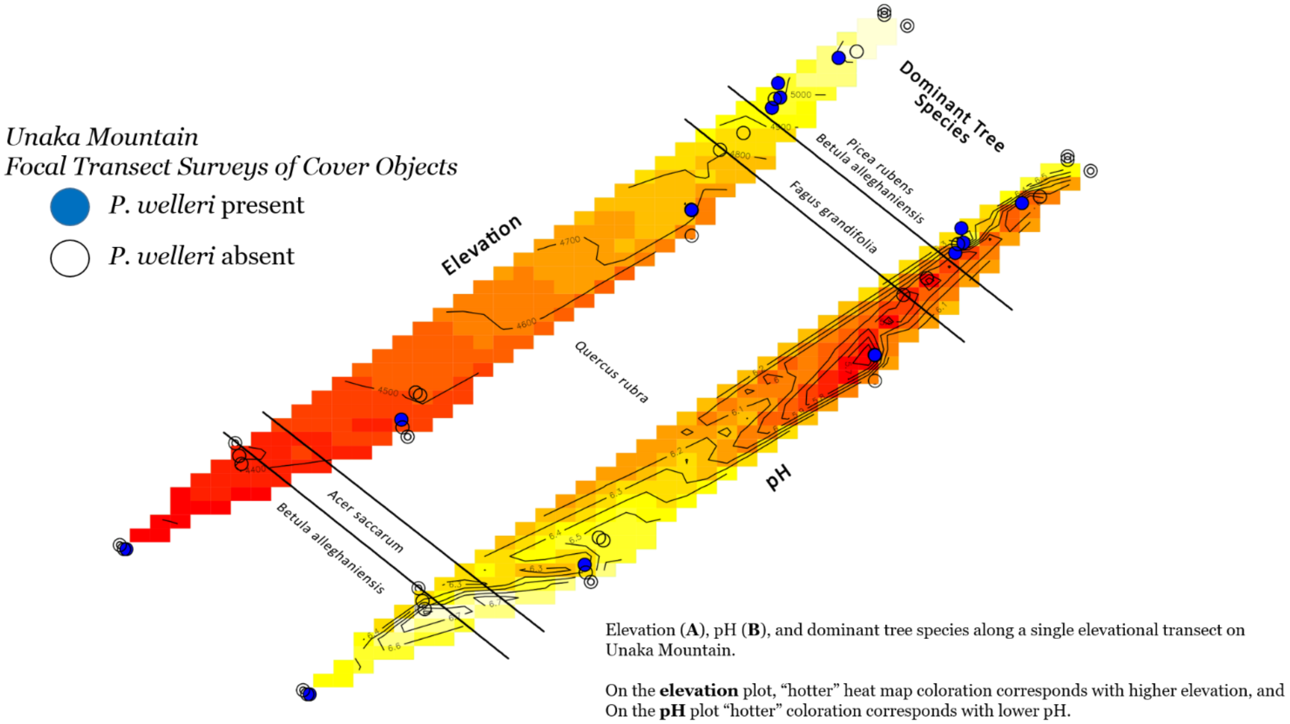

Figure 5. Elevation, pH, and dominant tree species along an elevational transect on Unaka Mountain.

On the elevation plot, "hotter" (light yellow) heat map coloration corresponds with higher elevation, and on the pH plot "hotter" coloration corresponds with higher pH (less acidic). Tree species are indicated across both. Blue dots indicate presence of *P. welleri*, empty circles indicate absence.

4. Discussion

The genetic similarity between the populations of *P. welleri* found on Unaka Mountain and Grandfather Mountain compared to the less-related population found in the Grayson Highlands of Virginia supports my hypothesis that populations of this species which are closer together spatially are also more closely related to one another. This may be due to vicariance events which have made areas between these populations inhospitable to salamanders attempting to cross from one area to another, preventing gene flow. Such events likely occurred in such a way that populations which are currently closer to one another remained contiguous longer than those farther away[6]. This may be related to the greater amount of lowland area which exists between the Grayson Highlands and the other two sites than between Grandfather Mountain and Unaka Mountain.

It is also interesting to note that the region I examined in this study displays relatively subtle differences between montane and lowland habitats, supporting the idea that *P. welleri* as well as other terrestrial salamander species are uniquely susceptible to small changes in habitat[6,16]. Thus, microhabitat may be especially important in determining whether an environment is suitable for *P. welleri* habitation.

Although the microhabitat data reported in this study do not provide adequate statistical strength from which to draw broader conclusions, they nevertheless suggest that a trend exists of *P. welleri* preference for areas of *Picea rubens* forest at higher elevations. They also suggest that a pH gradient may exist on Unaka Mountain, which may correlate with *P. welleri* presence (Fig. 5). However, more data will be needed to assess whether this is the case. These data will allow us to better understand the impacts that climate change and pollution will have on this species' ability to persist in its current habitat, which may become more acidic due to our changing atmosphere[4].

In addition to microhabitat, climatic changes may have exaggerated impacts on *P. welleri* populations. Due to the small of number and range of *P. welleri* populations, the loss of even a single population would significantly hinder the species' ability to survive[6]. Furthermore, the small degree of genetic variability between individuals within each population will likely further prevent them from adapting to environmental changes, making them even more vulnerable to small alterations to their environment[2]. Thus, to preserve this species, it is imperative that efforts be taken to minimize the impacts of both pollution and climate change on the areas which it occupies. Additionally, further research must be conducted into *P. welleri* microhabitat preferences to determine if any trends exist in the conditions which they require for survival, which may then be used to selectively target certain aspects of habitat conservation.

5. Acknowledgements and Funding

Genetic samples were initially collected in partnership with UNC Asheville alumnus Robert Chambliss, who also helped with microhabitat data collection and conducting salamander surveys. This project was funded by UNC Asheville Undergraduate Research Grants in the summer of 2016 and fall of 2018, as well as by the lab of Dr. R. Graham Reynolds.

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