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Population genetics of wood frogs (Lithobates sylvaticus) in a forested ridge-top wetland ecosystem

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POPULATION GENETICS OF WOOD FROGS (*Lithobates sylvaticus*) IN A FORESTED RIDGE-TOP WETLAND ECOSYSTEM

By

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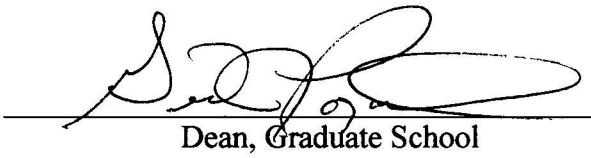

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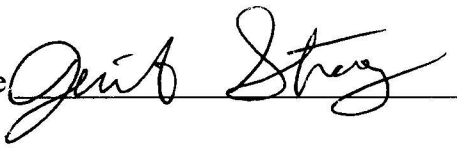
Signature 

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ABSTRACT

An important aspect of conservation biology is understanding how land-use changes impact biodiversity. Ridge-top wetlands are unique habitat for pond-breeding amphibians and the Daniel Boone National Forest (DBNF) contains natural forested ridge-top wetlands in close proximity to constructed wetlands intermixed across the same landscape. Genetic data can be used to address current population status, probability of persistence, and population connectivity. The objective of this study was to determine the amount and distribution of population genetic diversity of wood frogs in natural ridge-top wetlands and what factors influence this. Genetic data were analyzed for nine microsatellite DNA loci from twenty-five wood frog egg clutches at each of five randomly selected natural wetlands. Overall, genetic variation was measured by calculating observed heterozygosity (0.250–0.960), expected heterozygosity (0.270–0.913), and mean allelic richness (8.83–11.95). The results from program STRUCTURE gave support for 3 genetic clusters, and overall F_{ST} was 0.054 ± 0.022 SE among populations. Three populations exhibited signs of a recent population bottleneck event within populations. Pairwise F_{ST} and D_{ST} values were correlated, with D_{ST} indicating slightly higher population divergence. Isolation by distance was significant ($P = 0.0354$; $R^2 = 0.445$), indicating that geographic distance between the wetlands was an important factor explaining genetic differentiation. Future work should focus on expanding the sampling to a larger scale and sampling both natural and constructed wetlands between the sites to understand more fully how the genetic variation is partitioned across the landscape. For example, wood frogs have been observed breeding in both constructed and natural wetlands, which may demonstrate source/sink dynamics, and predation on wood

frog eggs in constructed wetlands may decrease overall wood frog genetic diversity over time.

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CHAPTER 1

I. INTRODUCTION

An important aspect of conservation biology is understanding how land-use changes impact biodiversity (Schwenk and Donovan 2011; Richardson 2012). Patch size affects population size and distribution, and the degree of population connectivity among fragmented suitable habitats across the landscape is driven by types of land use between patches (Ricketts 2001; Cushman 2006; Cushman et al. 2006). Additionally, connectivity and response to changes in the landscape will vary among species because of their different life histories and abilities to disperse across various land-use types (Gibbs 1998; Graeter et al. 2008; Richardson 2012). Types of biodiversity include ecosystem diversity, functional diversity, species diversity, and genetic diversity.

One type of biodiversity, genetic diversity, aids in sustaining viable populations and, therefore, regional species diversity (Wake and Vredenburg 2008), and studies focusing on the distribution of genetic diversity provide estimates of effective migration and gene flow (Julian et al. 2003). Genetic diversity can be defined as a measure of genetic variability within a population, such as the number of alleles per locus (Hughes et al. 2008; Scherer et al. 2012). Impacts to genetic diversity include habitat fragmentation, genetic drift, and population age and size (Scherer et al. 2012).

Wetland systems are important for maintaining regional biodiversity and provide key habitat for pond-breeding amphibians (Curado et al. 2011; Brown and Richter 2012). The terrestrial upland habitat surrounding wetlands is equally important for amphibian biodiversity through protection of water resources and habitat for life-history functions, including feeding, overwintering, and juvenile dispersal (Guerry and Hunter 2002;

Semlitsch and Bodie 2003; Cushman 2006). One type of wetland, forested ridge-top wetlands, is found across the eastern part of the state and is a primary amphibian breeding habitat (Brown and Richter 2012). Ridge-top wetlands are unique because of their geographic isolation from other natural wetlands and streams, occurrence on flat terrain, and ephemeral hydrology, and they contribute to vital ecological and landscape services such as provide habitat for diverse flora and fauna and filter sediments from surface water (Brown and Richter 2012; Kirkman et al. 2012). Ephemeral ridge-top wetlands in eastern Kentucky support an amphibian community of twelve species including some specialists of ephemeral wetlands: marbled salamanders (*Ambystoma opacum*), four-toed salamanders (*Hemidactylium scutatum*), wood frogs (*Lithobates sylvaticus*), and eastern spadefoots (*Scaphiopus holbrookii*) (Denton and Richter 2013; Drayer 2011).

Wetland loss and other habitat destruction and fragmentation have contributed to the recent decline in biodiversity, especially of amphibians because of their need for a moist environment and small body size (Gibbs 1998; Hayes et al. 2010; Buck et al. 2011; Curado et al. 2011). In the United States, most natural wetlands have been lost or degraded; Kentucky has lost more than 80% of its historic wetlands (Dahl 2000; Brown and Richter 2012). Because anthropogenic modifications occur over shorter time frames than natural processes, they can lead to rapid population subdivision and reduced connectivity (Crosby et al. 2009; Scherer et al. 2012). Therefore, when wetlands or the surrounding habitat become degraded and fragmented, genetic diversity can be severely reduced (Andersen et al. 2004; Cushman et al. 2006; Greenwald et al. 2008; Richter et al. 2009; Rivera-Ortíz et al. 2014).

Genetic data can be used to address current population status (i.e. health, viability) and probability of persistence (Cosentino et al. 2011). Additionally, genetic data are useful in identifying population connectivity as it relates to landscape heterogeneity or geographic distance (Cushman 2006). Landscape features can influence population structure by facilitating or limiting individual dispersal (Crosby et al. 2009). Thus, suitable habitat, such as wetland density, can influence genetic patterns and connectivity across a landscape (Scribner et al. 2001). Highly variable genetic markers are required to detect fine-scale dispersal patterns, dynamics of metapopulations, interspecific interactions, and distribution of genetic variation (Newman and Squire 2001; Jehle and Arntzen 2002). One example of such markers is microsatellite DNA, which is short repeats of nucleotide sequences in non-coding regions of eukaryote DNA that differ among individuals in the number of repeats (Jehle and Arntzen 2002).

In the Daniel Boone National Forest (DBNF) wetland system, the number and distribution of natural wetlands provides an ideal situation to address how genetic diversity is distributed across the landscape and what factors influence this. The DBNF contains natural forested ridge-top wetlands, which have temporary hydrology, and constructed wetlands, most of which have permanent hydrology, that are intermixed across the same landscape (Brown and Richter 2012). Wood frogs (*Lithobates sylvaticus*) are good representative species of the natural wetland community because they breed in temporary wetlands, have low to no reproductive success in constructed wetlands, and are widely distributed throughout the DBNF and eastern North America (Berven 1990; Newman and Squire 2001; Drayer 2011; Kross 2014). Therefore, my study focused on wood frog populations in natural wetlands.

The objective of this study was to determine the amount and distribution of population genetic diversity of wood frogs in natural ridge-top wetlands and what factors influence this. I predicted there would be a direct relationship between genetic diversity and the number of clutches deposited per wetland and the number of natural and constructed wetlands in close proximity to each sampled population. I also predicted that there would be evidence of fine scale genetic structure and that isolation by distance would best explain genetic structure of *L. sylvaticus*.

CHAPTER 2

II. MATERIALS AND METHODS

Study Species

Wood frogs are widely distributed throughout eastern North America from the southern Appalachians to the Arctic Circle, and reach as far west as Colorado (Redmer and Trauth 2005). Females mate with only one male, and males mate with multiple females if there is an opportunity (Berven 1981; Howard and Kluge 1985). Wood frogs typically breed in temporary pools in early spring with an egg-laying period of around one week and an average clutch size of 600 to 1,000 eggs, which hatch after four days to four weeks (Harding 1997; Berven 1988). Wood frogs have a larval period between 73 and 113 days, and sexual maturity is reached one to two years following metamorphosis (Berven 1990; Newman and Squire 2001).

Larvae develop in ponds and then metamorphose and disperse into the uplands (Redmer and Trauth 2005). Post-metamorphic wood frogs disperse in late spring and summer from the ponds and return to breed the next spring (Berven and Grudzien 1990). Juveniles are able to disperse as far as 2.5 km in a generation with a mean dispersal of 1.2 km (Berven and Grudzien 1990). Once they are sexually mature, some individuals return to their natal ponds, and others disperse to other breeding sites (Berven and Grudzien 1990; Squire and Newman 2002). Most adults return to breed in the same pond where they first bred, which suggests they have well-developed homing abilities (Berven 1982; Berven and Grudzien 1990).

Previous studies of wood frog dispersal found ponds within a fine-scale radius (1,000 m) did not exhibit genetic differentiation (Berven and Grudzien 1990; Newman

and Squire 2001). Individuals can disperse over 200 m from a breeding pond, but may have restricted dispersal due to human land use such as roads, fields, or agriculture (Windmiller 1996; Homan et al. 2004). Regosin et al. (2005) found adult wood frogs tend to move toward breeding ponds during the fall and remain close to the ponds through the winter until they breed in the late winter to early spring.

Possible predators of larval *L. sylvaticus* include *N. viridescens*, insects, and spotted salamanders (*A. maculatum*) (Berven 1982). Adult wood frogs feed on invertebrates while larval diet includes algae, diatoms, decaying plant matter, and eggs and larvae of other amphibians (Harding 1997). Post-metamorphic wood frogs have an equal likelihood of mortality during the rest of their life span following a Type II survivorship curve (Berven 1990). *Lithobates sylvaticus* have an average life span of four to five years (Redmer and Trauth 2005).

Site Selection and Wetland Sampling

Many natural forested ridge-top wetlands exist in the DBNF, and five were randomly selected for the study within the Cumberland Plateau in Jackson County, Kentucky from 2011 to 2013 (Figure 1[1]). Natural wetlands were randomly selected from areas where the wetland was at least 1 km from a constructed wetland. From each wetland, one egg from each of twenty-five *L. sylvaticus* egg clutches were collected and stored in 95% ethanol. Eggs were collected instead of larvae to decrease the risk of sampling closely related individuals.

[1] All Figures are located in Appendix B.

Genetic Data Collection

DNA was extracted from each tissue sample using QIAGEN DNEasy tissue protocol. Twelve loci (C11, C23, D25, D32, D40, C41, C52, C63, D70, D77, C83, and D88) were used for *L. sylvaticus* following the protocol of Julian and King (2003). DNA was amplified using polymerase chain reaction (PCR), and 3 loci were pooled per sample for genotyping using an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, CA). Allele lengths were scored using GeneMapper v. 3.0 (Applied Biosystems, Inc., Foster City, CA).

Three-primer PCR protocol was used to fluorescently label PCR products with FAM, HEX, and NED dyes. Total volume was 50ul with 10ul of DNA. An initial denaturation step of 2 min at 94°C was used followed by 38 cycles of 94°C for 45 s, 53°C for 45 s, and 72° for 1.5 min, which was followed by a final polymerization step of 72° for 2 min.

Genetic Analyses

Tests for departure from Hardy-Weinberg equilibrium (HWE) at each locus per population, linkage disequilibrium (LD) between all pairs of loci within each population, and calculation of allelic richness using rarefaction was performed using FSTAT v 2.9.3 (Goudet 1995). Null allele frequency was estimated with Micro-Checker v 2.2.3 (Van Oosterhout et al. 2004), and loci with >15% null allele frequency for the majority of the populations were removed (Richardson 2012). The loci with a null allele frequency of 15% or higher in only one or two wetlands were retained in analyses because when removed, they did not change the results of analyses. Observed heterozygosity (H_O),

expected heterozygosity (H_E), number of alleles, and Wright's inbreeding coefficient (F_{IS}) were calculated using GenAlEx 6.5b5 (Peakall and Smouse 2006). Additionally, F_{ST} for pairwise comparisons were calculated in GenAlEx. The significance of D_{ST} (Nei 1973) and overall Weir and Cockerham's (1984) estimator (θ) of Wright's F_{ST} was calculated in FSTAT v 2.93. D_{ST} detects genetic structure on a more historic landscape scale than does F_{ST} (Landguth et al. 2010). F_{ST} and other fixation indices are used to identify and quantify the degree of genetic differentiation among and within populations and assess the way genetic variation is distributed in natural populations. This is similar to an analysis of molecular variation (AMOVA), which uses a matrix of genetic distance among individuals that is subdivided by population samples (Bird et al. 2011).

Genetic isolation by distance (i.e., relationship between Euclidean distance and genetic distance) was tested using a Mantel test with 10,000 permutations in FSTAT v 2.9.3 (Mantle 1967; Goudet 1995). Euclidean distance was measured without consideration of surrounding habitat or geographic barriers. Additionally, regression analyses were performed in SPSS v 16.0 22 (IBM Corporation, Armonk, NY) to determine if wood frog clutch size or number of wetlands in a 1,000-m buffer explained the variance in genetic diversity measured by allelic richness, observed heterozygosity, and expected heterozygosity. Prior to analyses, data transformations were performed taking the square root of the allelic richness and the arcsin square root of the observed heterozygosity and expected heterozygosity.

Evidence of a recent bottleneck was tested by looking for significant heterozygosity deficiency using the software BOTTLENECK based on 5,000 replications using all three models of mutation (infinite alleles model, stepwise mutation model, and

the two-phase mutation model) (Cornuet and Luikart 1996; Luikart and Cornuet 1998). The TPM was used with 95% single-step mutations and a variance among multiple steps of 12% (Piry et al. 1999). Significance was assessed using the Wilcoxon's test. Allelic frequency distributions were also assessed using the mode-shift indicator described by Luikart et al. (1998). The presence of an L-shaped frequency indicates a healthy population with a high proportion of low-frequency alleles present.

A Bayesian clustering approach in STRUCTURE v. 2.3.4 was used to determine the number of distinct genetic groups (K) and to assign individuals to groups using an admixture model (Pritchard et al. 2000). The program STRUCTURE uses genotypic data and a model-based clustering approach to infer population structure. Models assume there are K populations characterized by a set of allele frequencies at each locus and individuals are assigned to populations (Pritchard et al. 2000). STRUCTURE analysis was carried out with a burn-in of 200,000 Markov chain Monte Carlo (MCMC) iterations followed by 500,000 iterations. For each value of K, five replications were performed and the value of K that best fit the data was determined by averaging the five replicates. Two separate analyses were performed with consideration of prior location and without prior location. Delta K, the average log likelihood of data, and the value of K were estimated in STRUCTURE HARVESTER (Evanno 2005; Earl and vonHolt 2011) and assignment of individuals to genetic clusters were visualized using plots in STRUCTURE.

CHAPTER 3

III. RESULTS

Microsatellite Diversity and HWE

An average of one hundred and ten wood frogs from five localities were genotyped at 12 polymorphic microsatellite loci (Table 1[2]). Micro-Checker estimated null allele frequency at >15% for the majority of the populations at three loci (C11, C23, and D40); thus the three loci were removed from further analyses. For the remaining nine loci, a total of 181 alleles were observed with an average of 20.1 alleles per locus (range 6–32) (Table 1). After rarefaction, allelic richness was highest at the RF wetland site and lowest at SG (Table 2). Observed heterozygosity (H_O) and expected heterozygosity (H_E) among loci was variable: 0.250–0.960 and 0.270–0.913, respectively (Table 2) (Figure 2). Five loci were out of Hardy-Weinberg equilibrium in three of the five populations (Table 2). Deviations from Hardy-Weinberg expectations could be explained by insufficient sample size, substructuring such as a Wahlund effect, inbreeding, or presence of null alleles. No evidence of linkage disequilibrium was observed across all pairs of loci.

Evidence was found from a Wilcoxon's test in BOTTLENECK through the S.M.M. model to suggest there was a recent bottleneck event in D30 (P = 0.002), LP (P = 0.014), and RF (P = 0.006) (Table 3) because of a heterozygote deficiency. The mode

[2] All Tables are located in Appendix A.

shift test indicated low-frequency alleles were present in all populations, indicated by an L-shaped frequency (Figure 3).

Population Differentiation

Estimates of overall F_{ST} indicated significant levels of genetic differentiation among populations (F_{ST} = 0.054 ± 0.022; 95% CI = 0.017–0.096). Pairwise F_{ST} ranged from 0.020 to 0.053 (Table 3). Pairwise F_{ST} and D_{ST} values showed a similar pattern, with D_{ST} indicating slightly higher population divergence, ranging from -0.004 to 0.126 (Table 4). The Mantel test for IBD showed there was a significant positive relationship between pairwise F_{ST} and geographical distance (P = 0.035). Percent of the variance of F_{ST} explained by geographic distance was 44% (Figures 4).

There was no significant relationship between number of clutches deposited in each wetland and genetic variability measured by allelic richness (F = 0.006, df = 4, P = 0.945), expected heterozygosity (F = 0.058, df = 4, P = 0.825), and observed heterozygosity (F = 0.77, df = 4, P = 0.799) (Table 5). Additionally, there was no significant difference between the number of wetlands within a 1,000-m buffer and genetic diversity measured by allelic richness (F = 0.308, df = 4, P = 0.618), expected heterozygosity (F = 0.395, df = 4, P = 0.574), and observed heterozygosity (F = 0.460, df = 4, P = 0.546) (Table 5). Wetland size was not used in statistical models because it was correlated to number of clutches size (Pearson r = 0.713).

Under the admixture model, STRUCTURE determined the mean log probability of the data was greatest for K = 3, with three distinct groups of *L. sylvaticus* (Table 6). Both models, with sampling location included as a prior and without, gave similar results

and values did not change greatly when sampling location was included as a prior (Table 6; Figure 5). When results were visualized using STRUCTURE HARVESTER, more migrants were indicated based on the model without prior sampling location (Figure 5).

CHAPTER 4

IV. DISCUSSION

The results of my study indicated that genetic diversity measured by allelic richness, observed heterozygosity, and expected heterozygosity is similar to other wood frog populations. However, there was evidence of a recent bottleneck event in three of the five populations. There is weak genetic structure among *L. sylvaticus* populations and three distinct genetic groups, suggesting habitat fragmentation or landscape features are affecting the patterns of genetic variation.

Population Genetic Diversity

Overall, populations had similar genetic variation; however, the SG population had a slightly lower mean allelic richness value (8.83) and RF had a slightly higher value (11.95) than the other three populations (Table 2). Genetic diversity was similar to that of other studies. The mean allelic richness (8.83–11.95) is slightly higher compared to Peterman et al. (2013), who found a range of allelic richness from 4.25–5.5, and Crosby et al. (2009), who found a range of 5.88–10.98. However, mean observed heterozygosity (0.581–0.719) and expected heterozygosity (0.736–0.780) is similar to Crosby et al. (2009): observed heterozygosity (0.661–0.798) and expected heterozygosity (0.633–0.800) and Peterman et al. (2013): observed heterozygosity (0.66 ± 0.10).

The DBNF contains natural forested ridge-top wetlands and constructed wetlands intermixed across the same landscape because the initial purpose of constructing wetlands was to provide permanent water sources for game wildlife in an ecosystem in which natural wetlands dry during the summer (Brown and Richter 2012). Genetic

diversity is still relatively high despite the number of constructed wetlands in close proximity to natural wetlands. This may mean there is little adverse effect on genetic diversity in wood frogs that breed in natural wetlands that are in close proximity to constructed wetlands that contain predators such as eastern newts (*Notophthalmus viridescens*) that predate on the eggs laid there (Kross 2014). However, because the constructed wetlands have been introduced in the relatively recent past (most of them within a 30-year period), the effect on wood frog genetic diversity may be too recent to detect.

There was evidence of a recent bottleneck event in three of the five populations. When the constructed wetlands were placed in close proximity to natural wetlands, individuals might have begun breeding in the constructed wetlands, which would act to subdivide the historically larger population. The natural wetlands may be acting as population sources, and extinction and recolonization dynamics may play an important role in the system. Habitat loss and fragmentation can cause a decrease in allelic richness and cause population bottlenecks (Rivera-Ortíz et al. 2014). For example, Scherer et al. (2012) found evidence of a recent bottleneck event in wood frog populations, which was explained by recent disturbances and fragmentation. However, other similar studies found no evidence of bottleneck events when they were predicted based on land-use change (Zellmer and Knowles 2009; Peterman et al. 2013). More sampling from wetlands in the area and analysis of historic land use is needed to fully understand why bottleneck events occurred.

Clutch size and number of wetlands within a 1,000-m buffer were not shown to explain the variation seen in genetic diversity. This is different than what was expected

because D30 and LP wetlands with the highest clutch size and most breeding pairs were predicted to have more genetic diversity than the other wetlands measured by allelic richness, observed heterozygosity, and expected heterozygosity. The number of wetlands within a 1,000-m buffer was used as a factor to explain genetic variation because increased wetland density was predicted to have a positive effect on genetic diversity, but no patterns were found.

Genetic Differentiation

The overall F_{ST} value (0.054) with a geographic scale of < 13 km (3.8–12.2 km between sites) can be compared to other similar studies of *L. sylvaticus* genetic structure. Some studies found little evidence of genetic structure in wood frog populations with distances between them of 50 m–20 km (Newman and Squire 2001; Squire and Newman 2002; and Julian and King 2003). Richardson (2012) found the overall F_{ST} value 0.016, which is lower than my study. This is most likely because sampling was concentrated along one ridge with a high density of wetlands compared to the less continuous landscape in the DBNF. Population differentiation, measured by pairwise F_{ST} values, in my study (0.020–0.053) were similar to that of other studies which suggests relatively high connectivity and little genetic structure (Gabrielsen et al. 2013). Zellmer and Knowles (2009) found a similar pairwise F_{ST} range of -0.008–0.087 with a larger scale range of around < 1–25 km. Peterman et al. (2013) had a pairwise F_{ST} range of 0.0001–0.071 and a scale range of 0.961–22.971 km. Genetic differentiation might be low in my study because the sites are distributed across a forested landscape and have many natural

wetlands located in close proximity, making a higher density of populations and more likely to share genes, have less genetic differentiation, and higher connectivity.

Although IBD was statistically supported, the genetic clustering results in STRUCTURE gave the most support for three genetic groups of wood frogs in the study system. Visualizations of the no prior location graph (Figure 5) gave support for recent migrants between D30 and LP populations. Although the two populations have a relatively large distance between them (Table 4), they are part of one distinct genetic group. This could be because there are wetlands interspersed between the two populations that allow for gene flow between the populations, and if sampled would show recent migrants from the two populations (Figure 7). Juveniles are the main dispersers of the species, and are able to travel up to 2.5 km, which may explain why there is low genetic structure between the five sites if gene flow occurs between wetlands over several generations. Because D30 and LP are part of one distinct genetic group, the wetlands between them could be sampled to determine if juveniles are facilitating gene flow, or historically the populations were more connected and have recently been fragmented. Additionally, D30 and LP are the largest natural wetlands in the study, and produced the highest number of egg clutches (Table 5) and may be acting as population sources for the surrounding wetlands. Populations SG and HK were shown to be one genetic group. This was expected because the wetlands are in close proximity to one another, and there is a large distance between the two wetlands and the other three natural wetlands (Figure 6). The RF population was a distinct genetic population. Although it is geographically close (6.1 km) to the wetland D30, there is urban development directly adjacent to RF between it and D30 (Figure 8). This could be preventing dispersal to the intervening wetlands.

Previous studies at the same geographic scale showed similar results. Scherer et al. (2012) found two distinct genetic groups of wood frogs in the Rocky Mountain National Park, Colorado. Over a larger scale, Richardson (2012) found three distinct genetic groups of wood frogs in Connecticut.

Conservation and Future Work

This research aids in the overall understanding of wood frog population genetics and specifically increases the knowledge of genetic diversity and structure of populations of wood frogs in the DBNF, Kentucky. No research has been conducted on population genetics of amphibians in the DBNF, and the data collected can be used as a reference to compare to future studies focusing on genetic structure and overall health of amphibian populations. In addition to genetic data, wetland size and wood frog clutch size can be used to monitor future populations of wood frogs in the system.

Future work in the DBNF should include looking at the relationship of constructed wetlands and natural wetlands. Because constructed wetlands have been built in close proximity to natural wetland and support a different community (Denton and Richter 2013; Drayer 2011), there are potential interactions between the constructed and natural species. The outcome of these interactions could include disease transfer or predation of the natural species such as wood frogs by species in the constructed wetlands (Richter et al. 2013; Kross 2014), which could affect population structure and genetic diversity. Constructed wetlands may be acting as population sinks, and natural wetlands acting as sources. In addition, more wetlands should be studied across a larger scale to

determine if there is a relationship between the density and distribution of natural and constructed wetlands and population genetic diversity of wood frogs.

Habitat fragmentation can influence long-term persistence of amphibian populations (Cushman 2006), and should be taken into consideration in any future studies. In the future, landscape features such as distance, roads, topography, aspect, and land cover should be analyzed to see if there is a relationship to genetic structure. Landscape genetics focuses on geographic and environmental features to explain population structure (Crosby et al. 2009). It is important for conservation biology to understand how habitat alteration affects populations so successful management strategies can be implemented and preserve the remaining amphibian species.

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APPENDIX A:

List of Tables

Table 1. Primer information for twelve *Lithobates sylvaticus* microsatellite DNA loci. Primers were developed by Julian and King (2003).

Locus	Repeat motif	Size range (bp)	No. of alleles	*n*
*Rsy*D25	$(TAGA)_{18}$	124–200	16	110
*Rsy*D32	$(TAGA)_{11}$	148–232	17	110
*Rsy*C41	$(TACA)_{8}$	104–160	18	109
*Rsy*C52	$(TACA)_{17}$	129–217	23	117
*Rsy*C63	$(TACA)_{12}$	145–237	21	115
*Rsy*D70	$(TAGA)_{17}$	140–340	27	116
RsyD77	$(TAGA)_{15}$	165–241	32	115
*Rsy*C83	$(TACA)_{10}$	115–147	6	113
*Rsy*D88	$(TAGA)_{13}$	114–226	21	112

Table 2. Observed (H_O) and expected (H_E) heterozygosity, F_{IS}, and D_{ST} for each locus and mean allelic richness for each *Lithobates sylvaticus* population in the Daniel Boone National Forest, Kentucky. Loci out of HWE within populations are shown in bold.

	D30		LP		HK		SG		RF				
n	0		0		0		0		0				
Mean allelic richness	10.26		10.82		10.05		8.83		11.95				
SE	1.17		1.22		1.38		0.99		1.31				
Locus	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	*n*	F_{IS}	D_{ST}
*Rsy*D25	0.565	0.675	**0.500**	**0.770**	0.636	0.773	0.591	0.777	**0.348**	**0.733**	20-23	0.292	-0.004
*Rsy*D32	0.727	0.786	0.773	0.869	0.727	0.884	0.783	0.871	0.857	0.764	21-23	0.074	0.021
*Rsy*C41	0.739	0.868	**0.450**	**0.851**	0.909	0.871	0.739	0.827	0.857	0.907	20-23	0.146	-0.001
*Rsy*C52	0.636	0.794	0.680	0.879	0.682	0.913	0.640	0.777	**0.391**	**0.892**	22-25	0.288	0.043
*Rsy*C63	**0.682**	**0.892**	0.762	0.889	0.833	0.911	0.625	0.867	**0.500**	**0.906**	21-24	0.238	0.000
*Rsy*D70	**0.625**	**0.844**	**0.619**	**0.876**	0.609	0.436	0.538	0.476	0.682	0.876	21-26	0.124	0.126
RsyD77	0.960	0.758	0.762	0.891	0.826	0.776	0.636	0.787	0.750	0.869	21-25	0.036	0.059
*Rsy*C83	0.538	0.596	0.500	0.453	0.583	0.521	0.348	0.421	0.250	0.270	20-26	0.018	0.006
*Rsy*D88	0.550	0.641	0.826	0.543	0.667	0.868	0.739	0.821	0.591	0.773	20-24	0.075	0.068
Mean	0.669	0.761	0.652	0.780	0.719	0.772	0.627	0.736	0.581	0.777		0.143	0.035
SE	0.044	0.035	0.047	0.055	0.038	0.059	0.044	0.056	0.074	0.067		0.035	0.014

Table 3. Results from a Wilcoxon's test preformed using BOTTLENECK for five populations of *Lithobates sylvaticus* under three mutation models. Results shown are P-values based on 5000 replicates. I.A.M. = Infinite alleles model, T.P.M. = Two-phase mutation model, and S.M.M = Stepwise mutation model

Population	I.A.M.	T.P.M.	S.M.M.
D30	1.0000	0.0039	0.0020
LP	0.4961	0.0273	0.0137
HK	0.0273	0.7344	0.5703
SG	0.4961	0.1641	0.1641
RF	0.4961	0.0195	0.0059

Table 4. Genetic distance values for Lithobates sylvaticus populations in the Daniel Boone National Forest, Kentucky. Pairwise F_{ST} values are reported below the diagonal, and geographic distance (km) values are above diagonal.

	D30	LP	HK	SG	RF
D30	-	11.108	7.627	7.162	6.077
LP	0.033	-	8.580	12.167	6.140
HK	0.045	0.045	-	3.845	8.449
SG	0.042	0.053	0.020	-	10.532
RF	0.032	0.036	0.050	0.043	-

Table 5. Wetland variables used to explain the variation of genetic variability.

Wetland	Clutch size	Wetlands within 1,000m buffer	Wetland size (m^2)
D30	636	5	785
LP	579	1	943
HK	45	2	597
SG	133	2	274
RF	52	1	628

Table 6. Evanno statistics including log likelihood and delta K for the detection of *Lithobates sylvaticus* populations using STRUCTURE v 2.3.4. In both the prior and non prior sampling runs, a K value of three was most supported

K	Mean LnP(K)	SD LnP(K)	Delta K
	No prior on Sampling Site		
1	-4679.560	0.631	–
2	-4608.840	24.268	3.112
3	-4462.600	3.752	112.904
4	-4739.940	114.274	3.138
5	-4658.680	118.462	–
	With prior on Sampling Site		
1	-4680.140	0.385	–
2	-4565.320	7.163	6.955
3	-4400.680	4.261	36.873
4	-4394.260	12.563	4.674
5	-4446.560	80.478	–

APPENDIX B:

List of Figures

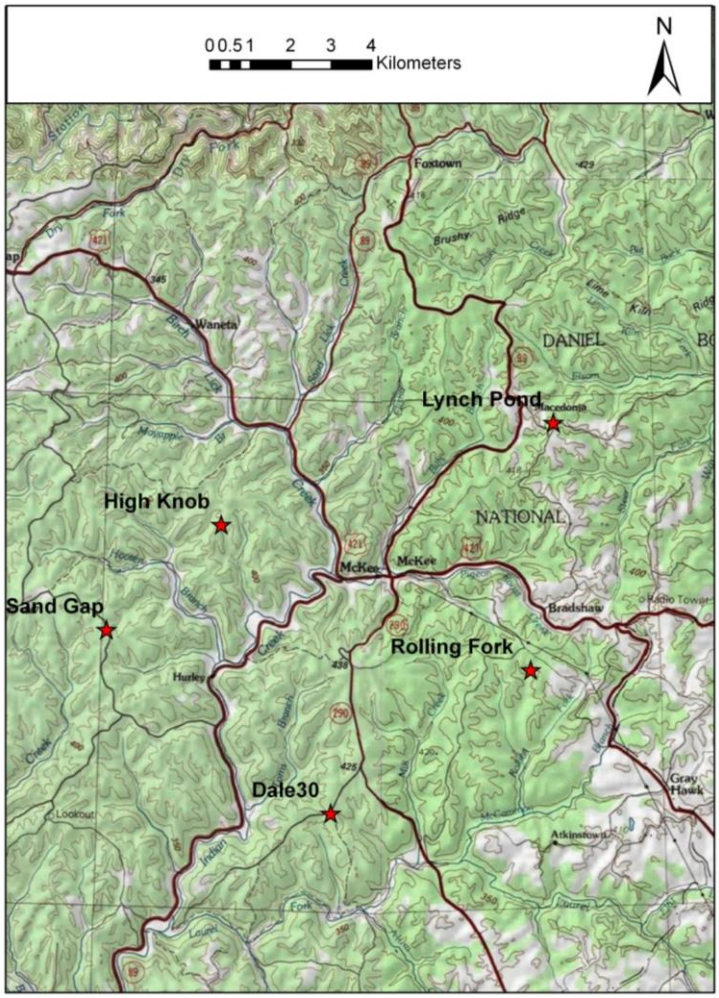


Figure 1. Topographic map of Jackson County, Kentucky with five natural wetlands indicated by stars.

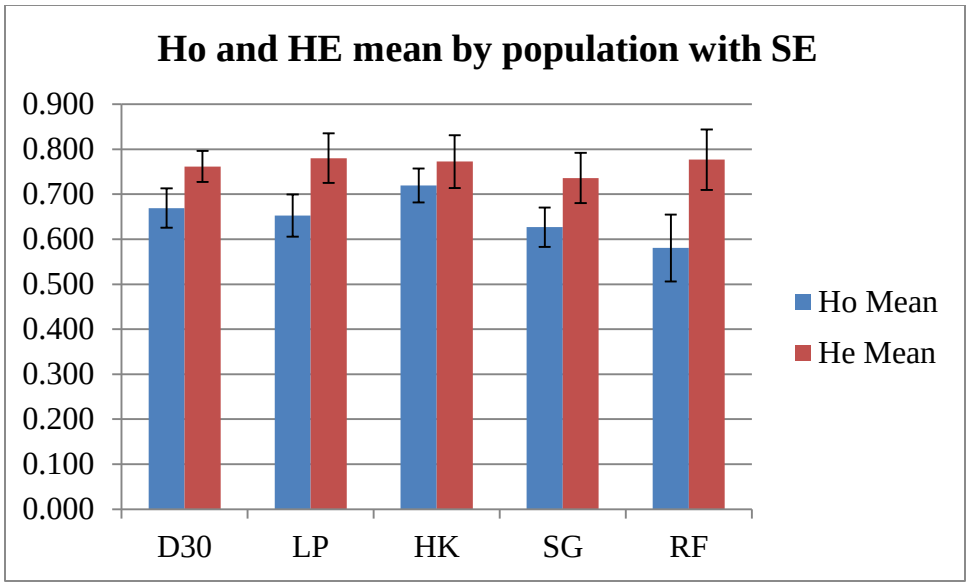
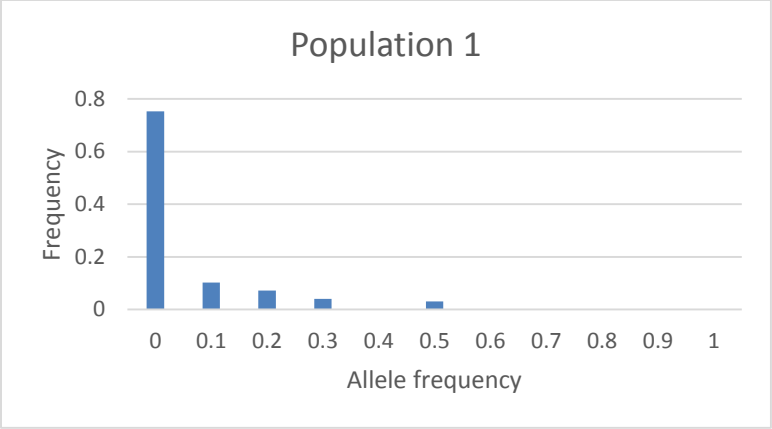
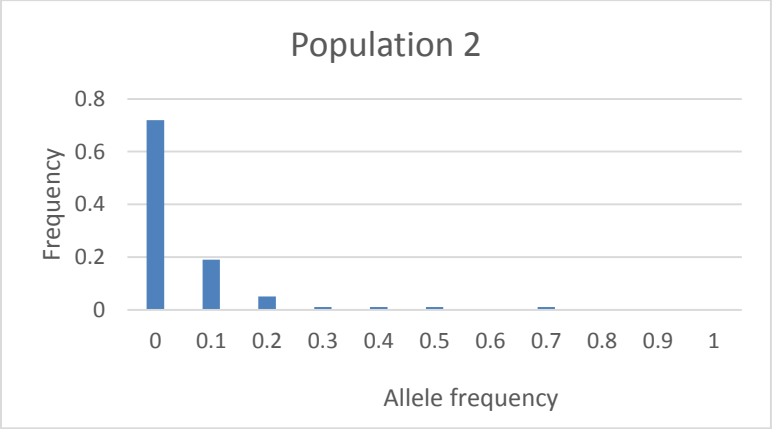


Figure 2. Mean (± 1 SE) observed heterozygosity and mean expected heterozygosity for five populations of *Lithobates sylvaticus* in the Daniel Boone National Forest, Kentucky.





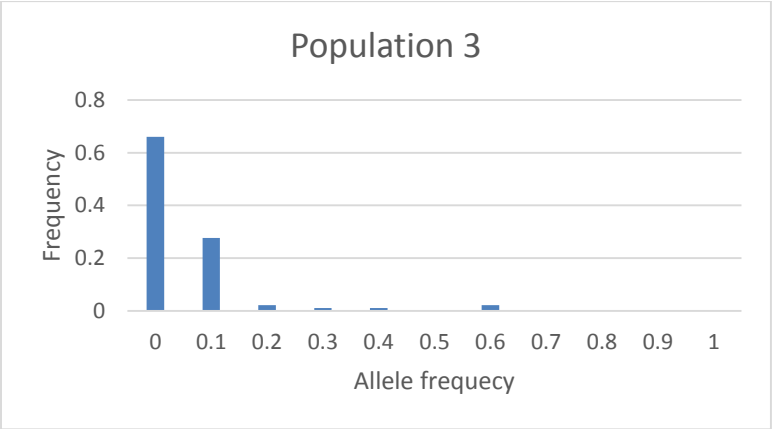
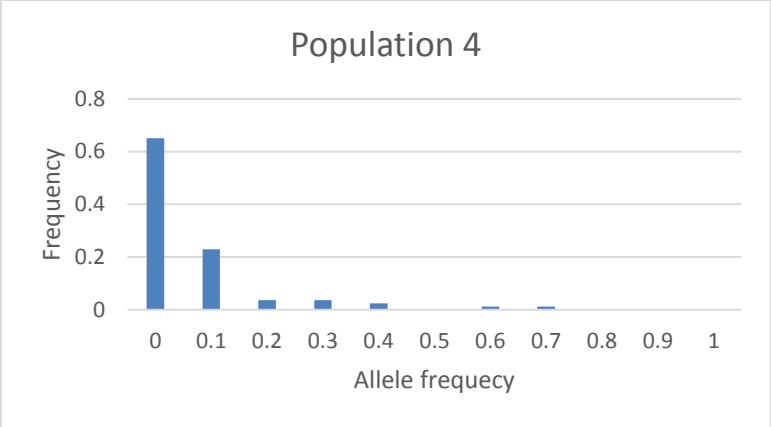


Figure 3. Histogram of allele frequency distribution for all genotyped loci by each of the five populations of *Lithobates sylvaticus* in the Daniel Boone National Forest, Kentucky. Allele frequencies are grouped into 0.1 class intervals along the x-axis.



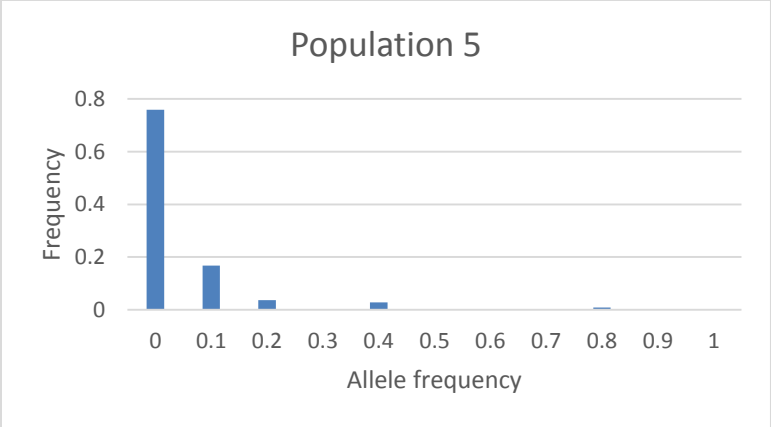


Figure 3 (continued). Histogram of allele frequency distribution for all genotyped loci by each of the five populations of *Lithobates sylvaticus* in the Daniel Boone National Forest, Kentucky. Allele frequencies are grouped into 0.1 class intervals along the x-axis.

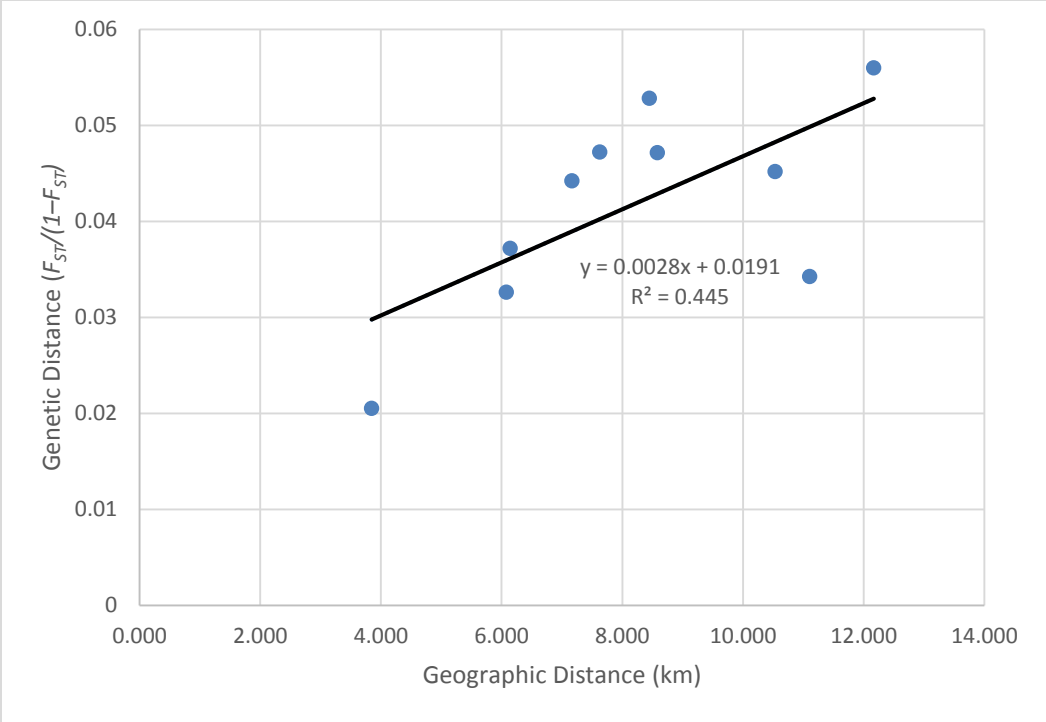


Figure 4. Genetic distance ($F_{ST}/(1–F_{ST})$ plotted against geographic distance (km) for all sampling sites in the Daniel Boone National Forest, Kentucky. The solid line represents the best-fit linear regression and R^2 value.

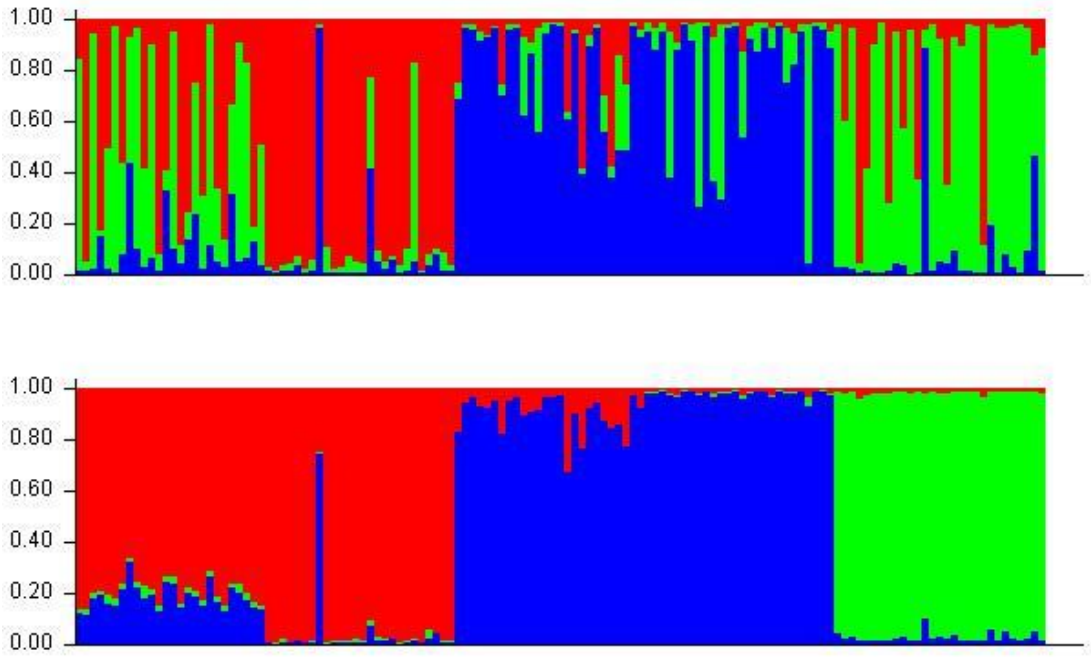


Figure 5. Visualizations of *Lithobates sylvaticus* individuals using STRUCTURE HARVESTER of the most supported value of K=3. The top graph shows individuals assigned to groups without prior location knowledge, and the bottom graph shows individuals assigned to groups with prior location.

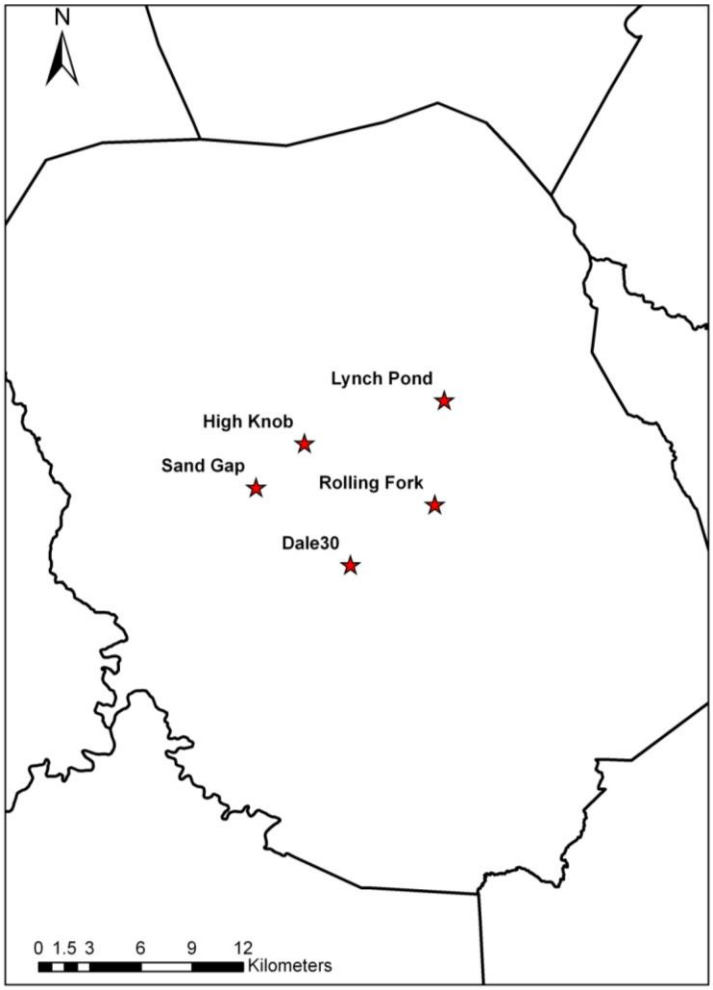


Figure 6. Map of Jackson County, Kentucky. Natural wetlands are labeled as stars Dale30 (D30), Lynch Pond (LP), High Knob (HK), Sand Gap (SG), and Rolling Fork (RF).

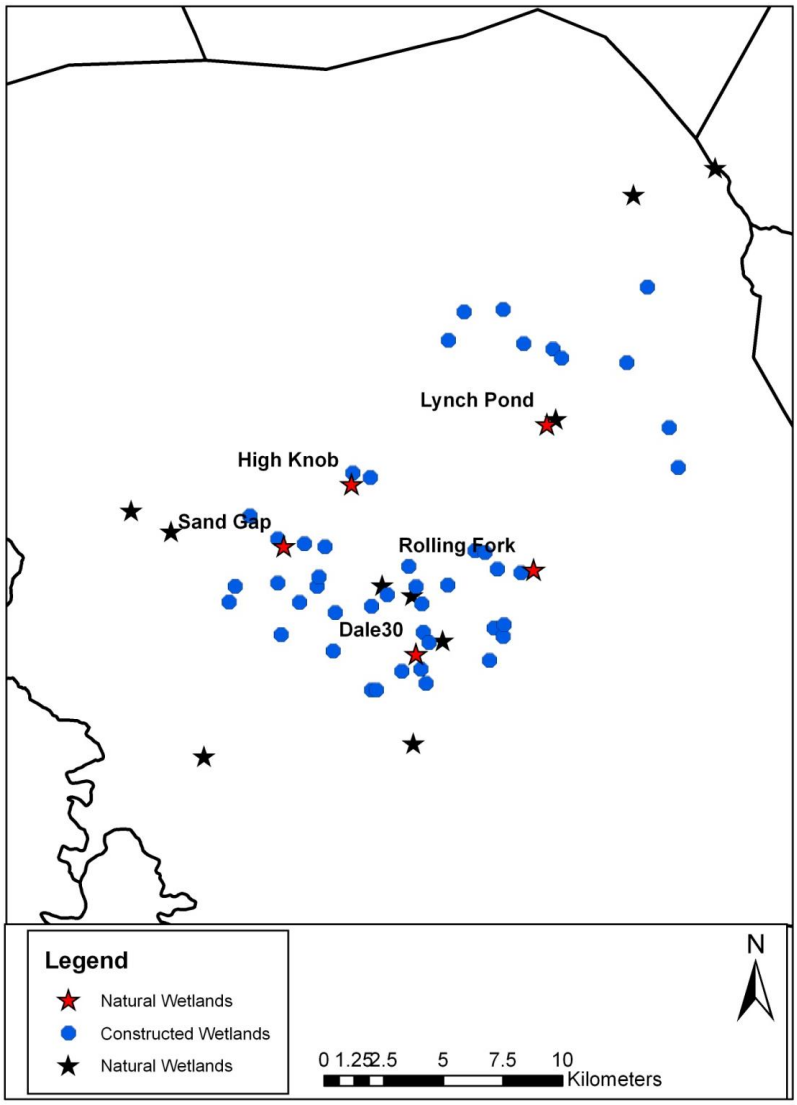


Figure 7. Map of Jackson County, KY. Natural wetlands used in my study are labeled with red stars, other natural wetlands are labeled as black stars, and constructed wetlands are labeled as blue circles.

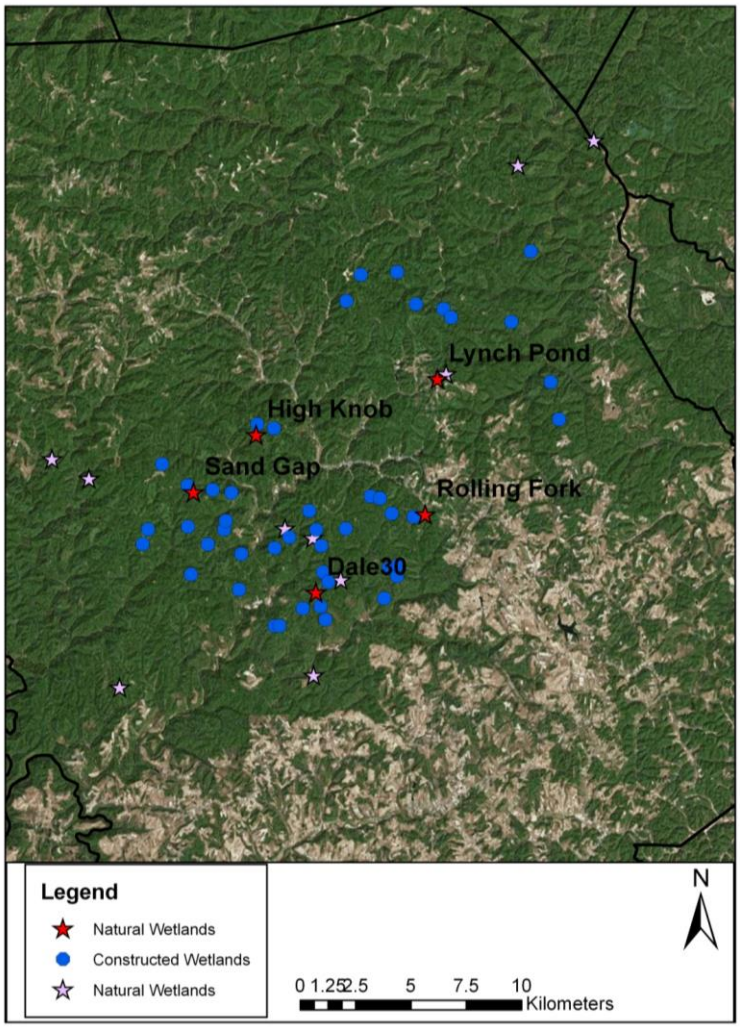


Figure 8. Aerial view of Jackson County, KY. Natural wetlands used in my study are labeled as red stars, other natural wetlands are labeled as black stars, and constructed wetlands are labeled with blue circles.