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Documenting the Southern Range Terminus of the Wood Frog (Lithobates sylvaticus) in North America

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Documenting the Southern Range Terminus of the Wood Frog (*Lithobates sylvaticus*) in North America

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Abstract

The Wood Frog (*Lithobates sylvaticus*) holds a remarkable position in North American amphibian biology, with its range extending from the Arctic Circle down to the near sub-tropical southeastern United States. This thesis presents a novel quantitative polymerase chain reaction analysis (qPCR) primer specific to *L. sylvaticus* and a survey effort regarding the southernmost distribution and detection of this species in Alabama through the application of environmental DNA (eDNA) sampling techniques. By investigating historical data and employing advanced genetic methodologies, this research provides insights into the contemporary status and distribution of the Wood Frog. This research is important to shed light on its adaptability to changing climates and habitats of a presumably thermosensitive species.

Keywords: Wood Frog (Lithobates sylvaticus), Environmental DNA, Quantitative PCR, Range Terminus, PCR Primer

Dedication

This thesis is dedicated to the memory of Dr. George Cline, whose passion for biodiversity conservation and tireless dedication to teaching and research continue to inspire. Dr. Cline's legacy of excellence in academia and his profound impact on environmental science serve as a gold standard for all who seek to better understand the field of herpetology. Additionally, I extend my heartfelt gratitude to Jacksonville State University for providing the academic environment and resources that have enabled me to pursue my research endeavors.

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-Christian Shane Braswell

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Chapter I

Introduction

North America is a continent of diverse climates and topography, extending from the Arctic Circle at its higher latitudes to the Tropic of Cancer in the south. This extreme diversity in climate zones is reflected in the biodiversity of the organisms living in the various habitats of North American landscapes. Very few species can survive in all climate zones of this continent, either being specialized for certain biotopes or elevation ranges, having certain dietary restrictions or migratory limitations (Blackburn et al 2001; Frost 1985). A standout in North American biodiversity, the Appalachian region is renowned for its rich biodiversity, harboring a plethora of plant and animal species within its diverse landscapes. Stretching from the southeastern United States up to the Canadian province of Newfoundland and Labrador, the Appalachian Mountains form a biogeographic feature that has facilitated the evolution of unique species and ecosystems over millions of years (Graham et al 2010). A defining aspect of Appalachian biodiversity is the high level of endemism across this region, with many species found nowhere else on Earth. Geographic isolation, complex topography, deep geologic time, and diverse habitats have fostered the evolution of numerous rare endemic species, ranging from salamanders and freshwater mussels to plants and songbirds (Graham et al 2010; Isenhower 2017).

The biodiversity of the Appalachian region has far-reaching implications for surrounding areas, including the southeast. The higher relative elevations of the southern Appalachian range provide a sliver of montane via the Talladega Upland habitat that extends into much of the southeastern United States, terminating in Alabama (Duncan 2013; Dodd 2023). By being located at this southern extent of the Appalachian Mountains, Alabama is influenced by the

ecological processes and species interactions that originate in the Appalachians (Smith et al 2019). As a result, the state boasts a remarkable diversity of ecosystems and species, ranging from the coastal plains of the Gulf Coast to the rugged terrain of the Appalachian foothills (Duncan 2013). The Appalachian influence on Alabama's biodiversity can be seen in various ways. For example, many plant and animal species found in Alabama have their southern range terminus in the Appalachian foothills of Alabama, reflecting historical patterns of species dispersal and colonization (Duncan 2013; Smith et al 2019). Additionally, the Appalachian Mountains serve as a source of genetic diversity for species that inhabit Alabama, contributing to the overall resilience and adaptability of regional ecosystems (Smith et al 2019).

While the Appalachians are rich in biodiversity, there are still some species we know very little about. For instance, the Wood Frog (*Lithobates sylvaticus*) is widespread with species found from mid- to high- latitudes. It can withstand considerably harsher climates in Canada where it is the only frog with a range extending past the Arctic Circle (Dodd 2013). This ability to endure climate extremes is due to the frog's ability to freeze up to 65-70% of its total body water mass using a biological anti-freeze in its blood to survive until the spring, where it thaws and resumes its activities (Costanzo et. al. 2015; Storey et al 2021).

Furthermore, the presence of *L. sylvaticus* in these areas has been poorly studied in the southeastern U.S., leaving gaps in our understanding of their distribution and ecology (Davis and Folkerts 1986). Despite the current range of *L. sylvaticus* extending into central Alabama, the Wood Frog was not detected in Alabama until 1974, where it was found in Cleburne County, extending the previously known range by 160 km (Davis et. al. 1986) from neighboring Georgia. Some specimens are known from northeastern Alabama, but only in the Appalachian Mountain foothills, notably the Piedmont and Ridge and Valley physiogeographic provinces (Davis &

Folkerts 1986). The range of *L. sylvaticus* has been thoroughly documented at its higher latitudes where it is a much more conspicuous species on the landscape and sometimes the only amphibian present in the environment (Martof, 1970; Spangler et al 2017; Dodd 2023). Whether the species in the southeastern United States is rarer, harder to detect, or both, remains unclear, but little is known about the species at their southern terminus.

Throughout the rest of its range, the Wood Frog has had such a significant amount of research done that it is considered a model organism and utilized in many other research and academic applications (Fitzpatrick et al. 2019; Mundy et al. 2019). *Lithobates sylvaticus* serves as an ideal model organism in ecological and evolutionary research due to its widespread distribution and well-studied physiological adaptations. Its ability to tolerate extreme environmental conditions, such as freezing temperatures, makes it an ideal subject for studying cryoprotective mechanisms (Costanzo et al., 2015). Additionally, the species' ecological significance as a prey item and indicator of environmental health further enhances its suitability as a model organism for broader ecological studies (Fitzpatrick et al., 2019). Little current research has been done on the species' southern peripheries as it seemingly becomes more fragmented on the landscape, and ranid diversity increases, making the species, even when calling, less conspicuous. Extensive surveys were last conducted in the 1970's. These surveys found 42 records of Wood Frogs in Alabama, mostly from the Talladega National Forest in Calhoun, Clay, Cleburne and Tallapoosa counties (VertNet, Davis et. al. 1986). Their conservation status is considered "not listed" in Alabama, however no recent formal surveys have been conducted in the almost 40 years since the Davis and Folkerts (1986) publication to evaluate the status of this enigmatic species. Because of their short breeding phenology and cryptic behaviors outside of the breeding season

in the southeastern US, the true contemporary conservation status of the species in Alabama is unknown.

One such way to sample rare or elusive taxa is through use of environmental DNA (eDNA) techniques (Ficetola et al 2008). A revolutionary methodology allows researchers to collect presence/absence data for target species that are cryptic, endangered, or inaccessible, without ever seeing or capturing their species of study. This technique uses filters to sample water, air, soil, or other suitable medium that the species is thought to frequent or inhabit and collect minute traces of DNA from mucous, shed cells, or other bodily secretions (Ficetola et al 2008; Ma et al 2017; Kirse et al 2021; Lynggaard et al 2022). This method can detect trace amounts of DNA and, through polymerase chain reaction (PCR) techniques, extrapolate enough DNA for amplification.

The primary objective of this project is to update the range and document extant populations for *L. sylvaticus* in Alabama. By using eDNA techniques, this frog's presence can be determined across many sites with relative ease and cost-effectiveness. We resampled historical sites in an attempt to update the known distribution of *L. sylvaticus* in Alabama. This approach is an efficient means of determining the presence of the species, which is notoriously difficult to detect outside the breeding season, across numerous sites in eastern and northeastern Alabama, shedding light on its current status and persistence.

While eDNA primers for this species are published in the existing literature, these primers were developed in Alaska (Spangler et al 2017). As it turns out, these primers are not specific to *L. sylvaticus* in Alabama. In Alaska, this is not an issue as *L. sylvaticus* is the only Ranid species present, but in Alabama, preliminary testing found that the primers amplify all native Ranids (*Lithobates clamitans* (Green Frog), *L. catesbeianus* (American Bullfrog), *L.*

sphenocephalus (Leopard Frog), and *L. palustris* (Pickerel Frog)). Thus, the objective was to design eDNA primers specific to Southern populations.

Methods

Primer Design

Upon realizing the inadequacy of the existing primer set for this study, we focused on creating a primer with increased specificity to *L. sylvaticus*. The main challenge was the unintended amplification of multiple species of ranid frogs that coexist with *L. sylvaticus*. To solve this problem, we needed to find a genomic region that could serve as a target for a primer with minimal cross-reactivity with other frog species' environmental DNA (eDNA).

We compared mitochondrial gene sequences across *L. sylvaticus* and closely-related species. Mitochondrial DNA (mtDNA) is particularly advantageous due to its higher abundance in the environment and stability compared to nuclear DNA. This is attributed to the presence of multiple mitochondria per cell, each containing several copies of mtDNA, along with the protective extra membrane of mitochondria (Kelly et al., 2019). We looked at published sequences for Alabama ranids on GenBank, specifically focusing on *NAD2* and *CytB* genes. Genes were aligned and visualized across species using Molecular Evolutionary Genetics Analysis Version 11 (MEGA11; Tamura, Stecher, and Kumar 2021) to identify potential areas of primer design where species exhibited genetic dissimilarity. By comparing the ribosomal DNA sequence of *L. sylvaticus* in GenBank with the corresponding regions of its closest relatives, such as *L. catesbeianus*, we identified a region within the *L. sylvaticus* genome that showed divergence from its relatives and was absent in other closely related species. For the development of the probe, we used the following sequence: 5’-

/FAM/CCACCCTTGCTCTAACCCTT/Q/-3'; then we used 5'-CCAGTTCGCCCATCAACATC -3' for the forward primer, and 5'-GAATAGGGGATTGGGAGGGG-3' for the reverse primer. Using this distinctive region, we hoped to develop a new primer set specifically for *L. sylvaticus*.

Primer Optimization

The quantitative polymerase chain reaction (qPCR) method is fundamental in molecular biology research for its ability to accurately quantify DNA molecules (Ma et al. 2020). In this study, qPCR was employed using the new primer to detect and quantify eDNA from water collected across sample sites. This technique offers exceptional sensitivity and specificity, allowing for the detection of trace amounts of DNA from target organisms (Waits & Paetkau, 2005). To ensure reliable results, I used standardized protocols and optimized reaction conditions as our guidelines when formulating our protocol (Wilcox et al., 2013; Kelly et al., 2019). We optimized the primers across several different annealing temperatures to calibrate specificity. We used PCR Master Mix (2X) reagent, along with ~50 ng of DNA in the samples used for the reaction. Ultimately, the qPCR protocol that I optimized amplification had an initial melt cycle at 95°C for 150 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Additionally, the primer concentration was carefully adjusted to a final concentration of 0.5 µM for each primer. This protocol focused on the accuracy and reproducibility of our qPCR assays in detecting the presence of *L. sylvaticus* in the extracted samples. I collected tissue samples from all other ranid species that were located in this region (N = 5). These were extracted using the protocols provided on the QIAGEN DNeasy Blood and Tissue Kit, and we tested these species using the same conditions optimized for Wood Frogs.

Results

Because published primers had unintended amplification of multiple species of ranid frogs that coexist with *L. sylvaticus*, we needed to find a genomic region that could serve as a target for a new primer with minimal cross-reactivity with other frog species' eDNA. The primer set tested in our experiments failed to amplify the genetic material of closely related frog species, including *Lithobates catesbeiana*, *Lithobates clamitans*, *Lithobates palustris*, and *Lithobates sphenocephalus*. There was a small, but overall insignificant, amplification of *L. clamitans*. This suggests that the qPCR reaction employing these primers would selectively amplify the target species if utilized in an eDNA sample containing Wood Frogs and closely related species. This careful primer design process has been crucial in ensuring accurate detection of *L. sylvaticus* eDNA in our study (Wilcox et al 2017).

Despite the positive specificity results in the primer tests, the initial trial run with all the eDNA samples yielded no amplification signals in any of the runs. However, it is noteworthy that the positive control, consisting of tissue-extracted *L. sylvaticus* DNA, consistently yielded successful high amplification signals. This discrepancy between the performance on the positive control and the eDNA samples warrants further investigation into potential factors contributing to the lack of amplification in the samples.

Discussion

One of the key challenges encountered in this study was the design of specific eDNA primers for *L. sylvaticus* for use in southeastern population studies. While existing primers were available, they proved to be inadequate for detecting the species in Alabama due to cross-reactivity with closely related Ranid frogs (Spangler et al., 2017). To address this issue, a new primer set was developed based on a distinctive genomic region of *L. sylvaticus*, identified

through comparative analysis of mitochondrial gene sequences. The effectiveness of these primers was validated through PCR tests, which demonstrated their specificity in targeting *L. sylvaticus* while avoiding amplification of DNA from related species.

The development of these new specific eDNA primers for *L. sylvaticus* represents a significant advancement in the conservation and population monitoring for this species, filling a critical gap in the literature. Prior to this study, the existing primers lacked the necessary specificity to accurately detect *L. sylvaticus* populations in Alabama, hindering comprehensive assessments of their distribution and abundance. In recent years, detection of the frog species has been due mostly to chance encounters, but otherwise this species still remains an enigma 50 years after its initial detection in the state. By addressing this limitation, the newly developed primers provide researchers with a reliable tool for precisely identifying *L. sylvaticus'* presence, enabling more accurate assessments of population dynamics and habitat suitability. Moreover, the application of these tools extends beyond the study area, offering opportunities for widespread use throughout additional portions of Alabama and the southeast where Wood Frog populations may exist but remain under-studied in Alabama and neighboring states. This expanded application enhances our ability to monitor and manage Wood Frog populations across diverse habitats, ultimately contributing to more effective conservation strategies and informed decision-making. The application of these newly developed eDNA primers are more than just a new way to detect this species in the region, they have the capacity to offer a valuable tool for monitoring Wood Frog presence and absence dynamics over time. By repeatedly sampling targeted sites and analyzing eDNA, researchers can track changes in Wood Frog occupancy, providing insights into population trends, habitat suitability, and the effectiveness of conservation efforts.

Further testing and considerations are warranted to evaluate the primer's performance across different environmental conditions and sample types. This ongoing research will ensure the reliability and accuracy of species detection when employing these primers in future eDNA-based studies. Additionally, efforts should be made to optimize the primer design to strike a balance between specificity and applicability in broader ecological contexts

Chapter II

Introduction

The Wood Frog (*Lithobates sylvaticus*) is a small to medium-sized amphibian with an extensive range across North America, stretching from Alaska to Alabama. However, its presence and range in the latter state was not confirmed until the late 1980s by Davis and Folkerts of Auburn University. Extensive surveys found the frog to be present, though enigmatic, in several counties in Alabama, primarily in the Talladega National Forest in Clay, Tallapoosa, and Cleburne counties (Davis & Folkerts, 1986). These records indicate the presence of the species in the state, although comprehensive studies on *L. sylvaticus* populations in Alabama are lacking. Known mostly for their freeze tolerance, these frogs are found in much colder climates in regions much farther north of Alabama, such as Canada and areas beyond into the Arctic Circle (Costanzo et al., 2015). While their range extends along the Appalachians into the southeastern United States, including Georgia and Alabama, their distribution becomes patchier in the southernmost areas (Fitzpatrick et al., 2019). Limited information exists regarding *L. sylvaticus* populations at their southern terminus in Alabama, necessitating further research to elucidate their distribution, abundance, and conservation status (Graham et al., 2010).

An earlier aspect of this study involved the design of eDNA primers tailored specifically to this species in Alabama. Environmental DNA (eDNA) refers to the genetic material shed by organisms into their surrounding environment, such as soil, water, or air. By sampling and analyzing this genetic material, researchers can detect the presence or absence of species without directly observing them. This approach offers a non-invasive and sensitive method for species detection and monitoring (Ficetola et al, 2018). Using these techniques, we conducted targeted sampling at sites identified in the only other study of the species in the state by Davis and

Folkerts (1986), aiming to provide updated insights into Wood Frog distribution and abundance in the state. The novelty and utility of environmental DNA (eDNA) methodologies in ecological research cannot be overstated. By harnessing eDNA techniques, researchers can detect the presence of species with increased efficiency compared to traditional survey methods. In the context of *L. sylvaticus* studies in Alabama, the development of novel and specific eDNA primers represents a crucial advancement.

By revisiting these sites and employing modern environmental DNA sampling techniques, I aim to provide updated information on the distribution and presence of *L. sylvaticus* in the state. This approach allows for an efficient and cost-effective assessment compared to traditional survey methods, offering the potential to detect *L. sylvaticus* even in areas where they may be cryptic or difficult to observe (Jerde et al., 2010). Additionally, by comparing our findings to historical data, any changes in the species' current distribution can be evaluated and any future assessments of environmental change or habitat alteration can be better informed (Ficetola et al., 2008). One of our primary objectives is to contribute valuable insights into the current status and conservation needs of *L. sylvaticus* populations in Alabama, informing effective management strategies for this species.

Methods

Water Sampling

The sample sites were chosen through a combined review of historical collection locations taken from the archives of the Auburn University Museum, historically published sites, iNaturalist.com observation datapoints, and anecdotal reports from foresters of the United States

Forest Service that provided us proper directions to the historical sites found in the Davis and Folkerts publication (pers. comm).

We used a Masterflex® E/S® portable water sampler to attempt to filter 1L of water from each sampling site visited. The pump is relatively small, allowing for ease of transport to the often significantly off-trail sites. To ensure good coverage of the sampling sites, at least 3 water samples were taken per area. The water samples were placed on ice in a cooler for transportation to the lab for extraction. Filtering the samples was accomplished through a 45-micron acetate filter, and then extracted within 24 hours using a PowerWater® DNA Isolation Kit from MO BIO Laboratories to extract any DNA present on the filter (Metcalfe, 2018). This filter proved difficult to extract a full liter of water at each site, and some sites were only able to pass around 300ml of water through the filter before it became too clogged to continue.

Filter Extraction

To prepare the water samples for DNA extraction, I followed the standardized published protocol outlined in the MO BIO Laboratories PowerWater® DNA Isolation Kit. Initially, Solution PW1 was warmed to 55°C for 5-10 minutes, and Solution PW3 was checked and warmed if necessary. The filtered membranes were rolled into cylinders and inserted into PowerWater® Bead Tubes. Following the addition of Solution PW1 and vortexing, the tubes were centrifuged, and the supernatant was transferred to clean collection tubes. Subsequent steps involved the addition of Solution PW2, centrifugation, addition of Solution PW3, loading of supernatant onto Spin Filters, and centrifugation to discard flow through. This process was repeated with Solution PW4 and Solution PW5. Finally, Solution PW6 was added to the filter membrane, centrifuged, and the Spin Filter basket was discarded, leaving the DNA ready for downstream applications. The samples were finally placed in a freezer for long term storage.

Results

The points were visited once each during the sampling period. One of the points was not usable for my study due to its destruction by recent development, so only 13 of the 14 sites were sampled. Of these localities, only the Duggar Mountain study area was observed to have a breeding population of L. sylvaticus. Figure 1 shows an adult in situ at this site photographed on January 12, 2023, near the edge of the vernal pool that was sampled for the study. Figure 2 shows the resulting egg mass observed floating on one end of the pool on January 23, 2023. All the sites were compiled on a map showing their distribution in the state (Figure 3). This map shows the sample site locations along with the nearby urban and geographic areas of Alabama.

In the extraction procedures, I employed the standard protocol outlined for the MO BIO Laboratories PowerWater® DNA Isolation Kit. The extraction yielded promising results, with the samples appearing to have been extracted successfully with detectable amounts of eDNA present. This suggests that the DNA isolation protocol effectively recovered genetic material from the water samples, but the issues with the qPCR not amplifying any *L. sylvaticus* DNA, even from sites I knew a population was present, remains an issue. Upon a review of the DNA concentrations in the samples using a NanoDrop Spectrophotometer, it was found that most of the samples could be categorized as “very poor concentrations” as they were below 10 ng/μL (mean = 18.6 ± 16.3 ng/μL; García-Alegría et al 2020). This did not come as a surprise as the average amount of water we were able to sample from each site was 341.02 mL (s.d. = 190.6 mL). This is likely because these ephemeral pools had significant suspended particulate organic matter, which is typical in aquatic systems of this kind (Boeckman and Bidwell 2007)

Discussion

The objective of our study is to develop a novel eDNA primer set tailored specifically to Wood Frogs inhabiting the Southern United States and use it to evaluate the current range of the species in the southernmost terminus if its range. We did this to facilitate broader and more comprehensive sampling of this enigmatic frog species within the region. This endeavor is particularly significant considering the potential vulnerability of Wood Frogs to the impacts of climate change. For instance, recent research has highlighted the susceptibility of Wood Frog populations to the effects of shifting climatic conditions (Arietta et al 2020; Arietta et al 2021). Therefore, by enhancing our ability to detect and monitor Wood Frog populations using eDNA techniques in the Southeast, we can better understand their responses to environmental changes and inform targeted conservation efforts.

Two scenarios exist with the application of eDNA for Wood Frogs in Alabama. There is the potential to document instances of *L. sylvaticus* in novel parts of Alabama through eDNA techniques, and subsequently the known southern terminus of this species' range may be altered, making it one of the few terrestrial species on Earth that has a range extending from the Arctic Circle to the near tropics. However, the inverse may be true if the findings indicate a reduction in their range northward, since climate change or habitat loss may have a significant impact on this species adapted for colder climates. *L. sylvaticus* is a temperature sensitive species, and the steady increase of average temperatures due to climate change may have a severe impact on their southern range (Arietta et al, 2020) The potential shifts in the range of *L. sylvaticus* underscore the urgency of precisely documenting its distribution in Alabama, a task central to our project's objectives.

The previously mentioned failure of primers to amplify the eDNA samples poses a significant challenge and warrants careful consideration of potential explanations. Several factors may contribute to this lack of amplification as eDNA sensitivity can vary across taxa and across different sites (Furlan et al. 2015). Firstly, it is possible that the targeted populations were absent or present in extremely low densities within the immediate sampling areas chosen. In the sites with lentic systems characterized by limited water flow, the dispersal of eDNA may be restricted, (or spatial clumping of the target species) making it difficult to detect species that are shedding DNA into the environment over a limited travel area (Furlan et al. 2015; Harper et al 2018; Moyer et al 2014; Roussel 2018).

Additionally, the small pore size of the filters used for water sample filtration (45 microns) seemingly imposed limitations on the volume of water that could be effectively filtered (Kumar et al. 2021). The optimization of filter size, volume of water filtered, reduction of inhibitors and amount of eDNA captured is an ongoing challenge to eDNA studies (Turner et al. 2014; Sanches and Schreier 2020). In this study, the filters became clogged after only half of my target amount of 1L was filtered, and even less in some cases. This reduced sampling volume could have decreased the likelihood of capturing detectable amounts of eDNA, reducing the sensitivity of the primers, particularly if the target species' DNA concentration in the sampled water was already low (Furlan et al. 2015).

Another possible factor is the presence of inhibitor compounds such as tannins that are often highly concentrated in these types of lentic environments which may have interfered with the DNA extraction procedure (Uchii et al 2019; Sanches and Schreier 2020; Lance & Guan 2021). These inhibitors can potentially inhibit PCR amplification by binding to DNA or interfering with enzymatic reactions involved in the extraction process. In combination with the

limited sample volume, the presence of inhibitors could have further hampered the detection of eDNA in the samples.

Conclusion

The 13 sites we collected eDNA samples from were all selected as promising locations where remaining *L. sylvaticus* populations had the highest probability of existing. The site near Dugger Mountain still has an active breeding population, and through iNaturalist.com observations and some other anecdotal reports from near Mt. Cheaha more are suspected of existing in Alabama. However, all of the results for the qPCR tests on the samples taken at these sites, including the one where *L. sylvaticus* is known to be, all returned negative results. The failure of the primers to amplify the eDNA samples underscores the complexity of environmental DNA-based surveys and highlights the importance of considering various environmental factors and methodological limitations when interpreting results. Further research, increased sample sizes, and methodological refinement may be necessary to increase the sensitivity of these primers and to overcome these challenges and improve the effectiveness of eDNA-based monitoring for populations in stagnant, lentic environments.

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Appendix A

Figures

Figure 1: An adult *Lithobates sylvaticus* in situ from the Dugger Mountain population (Site 10).

Figure 2: *Lithobates sylvaticus* egg mass at the Dugger Mountain site.

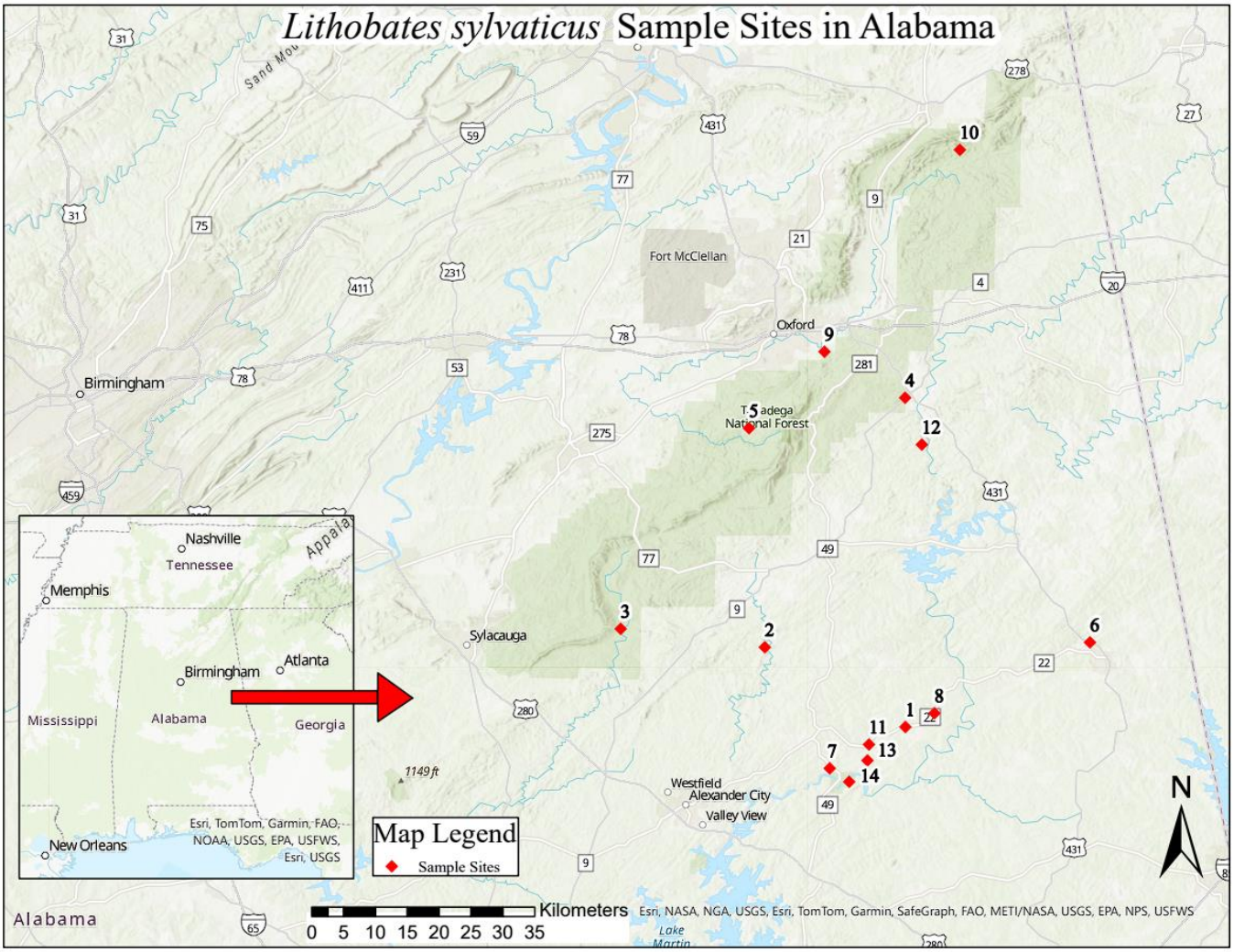

Figure 3: The map of the study area showcases the sample localities and their locality relation to geographic and anthropogenic features.