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Research Article

The Type Locality Project: collecting genomic-quality, topotypic vouchers and training the next generation of specimen-based researchers

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DNA evidence is often critical for taxonomic studies; however, many historical type specimens lack corresponding genetic samples, which limits contemporary molecular research questions and may restrict conservation and management decisions. We conducted a pilot Type Locality Project to collect voucher specimens and genomic-grade samples from amphibian type localities in the state of Virginia, USA. These samples can serve as proxies for cases in which obtaining genomic data from the type specimen is not possible. Undergraduate students participated in all aspects of the project including fieldwork, DNA barcoding, and incorporating specimens into the Smithsonian Institution's National Museum of Natural History research collection. The Type Locality Project is an excellent platform for providing undergraduate students with hands-on research experience and training in taxonomy and systematics. Other institutions could easily adapt our approach to obtain genomic-quality, topotypic vouchers for other taxa and simultaneously create authentic undergraduate research experiences in field, laboratory, and natural history museum settings.

Key words: amphibian, DNA barcode, frog, museum science, salamander, taxonomy, Virginia

Introduction

In systematics and taxonomy, the value of type materials cannot be overstated. Type material, from which species are described, anchors a taxon in time and space and serves as a tangible reference point to which all future comparisons are made. While it is common in contemporary taxonomic studies to include DNA evidence to validate new species, many historical type specimens do not have genetic samples associated with them. The absence of DNA-grade tissue samples from type materials significantly limits contemporary molecular research questions and may restrict conservation and management decisions in cases when species boundaries are contentious or challenging to delimit (e.g., Stuart & Fritz, 2008). Although a few recent studies demonstrate success with extracting and sequencing DNA from formalin-fixed and historical specimens (e.g., Austin & Melville, 2006; Ruane & Austin, 2017; Turvey et al., 2019), these samples typically do not perform as well as tissue samples that were explicitly preserved for genetic analysis. Furthermore, because removing tissue from specimens for genetic analysis can compromise the integrity and future utility of the specimen, many institutions restrict destructive sampling of type material. Consequently, this limits the utility of

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museum specimens (and type materials specifically) for which there are no specific DNA-grade tissue samples.

As an alternative to extracting low quality genetic data from type specimens, we propose collecting new specimens from type localities to serve as topotypic vouchers. These vouchers can be preserved with the goal of obtaining both high-quality morphological specimens, as well as high-quality tissue samples for genomic research. To accomplish this, we propose establishing collaborative networks on a local scale and in particular, leveraging the strengths of local undergraduate institutions. This approach has the added benefit of providing a platform to train students in museum science, field and laboratory techniques, and systematics research, and thereby provide them with tools that can be applied to future employment and research opportunities (Hiller et al., 2017). As a proof of concept, we assembled a team of biologists from the Smithsonian Institution's National Museum of Natural History (NMNH), U. S. Geological Survey (USGS), James Madison University, and Eastern Mennonite University/ Wilson College, developed a full sampling protocol, and applied it to obtain genomic-quality, topotypic vouchers from amphibian type localities in Virginia.

The choice to focus on amphibians to develop this broader project reflects the taxonomic expertise of the authors, accessibility of frogs and salamanders to student researchers, and high local diversity and abundance of amphibians. In addition, we determined that among natural history museums in the United States with significant herpetological collections, the vast majority of U.S. amphibian specimens in these collections do not have associated tissue samples (Fig. 1). Virginia is home to 84 species of amphibians (Virginia Herpetological Society; https://www.virginiaherpetologicalsociety.com/) and the type localities for 17 species and subspecies occur in the state. Amphibian diversity and endemism contributed to the Appalachian region's recognition as a Biodiversity Hotspot (Milanovich et al., 2010) and yet, a number of cryptic species complexes remain unresolved across this relatively well-studied region (e.g., Beamer & Lamb, 2008; Felix et al., 2019; Jones & Weisrock, 2018; Kozak et al., 2006; Kuchta et al., 2018; Tilley et al., 2008). Furthermore, some amphibian populations that were described as species were subsequently synonymized (e.g., Highton, 1962; Muchmore, 1955), while others that were described as subspecies are now recognized as full species in light of molecular evidence and a closer examination of morphology (e.g., Felix et al., 2019; Kuchta et al., 2018). Thus, this focal region provides a representative set of case studies for demonstrating how topotypic genetic material can be useful for addressing unresolved taxonomic issues. In addition, amphibians are currently facing population declines and extirpations as a result of habitat degradation, habitat fragmentation, infectious disease, and climate change (Highton, 2005; Milanovich et al., 2010; Wake & Vredenburg, 2008). Thus, when possible we incorporated community-level surveys and pathogen screening for the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) into our field protocols to provide students with experience in implementing these standard techniques.

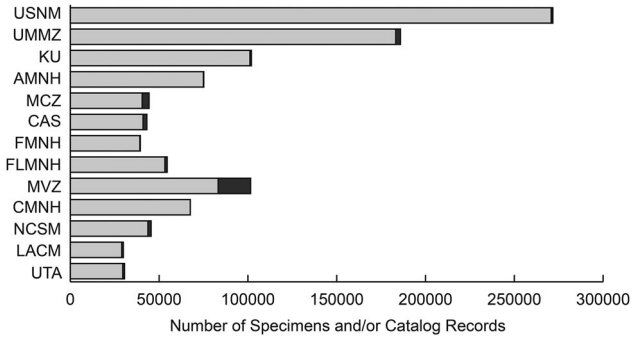

Fig. 1. The 13 largest herpetological natural history collections in the U.S and the corresponding number of specimens and/or catalogue records of amphibians housed within each of these collections that were collected in the continental U.S. The proportion of morphological vouchers without tissues (light grey) and vouchers with tissues and/or tissues without morphological vouchers (dark grey) are indicated. The museums are presented in ranked order from largest to smallest size of their respective overall herpetological collections (worldwide holdings of amphibians and reptiles): USNM (U.S. National Museum of Natural History), UMMZ (University of Michigan Museum of Zoology), KU (Kansas University), AMNH (American Museum of Natural History), MCZ (Museum of Comparative Zoology), CAS (California Academy of Sciences), FMNH (Field Museum of Natural History), FLMNH (Florida Museum of Natural History), MVZ (Museum of Vertebrate Zoology), CMNH (Carnegie Museum of Natural History), NCSM (North Carolina Museum of Natural Sciences), LACM (Los Angeles County Museum), UTA (University of Texas, Arlington).

Here we detail our approach to jointly (1) collect genomic-quality tissues from specimens collected at type localities (topotypic materials) of amphibians in Virginia and curate them in a public research institution, (2) generate topotypic DNA barcodes and deposit them in standard genetic data repositories, and (3) train undergraduate researchers in museum science, field and laboratory techniques, and systematics research (Fig. 2).

Methods

More detailed instructions and planning notes for all aspects of the project are included in the Supplemental Materials. During the spring semester of 2017, nine undergraduate and two graduate students enrolled in Herpetology (BIO 427/526) at James Madison

University (JMU) actively participated in multiple aspects of this project as part of the required coursework. Course-based activities included training in field sampling techniques, collection and preparation of museum specimens, literature reviews, reviews of synonymies, and identification of specific localities for sampling. One JMU undergraduate student (GS) and one Eastern Mennonite University student (CM) were offered paid internships for summer 2017 during which they organized and led fieldwork, and worked with project mentors in laboratory and museum settings.

We used Amphibian Species of the World (ASW; Frost, 2018), field guides, and local, state and county species lists, to determine currently recognized taxa and synonyms for our focal taxa and region (amphibians in Virginia). Only taxa and synonyms that were described from our focal region (Virginia) were included in resurvey efforts. For instance, the green tree frog, *Hyla cinerea*, occurs in Virginia but the type locality of the species is "Carolina" (Schneider, 1799), thus *H. cinerea* was not included in our list of taxa. Conversely, *H. evittata* is a synonym of *H. cinerea* and its type locality is in Virginia (Miller, 1899); thus, we included *H. evittata* in our list of taxa. We included sub-species and synonyms in our sampling efforts because historically these taxonomic designations were often made without genetic data and topotypic genetic material may be useful for resolving any lingering taxonomic uncertainty. Our final list of currently recognized taxa and synonyms included 17 species and subspecies (Table 1).

To identify the type locality for each taxon, we used ASW (Frost, 2018), taxonomic monographs, and museum databases that include further details about type localities. Confirming localities from the primary publication is important because secondary sources may be incomplete or introduce errors by repeating unjustified or incorrect modifications from previous publications. Likewise, taxonomic revisions often include justified clarifications or restrictions of type localities and may provide additional information for more precisely placing a particular locality. For instance, the type locality of *Desmognathus planiceps* presented in the original publication (Newman, 1955) was restricted to a one-mile radius, but was open to interpretation at a finer scale. In their taxonomic analysis of a portion of the *D. fuscus* group, however, Tilley et al. (2008) visited the specific collecting locality with one of the original collectors and provided the updated locality in their study. For more recent, geocoded type localities, we used Google Earth to physically place the localities. We took more care in physically placing older type localities that were originally based on verbal descriptions because the names and locations of towns and roads often change through time, which makes this process more prone to error. Whenever possible, we used maps from approximately the time that the specimen was collected and relied on the USGS Geographic Names Information System (GNIS; https://geonames.usgs.gov/domestic/index.html). Our final list included 16 type localities that represent the 17 amphibian species and subspecies (Table 1, Fig. 3).

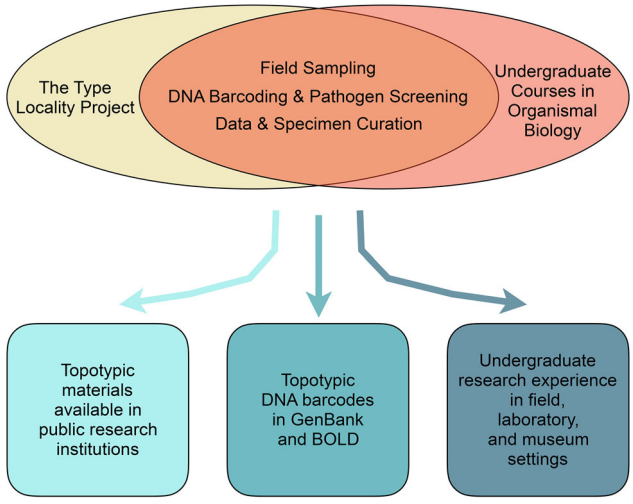

Fig. 2. Conceptual framework and outcomes of the Type Locality Project and areas of overlap with learning objectives in undergraduate organismal biology courses.

Planning and executing fieldwork

The 16 type localities occurred across public, protected, and private land; therefore, we obtained collecting permits at the Virginia State and US National Forest levels, and contacted city park officials and private landowners to ask for permission to access the sites. Two of the type localities are in Shenandoah National Park, which would have required an additional permit, but we obtained topotypic materials for these taxa through an ongoing conservation genetics project conducted by the USGS and the Smithsonian Conservation Biology Institute (SCBI) and thus did not revisit these localities. To locate contact information for private landowners, we used local property tax record databases. For sites at which our permits allowed general collecting of amphibians, we sampled non-target amphibian taxa as well to bolster our DNA barcode reference library.

To support the development of leadership and organizational skills, students (GS and CM) were encouraged to take responsibility for planning and executing fieldwork with mentors playing a supporting role. Students were responsible for contacting private landowners, communicating trip details to the team, and determining the logistics and equipment/supplies required for day and overnight trips. To develop an efficient field

Table 1. Amphibian taxa, taxonomic authority, and corresponding type localities in Virginia.

Taxon and authority	Locality	Sampled
Desmognathus monticola jeffersoni Hoffman, 1951	Albemarle County: Saddle Hollow on Jarman's Mt., 2 mi west of Crozet, 1600 ft. elev.	Y
Desmognathus orestes Tilley & Mahoney, 1996	Smyth County: headwaters of Daves Branch, along Elk Garden trail just north of Elk Garden on divide between Mt. Rogers and Whitetop Mt., 1329 m elev.	Y
Desmognathus organi Crespi et al., 2010	Smyth County: north-facing slope of Whitetop Mt. (36°, 38.342′ N, 81° 36.549′W), 1672 m elev.	Y
Desmognathus planiceps Newman, 1955	Patrick County: stream (approx. 2800 ft. elev.) … gorge below the Dan River Dam near Meadows of Dan	Y
Eurycea bislineata wilderae Dunn, 1920 *(Eurycea wilderae)*	Grayson County: Whitetop Mt., 4000 ft. elev.	Y
Hyla evittata Miller, 1899 *(Hyla cinerea)*	Fairfax County: Four Mile Run, Alexandria	N
Plethodon huldae Grobman, 1949 *(Plethodon cinereus)*	Madison County: along foot trail to Hawksbill Mt. … 3500 ft. elev.	(Y)
Plethodon dixi Pope & Fowler, 1949	Roanoke County: Dixie Caverns	Y
Plethodon hoffmani Highton, 1972	Alleghany County: Clifton Forge	N
Plethodon hubrichti Thurow, 1957	Bedford County: by the Blue Ridge Parkway at about 3100 ft. [elev.], 0.9 mi south of cement milepost 80	Y
Plethodon montanus Highton & Peabody, 2000	Grayson County: Deep Gap (36° 39′ 28″N, 81° 33′ 25″W), 1 km west of top Mt. Rogers, 1500 m elev.	Y
Plethodon richmondi popei Highton & Grobman, 1956	Grayson County: Comers Rock, Grayson-Wythe County line	Y
Plethodon richmondi shenandoah Highton & Worthington, 1967 (*Plethodon shenandoah*)	Page County: Hawksbill Mt., Shenandoah National Park	(Y)
Plethodon sherando Highton, 2004	Augusta County: NW slope Bald Mt. (37° 55′ 09″ N, 79° 04′ 00″ W)	Y
Plethodon jacksoni Newman, 1954	Montgomery County: Trilium Vale, 2100 ft. elev., approx. 1 mi east of Blacksburg	Y
Plethodon welleri ventromaculatum Thurow, 1956	Grayson County: Mt. Rogers, 5500 ft. elev.	Y
Pseudotriton ruber nitidus Dunn, 1920	Grayson County: Whitetop Mt., 4000 ft. elev.	N

In the time since their original description, some sub-species have been elevated to full species status and some described species have since been synonymized. For these taxa, the presently recognized taxonomy is given in parentheses following the original name. Topotypic sampling status: Y – yes, this study, (Y) – yes, via collaboration, N – unsuccessful.

sampling plan, the students plotted the type localities on topographic maps and estimated travel times between sites to determine which sites could be surveyed jointly. For instance, seven of the targeted type localities were within close proximity to one another in southwest Virginia (*Desmognathus organi, D. orestes, Eurycea wilderae, Plethodon montanus, Pl. richmondi popei, Pl. welleri ventromaculatum,* and *Pseudotriton ruber nitidus;* Fig. 3) so the students planned a three-day trip to survey these sites and identified nearby Grindstone Recreation Area Campground as a convenient home base. Likewise, the type localities for *D. monticola jeffersoni*, *Pl. dixi*, *Pl. hubrichti, Pl. sherando*, and *Pl. hoffmani* are located along the Blue Ridge Mountains (Fig. 3) so the students planned a two-day trip to survey these sites and identified Douthat State Park as a convenient site to camp. Mountain Lake Biological Station served as a home base for surveys of *D. planiceps* (Patrick County) and *Pl. jacksoni* (Montgomery County; Fig. 3). Finally, given that the type locality for *H. evittata* is within close proximity to the NMNH, museum staff and research volunteers searched this site when weather conditions were favourable for detection. Prior to the first collecting trip, students were trained in field sampling techniques (including collection, euthanasia, tissue sampling, swabbing for pathogens, specimen fixation and preservation), photography, morphometric data collection, and recording data in Grinnell-style field notes (Simmons, 2015). Students were also provided with necessary equipment, supply lists, and field safety guidelines. University faculty and/or federal scientist mentors accompanied students on the initial excursions to provide guidance when needed; however, the undergraduate researchers also conducted one trip

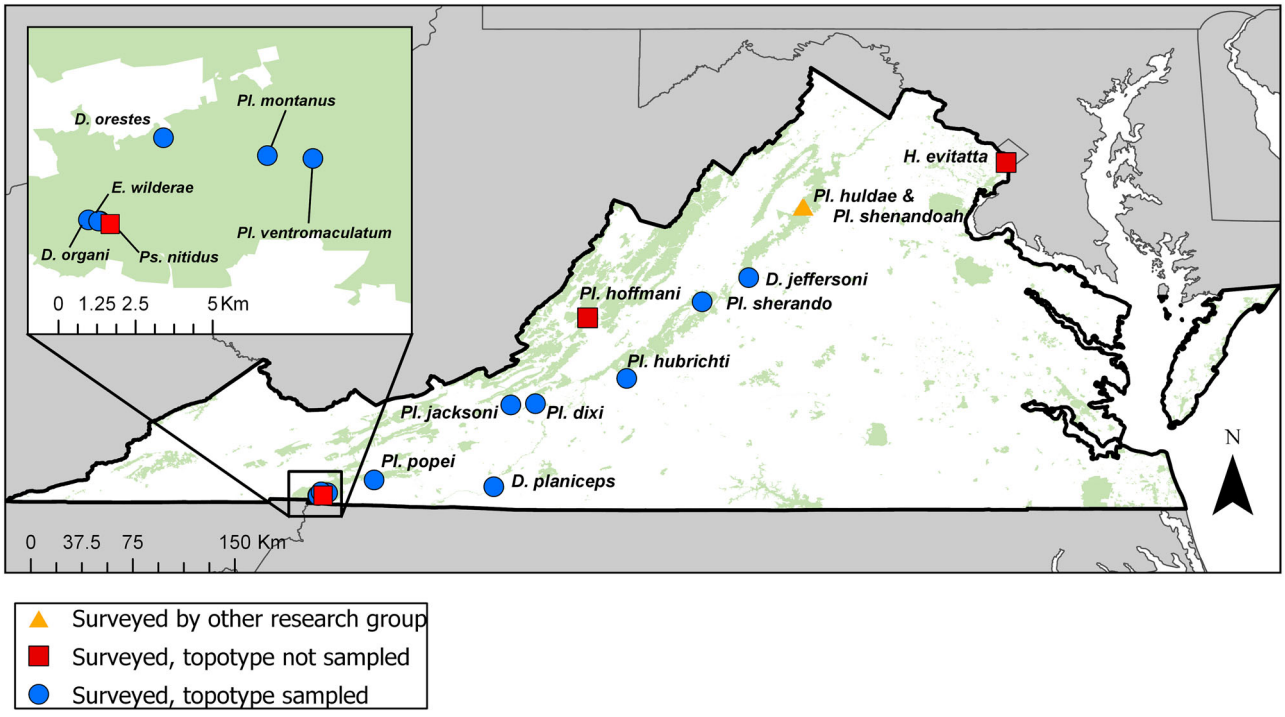

Fig. 3. Type localities of amphibians in Virginia and our success in obtaining topotypic genetic samples. The green areas indicate lands in public and private protective management, with the exception of conservation easements (data from Boyd, 2018). Localities for *Eurycea wilderae* and *Pseudotriton ruber nitidus* are slightly offset for clarity, but the same area (Daves Branch Creek) was sampled for both taxa.

independently. In total, five undergraduate students planned and participated in field surveys.

Sampling was primarily conducted as visual encounter surveys (following Guyer & Donnelly, 2012) in which two or more observers intensively searched the type locality for the target species (Fig. 4). Cover objects (natural or artificial) were flipped and returned to their original position after searching. On tree falls, bark and rotten wood were searched for salamanders. Fine mesh nets were used to collect aquatic salamanders and larvae. At sites where our permits restricted general collecting, any non-target species we encountered was released at the point of capture and its presence was recorded in field notes. For *Hyla evittata*, the one anuran in our study, we used a combination of active searching and PVC frog shelters (McGrath et al. 2020) to survey the study area. We placed ten frog shelters in a wooded area at the inferred type locality (38.84164, −77.05956; WGS84) on 10 April 2018. We checked the shelters and surveyed the surrounding habitats during both day and night at least once a month over the next several months (April–August) targeting dates during and after rains to maximize chances of detection.

All research activities (animal capture, tail clipping, euthanasia) were conducted under approved IACUC protocols (NMNH Jacobs 2015-03 and Bell 2018-01). Specimen preparation and tissue sampling followed the recommendations of McDiarmid (1994) and Simmons (2015). Preliminary identifications were recorded in the field to the species or subspecies level by examining morphological characters and referencing field guides. A standardized field data sheet was provided by the Division of Amphibians and Reptiles of the NMNH and used by all personnel in the field (Table S1). All specimens were photographed on a neutral background and with an X-Rite Color Checker Passport Photo (colour standard) using an Olympus TG-4 waterproof digital camera equipped with a ring-type flash diffuser (Fig. 4). In addition, standardized photographs of specimens included field numbers and a metric ruler to facilitate identifications. At a subset of study sites, each animal was swabbed 30 times using sterile dry swabs (Medical Wire and Equipment - Pt.#MW113): five times each on the right ventrolateral surface of the belly, right ventral surface of the thigh, and right ventral surface of the foot, and then repeated on the left side. Each swab was placed in a 2 ml Nalgene cryotube with 95% ethanol. After being photographed and swabbed, specimens were euthanized in a dilute bath of MS-222. Because of permitting restrictions in protected areas, we could not collect morphological vouchers of some species; therefore,

Fig. 4. Sample photographs for "image-tissue only" topotypic genetic voucher USNM-HI 2897 *Plethodon sherando* collected at Bald Mountain, Blue Ridge Parkway, Augusta County. Because salamanders were photographed alive, it was often challenging to feature morphological traits that are needed to confirm species identifications but here we illustrate dorsal coloration, number of costal grooves (A) and ventral coloration (B) all of which are useful for distinguishing *P. sherando* from closely related species. Students and mentors searching for topotypic *P. dixi* at Dixie Caverns, Roanoke County (C). Topotypic *P. dixi* photographed in situ at Dixie Caverns, Roanoke County (D). Students collecting data for topotypic *P. hubrichti* at Onion Mountain, Blue Ridge Parkway, Bedford County (E).

we non-lethally collected tissues (tail tips) and photographed the animals to serve as photo vouchers for these "tissue-image-only" samples. These samples were ultimately assigned USNM Herp Image numbers (USNM-HI) in the USNM catalogue. Tissue samples (liver, muscle, tail tips) from euthanized and non-lethally sampled animals were preserved in DMSO/EDTA or 95% ethanol; however, other preservation approaches such as RNAlater or flash freezing in liquid nitrogen would allow for an even wider set of downstream applications including transcriptome sequencing and the maintenance of viable cell lines. Specimens were fixed in 10% neutral buffered formalin for a minimum of 24 hours and transferred to 70% ethanol for permanent storage.

DNA barcoding and confirming field identifications

Two undergraduate students (GS and CM) worked with NMNH molecular lab personnel to obtain DNA barcodes from tissue samples, confirm field identifications, curate sequence data, and make the data publicly available in GenBank and the Barcode of Life Database (BOLD). Colleagues at the USGS and the SCBI provided sequences for *P. shenandoah* (GenBank numbers MK493171 and MK493174; Mulder et al., 2019) and *P.* [*huldae*] *cinereus* (MN483063; KP Mulder, unpublished). For all other samples, DNA extractions were performed on an AutoGenprep 965 (AutoGen, Inc.) with an overnight digestion in proteinase-K and M1–2 buffers

and the standard animal protocols. The barcode portion of the COI gene was amplified using Bioline Taq in 10 µl following standard DNA barcoding procedures (Weigt et al., 2012). Currently, there are few published COI DNA barcodes for North American salamanders and many of those currently available are problematic (see Discussion). Therefore, we also sequenced portions of the 16S and Cytochrome b (Cyt b) mitochondrial genes as these are more widely used by the North American amphibian research community (primer and amplification details in Table S2). PCR products were purified using ExoSap-IT™ and sequenced in both directions using a BigDye® Terminator v3.1 Cycle Sequencing kit on an Automated ABI3730 Sequencer (Life Technologies). We edited raw chromatograms and translated protein-coding genes to check for stop codons in Geneious (v.10.0.9; Biomatters Ltd., 2005–2017). To contextualize our data in a more geographically and taxonomically comprehensive dataset, we downloaded sequences for conspecific and closely related species from GenBank and/or BOLD to include in our gene tree analyses. We aligned the sequences for each gene with the MUSCLE plug-in and generated neighbour-joining trees in Geneious (v.10.0.9; Biomatters Ltd., 2005–2017). We also used the barcode index number system (BINs), which is a method that clusters sequences algorithmically and has been adopted by the BOLD community to verify species identifications and document cryptic taxonomic diversity. These clusters typically show high concordance with species; thus, we combined our sequences with those from GenBank and BOLD to test whether sequences attributed to a given species form unique clusters. Sequences generated for this study are accessioned in GenBank (COI: MK037288–MK037383; 16S: MK029509–MK029565; Cyt b: MK029458–MK029508) and Barcode of Life Database (BOLD; BISON001-17–BISON056-17 and BISON057-18– BISON098-18).

Pathogen screening

One undergraduate student (CM) worked with JMU faculty to determine the presence of the amphibian chytrid pathogen *Batrachochytrium dendrobatidis* DNA on skin swabs collected from animals. Swabs were extracted with Prepman Ultra (Applied Biosystems, Part No. 4318903), diluted 1:10 in sterile water, and stored at −20 °C prior to qPCR. *Bd*-positive and -negative controls were included in each batch of extractions. Quantitative detection of *Bd* followed Retallick et al. (2006) using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Samples were considered positive if more than one dilution-corrected zoospore genomic equivalent (ZGE) of the pathogen was detected. To reduce the likelihood of false *Bd* positives, samples that tested positive were rerun and considered true positives if *Bd* DNA was detected in at least one triplicate well in the subsequent run (Kriger et al., 2006). Reported ZGE were corrected to account for the total amount of DNA isolated per swab.

Cataloguing and curating specimens and associated field data

All specimen vouchers, tissues, and associated data were deposited in the research collection of the Smithsonian Institution's National Museum of Natural History (NMNH). Because of permitting restrictions in protected areas, we were allowed to collect tissues (tail tips), but not specimens, for some of our target taxa and therefore have photographic vouchers for these "tissue-only" samples. Fortunately, the NMNH catalogues photos and associated data (including genetic material) without physical specimens, but this practice varies widely across institutions.

Two undergraduate students (GS and CM) worked with NMNH collections personnel to process the specimens from arrival at the museum through cataloguing, installation into the Division of Amphibians and Reptiles collection, and making the data publicly available. After transferring the formalin-fixed specimens to 70% ethanol, we first checked field identifications of each specimen (or photograph for "tissue-only" samples) by looking for diagnostic morphological characters. We used a combination of the morphological identification and DNA barcodes to confirm species identifications. Once the species identifications were confirmed, we curated the associated field data and photographs. The catalogue data and photographs are accessible online (https://collections.nmnh.si.edu/search/herps/), and the topotypic specimens and tissues are available to the research community following the Division's standard loan terms (https://naturalhistory.si.edu/research/vertebrate-zoology/amphibians-reptiles/collections-access/specimen-loans).

Results

Successful sampling efforts

We successfully collected topotypic material for 12 of the 17 species and subspecies (Table 1, Fig. 3). Through fieldwork conducted by colleagues at the USGS and the SCBI, we obtained topotypic material for two additional species with type localities in Shenandoah National Park bringing our combined topotypic sampling to 14 of 17

target species and subspecies. Most of our sampling efforts were concentrated into three trips in summer 2017 and two trips in fall 2017.

Unsuccessful sampling efforts

Hyla evittata – The type locality, Four Mile Run District Park in Alexandria, is in a flood plain forest at the edge of Four Mile Run stream, just upstream of where it feeds into the Potomac River. The surrounding area is heavily urbanized; however, the park includes a roughly six-hectare area of mixed hardwood stands, ephemeral channels, and vernal ponds. Because it is less than half a mile from the Potomac River, the area is highly influenced by tidal changes and the area floods during high summer tides and heavy rains. We used a combination of active searching and frog shelters to survey areas throughout the park. Although we did not find *H. evittata (H. cinerea)* at the study site, we did find several *H. chrysoscelis* at a vernal pond and one in a frog shelter. Two additional surveys were conducted at a small, managed pond system ~5.6 km upstream in Glencarlyn District Park. We found juvenile *Lithobates* but no hylid frogs at this second site, even on the day that *H. chrysoscelis* were found calling at Four Mile Run.

Plethodon hoffmani – Based on the original description, the type specimen (USNM 135203) was taken from "Clifton Forge, Alleghany County, Virginia" (Highton, 1972). This general identification of a small city as the type locality presented us with logistical problems in knowing where to direct our search efforts and provided no way of knowing if we had in fact collected specimens from the true type locality. We were fortunate to be able to communicate directly with the original author who clarified the location as being an "unnumbered road that leaves the centre of Clifton Forge and climbs the mountain toward Hot Springs. On that road you come to an intersection of Rose Ave. and Revere St. Continue north for 0.6 miles and you see a little stream on the east (right) side of the road" (Richard Highton, University of Maryland, College Park, MD, personal communication, 7 September 2017). Dr. Highton also recommended that we survey the site in October after a rain. Although his description provided much needed detail for locating the precise type locality, the surrounding landscape has changed substantially over the last 40+ years, including the construction of an elevated interstate highway bridge at this site subsequent to the description of the species. Although it appears that suitable habitat for *P. hoffmani* persists at this site, no specimens were encountered during multiple (five) nocturnal and diurnal searches during May, June, and early November of 2017 and 2018. Samples of *Desmognathus fuscus* and *Eurycea cirrigera* were collected at this site, and the latter was relatively abundant.

Pseudotriton ruber nitidus – Based on the original description (Dunn, 1920), the type locality was "Whitetop Mt., Va., 4000 feet (under a log in the woods)." This same locality description was provided by Dunn for *Eurycea bislineata wilderae*. Topographic maps were used to identify locations at Whitetop matching the elevation and habitat requirements for this semi-aquatic salamander. A suitable location at Daves Branch stream was located along Whitetop Road (36.6541, −81.5841) and was searched once in 2017. We searched for both adults (terrestrial habitats) and larvae (aquatic habitats). Despite finding *Eurycea* at this location, as well as *Desmognathus quadramaculatus* and *Gyrinophilus porphyriticus*, we did not find any *Pseudotriton* adults or larvae. *Pseudotriton* and *Gyrinophilus* larvae are very similar in appearance and can be found together in rocky mountain streams (DSM, personal observation); thus, it appears that there is suitable habitat for *Pseudotriton* at this site and that the species will be encountered there in future surveys.

DNA barcoding and confirming species identifications

We obtained DNA sequences for 98 individuals (97 salamanders and one anuran) with 96 COI barcodes, and 57 16S and 51 Cytochrome b sequences. Based on our approach for confirming species identifications, our sampling encompassed 24 taxa, including 14 of our focal topotypes (Table 1) and several non-target taxa we sampled through general collecting efforts (Table S3). We obtained COI barcodes for every species and nearly every individual we sampled (Fig. 5). Although we were unable to sequence COI for one specimen of *Desmognathus orestes* and one specimen of *Plethodon jacksoni*, Cytochrome b sequences and morphological traits confirmed the identity of these specimens.

In most cases, all COI barcodes for a given species were placed into single barcode index numbers (BINs) in the combined dataset of sequences from our study and those available in GenBank and BOLD. The exceptions were non-target taxa we collected as part of our general sampling surveys (*Desmognathus fuscus, D. quadramaculatus,* and *Eurycea cirrigera*). Each of these taxa are considered species complexes that are not monophyletic based on mtDNA sequence data (Beamer & Lamb, 2008; Kozak et al., 2006). Correspondingly, sequences from our sampling, GenBank, and BOLD clustered into two BINs for each species complex: *D. fuscus* USNM 589853 was placed in its own BIN with

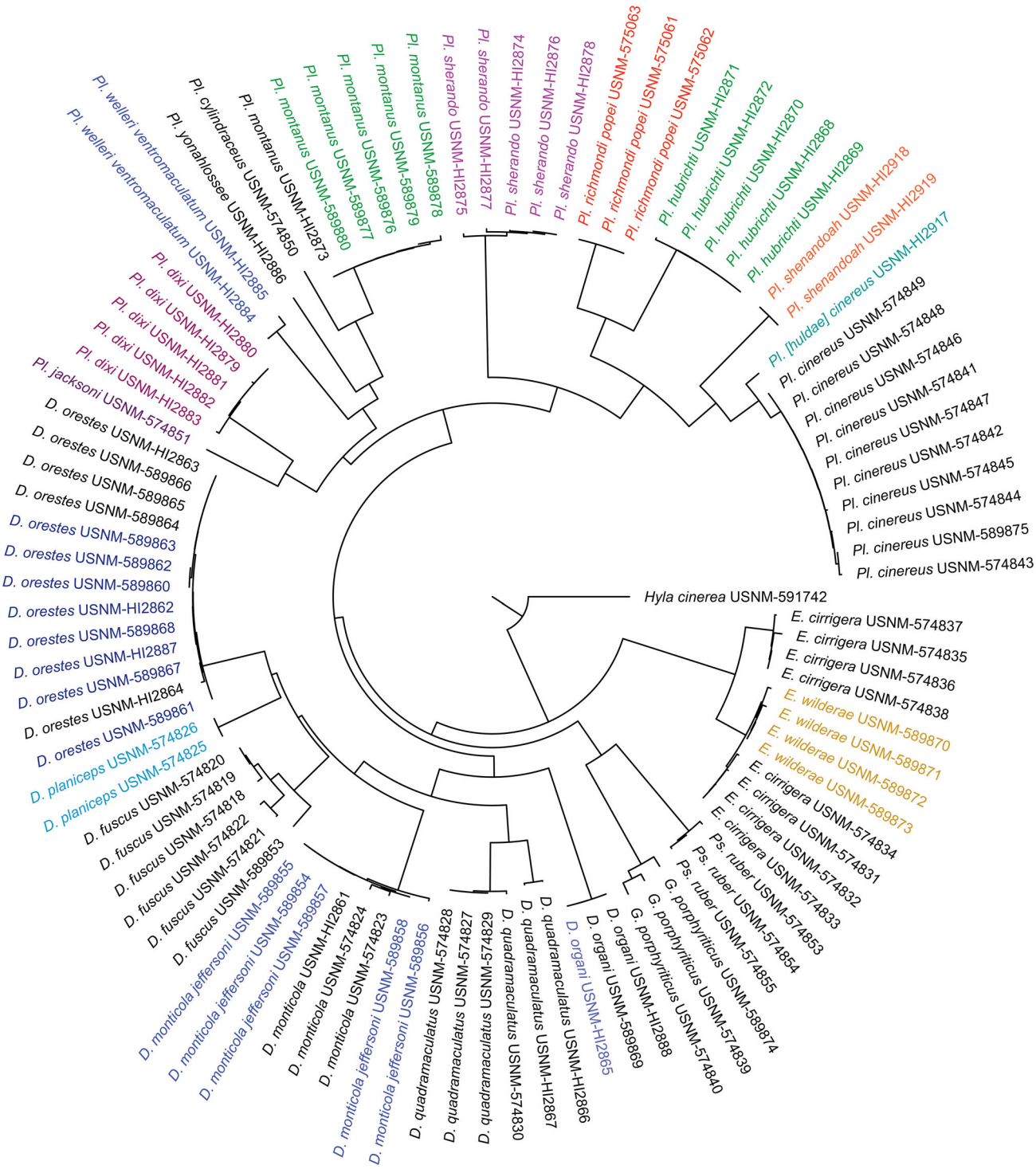

Fig. 5. Neighbour-joining tree based on the mitochondrial COI gene from specimens collected for the present study. Sample names in colour represent topotype target taxa, while those in black are non-target taxa or non-topotype localities that we sampled to contribute to the DNA barcode library. USNM-HI refers to Herp Image numbers, for tissue-image records only, as opposed to the standard USNM Herp catalogue numbers for voucher specimens.

the remaining *D. fuscus* in a separate BIN, and the two clades of *D. quadramaculatus* and two clades of *E. cirrigera* (Fig. 5) were each placed in their own BIN. Some specimens (USNM 574831–34), from Blacksburg, Montgomery Co, VA, that we initially identified as *E. cirrigera* were placed in the same BIN as the topotypic

E. wilderae specimens (same clade in Fig. 5); these two species are difficult to distinguish morphologically and are indistinguishable based on mtDNA (Kozak et al., 2006).

Although the remaining sequences from our study were placed in single BINs per species, our species identifications did not always match those of GenBank and/or BOLD sequences that were placed in the same BIN. For example, our topotypic samples of *Desmognathus monticola jeffersoni* (USNM 589854–58) were placed in one COI BIN (BOLD:AAC2421) along with our three non-topotype samples, eight sequences from GenBank identified as *D. monticola* [four from Rissler et al. (2004) and four from Beamer and Lamb (2008)], and a single GenBank sequence (KU986016) identified as *D. marmoratus* (UAHC 16123; Fig. S1). This previously published *D. marmoratus* sample has no locality data other than "United States" and is part of a large North American amphibian and reptile DNA barcoding project (Chambers & Hebert 2016). Based on our combined dataset, a number of specimens from the Chambers and Hebert (2016) project appear to be misidentified (see Supplemental Materials).

Though we were not able to find *Hyla evittata* from the type locality, we did collect one specimen of *Hyla cinerea* in the District of Columbia approximately 6.5 km northeast of the type locality of *H. evittata*. This specimen was phenotypically intermediate for the striped patterns described for *H. evittata* and *H. cinerea* (short lateral stripe over both shoulders, faint lateral stripes along body, and little upper labial stripes), and was placed in a BIN (BOLD:AAK3512) with five other individuals of *Hyla (Dryophytes) cinerea* from BOLD.

Pathogen screening

We swabbed 37 individual salamanders representing twelve plethodontid species from our surveys in Augusta, Alleghany, Montgomery, and Patrick counties (Table S3). Two salamanders tested *Bd* positive (overall prevalence Clopper Pearson 95% CI: $0.01 \leq 0.05 \leq 0.18$): a *Eurycea wilderae* in Montgomery County and a *Desmognathus quadramaculatus* in Patrick County (Table S3). The two positives contained mean *Bd* zoospore loads of 16,000 and 1,700 ZGE, respectively (Table S3). Positive and negative qPCR controls indicated no evidence of PCR inhibition or contamination, respectively.

Discussion

The overarching scientific objective for this study was to develop an adaptable protocol for collecting topotypic genetic samples that can be applied to all vertebrate taxa and beyond. In parallel, we leveraged the multifaceted nature of this project to provide training to the next generation of researchers in museum science, systematics, and taxonomy. Here we summarize the outcomes for the scientific and mentoring goals of the project and offer recommendations for those who we hope will pursue similar endeavours in the future.

The present status of amphibian type localities in Virginia

Re-locating historical study sites, especially those used by other researchers, can be challenging, particularly if anthropogenic development has occurred at or near the study site. Nevertheless, the ability to accurately identify and locate the type locality is essential to this kind of study. Fortunately, most of the type localities for our study were situated in protected and/or managed areas (e.g., Shenandoah National Park, George Washington and Jefferson National Forests, Dixie Caverns) and the habitats surrounding these historical study sites likely are not dramatically different today from when the species were originally collected and described. Correspondingly, we successfully obtained topotypic samples for most localities situated within non-urbanized, protected areas and in many cases the target taxa were locally abundant. One exception was the Daves Branch site near Whitetop Mountain. Despite finding many species at this location (including topotypic *Eurycea wilderae*), during a diurnal search we were unable to find *Pseudotriton ruber nitidus*. Adults of *Ps. ruber* are known to shelter in burrows and under cover objects during the daytime; thus, nocturnal surveys of this site may be more fruitful for future sampling efforts. Some type localities were more challenging to pinpoint (e.g., Four Mile Run and Clifton Forge), in part due to major regional development in the decades since the species were described (*H. evittata* in 1899 and *Pl. hoffmani* in 1972). The portion of Four Mile Run that we determined to be the type locality based on the original habitat description is presently managed as a city park and is surrounded by extensive urban development including Ronald Reagan Washington National Airport and the Arlington Pollution Water Control Plant. Whereas our surveys confirm that suitable amphibian habitat exists at this site, we did not detect *H. evittata* despite repeated attempts. We did, however, collect one specimen of *H. cinerea* in the driveway of the National Museum of Natural History, in the District of Columbia,

ca. 6.5 km NE of the type locality, and found an iNaturalist record of *H. cinerea* near Four Mile Run (dated June 2017; https://www.inaturalist.org/observations/6802148) indicating that the species does occur in nearby, urbanized landscapes. We recommend further surveys at these sites to assess whether the species have been truly extirpated from their respective type localities.

Presence and prevalence of Bd infections

Bd infection prevalence across our samples was relatively low (Clopper Pearson 95% CI: $0.01 \leq 0.05 \leq 0.18$), aligning with previous surveys of Appalachian plethodontids that found similarly low *Bd* prevalence (e.g. Chinnadurai et al., 2009; Gratwicke et al., 2011; Muletz et al., 2014). Muletz et al. (2014) discuss hypotheses for the underlying causes of this relatively low prevalence, which may be related to pathogen detection issues or host resistance. Previous studies have also detected evidence of *Bd* infections in *Eurycea* spp. and *Desmognathus quadramaculatus* (e.g. Byrne et al., 2008; Gratwicke et al., 2011; Timpe et al., 2008), however our surveys may be the first reported detection of *Bd* in *E. wilderae* (see Hughey et al., 2014 for review). The level of *Bd* infection intensities we detected were relatively high compared to other previously reported infections in wild plethodontids (e.g. Muletz et al., 2014); however, infection intensity estimates derived from qPCR should be interpreted with caution given the wide variation in ITS copy number among *Bd* strains (Longo et al., 2013). Given the extensive variation in pathogen susceptibility and disease outcomes among and within amphibian species, we strongly advocate for sustained efforts to document the presence, prevalence, and effects of *Bd* infections in the amphibians of Appalachia and beyond.

Recommendations for preparing students to lead fieldwork

One of the goals of this project was to provide a semi-structured research environment that allows students to grow as independent researchers while also ensuring their safety. Meanwhile, fieldwork also needs to be planned such that productive sampling can be accomplished in the limited seasons in which amphibians are active. Thus, the project mentors aimed to strike a balance between communicating responsibilities to the undergraduates while not micromanaging the planning process. In the typical classroom setting, students are provided with equipment and directions; however, in this field setting, mentors expected students to coordinate their own logistics, pack necessary supplies, and plan for unforeseen circumstances that might arise in the field. We recommend that students prepare a written plan for the first trip outlining a detailed itinerary and packing list for review/approval by project mentors. Project mentors can then identify items/tasks that the students have overlooked, point out potential problems or issues, and assess how well student responsibilities have been communicated. A written plan also allows multiple student researchers to work out amongst themselves individual responsibilities and assignments, and serves as a template for subsequent trips. Even in a rather developed state like Virginia, fieldwork often occurs in fairly remote locations and we found that students may be unfamiliar with the challenges this poses. Accordingly, mentors may need to remind students that they may not have cell phone service at times or easy access to shopping centres and restaurants. Although project mentors were present for the initial trips and several others over the course of the project, the mentors adopted a supporting role as the students faced minor logistical challenges in the field. This structure helps build student leadership and confidence essential for independent decision making and planning, but also ensures that more experienced researchers are present to ensure safety and timely progress.

Our inclusion of undergraduates offered them the opportunity for meaningful participation in a project addressing real-world issues related to biodiversity conservation and provided a connection and networking point to professional biologists (Hiller et al., 2017). Undergraduates were active participants in most aspects of the project and we found that undergraduates in the herpetology course at JMU benefitted from course-based research in several ways. All students gained experience in species identification, general field-sampling strategies, literature reviews, and mapping type localities. For many, this was their first exposure to herpetology, field-based studies, museum science, and more generally, the relevance of natural history collections. This exposure provided them with opportunities to explore, understand, and participate in the process of science in a personally relevant way. Subsequently, the smaller group of students who ultimately conducted the study gained deeper experience with a breadth of research skills including field techniques related to specimen collection, bioinformatics and molecular techniques related to DNA barcoding and pathogen screening, and museum collection experience incorporating the voucher specimens and tissue samples into their final repository. This experience planning and executing biological research will aid these students in future projects across a range of potential career paths. For instance, some students

did not have experience identifying specimens in the field and found this to be one of the most challenging, but rewarding aspects of the project. Likewise, students learned that just because data are published and/or sequences are deposited in databases such as GenBank or BOLD, they may not be correctly identified, so all results should be carefully examined and verified. Finally, through trial and error, students realized the importance of having clear and concise objectives for travel planning, data management, and group communication. Over the course of the project, they established what each of their strengths were in the field and in the lab, and found that dividing responsibilities amongst themselves was helpful for planning their workflows and becoming increasingly independent from the project mentors. Beyond the ample set of research skills the students gained through this project, having a diverse team of professional scientist mentors also increased the benefits undergraduates received from this experience (Russell et al., 2007). Working in a museum setting specifically, among current taxonomists, helped to demonstrate the value of such work to a future generation of researchers.

Issues with misidentified sequences in genetic databases and the utility of topotype barcodes

In comparing sequences from this study with those in BOLD and GenBank, we noticed some specimens in these databases appear to be misidentified, while others may be correctly identified, but known issues of paraphyly and/or hybridization result in mixed (interspecific) BINs. Initially this created a frustrating and confusing situation for the project mentors and students, but ultimately served to reinforce several core systematics concepts (e.g., monophyly, paraphyly, mito-nuclear discordance), and the importance of combining morphological and genetic data for verifying species identifications. We noticed that a large number of misidentified specimens in BOLD come from a single study (Chambers & Hebert, 2016) and we provide a detailed explanation of the inconsistencies in the Supplementary Materials. Chambers and Hebert (2016) set out to extend the DNA barcode reference library for North American amphibians and reptiles by borrowing tissues from museums; however, it appears that the authors did not examine the voucher specimens of the material used and relied on museum identifications without further verification. Many institutions, including USNM, provide a disclaimer when loaning tissues not to rely on museum identifications without verification. Several of the apparently misidentified specimens from Chambers and Hebert (2016) confounded our research by creating mixed BINs that contained multiple species, or split BINs such that a single species occurred in several BINs. To sort out this confusion, we generated a neighbour-joining (NJ) tree (Fig. S1) with our COI sequences and those collected by Chambers and Hebert (2016) for the family Plethodontidae. We found that sequencing additional mtDNA markers was useful for sorting out some of the confusion caused by misidentified sequences in GenBank and BOLD. For instance, Chambers and Hebert (2016) deposited one COI sequence of *Pseudotriton ruber* in BOLD, and we sequenced three specimens of *Ps. ruber* that we collected as our non-target species during fieldwork. The Chambers & Hebert "*Ps. ruber*" was not closely related to our *Ps. ruber* and instead was 99.2–99.7% identical to our *Pl. welleri* sequences, and thus placed in our *Pl. welleri* clade (and BIN). To determine which set of *Ps. ruber* were correctly identified, we sequenced Cyt b for our samples so we could compare them to sequences in a recently published phylogeographic study of the species that used Cyt b (Folt et al., 2016). Our specimens were 98–99% identical to the Folt et al. (2016) sequences, highlighting that sequencing supporting markers in BOLD projects can be useful when limited COI barcode data are available for a given taxon.

Another utility of the topotype DNA barcodes, including those of synonyms, is in the identification of newly identified species from species complexes. For example, two of our topotype targets were synonyms of the species *Plethodon wehrlei* (*Pl. dixi* and *Pl. jacksoni*). This species complex has received recent attention, and these names (*Pl. dixi* and *Pl. jacksoni*) were recently elevated to specific status (Kuchta et al., 2018 and Felix et al., 2019, respectively). Prior to the publication of these recent studies, our sequences for these taxa were placed into separate BINs, demonstrating the utility of the DNA barcodes for identifying even closely related species. Conversely, some species cannot be identified to species-level based on DNA barcodes alone. For example, we included *Eurycea wilderae* as one of our topotype target species and also collected several non-target *E. cirrigera*, from two localities, Montgomery and Alleghany Counties, VA. We initially identified the Montgomery County specimens as *E. cirrigera* because they were collected within the greater range of *E. cirrigera*; however, this collecting site is in the Blue Ridge physiographic province, a known region for *E. wilderae* (and not *E. cirrigera*), but the site is >120 km NE of the known distribution of *E. wilderae*. Consequently, the specimens we collected may represent *E. wilderae* and not *E. cirrigera*, but further fine-scale taxonomic and geographic revision is needed to resolve this multi-

species complex composed of several mtDNA clades (Kozak et al., 2006). Thus, because some populations of *E. cirrigera* are indistinguishable from some populations of *E. wilderae* based on mtDNA data, including the topotype specimens (Kozak et al., 2006), this complex represents species that cannot be distinguished based solely on COI barcode data.

Conclusion

Taxonomy (the science of identifying, describing, and naming the Earth's biodiversity) is a branch of science that has been in decline for the past half century, but is needed now more than ever (Agnarsson & Kuntner, 2007; Cotterill & Foissner, 2010). We currently operate in an era in which demands for taxonomic services are increasing based on the needs of ecologists, conservation scientists, and resource managers, and yet we face a declining population of taxonomists, a lack of support for biocollections, and a dearth of programs to train the next generation of taxonomists and museum scientists (Anderson, 2017; Kim & Byrne, 2006; Tewksbury et al., 2014). Given the importance of museums as repositories of critical information about temporal and spatial patterns of biodiversity (Bradley et al., 2014; Suarez & Tsutsui, 2004), there is a growing need for trained professionals in natural history collections (Bradley et al., 2014). Natural history museums can help fill this growing need by providing training to undergraduates through hands-on research experiences, such as this project, and other immersive opportunities that can be formative in the careers of the next generation of researchers (Cook et al., 2014; Hiller et al., 2017; Powers et al., 2014; Russell et al., 2007). Furthermore, we consider science to be a community-driven enterprise and see the collection of DNA-quality topotypic materials and vouchers to be an opportunity for individuals and organizations to contribute to common resources that will facilitate current and future research efforts, and that these efforts can also serve as a platform to train undergraduate researchers.

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Supplemental material

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