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Conservation of the Ambystoma jeffersonianum Complex

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RIT

Conservation of the *Ambystoma jeffersonianum* Complex

By

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Table of Contents

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Abstract

The Great Lakes region is home to the diverse genus *Ambystoma*, including the Blue Spotted Salamander (*Ambystoma laterale)* and Jefferson's Salamander (*Ambystoma jeffersonianum).* These two species are relevant to conservation efforts because of unique hybrid populations. This hybridization creates offspring populations that are morphologically similar to the parental species. The hybrid populations are primarily all female lineages and are known as the *Ambystoma jeffersonianum complex*. Conservation interest in this hybrid complex stems from the fact that hybrid populations are not taxonomically recognized as separate species and therefore are not protected under the Endangered Species Act. To assess the contribution of hybrid populations to ecosystems, hybrids must be distinguished from non-hybrid salamanders. The goal of this project is to use molecular techniques to identify hybrids from parentals and gather more information about their distributions in a small sub-population on the Rochester Institute of Technology campus. Geographic Information System techniques were used to initiate characterization of the habitat associated with this population. In this project, 50 tissue samples were analyzed via microsatellite PCR with two loci, and gel electrophoresis. Of the 50, 29 were *A. laterale,* 19 were hybrids, and two samples failed to amplify. The habitat suitability model explored the predicted locations for where populations may be found on the RIT campus. Two of the 25 sample clusters (8%) were outside of the predicted areas generated by the model. The habitat model also successfully confirmed suitable habitat for 48 of the 50 salamanders sampled, while showing potentially suitable habitat in areas on the RIT campus and Monroe County that have not been sampled. Therefore, this predictive model could be used to identify additional sampling areas within the county to continue this research.

Introduction

The genus *Ambystoma* is diverse within the family Ambystomatidae containing a number species of mole salamanders throughout North America. Two species in the eastern/central U.S. and Canada are commonly known as the Blue Spotted salamander (*Ambystoma laterale*) and Jefferson's salamander (*Ambystoma jeffersonianum*). These are diploid and sexually reproducing species that can hybridize to produce all female lineages that are morphologically intermediate to both parent species and are not easily distinguished (Ramsden,2006). Due to the fact that the parental species and their hybrid lineages are morphologically similar, information about their distribution in the Great Lakes region is limited. Currently, the extent of the distribution of the hybrid *Ambystoma* lineages appears to be most prevalent in the Great Lakes basin and surrounding drainages (Bogart, 2007). Even though found locally in this region, the hybrid lineages have not been researched in depth. Throughout their range, the sexual parental species are protected, while the hybrid lineages are not, since they are not legally considered independent species under the Endangered Species Act. The accurate identification of the hybrid and parental populations is a crucial part of conservation efforts because proper identification informs accurate protection levels both federally and locally.

Conservation efforts are complicated due to hybrid salamanders being morphologically indistinguishable from the parentals (Ramsden, 2005). To address this, researchers have developed non-lethal tissue sampling protocols (toe and tail clips) to properly distinguish species without sacrificing the specimen (Ramsden, 2005). Prior to using this method, salamander species in this genus were identified primarily by coloration and the presence of blue spots. However, due to salamanders of the complex looking similar to the diploid species, this method is inaccurate and may lead to

misidentification. Given that salamander populations are rapidly declining worldwide, a nonlethal method of identification is advantageous and avoids unneeded population depletion for biodiversity studies (Yap, 2015). This project implemented a nonlethal tissue sampling method with standard DNA extraction, purification and amplification methods and a polymerase chain reaction (PCR) based identification method for salamanders to accurately identify *Ambystoma* parent species and hybrid populations of the *Ambystoma jeffersonianum* complex on the RIT campus. The individual distributions were also mapped on the RIT campus in order to learn more about the hybrid distribution to inform conservation efforts and potentially contribute to the development of a regional habitat suitability model.

Background

There are two parental species of salamanders that comprise the *Ambystoma laterale jeffersonianum* complex, the Jefferson salamander (*Ambystoma jeffersonianum)* and the Blue-Spotted salamander (*Ambystoma laterale*). The latter species of *Ambystoma laterale* originated from the post-glacial period, known as the Wisconsinan period, and were the primary colonizer of this period (Demastes, 2019). Jefferson salamanders are found throughout eastern North America in deciduous forests, where they often breed in vernal pools (Connecticut, 2016). Locally, this species is rare, presumably due to restricted suitable areas for breeding (Noel, 2008). This also may be caused by degradation of habitat and poor water quality (Species Status Assessment, 2013). The hybrids breed in vernal pools and wetlands as well, therefore, loss of this critical habitat may also contribute to its rarity (Hoffman, 2017).

Early work on this complex indicated that the only differences among the unisexual female lineages were at the cellular level, as polyploids have a larger cell and nuclei size, to accommodate additional sets of chromosomes they possess (Uzzel, 1964). Further, it was assumed at the time that the hybrids mated with either parental sexual species in order to produce viable offspring (Uzzell,1964). More recently, general blood serum sampling was completed to determine the genetic identification of the organism, representing the first molecular attempt to identify these lineages. This led to the discovery that gynogenesis (parthenogenesis) may be used without the contribution of male genetic information for reproduction (Bogart, 1982).

Hybrid Jefferson salamander populations have been described as lineages rather than asexual or parthenogenetic. These salamander populations of the *Ambystoma* complex participate in kleptogenesis, with DNA only being used a fraction of the time (Bogart, 2007). However, this idea has been challenged with the discovery that sperm is necessary for reproduction, even if it is not incorporated into the final offspring. Instead, replacement of the genome can occur if the new genome could be beneficial for mating, by providing benefits to the offspring that would aid in obtaining a mate (Bogart, 2019).

With all of these new discoveries come new questions related to the overall health and fitness of these lineages. It is hypothesized that polyploid unisexual populations (hybrids) may have fitness advantages relative to diploid populations, since these hybrids can reproduce with sperm from any other *Ambystoma* lineages (parthenogenesis). Males from different species can activate the eggs of hybrid females, providing an evolutionary advantage for the hybrid lineages (Bogart, 2009). However, recombination may be limited because the male sperm only chemically

activates development but doesn't contribute DNA or chromosomes to the offspring (Bogart, 2009). The sperm of the male bisexual parental species is used to simulate egg development by gynogenesis or be incorporated by the female lineage to replace of the female's haploid genomes (Bogart, 2019).

The hybridization of *A. jeffersonianum* and *A. laterale* has resulted in all-female clonal lineages composed of phenotypically identical offspring that reproduce asexually (Bogart, 2009). The all-female lineages closely resemble the sexual parental counterparts phenotypically, making identification difficult (Julian, 2003). The occurrence of these hybrid lineages is due to the geographic overlap of the two parental species during the postglacial colonization and is possible due to the two species being phenotypically and genetically similar enough to produce viable offspring (Demastes, 2007). Because colonial lineages are unique, they tend to be the focus of research in reproductive genetics and species boundaries. Hybrid populations of the Ambystoma complex may exhibit haploidy, diploidy, triploidy, tetraploidy and even pentaploidy (Noel, 2008). Geographically, this complex and the lineages it produces are endemic to the Great Lakes Basin of North America (Wee, 2017).

Identification of salamanders of Ambystomatid salamanders in this complex is difficult because polyploid hybrids are often morphologically indistinguishable from diploids. Additional methods of identification have been developed using taxon-specific primers based upon microsatellite DNA loci to distinguish polyploid hybrids from diploid species and to determine hybrid ploidy. Polymerase Chain Reaction (PCR) is also used to identify the ploidy of the specimens (Ramsden, 2006).

Conservation status of the parental species varies by nation and state throughout the range of each. In Canada, where much of the recent research regarding this complex originates, *A. jeffersonianum* is listed as an endangered population (Bogart, 2017). Meanwhile, *A. laterale* is considered to not be at risk (Canada, 2017). A species of concern is any species that does not meet the criteria for endangered or threatened but is considered vulnerable to eventually needing listing. In New York State both are listed as species of concern, but this varies in other parts of the eastern U.S. The Blue Spotted Salamander (*A. laterale*) is decreasing in North America overall by at least 30% (Species Status Assessment, 2013). Some states list the species of concern as the *"A. laterale-jeffersonianum complex"* or simply "*A. jeffersonianum complex"* due to the difficulty differentiating between parental individuals and those of the complex. Unfortunately, because the Endangered Species Act excludes hybrids, many states do not list the *A. jeffersonianum* complex for any special protections (Haig, 2006). Hybrids are not considered a species under the Endangered Species Act, creating a "grey" policy area for these lineages. Indeed, in some literature, hybrid conservation is discouraged because hybrids conservation could interfere with listed species conservation (Haig, 2006).

Salamanders of the *A. jeffersonianum* complex need various habitat requirements for their life cycles. In the ecoregion of the Great Lakes, within the southeastern Lake Ontario watershed basin, these organisms occupy most forested habitats nearby vernal pools, all with mineral soils (Forest Dependent Species, n.d). Both species and hybrids also are found in forests adjacent to disturbed and agricultural lands. In addition, they prefer vernal pools and ponds free of fish, with emergent

vegetation (Gibbs et al., 2007). The main difference between the two diploid parentals appears to be in the preference for more upland forests (non-wetland forests) in *A. jeffersonianum* and lowland forests (wetland forests) for *A. laterale* (pers. comm. S. Morse).

Objectives

The first objective of this project was to identify individual salamanders sampled on the Rochester Institute of Technology (RIT) campus as polyloid hybrids or diploid parental individuals from the *Ambystoma jeffersonianum* Complex through PCR of microsatellites. I hypothesized that both diploid parentals and hybrid lineages are present on campus, due to the types of potential habitat present. The PCR test will allow for determination of the species identification and the proportion of individuals in each group on campus.

The second project objective was to map the distribution of the *Ambystoma* diploid and hybrid lineages determined by the part of the project on the RIT campus. I hypothesized that the populations on the campus would be mixed hybrids and diploids, which would not be separated physically from one another.

The third project objective was the construction of a habitat suitability model. The model uses intersections between habitat parameters to determine potential habitat on and around the RIT campus and within Monroe County, NY. This allows for the development of recommendations on future sampling and to inform conservation efforts of those areas. I hypothesized that there are additional suitable habitat locations on campus, other than those sampled in this study, based on soils, vegetation, hydrology, and campus topography.

Methods

Sample Collection

In the wetlands of the Rochester Institute of Technology Campus, West Henrietta New York located in the Great Lakes' Basin, 104 samples were collected. These samples were collected in May and October of 2015 by a former faculty member. For each salamander sample, a nonlethal, interphalangeal toe clip or a tail clip (NYSDEC permit #644) was obtained and preserved in a DNA buffer, then the specimen was released as described in Lowcock et al. (1991). Using a handheld GPS, the location and date collected were recorded along with the sample number and then samples were frozen at -20 C until analysis. All samples from the specimens collected were assumed to be unidentified members of the *Ambystoma* complex and were assumed to be either parent or hybrid.

DNA Analysis

DNA was extracted from the tissue samples following the E.Z.N.A. protocol. Tissue DNA Mini kit protocol from Omega Biotech. The extraction process involves incubation of tissues in buffer and proteinase K with subsequent removal of cell debris and isolation of purified DNA. Three microsatellite loci, AjeD94, AjeD346 and AjeD37 were used (Julian et al. 2003). Loci AjeD94 and AjeD346 amplify *A. laterale* and *A. jeffersonianum* with different size amplimers (amplification products) while locus AjeD37 amplifies both species but with overlapping size amplimers. AjeD37 was used specifically for ploidy determination (Ramsden, 2006). The PCR reaction includes12.5 uL GoTaq, 1uL forward and reverse of each primer, n9.5 uL nanopure water, and 1 uL DNA, per sample. Each PCR includes the following temperatures and cycling: a 1-min

initial denaturation at 94 ° C, followed by 30 cycles at 94 ° C/annealing T/72 ° C for 45/45/30 seconds with annealing temperatures of 57 ° C for Aje D346 and Aje D94 and 53 ° C for Aje D37 (Ramsden, 2006). Upon completion of the PCR, the samples were run on a 2% agarose gel, infused with gel red. If the bands were not separated enough to view the individual fragments, the gel percentage was increased to 3%. Once run to 70-80% of gel length at 200 volts, the gel was visualized using UV transillumination, and the fragments were compared against the ladder. Following this, if the PCR results are not visible, 1ul of $MgCl^2$ solution was added to each reaction of the PCR to lower reaction stringency and increase amplification products.

Habitat Suitability Model

Using spatial layers representing habitat parameters, a habitat suitability model was developed using ArcGIS software to identify suitable habitat in and around the study area, and to confirm the existing samples were located within or near suitable habitat. The process by which the habitat suitability model was performed, as well at the overall goal of the model, is shown in Figure 1.

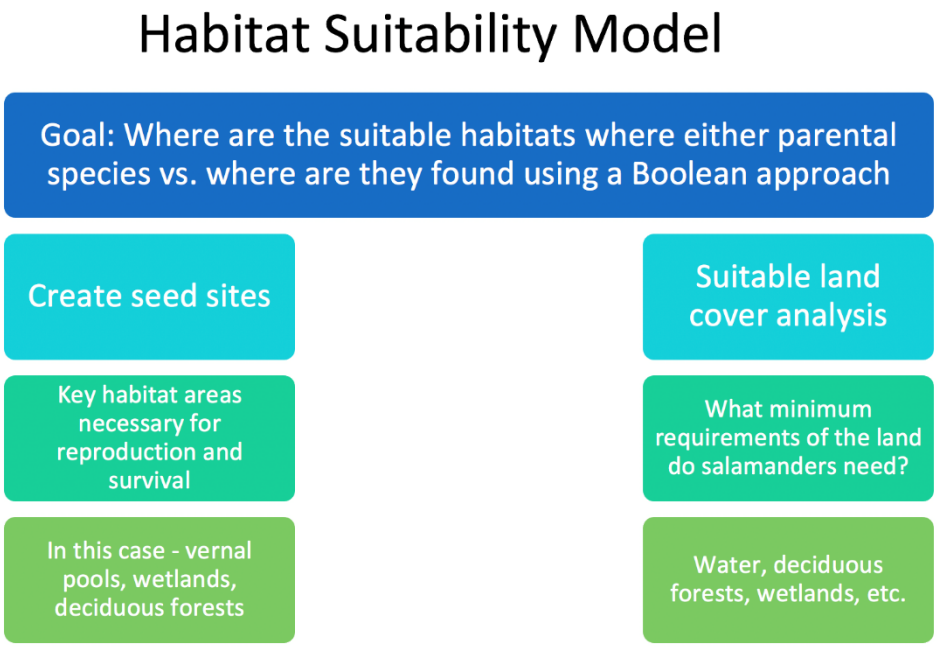

Figure 1: Habitat suitability model description of goal and process. A Boolean approach was used to determine the suitable habitat for salamanders of the complex based upon habitat criteria including the presence of wetlands and mineral soil.

Based upon literature from the New York State Department of Environmental Conservation, it was determined that the proper land cover habitat requirements for salamanders of the complex were as follows: mixed forests, deciduous forests, wooded wetlands and emergent wetlands. A Boolean approach was used, in order to analyze the geographical areas salamanders of the complex were likely to be found in Monroe County, New York. The Boolean approach was done through a reclassification of each layer, where the ideal habitat was rated numerically as a score of 1, while the rest of undesirable features were rated as a 0. An example of this is in Figure 2. The desirable habitats of mixed forests, deciduous forests and wetlands were given a value of 1, while other land covers such as highly developed land, scrub/shrub, cropland, etc. were placed with a value of 0.

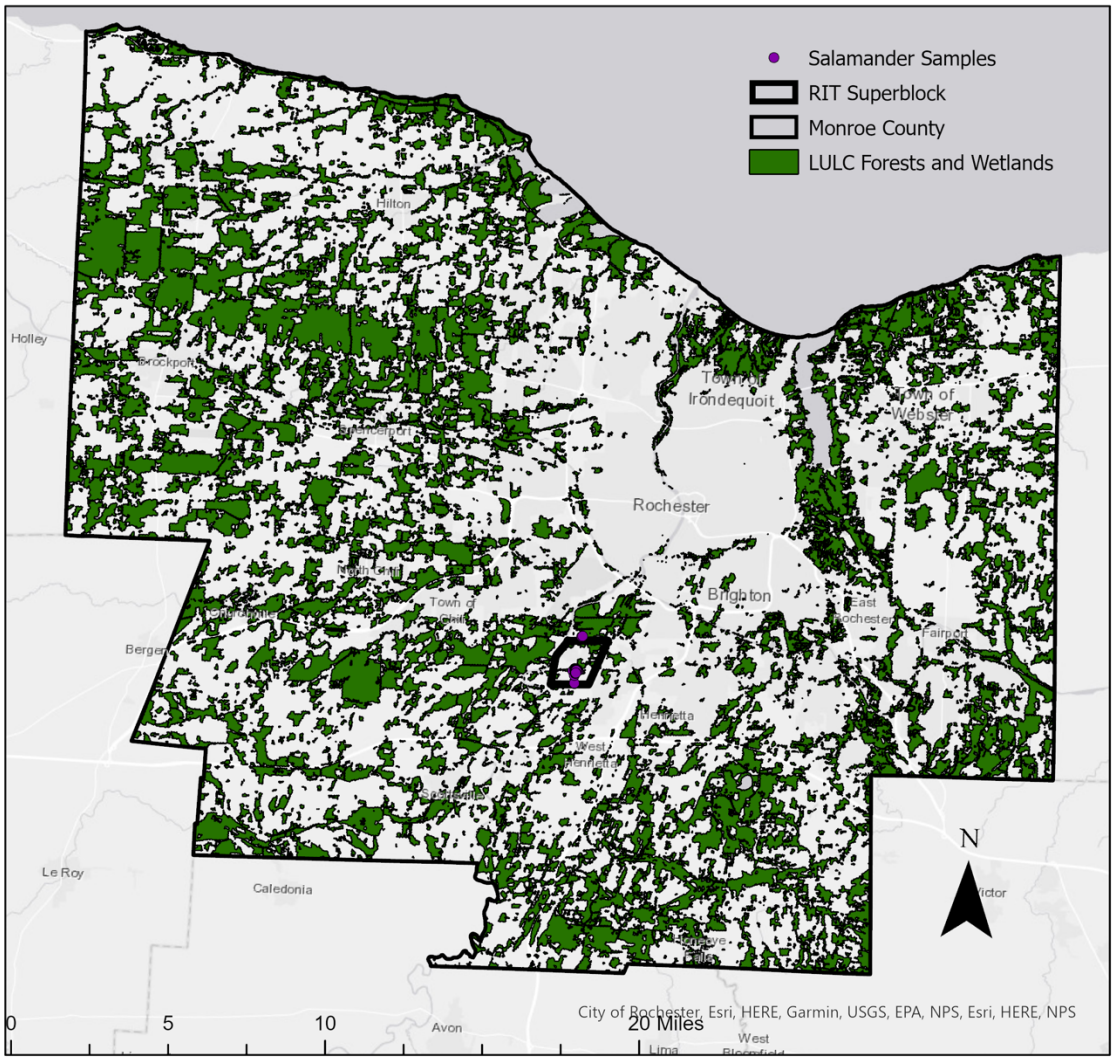

Figure 2. GIS layout of the land use and land cover data for the RIT campus and surrounding area. The purple circles represent sampled salamander locations while the green shows the land cover preferred by *Ambystoma* salamanders (deciduous forests, mixed forests and wetlands).

The ideal soil features for the model were selected differently. For the Monroe county soils layer, seen in Figures 3 and 4, all of the soils in the layer with a high organic content value (>50%) and that were nonhydric, were excluded using a definition query so that only hydric mineral soils with a low organic matter content (< 50%) were included.

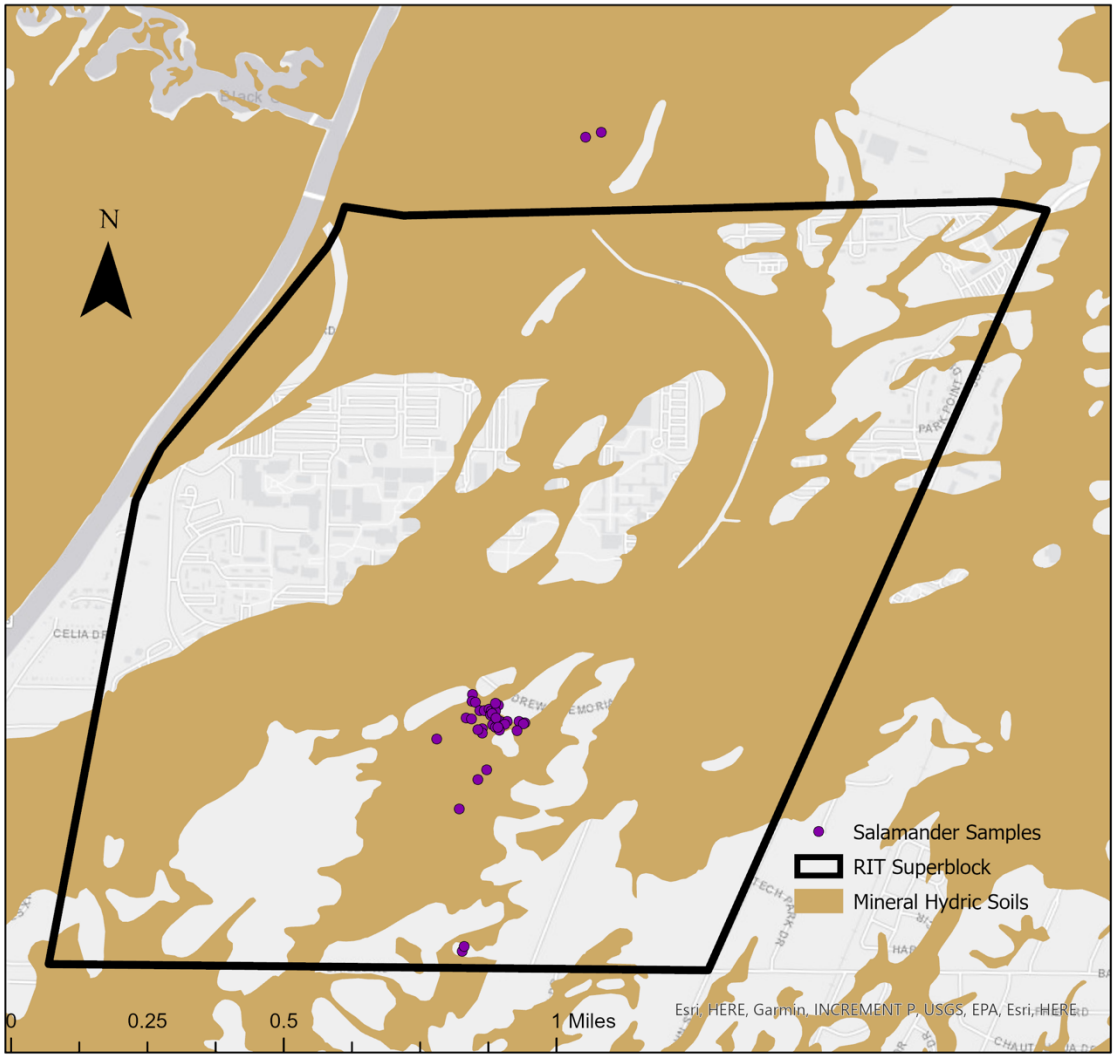

Figure 3. GIS layout of the areas surrounding RIT within Monroe County, NY where hydric mineral soil is present. The green represents soils with a low organic matter content (<50%) and that are hydric soils. The purple circles represents salamander sampling locations from 2015 on the RIT campus.

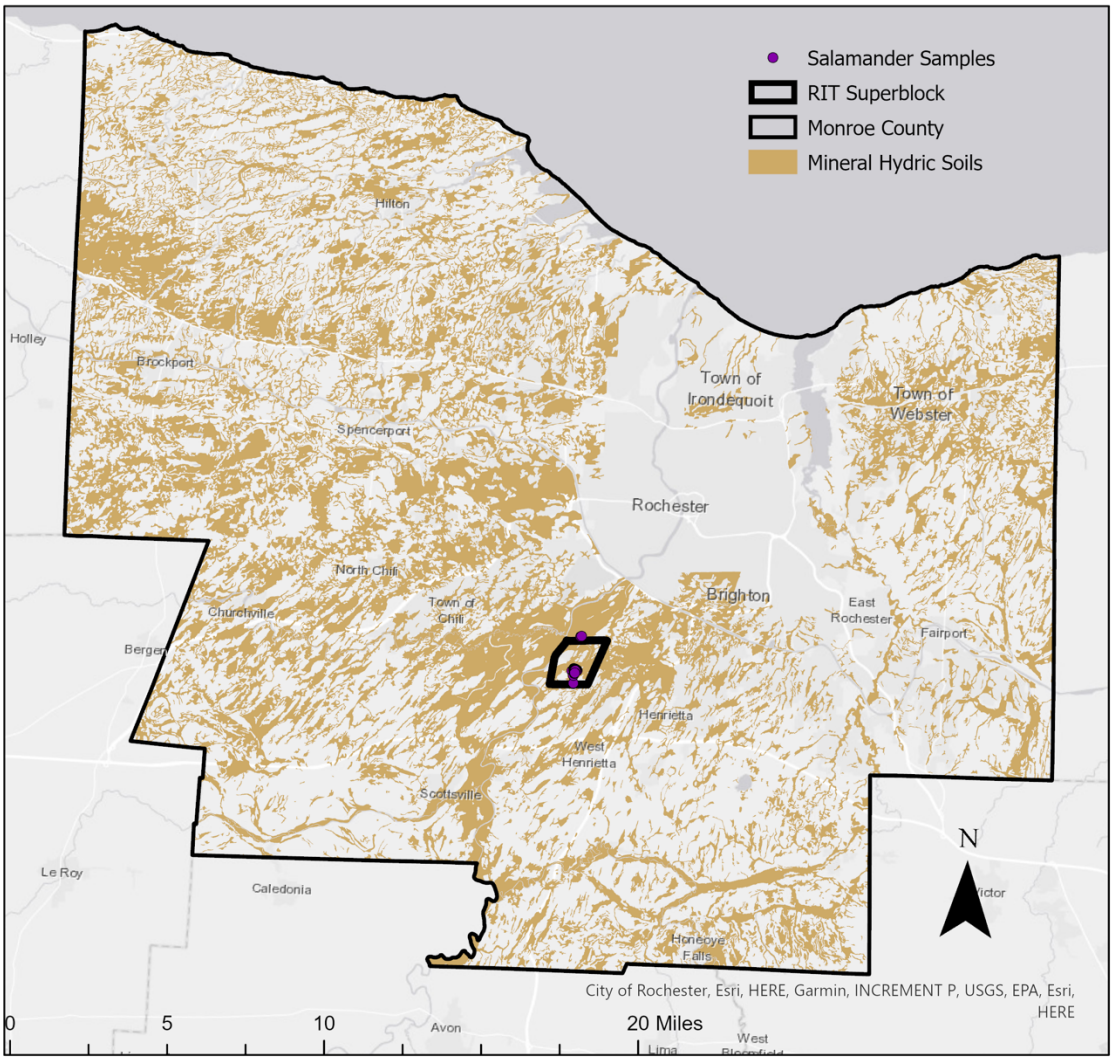

Figure 4. GIS layout Monroe County, NY where hydric mineral soil is present. The green represents soils with a low organic matter content (<50%) and that are hydric soils. The purple circles represents salamander sampling locations from 2015 on the RIT campus.

The wetlands on the RIT campus were used as an essential part of this habitat suitability model, as *Ambystoma* salamanders greatly rely on wetlands for reproduction and survival. The wetlands data were collected from the New York State Department of Environmental Conservation (NYSDEC) and were last updated in 2008. A Boolean approach was necessary with these data to identify wetlands as present or absent but

did not require the ranking of features. Figure 5 shows the wetlands on the RIT campus and surrounding areas. Note that the majority of the tissue sample points were within the critical wetland habitats. Two samples outside the habitat suitability model were in forests near agricultural lands. Figure 6 shows the NYSDEC wetlands for the county.

Figure 5. DEC wetland data from 2008 representing the presence of land that meets the criteria to be considered a wetland on the RIT campus. The light green coloring indicates the area of a wetland, while the purple circles show the locations of the salamanders sampled in 2015.

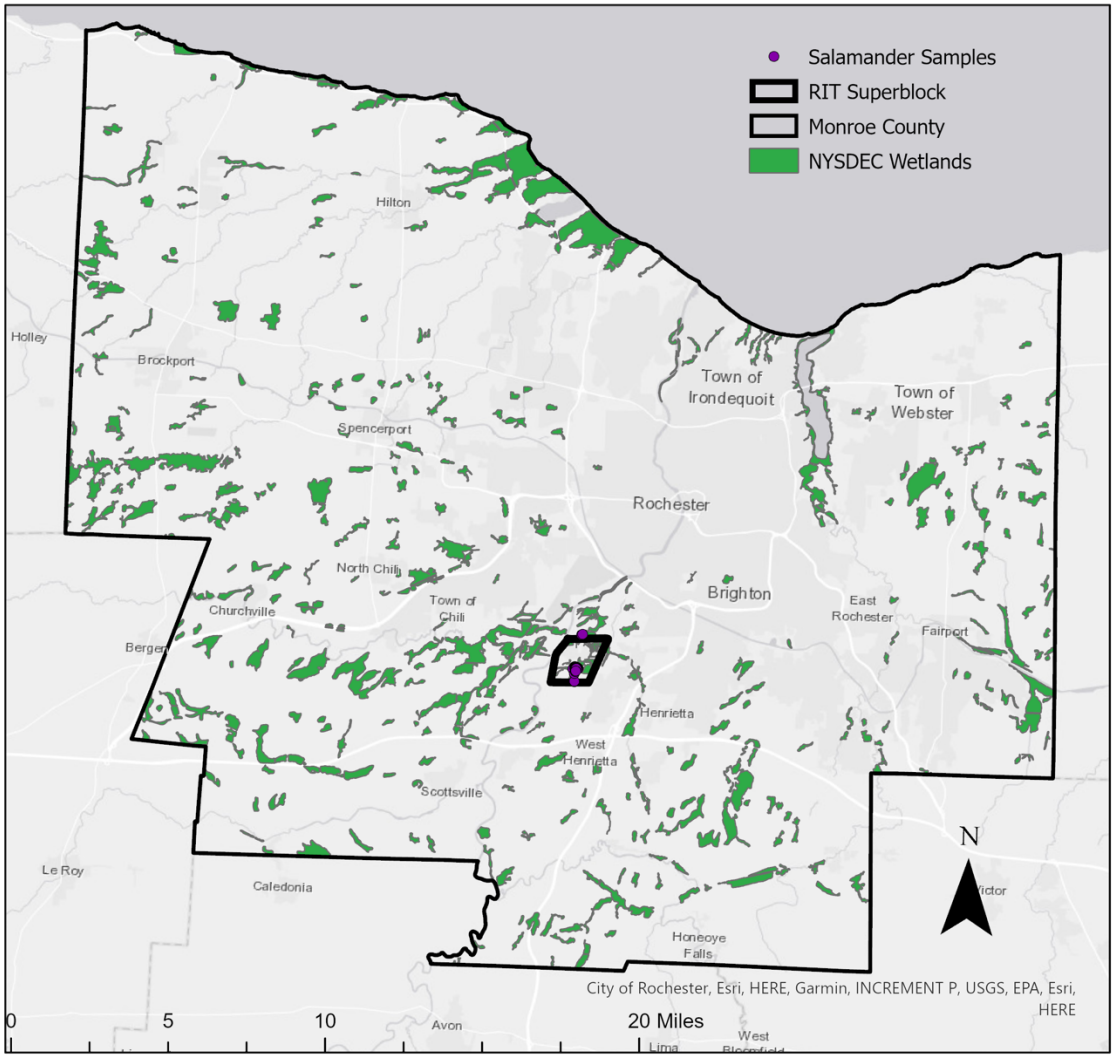

Figure 6. DEC wetland data from 2008 representing the presence of land that meets the criteria to be considered a wetland in New York State. The light green coloring indicates the area of a wetland, while the purple circles show the locations of the salamanders sampled in 2015.

Once each of the layers were reclassified or a definition query was applied, all of the raster data were then converted to polygon, and the features were exported to new layers. The two layers of soils and wetlands were intersected to create the habitat seed sites in the model.

Results

Sample collection

The locations of the samples collected for this study on the RIT campus in May and October of 2015 were verified and placed on an ArcGIS map (Figure 7). The specimens collected did not have positive field identification at the time of collection due to difficulties with non-diagnostic morphologic characteristics among the *Ambystoma* species.

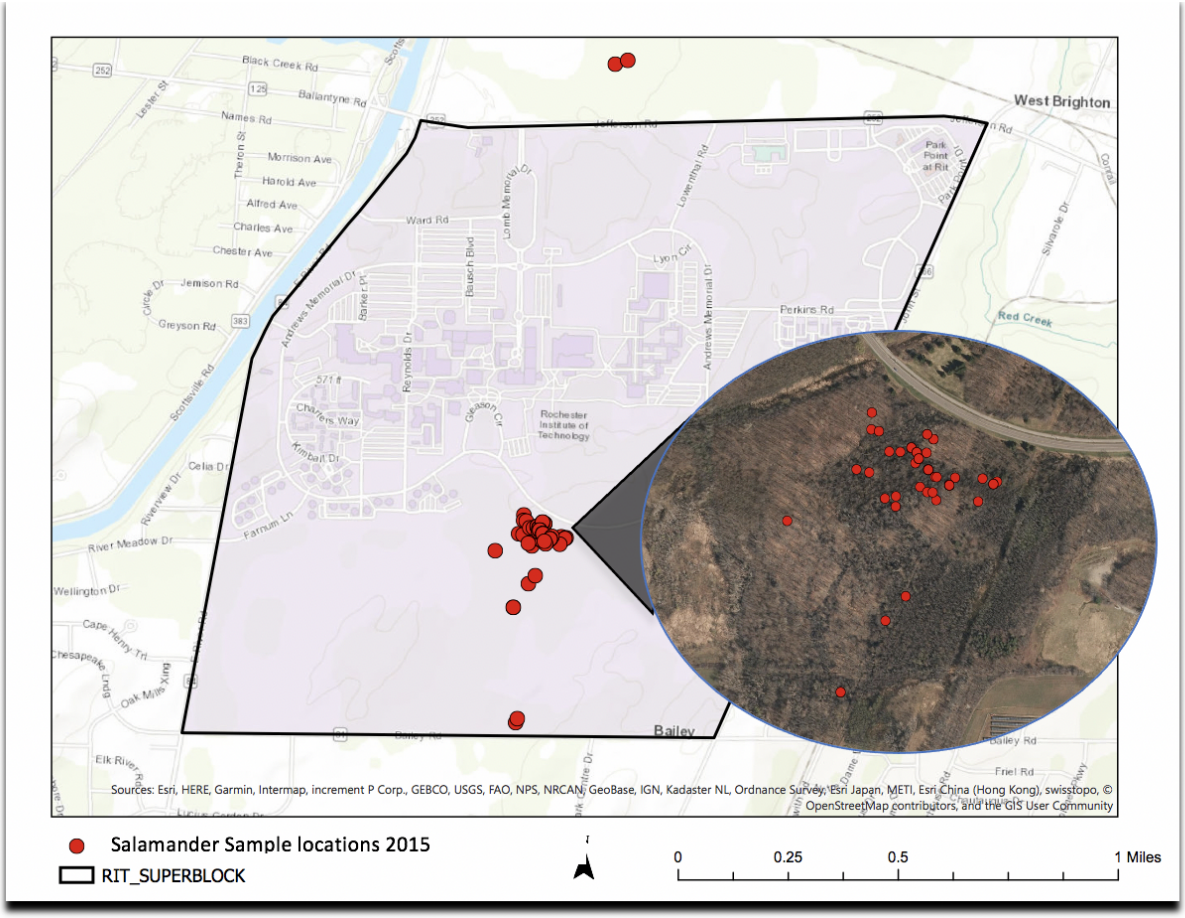

Figure 7: Locations of salamanders that samples were obtained from on the Rochester Institute of Technology Campus in the Great Lakes Basin. The zoomed in image shows habitat area being deciduous forest and forested wetlands on Andrew's Memorial Drive.

Sample Processing

Of the 104 collected samples, 50 tissue samples from the RIT campus were used in this study. Additional data on the individual salamanders (collection date, snout-vent and total length, GPS coordinates) accompanied the tissues and the DNA concentration and 260/280 values for each sample were also recorded (Table 1). Five of the 50 samples in the data set did not have a tissue sample date, coordinates, snout-vent or total length recorded, and one additional sample did not have a total length measurement (Table 1); however, these samples were still analyzed and genotyped. Multiple samples are matched with the same GPS coordinates; therefore, when mapping, they will be shown as one cluster.

Table 1. Identification of *Ambystoma* samples sampled on the RIT campus. The date collected ID, genetic ID (genotype), GPS coordinates are provided. The snout-vent length (S-V), and total length, are recorded in millimeters. The DNA concentration was measured in ug/uL. “NA” indicates data was not recorded.

DATE	ID	Genetic ID	LATITUDE	LONGITUDE	S-V	TOTAL	DNA Con.	260/280
10/14/2014	AI07	*A. laterale*	43.07881	-77.67292	46	85	64.8	1.98
10/15/2014	AI08	*A. laterale*	43.07881	-77.67222	62	118	144	1.90
10/15/2014	AI09	*A. laterale*	43.07878	-77.67200	46	89	140.6	1.94
10/15/2014	AI10	Sample Failed	43.07875	-77.67206	61	118	18.3	2.02
10/15/2014	AI11	Sample Failed	43.07875	-77.67206	56	112	35.3	2.01
10/14/2015	AL60	*A. laterale*	43.07948	-77.67393	44	79	40.6	1.90
10/14/2015	AL61	hybrid	43.07930	-77.67393	42	74	42.4	1.86
10/14/2015	AL62	*A. laterale*	43.07928	-77.67382	46	74	44.8	1.88
10/14/2015	AL63	hybrid	43.07907	-77.67365	78	151	84.3	1.89
10/14/2015	AL64	*A. laterale*	43.07907	-77.67348	41	71	46.4	1.89
10/14/2015	AL65	hybrid	43.07912	-77.67332	44	76	40	1.95
10/14/2015	AL66	*A. laterale*	43.07912	-77.67332	40	72	66.6	1.99
10/14/2015	AL67	*A. laterale*	43.07912	-77.67332	41	77	49.1	1.85
10/14/2015	AL68	*A. laterale*	43.07912	-77.67332	38	66	45.6	1.90
10/14/2015	AL69	*A. laterale*	43.07907	-77.67323	42	75	63.7	1.85

10/14/2015	AL70	hybrid	43.07907	-77.67323	40	72	23.5	1.80
10/14/2015	AL71	*A. laterale*	43.07907	-77.67323	34	60	45.4	1.87
10/14/2015	AL72	hybrid	43.07895	-77.67325	38	64	30.5	1.90
10/14/2015	AL73	*A. laterale*	43.07922	-77.67298	38	68	18.2	1.79
10/14/2015	AL74	*A. laterale*	43.07922	-77.67298	48	81	26.2	1.77
10/14/2015	AL75	hybrid	43.07907	-77.67308	48	68	42.2	1.88
10/14/2015	AL76	hybrid	43.07927	-77.67308	42	79	27.8	1.84
10/14/2015	AL77	hybrid	43.07927	-77.67308	45	81	36.1	1.89
10/14/2015	AL78	*A. laterale*	43.07900	-77.67320	42	77	54	1.88
10/14/2015	AL79	*hybrid*	43.07900	-77.67320	33	60	39.4	1.85
10/14/2015	AL80	*A. laterale*	43.07900	-77.67320	43	81	80.4	1.76
10/14/2015	AL81	*A. laterale*	43.07888	-77.67305	38	66	72.7	1.91
10/14/2015	AL82	*A. laterale*	43.07888	-77.67305	35	65	37.5	1.82
10/14/2015	AL83	*A. laterale*	43.07888	-77.67305	39	65	36.7	1.91
10/14/2015	AL84	hybrid	43.07888	-77.67305	43	NA	68.1	1.79
10/14/2015	AL85	*A. laterale*	43.07888	-77.67305	37	71	42.5	1.81
10/14/2015	AL86	*A. laterale*	43.07888	-77.67305	41	75	26.1	1.83
10/14/2015	AL87	*A. laterale*	43.07888	-77.67305	38	70	30.1	1.83
10/14/2015	AL88	*A. laterale*	43.07888	-77.67305	38	69	24.8	1.81
10/14/2015	AL89	*A. laterale*	43.07888	-77.67305	54	101	16.7	1.92
10/15/2015	AL90	*hybrid*	43.07881	-77.67263	40	71	38	1.84
10/15/2015	AL91	*A. laterale*	43.07872	-77.67272	37	64	30.6	1.69
10/15/2015	AL92	hybrid	43.07872	-77.67272	65	122	33.6	1.93
10/15/2015	AL93	hybrid	43.07872	-77.67272	65	125	59.8	1.83
10/15/2015	AL94	hybrid	43.07872	-77.67272	59	114	48.6	1.93
10/15/2015	AL95	*A. laterale*	43.07872	-77.67272	43	81	29.4	1.86
10/15/2015	AL96	hybrid	43.07872	-77.67272	50	104	35.5	1.86
10/15/2015	AL97	hybrid	43.07856	-77.67292	75	145	72.5	1.77
10/15/2015	AL98	*A. laterale*	43.07864	-77.67297	60	122	33.9	1.87
NA	AL100	*A. laterale*	NA	NA	NA	NA	40.8	1.89
NA	AL101	hybrid	NA	NA	NA	NA	25.7	1.78
NA	AL102	hybrid	NA	NA	NA	NA	177.7	1.89
NA	AL103	hybrid	NA	NA	NA	NA	109.4	1.83
NA	AL104	hybrid	NA	NA	NA	NA	99.8	1.87

The majority of salamanders sampled (58%) were identified as *A. laterale* and 38% of the samples were identified as hybrids (Figure 8).Two samples (4%) failed to amplify, and no samples being identified as *A. jeffersonianum.*

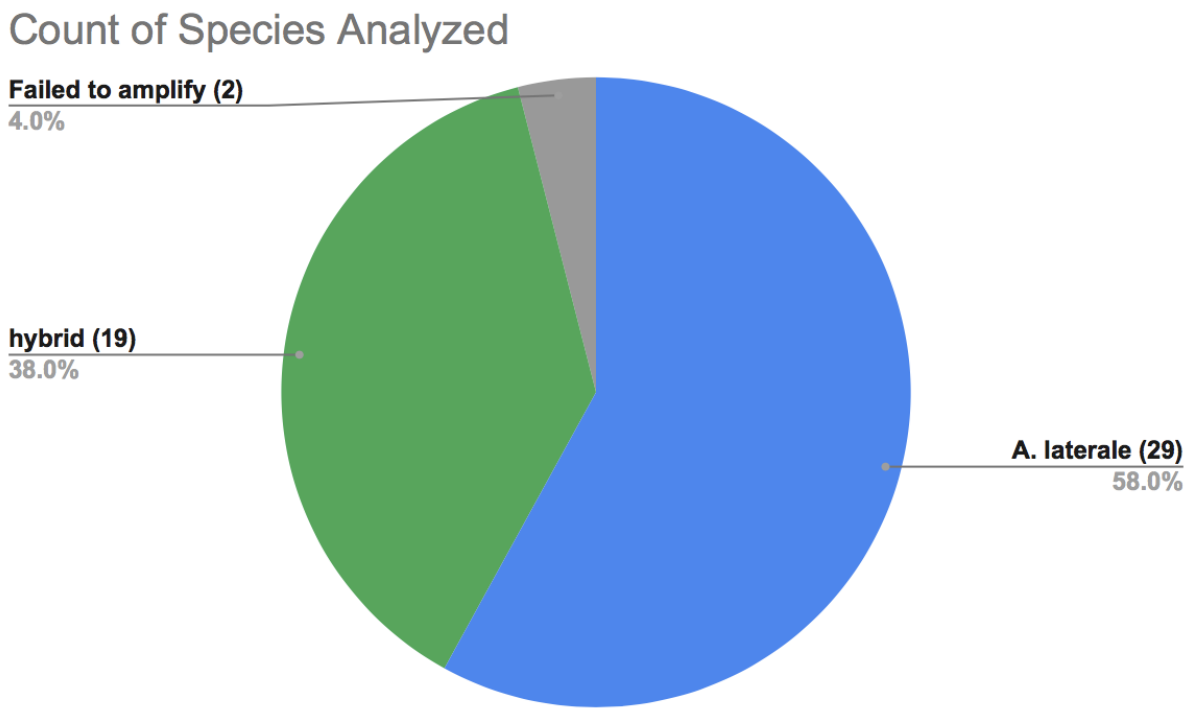

Figure 8. Identification of species sampled on the RIT campus. Each sample was classified as either a hybrid, failed to amplify, or as one of the parental species.

A subset of the 50 samples is shown in Figure 9 to visualize representative agarose gel results for the PCR identification. The highlighted areas indicate the genotype of each of the samples. *A. jeffersonianum* is shown in sample AL90 as having a single band as a larger DNA fragment at 180-250 base pairs. Sample AL69 represents *A. laterale,* having a single band at 140-155 base pairs representing a smaller DNA fragment. Lastly, sample Al102 presents a hybrid of the complex with two bands, one less than 155bp and another greater then 180bp, containing one allele from

each of the parental species.

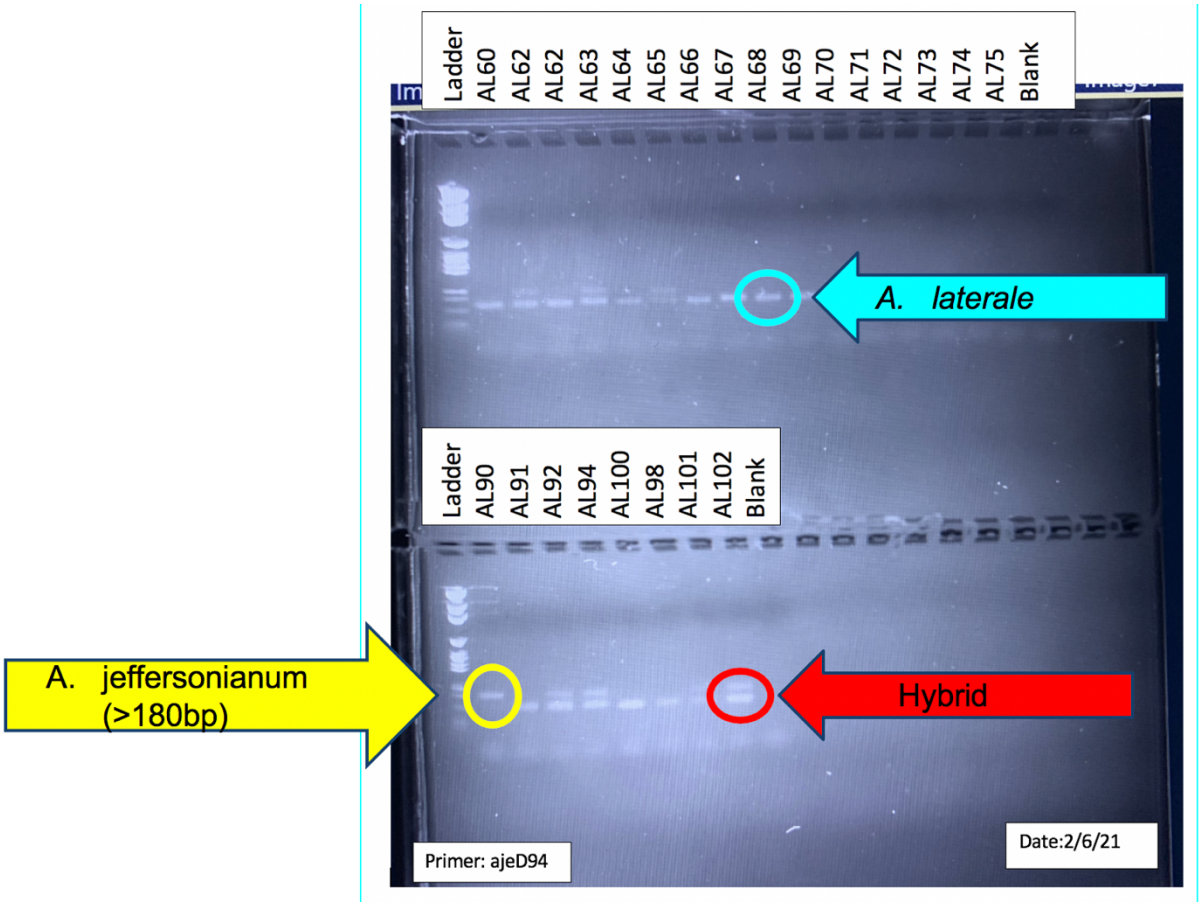

Figure 9. Agarose gel electrophoresis results from a subset of samples from the RIT campus. All samples were run using Primer AjeD94 which amplifies both *A. jeffersonianum* and *A. laterale. A. jeffersoninaum* is amplified from base pairs 180 -250 and A. laterale 140 - 155 using primer AjeD94. Three samples have been highlighted as hybrid, *A. laterale,* or *A. jeffersonianum.* A single band indicates a diploid parental, while two bands indicates a polyploid hybrid.

The two primers, AjeD37 and AjeD94, amplify different alleles. AjeD37 determines ploidy, while AjeD94 determines genotype. Figure 9 demonstrates the results obtained when four samples (AL104, LB188, AL80, AL98) were run using two loci (AjeD94-Sp.ID, AjeD37-ploidy). These results for samples #104 and #188 show three bands with locus D37, indicating triploid hybrid samples. The same samples possess two visible bands with locus D94, however the lower of the two bands is

significantly brighter indicating co-migration (they migrate together and look twice as bright) of two equal size alleles confirming the triploid result obtained with the D37 locus.

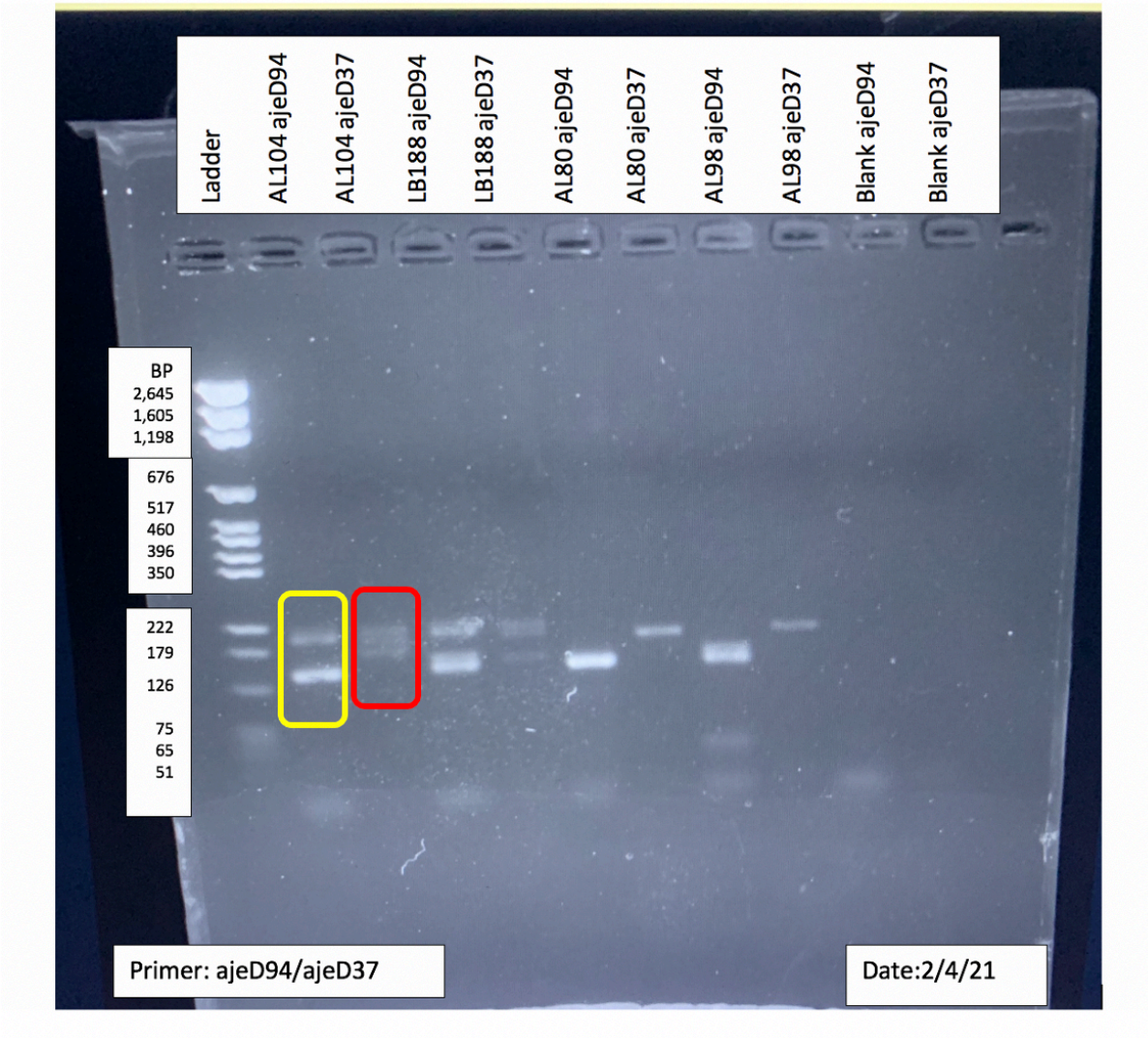

Figure 10. Agarose gel electrophoresis results from a subset of samples from the RIT campus. All samples were run using Primer AjeD94 which amplifies both *A. jeffersonianum* and *A. laterale. A. jeffersoninaum* is amplified from base pairs 180 -250 and A. laterale 140 - 155 using primer AjeD94. One sample has been highlighted in green as a hybrid with three bands.

Habitat Suitability Model and Mapping

Figure 11 shows individual genotype results and locations of each salamander analyzed using PCR in this study. The salamanders appear to be distributed within and along the edges of forests as seen in Figure 11.

Figure 11. GIS map of the RIT campus in Monroe County, NY showing the 2015 sample locations and confirmed genotypes as either *Ambystoma* salamanders or hybrids. The green circles represent those identified as the complex, the blue circles a confirmed *Ambystoma laterale,* and the red circle indicates samples without a positive identification. Each dot may represent more than one sample.

The habitat suitability model of the RIT campus in Figure 12 shows sampled salamander locations from 2015 relative to areas that are potentially suitable for salamanders of the complex. The map indicates (1) all study samples are in or near

suitable seed site habitat and (2) much more suitable habitat is present on campus than what was sampled in this study, suggesting additional areas to search for salamanders. The figure contains all the identifications of the salamanders analyzed and does not include those that were not analyzed or PCR genotyping.

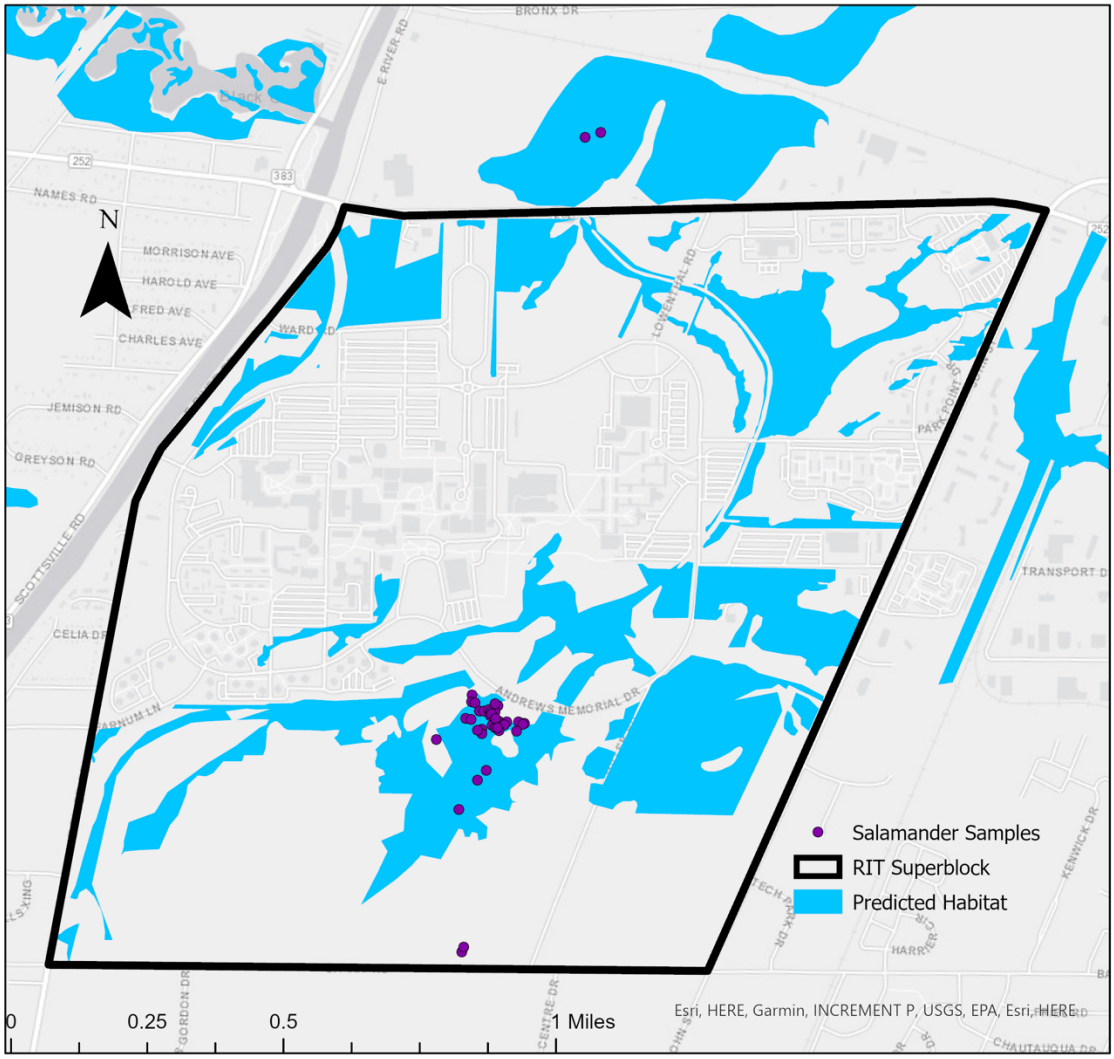

Figure 12. Sampled salamander locations on the RIT campus in 2015 and predicted areas of potentially suitable habitat generated by GIS located in Monroe County, NY for *Ambystoma sp*. and hybrid salamanders based upon criteria the habitat criteria of presence or absence of wetlands, deciduous or mixed forests, wooded or herbaceous wetlands and hydric mineral soil.

Figure 13 shows Monroe County and the potential suitable habitat for *Ambystoma* salamanders, based only on the intersection of hydric mineral soils and NYSDEC Wetlands data. The blue areas suggest that the areas may be prime habitat for *Ambystoma* salamanders.

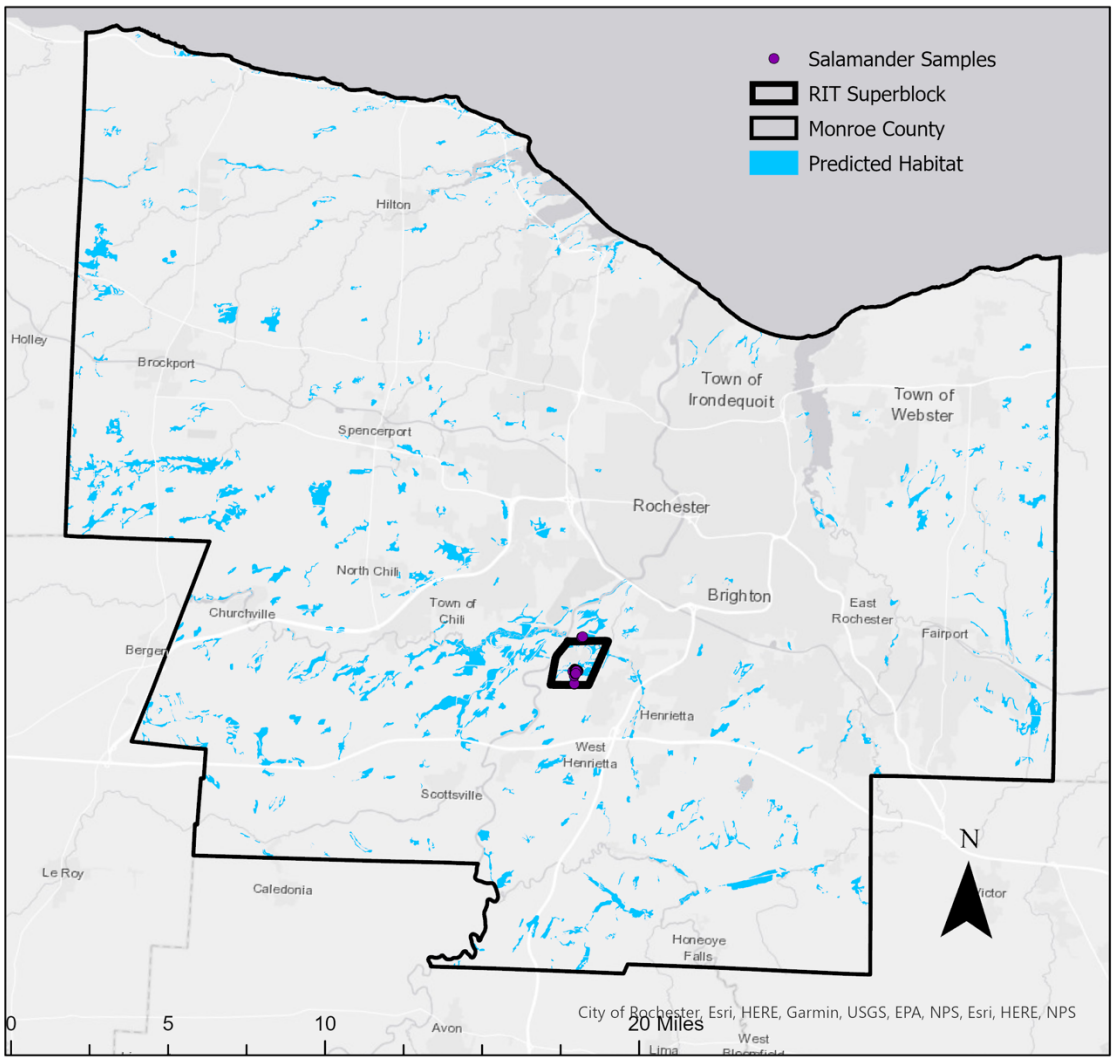

Figure 13. GIS habitat suitability model of Monroe County in New York State. The blue represents habitat predicted by the model to be suitable for the *Ambystoma* complex, given the habitat requirements of preferred land cover, presence of wetlands, and hydric mineral soils.

Discussion

Parental species (*A. jeffersonianum. A. laterale)* of the *Ambystoma* complex are distinct morphologically when observed in their diploid form. However, hybrid individuals can resemble either parental diploids morphologically. The results of this project identified no diploid *A. jeffersonianum* samples, 29 *A. laterale* samples, and 19 hybrid samples on the RIT campus. Therefore, ninety-six percent (48/50) of the RIT samples were unambiguously identified as members of a diploid parental species (*A. laterale)* or as a hybrid polyploid.

A habitat suitability model was created in order to predict where salamanders of the complex may be present on the RIT campus and the surrounding areas in Monroe County, NY. The habitat suitability model successfully confirmed suitable habitat in imagery for 48 of the 50 salamanders sampled in this study. When comparing the coordinates from 2015 samples with the areas the model predicted as suitable habitat in the GIS model, all but two of the sample data points were within the predicted habitat areas. Some of the 50 samples shared identical GPS coordinates, resulting in 25 separate clusters from which salamander tissue samples were collected. Of these 25 clusters, 16 were located directly within the seed site habitats identified by the model and seven samples were within 50 feet of the seed sites identified by the model. There are two remaining data points near the edge of the RIT campus boundary that did not fall within predicted suitable habitat seed sites generated by the GIS model, due to no NYSDEC polygon in this area (forest and mineral hydric soils were present).This supports previous documentation of that found salamanders of the *Ambystoma* complex

adjacent to disturbed habitat (Gibbs et al. 2007). Of the 50 samples in the study, 48 (96% accuracy) samples were correctly predicted by the GIS model.

In a future study, a model could be completed where the National Wetland Inventory (NWI) wetlands and New York DEC 2008 wetland layers are combined in order to take into account smaller mapped wetlands. NYSDEC wetland rules specify a minimum size of 12.3 acres (5 hectares), while NWI wetlands can be mapped as small as 0.25 acres. This project focused on the larger habitat areas produced by the NYSDEC wetlands data. Combining NWI and NYSDEC would likely generate additional potential habitat, such as may be present along Ridge Road in the Rochester area. The inclusion of this would lead to a more complex, but potentially more comprehensive and inclusive analysis with increased habitat being predicted.

Future work involving the habitat suitability model could include verification of the presence of *Ambystoma* salamanders in other predicted habitat locations of Monroe County and the RIT campus. Subsequently, additional tissue samples could be genotyped to determine which species or hybrids thrive in those regions. Future research may investigate whether different salamanders of the *Ambystoma* complex are present in the predicted locations, especially in relation to the soil pH, water quality and other factors. In addition, the model could be reanalyzed using high resolution data, such as 10m resolution rather than 30m databases. This would be beneficial for identifying potential habitats for these salamanders are smaller than the threshold 30m raster resolution, thus narrowing the scope of the habitat suitability model and allowing for prediction of more precise locations of potential habitat. Determining the home range extent of these salamander species and lineages with a mark-recapture study would

allow future researchers to determine how far individual salamanders may travel in a given year or time span, and allow for habitat quality assessments within their home range areas.

A land use, landcover analysis with forests and wetlands could also be completed for the salamander locations in the county, as seen in Figure 2. This would allow for a more refined look at the habitats these organisms require, in regard to where they may be found. The use of a home buffer and NWI wetland data could also lead to a more refined study.

Optimizing the PCR for genotyping may help to discriminate samples that had conflicting results when the different primers (AjeD94 and AjeD346) were used and determine why two separate genotypes were exhibited. This may involve adjusting PCR thermocycler condition (the number of cycles completed, annealing temperature, and annealing time). Ploidy determination, separate from using primer AjeD37, would be useful for classify samples by ploidy such as by triploid, tetraploid or pentaploid. Lastly, an interesting future direction could include determining if there are other *Ambystoma* species DNA within the samples on campus. Due to the fact that *Ambystoma* salamanders are capable of using sperm from five separate species, it would be interesting to investigate the possibility of samples containing additional genomes from those with populations here.

Protection for all salamanders of the complex, both parents and hybrids, especially *Ambystoma jeffersonianum*, needs to be considered because salamanders worldwide, regardless of legal conservation status, are facing rapid and great declines (Yap, 2015). Most importantly, clarification on hybrid protection in the U.S. is needed to

better understand how the loss of these organisms can be diminished. The biological species concept (BSC) is the basis for most federal and state species protections. Since the BSC identifies diploid lineages as species (binomial nomenclature names), hybrid lineages (polyploids) that remain without a binomial (genus/species) are often left unprotected because they are not assigned to a protected species. Polyploid hybrids are taxonomically unrecognized lineages that lie between diploid species that can be protected that hinders the protection of hybrid lineages and any populations that consist primarily of hybrids (Haig, 2006).

Overall, a more in-depth look at the distribution and prevalence of these species and hybrid assemblies needs to continue. The distribution of the complex is poorly understood and the extent to which the parental populations are distributed is also poorly understood. This is where the habitat suitability model may be beneficial, as it would confirm the distribution of these salamanders locally and provide insight as to where viable populations are located. A future model could also consider upland vs lowland habitats in the model, as there may be a preference with *A. laterale* living primarily in lowlands, such as RIT and Monroe county, and *A. jeffersonianum* having a preference towards uplands (S. Morse, pers. comm.). Therefore, the model could be expanded statewide, and determine what habitats the salamanders may be in, coupled by an in-field verification of their existence with genotyping.

Importantly, it is unknown what role these hybrids may play in ecosystems throughout their potential range, and why they appear to be successful in certain populations. Determining their locations, and factors that may encourage (or hinder) their success within those areas, may answer shed light on some of these roles. A

potential interesting question is whether biodiversity would decrease if these hybrids were to be extirpated. Although we know a great deal about their means of reproduction, it is still not known what ecological, fitness and reproductive advantages these methods provide. One additional question to consider for potential conservation efforts is whether the hybrids of this complex are out-competing the parental species in specific areas of the Great Lakes basin, and if so, what impacts do they have on each species and why are they more successful than others. These questions remain despite the previous research on this complex spanning nearly seven decades (Uzzel, 1964).

Conclusions

At the conclusion of this study, 29 *Ambystoma laterale* salamanders were identified, along with 19 hybrids of the *Ambystoma jeffersonianum* complex. Two samples were unable to be identified. The preliminary habitat suitability model accurately predicted known habitat areas on the RIT campus as well as potential areas to search for salamanders of the complex. In the future, toe and tail samples could be collected from these areas to determine what species or lineages exist within these local populations.

The validation of the salamander locations predicted by the habitat suitability model could be useful for future studies as it would confirm the model and provide locations as to where to search for these organisms. Performing a high-resolution model to fine-tune the predicted habitats. Considering the degree to which vernal pools and wetlands are necessary for reproduction for Ambystomatid salamanders, it is crucial that these habitats are protected. The loss of suitable breeding habitat, such as wetlands, needs to be addressed. Building on uplands when possible and leaving wetlands and vernal pools undisturbed is optimal (Canada, 2017). When not possible,

mitigated wetlands may be used as a replacement, although only when they closely resemble the wetland lost. Finally, reducing the uncertainty in the genotype of the three ambiguous samples, determining the ploidy of each sample, as well as seeing if other *Ambystoma* genomes exist within the populations would allow for more in-depth analysis of the local populations.

Research regarding the *Ambystoma jeffersonianum* complex is limited. It is known that their range extends primarily throughout the Great Lakes region, however, knowledge of specific whereabouts of these lineages is hindered due to similar morphological traits and the need for genetic methods for positive identification. An in-depth analysis of the hybrid ranges needs to be completed in order to determine their prevalence and population trends, as this information is unknown in New York State (Species Status Assessment, 2013). To better understand the distributions and ecosystem impacts of these lineages and the parental species, a range-wide in-depth analysis needs to be executed. In New York State, both species are of special concern (List of Endangered, n.d.). However, there is no protection for the hybrid lineages due to the stipulations of the Endangered Species Act against hybrid species (Haig, 2006), and *A. laterale* is not listed as endangered in Canada (Bogart, 2017). Actions need to be taken to address the lack of conservation efforts for hybrid lineages, and a more definitive meaning of hybrids needs to be taken into account. Policy changes may be necessary to include these challenges, as these organisms are not easily distinguishable from one another or diploid parental, outside of the lab. Because these organisms cannot always be discerned in the field and their natural habitat, the relatively simple and low-cost method of identification in the laboratory setting outlined

in this project should be encouraged. Conservation efforts need to include the lineages to protect the parental species. With current trends in habitat degradation, it is essential that more research is conducted to determine whether the hybrids lineages are as greatly affected as the parental species are, in order to inform conservation efforts.

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