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# *Trechus* (Coleoptera: Carabidae) of Appalachia: A Phylogenetic Insight into the History of High Elevation Leaf Litter Communities

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**Abstract:** Elevation gradients provide a wealth of habitats for a wide variety of organisms. The southern Appalachian Mountains in eastern United States are known for their high biodiversity and rates of endemism in arthropods, including in high-elevation leaf-litter taxa that are often found nowhere else on earth. *Trechus* Clairville (Coleoptera: Carabidae) is a genus of litter inhabitants with a near-global distribution and over 50 Appalachian species. These span two subgenera, *Trechus* s. str. and *Microtrechus* Jeannel, largely restricted to north and south of the Asheville basin, respectively. Understanding the diversification of these 3–5 mm flightless beetles through geological time can provide insights into how the litter-arthropod community has responded to historical environments, and how they may react to current and future climate change. We identified beetles morphologically and sequenced six genes to reconstruct a phylogeny of the Appalachian *Trechus*. We confirmed the Asheville Basin as a biogeographical barrier with a split between the north and south occurring towards the end of the Pliocene. Finer scale biogeography, including mountain-range occupancy, was not a reliable indication of relatedness, with group ranges overlapping and many instances of species-, species group-, and subgeneric sympatry. This may be because of the recent divergence between modern species and species groups. Extensive taxonomic revision of the group is required for *Trechus* to be useful as a bioindicator, but their high population density and speciose nature make them worth additional time and resources.

**Keywords:** endemic; bioindicator; sky island; soil biodiversity

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## 1. Introduction

The Appalachian Mountains of eastern North America are a region defined by old mountains and valleys. While lower in elevation than their western counterparts, the Rockies and Sierra Nevada, their age has allowed for organisms to establish and evolve in association with the abundant microhabitats in the region [1]. The Pleistocene glacial cycles were especially important to the development of the fauna of the present Appalachian Mountains [2]. While the Laurentide Ice Sheet covered Canada and the northern United States, the southern Appalachian region was affected by the climate cycles accompanying the glaciers further north [1]. Many biologists hypothesize that the glaciers and the cooler regions associated with them served as a connection for tundra-adapted species, allowing range expansion and gene flow. As glaciers melted and temperatures rose, these organisms were forced back north or into higher elevations, where climates were cooler [3,4].

Glaciation was not a static process; throughout the Pleistocene, areas of glacier expanded and contracted, allowing periodic migration between areas that were subsequently divided. When the glaciers finally receded at the end of the Ice Age, surviving organisms south of their typical ranges were confined to microhabitats where the remnants of their ideal habitat could be found, a movement pattern that can be modelled for both restricted and extended ranges [5,6] and often includes "sky islands", high-elevation areas that isolate organisms from one another across relatively short distances [7,8]. Whereas the mechanisms behind their origins may vary, "sky island" systems and the communities they sustain found in various parts of the world are facing similar challenges, requiring phylogenetic and conservation work to preserve them [9–11].

In pursuit of cool and wet environments, many montane Appalachian species also took refuge in caves and along streams [12,13]. Many of these taxa are still dependent on these geographical features, as they are now too low in elevation to survive elsewhere. Many of these lack obvious morphological adaptations to the dark and wet, closely resembling leaf-litter relatives [12].

Organisms are also limited in their ability to relocate or expand their ranges by geographical barriers, such as valleys and rivers, that change in form and degree over time. Whereas rivers themselves may not be absolute barriers, the lower elevation regions of unsuitable habitats surrounding them can be [14]. The Asheville depression, the modern valley of the French Broad River, is the classic example in the Southern Appalachians, but the Little Tennessee River and a range of smaller water courses no longer in existence created finer-scale genetic isolation [15].

Many microhabitat-limited Appalachian species are now short-range endemics under threat of extirpation by human activity, invasive species, and climate change [2,16,17]. Leaf-litter communities at these sites support high biodiversity, with frequent and ongoing studies continually bettering our taxonomic knowledge of these regions [15,18,19]. More broadly, systematic and taxonomic work in the southern Appalachian region has centered around rare, isolated, and habitat-specific taxa in an attempt to preserve the areas and species while they are still able to survive [3,13,20,21]. Whereas this is greatly informative of policy and crucial to the survival of rare species, including the spruce-fir moss spider, *Microhexura montivaga* [22], there are regional questions that may be better addressed by the common, highly speciose members of the community that have high variability in their environmental and climatic needs. Comprehensive regional conservation plans that take these under-documented elements of biodiversity into account will thereby become increasingly feasible.

The genus *Trechus* Clairville, 1806 has 52 species documented in southern Appalachia (Table 1). Adults are wingless and 3–5 mm in length, limiting dispersal ability. Where found, they can be incredibly abundant (hundreds of individuals) in litter samples and underneath rocks, providing a wealth of material for study. Because of their size, *Trechus* and beetles like them have been neglected as prospective ecological indicators in the region, where use of carabids is otherwise commonplace [23].

Three *Trechus* subgenera occur in the southeastern United States, two native, one introduced. *Microtrechus* Jeannel is found exclusively in Appalachia, typically southwest of the French Broad River in North Carolina, Tennessee, and northern Georgia. *Trechus* s. str. is a Holarctic subgenus with only a few members in Appalachia (in the regionally endemic '*T. hydropicus*' group), usually found northeast of the French Broad. *Trechus (Calotrechus) obtusus* Erichson, 1837 has been recently introduced into Appalachia and other areas and, unlike the endemics, is winged [24].

Trechine beetles have attracted considerable interest around the globe because of their adaptable nature and frequent association with cryptic microhabitats, including caves. Caves have served as lower-elevation refugia in a variety of trechines, some of which have evolved remarkable troglobitic traits including long legs, a loss of pigmentation, and reduced eye size [25–27]. Resolving the relationships of Appalachian *Trechus*, where such adaptations have not arisen, will help flesh out the picture of *Trechus* evolution globally.

In Appalachia, the genus *Trechus* has garnered particular interest because of their sheer diversity (over 50 species) and their fine-scale distributions across the central and southern Appalachians. In the early twentieth century, Jeannel described three species within this range: *Trechus beutenmuelleri* (1931) (which would become a subspecies of *T. hydropicus*), *T. vandykei* (1927), and *T. barberi* (1931) [28]. This group was then largely untouched until the 1960s, when Thomas Barr Jr. began publishing his adventures in the high peaks and caves of Appalachia. Barr established the species groups and their subgroups that evolved into the classification in use today (Table 1, Figure 1), based on geographic and morphological hypotheses [12,29–32]. His meticulous notebooks and specimens were left to the Carnegie Museum of Natural History, where they were made available to researchers. Over his career, Barr described 35 species of Appalachian *Trechus* and left many other potential new species from additional localities unpublished. Martin Donabauer, an amateur entomologist from Europe, would publish many of these localities and others in the early 2000s, describing 18 novel species and revising *Microtrechus* higher organization into what is currently accepted (Table 1) [33–35]. Donabauer is still contributing to the global trechine literature, with a focus on European representatives [36,37].

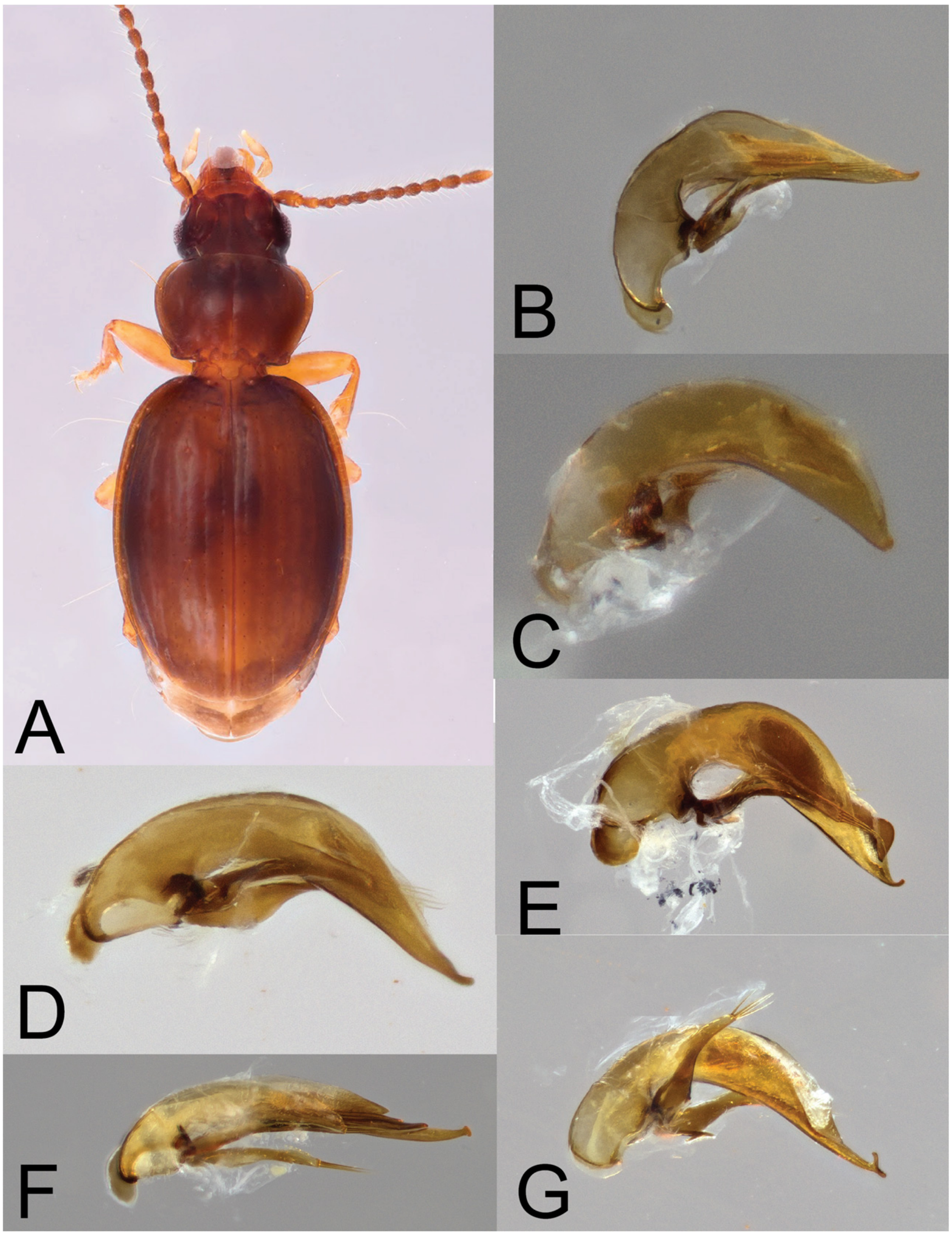

**Figure 1.** Appalachian *Trechus* (**A**). Adult habitus dorsal view (**B**–**G**). Aedeagi of group representatives (**B**). *Trechus barberi* (*T. barberi* subgroup) (**C**). *T. pisgahensis* (*T. vandykei* subgroup) (**D**). *T. bowlingi* (*T. bowlingi* subgroup) (**E**). *T. nebulosus* (*T. nebulosus* group) (**F**). *T. hydropicus* (*T. hydropicus* group) (**G**). *T. thunderheadensis* (*T. uncifer* group).

**Table 1.** Current taxonomy of Appalachian *Trechus* [30,32,33,35]. Taxa sampled in this study are indicated (*).

| Subgenus | Group | Subgroup | Species | Subspecies |
|---|---|---|---|---|
| *T.* (*Microtrechus*) | *T. vandykei* | *T. barberi* | *T. subtilis* Barr, 1962 * | |
| | | | *T. pseudosubtilis* Donabauer, 2009 * | |
| | | | *T. tonitru* Barr, 1962 | |
| | | | *T. barberi* Jeannel, 1931 * | |
| | | | *T. pseudobarberi* Donabauer, 2009 * | |
| | | *T. bowlingi* | *T. bowlingi* Barr, 1962 * | |
| | | *T. vandykei* | *T. tusquitee* Barr, 1979 | |
| | | | *T. pisgahensis* Barr, 1979 * | |
| | | | *T. vandykei* Jeannel, 1927 * | |
| | | | *T. haoe* Barr, 1979 * | |
| | *T. uncifer* | | *T. inexpectatus* Barr, 1985 * | |
| | | | *T. thunderheadensis* Donabauer, 2005 * | |
| | | | *T. uncifer* Barr, 1962 | |
| | | | *T. plottbalsamensis* Donabauer, 2005 * | |
| | | | *T. aduncus* Barr, 1962 * | |
| | | | *T. cowensis* Barr, 1962 | |
| | | | *T. toxawayi* Barr, 1979 * | |
| | | | *T. howellae* Barr, 1979 | |
| | | | *T. satanicus* Barr, 1962 * | |
| | | | *T. tusquitensis* Donabauer, 2005 * | |
| | | | *T. talequah* Barr, 1962 * | |
| | *T. nebulosus* | | *T. balsamensis* Barr, 1962 * | |
| | | | *T. cheoahensis* Donabauer, 2005 | |
| | | | *T. clingmanensis* Donabauer, 2005 * | |
| | | | *T. haoeleadensis* Donabauer, 2005 | |
| | | | *T. luculentus* | *T. l. cheoahbaldensis* Donabauer, 2005 |
| | | | | *T. l. joannabaldensis* Donabauer, 2005 * |
| | | | | *T. l. luculentus* Barr, 1962 * |
| | | | | *T. l. wayahensis* Barr, 1979 |
| | | | *T. nantahalae* Barr, 1979 | |
| | | | *T. nebulosus* Barr, 1962 * | |
| | | | *T. novaculosus* Barr, 1962 * | |
| | | | *T. pseudonovaculosus* Donabauer, 2005 | |
| | | | *T. ramseyensis* Donabauer, 2005 * | |
| | | | *T. rosenbergi* Barr, 1962 | |
| | | | *T. snowbirdensis* Donabauer, 2005 | |
| | | | *T. stefanschoedli* Donabauer, 2005 | |
| | | | *T. stupkai* Barr, 1979 | |
| | | | *T. tennesseensis* | *T. t. tauricus* Barr, 1962 * |
| | | | | *T. t. tennesseensis* Barr, 1962 * |
| | | | *T. thomasbarri* Donabauer, 2005 | |
| | | | *T. tobiasi* Donabauer, 2005 * | |
| | | | *T. tuckaleechee* Barr, 1962 * | |
| | | | *T. unicoi* Barr, 1962 | |
| | | | *T. valentinei* Barr, 1979 | |
| | | | *T. verus* Barr, 1962 * | |
| | | | *T. wayahbaldensis* Donabauer, 2005 * | |
| *T.* (*Trechus*) | *T. hydropicus* | | *T. hydropicus* | *T. h. hydropicus* (Horn, 1883) * |
| | | | | *T. h. avus* Barr, 1962 * |
| | | | | *T. h. beutenmuelleri* Jeannel, 1931 * |
| | | | | *T. h. canus* Barr Barr, 1962 * |
| | | | *T. schwarzi* | *T. s. schwarzi* Jeannel, 1931 |
| | | | | *T. s. scopulosus* Barr, 1979 * |
| | | | | *T. s. saludae* Barr, 1979 * |
| | | | *T. cumberlandus* Barr, 1962 * | |
| | | | *T. mitchellensis* Barr, 1962 * | |
| | | | *T. carolinae* Schaeffer, 1901 * | |
| | | | *T. roanicus* Barr, 1962 * | |
| | | | *T. calignis* Barr, 1985 * | |

Previous studies of *Trechus* have mostly focused on morphological variation in establishing the species limits and infrageneric taxa of these beetles. Whereas species groups are identifiable under the microscope through male genitalia and other somatic traits, relationships between them remain unknown. Kane et al., [25], a study evaluating the relationships of members of the *T. vandykei* and *T. bowlingi* subgroups using gel electrophoresis, is the only modern genetic assessment for at least a part of *Microtrechus. Trechus pisgahensis* was found to be the sister to the remainder, with *T. bowlingi* recovered as sister to *T. tusquitee*, and *T. vandykei* as sister to *T. haoe*. It was concluded that gene flow was nonexistent between species subgroups, and that altitude was a major factor in this isolation [25]. In a later taxonomic revision, it was speculated that the "*T. bowlingi*" sampled therein were a mixture of multiple species, bringing these results into question [35]. Whereas the species subgroup taxonomy and preferred genetic analyses have changed over the last 30 years, their hypotheses regarding the movement of *Trechus* across Appalachia have provided a perspective inaccessible through morphological analysis.

In this study, we evaluated the large-scale evolutionary history of Appalachian *Trechus* beetles. We first ask how many independent lineages are represented among Appalachian *Trechus*, as their current classification into two subgenera, one shared with other Holarctic taxa, does not suggest monophyly. We reconstruct a dated, multigene phylogeny for the southern Appalachian *Trechus* to examine environmental and historical factors implicated in their isolation and related evolutionary patterns, including the Pleistocene glacial cycles, earlier climatic events, and physical barriers such as the Asheville depression. By sampling across the region, we explore genetic differences between peaks and mountain ranges to determine the timings of possible patterns of dispersal and occupancy. This intensive look at how a tiny, flightless leaf-litter resident has navigated geological history provides a different perspective of regional changes.

## 2. Materials and Methods

### 2.1. Specimen Collection and Identification

Southern Appalachia includes Virginia, West Virginia, Kentucky, Tennessee, North and South Carolina, Georgia, and Alabama. *Trechus* is not known to reside in Alabama.

Adults and larvae were collected into ethanol and live in leaf-litter samples throughout the known range of Appalachian *Trechus*. Sampling localities were selected based on the previous literature, type localities for species of interest, and a general rule that *Trechus* are typically found above 5000′ elevation (though as we have found, this is not absolute). Leaf litter was sifted using an 8 mm mesh litter sifter and placed in Berlese funnels for 8–12 h to collect beetles. Adults were also hand-collected by flipping rocks and logs, peering under moss, and peeling apart leaf packs within and along streams.

Adult males were identified morphologically by aedeagus morphology using the primary literature (Table 1, Figure 1) [12,29–35]. A subset (3–5) of those collected from each site, including males, females, and larvae, were selected for sequencing, with more included where morphological variability, such as in body size or in aedeagal morphology (detectable when slightly extruded from the apex of the abdomen, or through the abdominal sternites of less darkly pigmented specimens) were apparent.

Extralimital *Trechus*, necessary for assessing the monophyly of Appalachian taxa, were sampled from other published studies, totaling 233 specimens. Because the monophyly of global *Trechus* is questionable, individuals from several related genera were included as well. The final data set included 200 Appalachian *Trechus* individuals, 214 extralimital *Trechus*, and 46 non-*Trechus* outgroups.

### 2.2. DNA Extraction and Amplification

Six genes were chosen for this study based on their prevalence in the worldwide trechine literature [37–39]. All Appalachian specimens were sequenced for the COI barcoding or mini-barcoding region following lab protocol [24]. Wingless, 18S, 28S, CAD4, and Topoisomerase were sequenced for a subset of specimens, one individual per species or species group (Table 2). Outgroup sequences were obtained from Genbank (National Library of Medicine, Bethesda, MD, USA) and other Trechini works in progress [40].

**Table 2.** Protocol for polymerase chain reactions (PCRs) conducted in-house. * Sequences for COI did not always render useable data, but the PCR was attempted for each individual.

| Gene | Forward Primer | Reverse Primer | Reference | Denaturing | Annealing | Cycle # | Sample Frequency |
|---|---|---|---|---|---|---|---|
| COI | LCO1490<br>BF2 | HCO2198<br>BR2 | [41]<br>[42] | 94 °C 0:30 | 50 °C 0:30 | 35 | Every Individual * |
| Topo | TP643F | TP932R | [43] | 94 °C 0:30 | 57°/52°/45° 0:30 | 6/6/36 | Species Group |
| | TP675F | TP932R | [43] | | | | |
| CAD4 | CD806F<br>CD821F | CD1098R2<br>CD1098R2 | [43]<br>[43] | 94 °C 0:20<br>94 °C 0:20 | 60 °C 0:20<br>55 °C 0:20 | 39<br>37 | Species Group |
| 18S | 18S5′ | 18Sb5.0 | [44] | 94 °C 0:30 | 50 °C 0:30 | 35 | Species Group |
| 28S | NLF184/21 | LS1041R | [45] | 94 °C 0:30 | 50 °C 0:30 | 35 | Species Group |
| Wingless | wg550F<br>wg578F | wgAbRZ<br>wgAbR | [43]<br>[43] | 94 °C 0:20<br>94 °C 0:20 | 52 °C 0:20<br>54 °C 0:20 | 37<br>35 | Species |

Beetles were extracted using a combination of spin-column (GeneJET, Thermo-Scientific, Waltham, MA, USA) protocols and magnetic bead (Omega Bio-Tek Norcross, Norcross, GA, USA) purification. Non-mitochondrial loci were commercially Sanger sequenced by Psomagen (Rockville, MD, USA), while COI sequences were generated through both Sanger and next-generation techniques (Nanopore [46] and Illumina (https://illumina.com accessed on 10 January 2024) sequencing). Sequences were trimmed in GENEIOUS 8.1.8 (https://www.geneious.com accessed on 10 January 2024) before being aligned both with MUSCLE [47] and manually in Mesquite [48].

### 2.3. Tree Building

Before analysis, individuals identical in all sequences were merged in PAUP* for simplification. To infer the topology and monophyly of the taxa in question, a maximum likelihood analysis of all sequences available was performed with IQ-Tree on CIPRES (www.phylo.org accessed on 10 January 2024) with 50 bootstrap replicates and the Test-Merge option, creating a consensus tree, returning a rooted phylogram with maximum-likelihood support values. Finding the monophyly of Appalachian *Trechus* to be strongly supported, the data set was then trimmed to Appalachian representatives and two outgroups (*Pseudanophthtalmus* Jeannel, 1920, and the extralimital *T. obtusus*), and the process was repeated. These outgroups were chosen over the more closely related *Duvalius* Delarouzée, 1859, because we were able to sequence all six genes from them. This trimmed phylogram was used as the model tree for BEAST2 analyses, constraining the topology to the above. Outgroups were limited in this analysis because of their variable genetic coverage. A dated phylogeny was created in BEAST2 with a standard carabid mutation rate for COI [49,50] of 0.0145 mutations per site per million years. A fossil parameter was included, creating a mean of 25 MYA for the separation of *Trechus* s.l. from other genera, with an offset of 15 and reverse log normal distribution. This was consistent with the dates obtained in the recent trechine literature [50,51]. The six genes were partitioned with unique rates and a single tree. The models were determined using IQ-Tree, which designated GTR for COI, TN94 for a concatenation of Topo and Wg, and Jukes–Cantor for the remainder of the genes (18S, 28S, and CAD4). Gamma site variability for all models was set to four categories. Bayesian analyses were completed on CIPRES in BEAST 2.6.6 with 100,000,000 mcmc generations and a tree-sampling frequency of 10,000. Trees were combined to obtain a consensus in TreeAnnotator, discarding 10% as burn-in.

A lineage-through-time plot was constructed to visualize the timing of diversification frequency and correlate these values with potential environmental cues. The plot was constrained to the interval between the Appalachian *Trechus* split and the timing of the shallowest branches with 90% ML support.

## 3. Results

The broadest maximum likelihood analysis provided 100% support for Appalachian *Trechus* monophyly, and for a sister group relationship between *Microtrechus* and the Appalachian members of *Trechus* s. str. (Supplementary Figure S1). Other genera were found to be interspersed with the *Trechus* outgroups but did not impact the taxa of interest's reciprocal monophyly.

Through Bayesian clock analyses on the constrained ML tree (Figure 2) from the reduced data set, we found that the split between *Microtrechus* and Appalachian members of *Trechus* s.str. is estimated at 3.8 MYA with a wide margin of error (2 MYA to 15 MYA). Species subgroups diverged from one another between 1 and 3 MYA, with species-level differentiation falling largely within the last million years (Figures 3 and 4). ESS values were high for all parameters except for COI tree likelihood (ESS = 126), which may demonstrate some incongruence between COI and the other genes.

The range of *Trechus* s.str. in Appalachia (the *T. hydropicus* group) was confirmed to largely be confined to the north of the Asheville depression. Those not conforming to this pattern were found in the Cumberland Plateau, where they are known exclusively from caves and cave entrances. This group also contains a few lower-elevation representatives from wet riparian areas (subspecies of *T. schwarzi* Barr).

*Microtrechus* was subdivided into five well-supported lineages that exhibit considerable geographic overlap, though mostly west of the Asheville depression, and thus seem to represent old and simultaneously diversifying groups in the region (Figure 5). Members of the *T. vandykei* subgroup, however, extend across the Asheville depression, where they overlap with the *T.* (*s.str.*) *hydropicus* group. The *T. bowlingi* subgroup was confined to the Great Smoky Mountains and overlapped with all other *Microtrechus* groups and subgroups. The *T. barberi* subgroup extends the furthest south, into the mountains of north Georgia, but also overlaps with all other *Microtrechus* groupings. The *T. uncifer* and *T. nebulosus* groups share a minor overlap in the Great Smoky Mountains, which otherwise serve as a border between their ranges.

The Great Smoky Mountains was the range exhibiting the highest diversity and overlap in subgroups, with members of all five *Microtrechus* subgroups present at Clingmans Dome or a neighboring peak (e.g., Mount LeConte). The time of speciation amongst and within these subgroups is inconsistent, as is the diversity within them. *Trechus subtilis* exhibited several haplotypes, even though most specimens were recovered from only Big Cataloochee Mountain. Its widespread sister, *T. barberi*, had low genetic variability within sample sites, leading to one haplotype representing most localities.

At lower levels, there are a number of clear geographical patterns in speciation. From north (Camp Creek Bald and Big Bald) to south (Mount Hardy and Toxaway Mountain), there is a clear split in the *T. vandykei* subgroup, dated at ~0.75 MYA. From east (Balsam Mountains and Toxaway Mountain) to west (Clingmans Dome and Huckleberry Knob), the *T. uncifer* group appears to have split at least 0.25 MYA.

The lineage-through-time plot demonstrates that Appalachian *Trechus*, after splitting at the end of the Pliocene, had limited diversification for the next 2 MYA, followed by a period of increasingly rapid diversification, totaling 46 sampled lineages at the ~species-level cut-off of 0.65 MYA. Nearly half of the total current-lineage diversity apparently arose within the past million years.

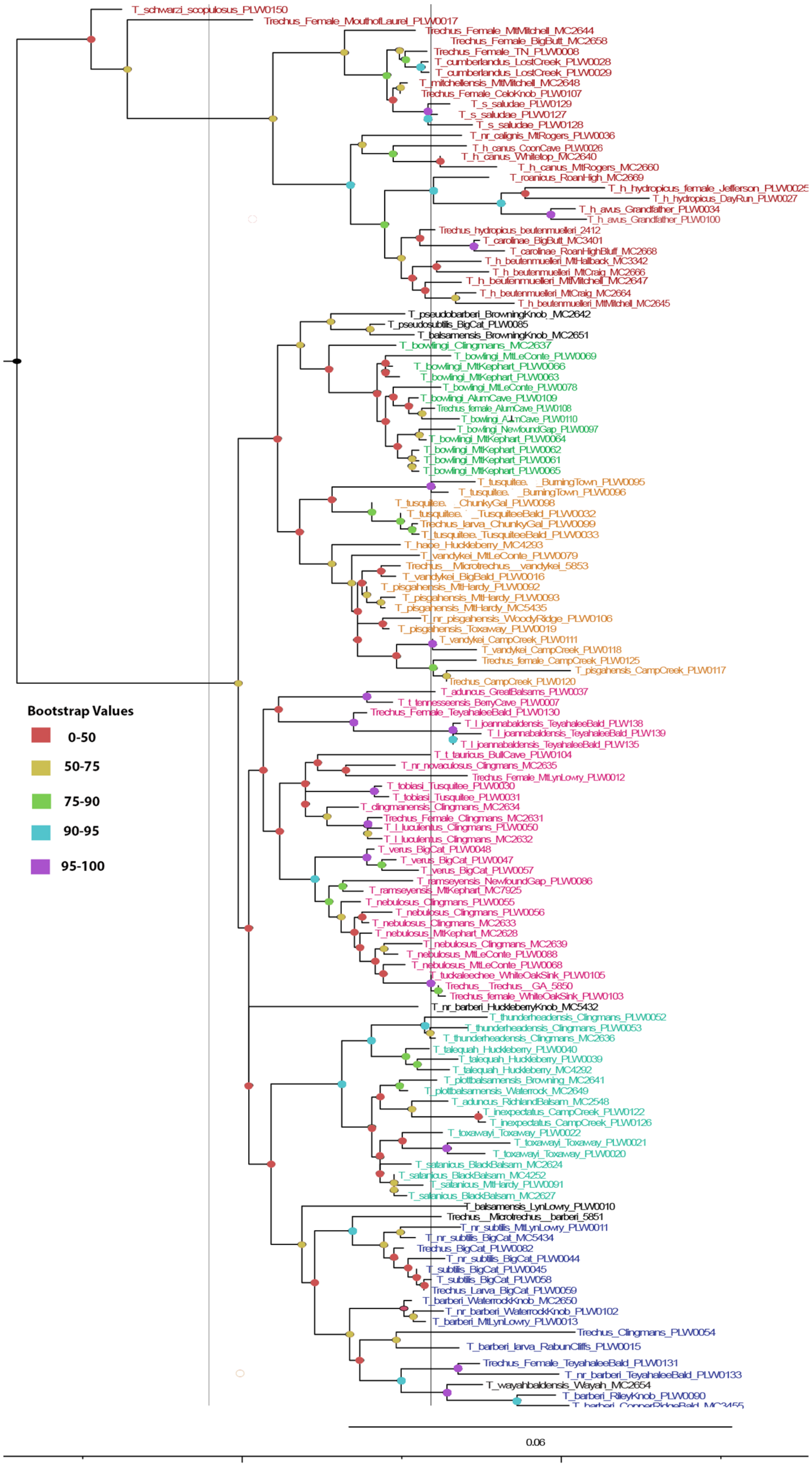

**Figure 2.** Maximum likelihood phylogram of Appalachian *Trechus*. Clades are coded to their species groups: red—*T. hydropicus* group, teal—*T. uncifer* group, pink—*T. nebulosus* group, orange—*T. vandykei* subgroup, green—*T. bowlingi* subgroup, and blue—*T. barberi* subgroup. Black-coded individuals fell outside their historical group. Bootstrap values are indicated at nodes. The tree is rooted at *T. obtusus* (not shown).

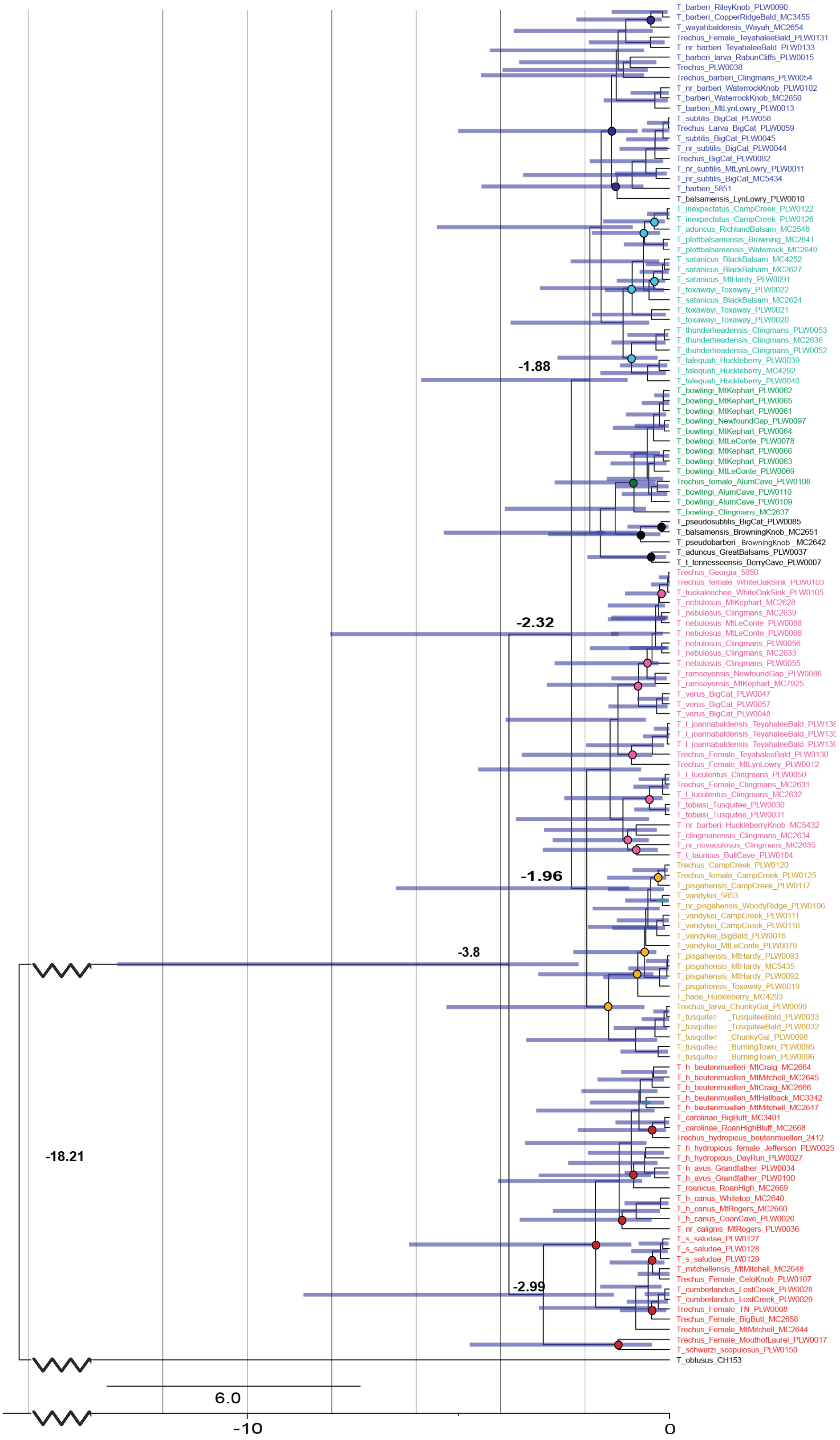

**Figure 3.** Bayesian-calibrated dated (MY) phylogram of Appalachian *Trechus* with a structure modelled from Figure 2. Circles indicate nodes of speciation colored by species group: red—*T. hydropicus* group, teal—*T. uncifer* group, pink—*T. nebulosus* group, orange—*T. vandykei* subgroup, green—*T. bowlingi* subgroup, and blue—*T. barberi* subgroup. Black-coded individuals fell outside their historical group.

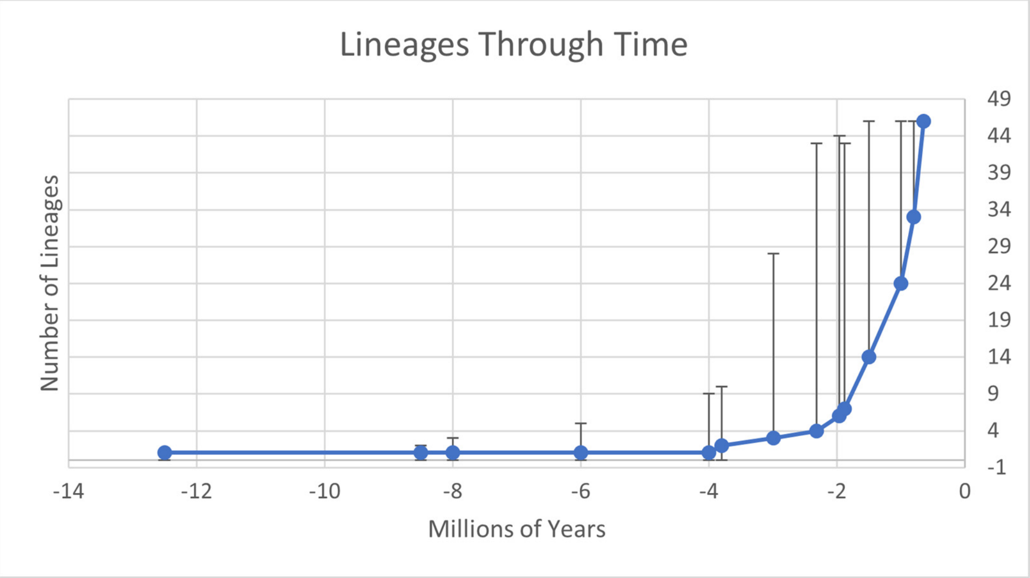

**Figure 4.** Lineage-through-time plot of Appalachian *Trechus* divergence. Error bars accommodate the uncertainty of dating reflected in the BEAST2 analysis.

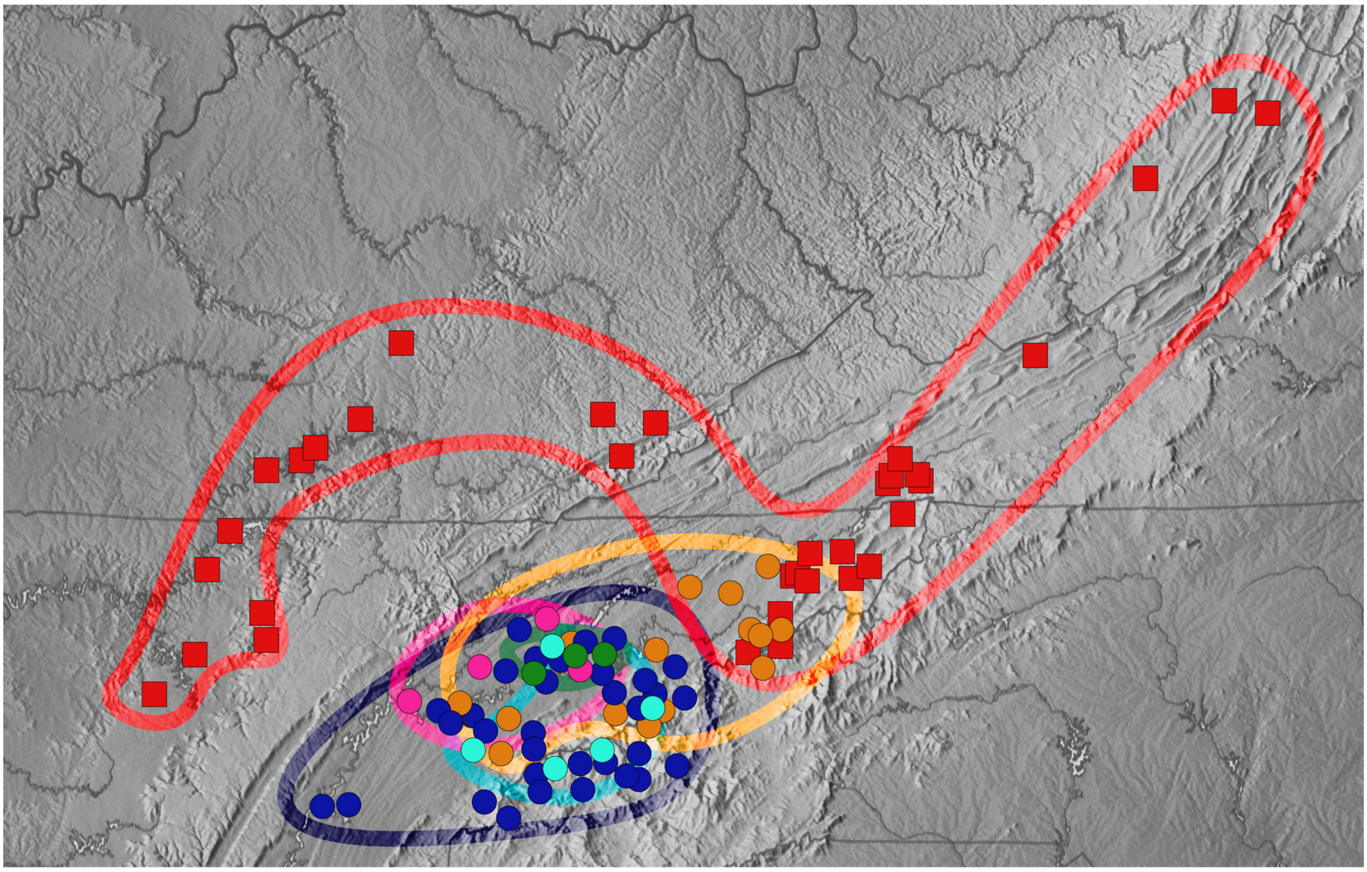

**Figure 5.** Map of southern Appalachia coded by species groups present. Dots indicate localities of previous collections. This includes those we collected, museum and databased specimens, and the previous literature on the group. Groups and subgroups are divided as denoted in Table 1. Squares are *Trechus s.str.* and circles *Trechus* (*Microtrechus*); the color code is as follows: red—*T. hydropicus* group, teal—*T. uncifer* group, pink—*T. nebulosus* group, orange—*T. vandykei* subgroup, green—*T. bowlingi* subgroup, and blue—*T. barberi* subgroup.

## 4. Discussion

In this study, we used the morphological and genetic traits of *Trechus* across southern Appalachia to better our understanding of how the Pleistocene glacial cycles, older climatic events, and physical barriers have shaped the diversification and distribution of extant *Trechus*.

Subgenera of *Trechus* in southern Appalachia are reciprocally monophyletic and are the result of one larger radiation, rather than two smaller ones or a polyphyletic species assemblage. The presence of *Microtrechus* and their relationship with the *T. hydropicus* group reinforces that global *Trechus* s.str. is not monophyletic, with the *Microtrechus* and the *T. hydropicus* group being sisters to one another [36,52].

The split between these two groups occurred approximately 4 MYA, in the late Pliocene, with finer divisions occurring throughout the Pleistocene. Further differentiation into the species groups and subgroups of *Microtrechus* recognized today occurred approximately 2 MYA, with *T. hydropicus* differentiating considerably earlier (3 MYA). The type-species of this group is multiply paraphyletic and will require more extensive revision (namely the elevation of the subspecies) than any of the groups of *Microtrechus* (Wooden and Caterino in prep).

The division into major lineages within *Microtrechus* and the *T. hydropicus* group occurred largely within the early Pleistocene epoch. The dates of speciation within these lineages are highly variable, consistent with multiple periods of gene flow and isolation across the region (Figure 3). Within *Microtrechus*, the *T. barberi* subgroup was recovered as sister to the *T. uncifer* group. This confounds the hypothesis of *T. vandykei* group monophyly (Table 1), and is interesting morphologically, as there is little genitalic difference between *T. barberi* and *T. bowlingi*, and *T. uncifer* group members have morphologically complex male genitalia (Figure 1). The sister relationship between the *T. vandykei* subgroup and the *T. nebulosus* group carries similar implications. The *T. uncifer* and *T. nebulosus* groups also speciated later than lineages exhibiting more conserved morphology (Figure 3), which may indicate disruptive selection pressure in the later Pleistocene, possibly related to a reintroduction into sympatry.

The groups and subgroups exhibit considerable overlap, so their ancestors were evidently able to move across the landscape during this time, with many populations becoming endemic as the ranges expanded and retracted (Figure 4). This is atypical for southern Appalachian fauna that are often found in population clusters, e.g., one mountain range, habitat type, or area with little to no sympatry [20–22,53,54]. This uniqueness upholds our expectation that studying a mix of narrowly distributed taxa within a widespread genus with multiple habitat preferences yields a different picture than a focus on small-range endemics. *Trechus*, while useful for reconstructing recent biogeographic events, did not differentiate substantially before the late Pliocene and therefore cannot provide a picture of older climatic events. This late Pliocene split coincides with the development of the Asheville Basin around 4 MYA, a pervasive physical barrier.

The *T. hydropicus* group, to the north and west, exhibits a much larger geographic range than *Microtrechus*. This may be because southern ancestors, unable to travel north due to this basin, were more dependent on refugial microhabitats than their northern counterparts, and this is reflected in the results where morphology has been largely conserved, and individuals at the periphery of the range (such as *T. cumberlandus* in central Tennessee) have recently split from their relatives. Southern *Microtrechus*, as post-Pleistocene climates warmed, moved up the same mountain ranges as their ancestors, whereas *T. hydropicus* was able to expand to the west.

The speciation of *Trechus* was rapid and recent when compared to the ancient mountains in which they reside (Figure 3). Some other studies of Appalachian fauna have similarly found major splits in the late Pliocene and early Pleistocene, such as the eastern tiger salamander (*Ambystoma tigrinum tigrinum*), whose clades became reciprocally isolated in the early Pleistocene and have undergone extensive population differentiation in the last 500,000 years [5]. Unlike many other arthropods, *Trechus* species groups largely overlap geographically, which may be due to this late explosion of speciation and dynamic migration patterns, resulting in the sympatry seen today (at Clingmans Dome, for example). Species groups diverged approximately 2 MYA (1–6 MYA according to confidence interval), but most species divergences occurred later, in the late Pleistocene, allowing for distantly related species to re-establish on and inhabit the same mountaintop. Smaller historical riverine barriers that limited movement for older radiations [4] cannot reliably predict cladistic relationships in this case.

A recent species explosion is not unprecedented for *Trechus*, as species in the Canary Islands have undergone a similar expansion within the last 4 million years [55]. In *Ptomaphagus* (Coleoptera: Leiodidae), with numerous cave and soil endemic species in the southeast, Appalachian representatives have been found to have diversified in similar waves, with a split between the Cumberland Plateau lineages (TN, AL) and rest of their Appalachian range (GA, KY) [56]. Whereas the Asheville depression is not implicated in the isolation of these beetles, the changes in the Cumberland Plateau that are hypothesized to be behind their isolation would have been a factor for western lineages of *Trechus* as well. In *Odontotaenius disjunctus* (Coleoptera: Passalidae), Appalachia is home to widespread sympatric lineages thought to be divided by niche-partitioning, allowing for sympatry [57]. Although this is not a high-elevation endemic, it was affected by Pleistocene cycles and experienced divergences similar to those seen in this study. This passalid has defied numerous geographical barriers in southeastern United States, but its lineages still felt the effects of glacial cycles [57].

The Asheville Basin, which contains the French Broad River, has long been considered a biogeographic barrier for high-elevation taxa that may have prevented their retreat to the north as climates warmed. The generalization that species of *Trechus* s. str. are found north of this barrier and *Microtrechus* largely to the south (Figure 4) agrees with the existing literature [3,5,15,18,19]. Previous *Trechus* work cites the Asheville Basin as the prominent divider in Appalachian species [25]. However, there are exceptions in both directions. *T. hydropicus* group members are found in the Cumberland Plateau to the west, where extant individuals are largely troglobitic. *Trechus vandykei* is found on both sides of the basin, indicating recent leakage. This anomaly has been a common source of debate, and it is thought that when met with the divide, members of the *T. hydropicus* group moved westward and *T. vandykei* east [25,35].

Clingmans Dome, the highest peak in the Smokies, and neighboring Mount LeConte, are common hosts for endemic flora and fauna. Sedges [8], grasses [54], and other sedentary taxa have members only present there, and the sympatry found in *Trechus* agrees. The variability in the time of differentiation in Clingmans Dome residents indicates multiple dispersal events to the mountain within the last million years, which is consistent with the rapid diversification seen in the lineage-through-time plot. Kane et al. [25] speculated that the Smokies were special because of their patchiness and high abundance of high-elevation microhabitats. They hypothesized that this may have caused rapid niche divergence in the region as populations competed for resources and space. We see evidence in support of this hypothesis in the region's sympatry, high diversity, and *T. subtilis*' high genotype diversity on Big Cataloochee Mountain. Our study upholds that the Smokies are an important focus for Appalachian conservation, and that further niche modelling is required to understand what makes this range so special.

Three lineages of Appalachian *Trechus* are known exclusively from caves. *Trechus cumberlandus* (*T. hydropicus* group) diverged approximately 0.3 MYA. *Trechus tennesseensis taurícus* (*T. nebulosus* group) diverged around the same period, and *T. t. tennesseensis* (which does not appear to be closely related to its other subspecies) was more recent, around 0.2 MYA. This supports previous work suggesting that *Trechus* migrated into caves opportunistically, rather than coming to the surface from them. This movement into caves from montane habitats is found in other Trechines (namely *Pseudanophthalmus*) and reflects multiple waves into the Cumberland Plateau from the Black, Craggy, and

Smoky mountains [29]. This recent deviation into caves is also supported by their lack of troglobitic morphology [32].

Two lineages are associated with riparian microclimates, found in lower elevations in leaf packs along and within riffles of perennial streams. *T. schwarzi saludae* (*T. hydropicus* group) and *T. luculentus joannabaldensis* (*T. nebulosus* group), while distantly related, both diverged around 0.25 MYA, implying that an environmental event, such as warming climatic cycles, may have sent them into these cooler, moist streams. Barr had also recognized that streams may be crucial to the lower-elevation success of these beetles, remarking that what he found below 4500 feet were proximal to mountain streams [29]. *Trechus schwarzi saludae*'s type and sampling locality are at a mere 1250 feet above sea level.

Appalachian *Trechus* contains multiple widespread species—namely *T. barberi*, *T. vandykei*, and *T. hydropicus*. There is little in their morphology to suggest why or for how long they have been more mobile than their relatives, and an ecological analysis is needed to understand the evolution of habitat preferences in the tolerant, riparian, and troglobitic members of the genus.

Southern Appalachian fauna have largely responded to the same geographical stressors, but the type of response and subsequent success of descendants is as varied as the region itself. Leaf-litter taxa are and continue to be a conduit for understanding the processes of the past and potential future. In pursuit of conserving the taxa known to science and describing those currently unknown to science, combining the perspectives of rare and common taxa in the context of phylogeography and niche partitioning may provide the most comprehensive picture for policy decisions in this microhabitat-rich landscape.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16040212/s1, Figure S1: Placement of Appalachian *Trechus* in global trechine phylogeny; Table S1: Table of GenBank accession number and notes on methodology.

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**Data Availability Statement:** All data generated during this study are available on GenBank (sequence data; Accession #s pending) or the Clemson University Arthropod Collection's search page on the SCAN (Symbiota Collections of Arthropods Network) database website (occurrence data).

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