
POPULATION GENETICS OF WOOD FROGS (*LITHOBATES SYLVATICUS*) IN AN ALTERED FORESTED RIDGETOP WETLAND ECOSYSTEM IN APPALACHIA

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Abstract.—Natural wetlands are important for maintaining regional amphibian biodiversity and their loss and degradation are a major cause of amphibian population declines. Population genetic data can be useful to conservation efforts by providing information on genetic variation, effective population size, and spatiotemporal patterns of gene flow. On the Cumberland Plateau in Daniel Boone National Forest, Kentucky, USA, ridgetops contain natural and constructed wetlands interspersed on the landscape. Our study objective was to determine population genetic diversity and structure of Wood Frogs (*Lithobates sylvaticus*) across this altered ecosystem and to evaluate what local and landscape factors influence patterns of genetic variation. We analyzed genetic data for nine microsatellite DNA loci from 20–26 egg clutches at five randomly selected natural wetlands across a 12.2-km landscape. Overall, we found considerable variability in genetic profiles of populations: observed heterozygosity = 0.581–0.719, expected heterozygosity = 0.736–0.780, F_{IS} = 0.069–0.252, and allelic richness = 8.83–11.95. Three populations exhibited a signature of a population bottleneck. There was support for three genetic clusters, and overall F_{ST} was 0.054 ± 0.022 standard error. Geographic distance significantly correlated to genetic distance ($P < 0.05$) and explained 42% of the variation in genetic distance among populations. Our study provides insight into current status and conservation needs of Wood Frogs in ridgetop wetland systems. Despite the abundance of constructed wetlands and potential for population sinks, genetic diversity is still relatively high, although this might be due to the recency of constructed wetlands on the landscape and the inherent lag time of genetic response.

Key Words.—amphibian; conservation biology; genetic diversity; landscape genetics; management; microsatellite DNA

INTRODUCTION

Amphibians are in global decline and are more threatened than birds or mammals (Stuart et al. 2004). Habitat loss, degradation, and fragmentation are major causes of amphibian declines because many amphibians need moist environments, are of small body size, have limited mobility, and tend to return to the same locations to breed (Gibbs 1998; Arens et al. 2006; Hayes et al. 2010; Buck et al. 2012). Wetland systems are important for maintaining regional amphibian biodiversity and provide key habitat for pond-breeding species (Curado et al. 2011; Brown and Richter 2012). The terrestrial upland habitat surrounding wetlands is equally important for maintaining amphibian biodiversity because it protects water resources and provides resources for life-history functions, including feeding, overwintering, and juvenile dispersal (Guerry and Hunter 2002; Semlitsch and Bodie 2003; Cushman 2006; Richter et al. 2013b). Unfortunately, most natural wetlands in the U.S. have been lost or degraded; for example, Kentucky has lost more than 80% of its historic wetlands (Dahl 2000; Richter et al. 2017).

In eastern Kentucky, forested ridgetop wetlands are a primary amphibian breeding habitat (Brown and Richter 2012), supporting an assemblage of 12 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 and Richter 2016). Forested ridgetop wetlands are characterized by their geographic isolation from other natural wetlands and streams, occurrence on flat terrain, and ephemeral hydrology. Ridgetop wetlands also contribute vital ecological and landscape services, such as providing habitat for diverse flora and fauna as well as filtering sediments from surface water (Brown and Richter 2012; Kirkman et al. 2012; Creed et al. 2017). The Daniel Boone National Forest (DBNF) wetland system in eastern Kentucky contains natural forested ridgetop wetlands and constructed wetlands intermixed on the landscape (Brown and Richter 2012). Because constructed wetlands have a permanent hydrology, they may be population sinks for species adapted to the temporary hydrology of natural wetlands,

and thus, extinction and recolonization dynamics may play an important role in the system.

Landscape features can affect populations by facilitating or limiting individual dispersal (Crosby et al. 2009), which can impact population dynamics and genetic structure (Clobert et al. 2001). Thus, the spatial configuration of suitable habitat can influence patterns of genetic variation and connectivity across a landscape (Scribner et al. 2001). The field of landscape genetics combines population genetic and landscape feature data to understand how landscape composition, configuration, and matrix quality affect spatial patterns of genetic variation and gene flow, and is especially useful for studying organisms, like amphibians, that are sensitive to environmental variation and habitat modification (Manel et al. 2003; Cushman 2006; Storfer et al. 2007).

The abundance and distribution of natural wetlands in DBNF provide an opportunity to examine how genetic diversity in wetland-breeding amphibian species is distributed across the landscape and what factors influence these patterns. Wood frogs are a representative species of the natural ridgetop wetland community in DBNF because they breed in temporary wetlands, have low to no reproductive success in constructed wetlands, and are widely distributed (Denton and Richter 2013; Drayer and Richter 2016; Kross and Richter 2016). Here we address the following questions: 1) What are levels of genetic diversity within Wood Frog populations and is there any evidence of recent bottlenecks as would be predicted if source-sink dynamics are occurring on the landscape? 2) Is there significant population differentiation and, if so, what are the spatial patterns of genetic structure in the ridgetop wetland system? 3) Do landscape features influence genetic variation and connectivity among populations?

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, USA (Redmer and Trauth 2005). It is important to consider aspects of the natural history of a species to help interpret observed genetic patterns. Wood Frogs are explosive breeders in late winter or early spring so females will mate with only one male per breeding season, whereas males may mate with multiple females if there is opportunity (Berven 1981; Howard and Kluge 1985). Larvae develop in ponds and metamorphose in late spring and summer. Post-metamorphic Wood Frogs then disperse from ponds to surrounding forested wetlands and upland forests as far as 2.5 km in a generation, with a mean dispersal of 1.2 km (Berven and Grudzien 1990; Redmer and

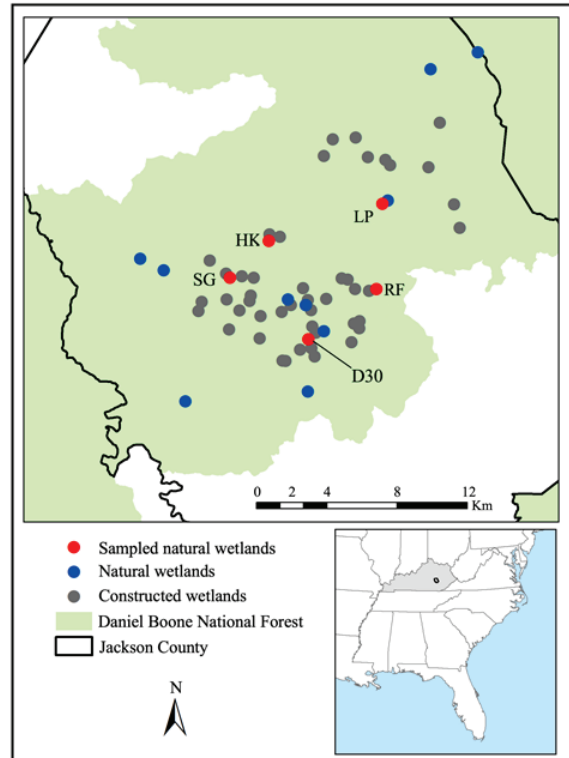


FIGURE 1. Map of a portion of Jackson County, Kentucky, USA. Features shown are county boundaries (black lines), Daniel Boone National Forest (green), natural wetlands included in the study (red circles), other natural wetlands (blue circles), and constructed wetlands (gray circles).

Trauth 2005; Baldwin et al. 2006a). The following spring, individuals return to breed in their natal ponds or 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 strong philopatry (Berven 1982; Berven and Grudzien 1990).

Site selection and wetland sampling.—We randomly selected five of the 15 known natural ridgetop wetlands in our study area within DBNF in Jackson County, Kentucky, USA (Fig. 1). During February–March of 2013, from each wetland, we collected one egg from 20–26 Wood Frog egg clutches ($n = 110$ total) and stored it in 95% ethanol. We collected eggs instead of larvae to avoid sampling full siblings, which would bias estimates of population genetic diversity.

Genetic data collection.—We extracted DNA from each egg sample using the QIAGEN DNEasy tissue protocol (QIAGEN, Valencia, California, USA). We genotyped 110 Wood Frogs from our five chosen wetlands at 12 polymorphic microsatellite loci following the protocol of Julian and King (2003; Tables 1 and 2). We amplified DNA using three-primer polymerase chain

TABLE 1. Mean allelic richness, F_{IS} , observed (H_o) and expected (H_e) heterozygosity, and variables used to explain the variation in genetic diversity for each Wood Frog (*Lithobates sylvaticus*) population in the Daniel Boone National Forest, Kentucky, USA. Values are presented \pm standard error (SE). The symbol # = number of.

	Population				
	D30	LP	HK	SG	RF
Mean allelic richness	10.26 \pm 1.17	10.82 \pm 1.22	10.05 \pm 1.38	8.83 \pm 0.99	11.95 \pm 1.31
F_{IS}	0.121	0.164	0.069	0.149	0.252
H_o	0.669 \pm 0.044	0.652 \pm 0.047	0.719 \pm 0.038	0.627 \pm 0.044	0.581 \pm 0.074
H_e	0.761 \pm 0.035	0.780 \pm 0.055	0.772 \pm 0.059	0.736 \pm 0.056	0.777 \pm 0.067
# Clutches	636	579	45	133	52
# Wetlands within 1,000-m	5	1	2	2	1
Wetland size (m ²)	785	943	597	274	628

reaction (PCR) to fluorescently label the PCR products with FAM, HEX, and NED dyes (Vartia et al. 2014). For all loci, we used an initial denaturation step of 2 min at 94° C followed by 38 cycles of 94° C for 45 s, 53° C for 45 s, and 72° C for 1.5 min, which was followed by a final polymerization step of 72° C for 2 min. Following PCR, we pooled three loci per sample (one of each dye) for genotyping using an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, California, USA). We scored allele lengths using GeneMapper 3.0 (Applied Biosystems, Inc., Foster City, California, USA).

Genetic analyses.—We used FSTAT 2.9.3 (Goudet 1995) to perform tests for departure from Hardy-Weinberg equilibrium at each locus per population, linkage disequilibrium between all pairs of loci within each population, and calculation of allelic richness using rarefaction. We used Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) to estimate null allele frequency and we examined loci with > 15% null allele frequency in one or more populations for potential removal from analyses (Richardson 2012). We calculated observed heterozygosity (H_o), expected heterozygosity (H_e), number of alleles (richness), and Wright’s inbreeding

coefficient (F_{IS}) using GenAIEx 6.5b5 (Peakall and Smouse 2006). We performed Linear Regression analyses in SPSS 16.0 (IBM Corporation, Armonk, New York, USA) to determine if the number of Wood Frog egg clutches or number of wetlands within a 1,000-m buffer explained the variation in genetic diversity of populations as measured by allelic richness, F_{IS} , H_o , and H_e . The number of clutches may be related to genetic diversity because it indicates how many breeding adults are at a wetland (Scherer et al. 2012). The number of nearby (< 1,000 m) wetlands may be related to genetic diversity because migration among sites in close proximity would maintain homogeneity, resulting in a larger effective population size (N_e ; Crosby et al. 2009). To meet the assumption of normality, we square-root transformed allelic richness and arcsin square-root transformed H_o , H_e , and F_{IS} prior to analyses.

We used two tests for recent population bottlenecks in the software BOTTLENECK (Cornuet and Luikart 1996; Luikart and Cornuet 1998). The first test examined data for significant heterozygote deficiency based on 5,000 replications and two models of mutation (Stepwise Mutation Model and the Two-phase Mutation Model [TPM]; Cornuet and Luikart 1996; Luikart

TABLE 2. Observed (H_o) and expected (H_e) heterozygosity for each locus from Wood Frogs (*Lithobates sylvaticus*) sampled in the Daniel Boone National Forest, Kentucky, USA. The number of individuals sampled from each population is presented as a range (n). Loci significantly ($P < 0.001$) out of Hardy-Weinberg equilibrium within populations are shown in bold. The abbreviation SE = standard error.

Locus	Population										n
	D30		LP		HK		SG		RF		
	H_o	H_e	H_o	H_e	H_o	H_e	H_o	H_e	H_o	H_e	
RsyD25	0.565	0.675	0.500	0.770	0.636	0.773	0.591	0.777	0.348	0.733	20–23
RsyD32	0.727	0.786	0.773	0.869	0.727	0.884	0.783	0.871	0.857	0.764	21–23
RsyC41	0.739	0.868	0.450	0.851	0.909	0.871	0.739	0.827	0.857	0.907	20–23
RsyC52	0.636	0.794	0.680	0.879	0.682	0.913	0.640	0.777	0.391	0.892	22–25
RsyC63	0.682	0.892	0.762	0.889	0.833	0.911	0.625	0.867	0.500	0.906	21–24
RsyD70	0.625	0.844	0.619	0.876	0.609	0.436	0.538	0.476	0.682	0.876	21–26
RsyD77	0.960	0.758	0.762	0.891	0.826	0.776	0.636	0.787	0.750	0.869	21–25
RsyC83	0.538	0.596	0.500	0.453	0.583	0.521	0.348	0.421	0.250	0.270	20–26
RsyD88	0.550	0.641	0.826	0.543	0.667	0.868	0.739	0.821	0.591	0.773	20–24
Mean	0.669	0.761	0.652	0.780	0.719	0.772	0.627	0.736	0.581	0.777	
SE	0.044	0.035	0.047	0.055	0.038	0.059	0.044	0.056	0.074	0.067	

and Cornuet 1998). We used TPM with 95% single-step mutations and a variance among multiple steps of 12% (Piry et al. 1999). We assessed significance with a one-tailed Wilcoxon Signed Rank Test, which is recommended for fewer than 20 loci and has a null hypothesis of no significant heterozygote excess, on average, across loci (Piry et al. 1999). In the second analysis, we examined allelic frequency distributions using the mode-shift indicator described by Luikart et al. (1998). Although this is not a statistical analysis, the presence of an L-shaped frequency distribution indicates a healthy population with a high proportion of low-frequency alleles present (Luikart et al. 1998).

To quantify the degree of genetic differentiation among and within populations, we calculated pairwise F_{ST} values in GenALEX and overall Weir and Cockerham's (1984) estimator (θ) of Wright's F_{ST} in FSTAT 2.93. Additionally, we used a Bayesian clustering approach in STRUCTURE 2.3.4 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). We carried out STRUCTURE analysis with a burn-in of 200,000 Markov chain Monte Carlo (MCMC) iterations followed by 500,000 iterations. We chose parameter values based on similar studies (Richardson 2012; Peterman et al. 2013a). We chose a value range of K of 1–5 to represent the five sites and for each value of K , we performed five replicates. We estimated ΔK , the average log-likelihood of data, and the best-fit value of K in STRUCTURE HARVESTER (Evanno et al. 2005; Earl and vonHolt 2011) and we visualized assignments of individuals to genetic clusters using plots in STRUCTURE PLOT (Ramasamy et al. 2014). We performed two separate analyses with consideration of prior location and without prior location.

Landscape connectivity analysis.—We performed a Supervised Landscape Classification with 30-m Landsat 8 data from June 2014 using ERDAS Imagine 2018 v16 (ERDAS, Atlanta, Georgia, USA) remote-sensing software with a parametric rule of maximum likelihood. We assigned pixels as forest, short vegetation, bare/built, or water. Using ArcMap 10.0 (Esri, Redlands, California, USA), we generated a raster in which we assigned each type of landcover a resistance value, based on the estimated difficulty a Wood Frog would have traversing that terrain using the high resistance values from Table A3 in Peterman et al. (2013a). We then used this raster to run a least-cost path analysis between each pair of our sampled wetlands and to generate a resistance matrix in Circuitscape 4.0 (Shah and McRae 2008) with

nodes at each of the five wetlands to act as both source and sink. Finally, we measured Euclidean distance between wetlands without consideration of surrounding habitat or geographic barriers. We ran Mantel tests in FSTAT 2.9.3 with 10,000 iterations to determine the strength of the correlation between genetic distance (F_{ST}) and each of the three models of dispersal: Least-cost Path Distance, Resistance Distance, and Euclidean Distance (i.e., isolation by distance). We then followed this with partial Mantel tests to evaluate the relationship between genetic distance (F_{ST}) and Least-cost Path and Resistance distances while controlling for Euclidean Distance (Mantel 1967; Goudet 1995).

RESULTS

Microsatellite diversity.—Micro-Checker estimated null allele frequency at > 15% in the majority of populations for three loci (C11, C23, and D40); thus, we removed these three loci from further analyses. For the remaining nine loci, we observed 181 alleles with an average of 20.1 alleles per locus (range, 6–32). After rarefaction, allelic richness was highest for population RF and lowest for SG (Table 1). Observed heterozygosity (H_o) and expected heterozygosity (H_e) of loci were variable: 0.250–0.960 and 0.270–0.913, respectively (Table 2). Five loci were out of Hardy-Weinberg equilibrium in at least one of the five populations and populations had between one and three loci out of equilibrium (Table 2). We observed no evidence of linkage disequilibrium across any pairs of loci.

There was no significant relationship between number of clutches deposited in a wetland and genetic variation as measured by allelic richness ($F_{1,4} = 0.006$, $P = 0.945$), H_o ($F_{1,4} = 0.077$, $P = 0.799$), H_e ($F_{1,4} = 0.058$, $P = 0.825$), and F_{IS} ($F_{1,4} = 0.022$, $P = 0.891$; Table 1). Additionally, there was no significant difference between the number of wetlands within a 1,000-m buffer and genetic diversity as measured by allelic richness ($F_{1,4} = 0.308$, $P = 0.618$), H_o ($F_{1,4} = 0.460$, $P = 0.546$), H_e ($F_{1,4} = 0.395$, $P = 0.574$), and F_{IS} ($F_{1,4} = 0.692$, $P = 0.466$; Table 1). We did not use wetland size (Table 1) in statistical models because it was correlated with number of clutches ($r = 0.713$). We found evidence for a recent bottleneck in populations D30, LP, and RF under both the Stepwise and Two-phase Mutation models ($P < 0.05$) because of heterozygote deficiency. The mode-shift test, however, did not support that bottlenecks occurred in any populations, as low-frequency alleles were present in all populations, resulting in an L-shaped frequency distribution.

Population differentiation.—Estimates of overall F_{ST} indicated significant levels of genetic differentiation among populations ($F_{ST} = 0.054 \pm 0.022$ [standard

TABLE 3. Genetic and geographic distances between Wood Frog (*Lithobates sylvaticus*) populations in the Daniel Boone National Forest, Kentucky, USA. Pairwise F_{ST} values are reported below the diagonal and Euclidean distances (km) are above the diagonal.

	D30	LP	HK	SG	RF
D30	-	11.11	7.627	7.162	6.077
LP	0.033	-	8.580	12.17	6.140
HK	0.045	0.045	-	3.845	8.449
SG	0.042	0.053	0.020	-	10.53
RF	0.032	0.036	0.050	0.043	-

error]; 95% confidence interval = 0.017–0.096). Pairwise F_{ST} ranged from 0.020 to 0.053 (Table 3). Under the admixture model, STRUCTURE determined the mean log probability of the data was greatest for $K = 3$, supporting three distinct populations of Wood Frogs. Both models, with sampling location included as a prior (mean $\text{LnP}(K) = -4,400.7$; $\Delta K = 36.9$) and without (mean $\text{LnP}(K) = -4,462.6$; $\Delta K = 112.9$), gave similar results and values did not change greatly when sampling location was included. When results are visualized, more migrants were indicated in the model without sampling location (Fig. 2).

Landscape connectivity.—The Euclidean distance and least-cost path distance were both significantly correlated with genetic distance ($P < 0.05$ for both), explaining 42% and 43% of the genetic distance between populations, respectively. Both resistance distance and genetic distance were not significantly correlated ($P = 0.155$), however. The positive relationship between Euclidian distance and genetic distance was strong ($r^2 = 0.445$; Fig. 3), and partial Mantel tests revealed that

least-cost path distance was no longer correlated with genetic distance after the effect of Euclidian distance was removed ($P = 0.780$).

DISCUSSION

Our results indicate that population genetic diversity, as measured by allelic richness and heterozygosity, was high and similar to other studies of Wood Frog populations across similar landscapes (Squire and Newman 2002; Crosby et al. 2009; Peterman et al. 2013a). Our mean allelic richness was higher than that of Crosby et al. (2009) and Peterman et al. (2013a), which could be due to differences in the loci used. The five study populations had similar levels of genetic variation; however, the SG population had a slightly lower mean allelic richness and RF had a slightly higher richness than the other three populations. This could be because RF has a larger wetland area (628 m²) compared to SG (274 m²). Larger areas are more likely to have higher colonization rates and less likely to be affected by local extinction events (Sherer et al. 2012). Deviations from Hardy-Weinberg expectations for a few loci in some populations could be explained by insufficient sample size, Wahlund effect (reduction of heterozygosity due to subpopulation structure), inbreeding, or presence of null alleles.

We expected wetlands with more breeding pairs would have higher genetic diversity, but we found no relationship between the number of egg clutches and genetic diversity. This could be because adults have high site fidelity and are not dispersing from their pond of origin (Berven and Grudzien 1990), leading to inbreeding. In support, we found moderately high

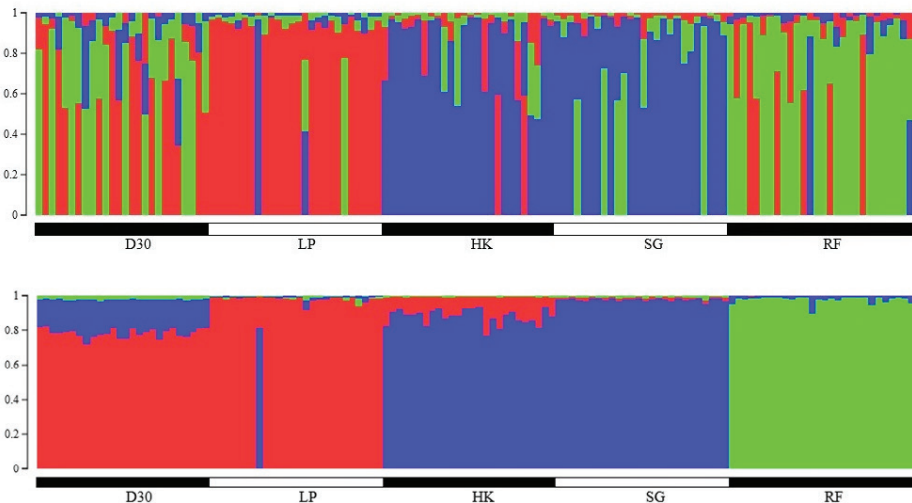


FIGURE 2. Visualizations of the ancestry assignment probabilities of individual Wood Frogs (*Lithobates sylvaticus*) under the most supported value of $K = 3$ in STRUCTURE PLOT. The top graph shows individuals assigned to groups without prior location knowledge and the bottom graph shows individuals assigned to groups with prior location. The letters along the bottom of graphs represent the wetland populations sampled. Each vertical bar represents an individual sampled, and the proportion of each bar colored in green, blue, or red indicates the probability of assignment (y axis) to one of three populations.

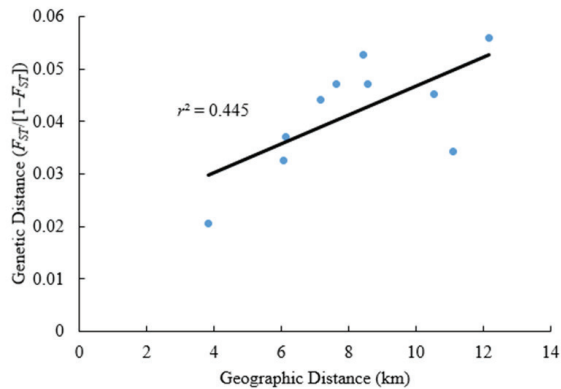


FIGURE 3. Genetic distance ($F_{ST}/(1-F_{ST})$) plotted against geographic distance (km) between pairs of sampled populations of Wood Frogs (*Lithobates sylvaticus*) in the Daniel Boone National Forest, Kentucky, USA.

levels of inbreeding in four wetlands, as measured by F_{IS} , and levels were unrelated to the number of egg clutches. Additionally, we expected that the number of wetlands within a 1,000-m buffer would positively relate with genetic variation, but we observed no relationship. Previous studies of Wood Frog dispersal found that ponds within a radius of 1 km did not exhibit genetic differentiation (Berven and Grudzien 1990; Newman and Squire 2001), and therefore ponds < 1 km may be intermediate areas used for dispersal. Genetic differentiation between ponds may thus appear at distances larger than 1 km (Kimura and Weiss 1964; Squire and Newman 2002) and ponds within 1 km may form a metapopulation, which should yield higher levels of genetic diversity because any alleles lost in one pond can be reintroduced from a nearby pond (Marsh and Trenham 2001; Rhoads et al. 2017). Our contrary results could be because Wood Frogs are not successfully breeding in all wetlands, as has been observed in the constructed wetlands in our ridgetop system (Denton and Richter 2013; Drayer and Richter 2016; Kross and Richter 2016).

We found evidence of a recent bottleneck in three of the five study populations. When the constructed wetlands were placed in close proximity to natural wetlands in our ridgetop system, individuals colonized and bred in the constructed wetlands, which would act to subdivide the historically larger population and could severely decrease population size if constructed wetlands are sink populations, as previous studies suggest (Denton and Richter 2013; Drayer and Richter 2016; Kross and Richter 2016). Genetic drift may also have a strong effect due to historic founder or extinction-colonization events (Zellmer and Knowles 2009; Peterman et al. 2013a). Additionally, habitat loss and fragmentation can cause a decrease in allelic richness and cause population bottlenecks (Rivera-Ortiz et al. 2014). For example, Scherer et al. (2012)

found evidence of a recent bottleneck in Wood Frog populations, which was explained by recent disturbances and fragmentation. A review of bottleneck tests by Peery et al. (2012) recommended sample sizes larger than 31–38 individuals and more than eight to nine loci to have sufficient power to detect a bottleneck event, and bottlenecked populations may go generations without being detected due to limitations of the sampling design. More sampling from wetlands in our study area, especially constructed wetlands, and analysis of historic land use is needed to fully understand why bottleneck events occurred.

Population differentiation ($F_{ST} = 0.054$) across our < 13-km (3.8–12.2 km between sites) ridgetop wetland landscape is comparable to other studies of Wood Frogs at similar spatial scales (0.96–25 km; Zellmer and Knowles 2009; Peterman et al. 2013a); however, some studies over similar distances (0.05–20 km) found little evidence of genetic structure in populations of Wood Frogs (Newman and Squire 2001; Squire and Newman 2002; Julian and King 2003). The pattern in our study and others (Gabrielsen et al. 2013) suggests high connectivity among populations and little genetic structure at small-to-moderate (< 25 km) spatial scales. Genetic differentiation might be low in the current study because our five sites are distributed across a forested landscape with many natural wetlands in close proximity, making a higher density of populations that are likely connected by terrestrial habitat that facilitates gene flow.

Results from STRUCTURE supported three genetic groups of Wood Frogs among our five sites. Although sites D30 and LP are moderately distant (11.1 km), they are members of the same genetic group. This could be because the three natural and roughly 15 constructed wetlands between them facilitate gene flow, either currently or historically. Juveniles are the main dispersers in Wood Frogs and are able to travel up to 2.5 km (Berven and Grudzien 1990), which may explain why there is little genetic structure between the five sites if gene flow occurs between wetlands over several generations. Additionally, D30 and LP are the largest natural wetlands in our study and produced the highest number of egg clutches and thus, these sites may be acting as population sources for the surrounding wetlands. Larger wetlands may also have longer hydroperiods and are able to support a higher breeding population (Baldwin et al. 2006b). The STRUCTURE results also indicated that sites SG and HK are a single genetic group. This was expected because these wetlands are close to each other (3.8 km), and there is a large distance (> 7 km) between these two wetlands and the other three. Nonetheless, there does appear to be some gene flow between HK and D30 and LP. The RF population was genetically distinct. Although RF is geographically

close (6.1 km) to D30, there is urban development directly adjacent to it, between it and D30, that could be impeding dispersal. Previous studies on Wood Frogs at geographic scales similar to ours also found significant genetic structure (Zellmer and Knowles 2009; Peterman et al. 2013a), but there is considerable variation. For example, Scherer et al. (2012) found two distinct genetic groups of Wood Frogs separated by 4 km in Rocky Mountain National Park, Colorado, whereas Richardson (2012) found three distinct genetic groups of Wood Frogs in Connecticut over a scale of approximately 225 km. There was strong isolation-by-distance (IBD) in these studies, however, and STRUCTURE results should be interpreted considering its limitations when IBD is present (Schwartz and McKelvey 2009).

The strong correlation between Euclidean (geographic) distance and genetic distance is likely because our study area had abundant habitat, was fairly homogeneous forest, and had minimal development. We found no correlation between resistance distance and genetic distance, and least-cost path distance was not correlated with genetic distance after controlling for Euclidean distance. This again is most likely because of homogeneity of the forested landscape and small spatial scale of our study. Coster et al. (2015a,b) found genetic distance was only weakly related to landscape variables in Wood Frogs because of high connectivity of populations; however, habitat alterations such as timber harvest may operate on a time scale that is too short to influence genetic structure of populations (Coster et al. 2015b). A similar study on a forest-dwelling salamander revealed population size was significantly correlated with forest patch area and pool area, with 74% of the population genetic distance explained by geographic distance (Rhoads et al. 2017). It should be noted the use of Mantel tests is controversial as they are prone to high Type I error rates (Raufaste and Rousset 2001; Cushman et al. 2013). Additional sampling across a larger spatial or temporal scale would be beneficial for identifying landscape variables affecting gene flow, especially those related to habitat alteration. It would also increase power to detect landscape effects, which is especially important for resistance analyses.

Conservation and future work.—This research adds to our general understanding of Wood Frog population genetics, but specifically increases our knowledge of genetic diversity and structure of populations of Wood Frogs in the DBNF, Kentucky, which could be incorporated into a species conservation plan. Future work in the DBNF should include looking at the relationship of constructed wetlands and natural wetlands, and potential colonization-extinction events. Because constructed wetlands were built in close proximity to natural wetlands and support a different

assemblage of amphibians such as Eastern Newts (*Notophthalmus viridescens*; Denton and Richter 2013; Drayer and Richter 2016), there is potential for negative interactions between species in constructed and natural wetlands, including disease transfer (Richter et al. 2013a) and predation of eggs and larvae of species typically found only in natural wetlands (e.g., Wood Frogs; Kross and Richter 2016). This could affect population structure and genetic diversity, especially if constructed wetlands are acting as population sinks and natural wetlands are acting as sources. A larger spatiotemporal-scale study in DBNF could reveal strong source-sink dynamics similar to a previous study that found Wood Frog populations have strong source-sink dynamics with 23 ponds acting as sinks and 11 ponds acting as sources over a time span of 6 y (Peterman et al. 2013b). Although genetic diversity is still relatively high in the DBNF ridgetop system, despite the number of constructed wetlands in close proximity to natural wetlands, it could be because the constructed wetlands are too recent on the landscape (most < 30 y) to detect effects on the genetics of Wood Frog populations. Genetic diversity and structure can represent the evolutionary potential and ability of a species to respond to environmental change (Emel and Storfer 2012). Therefore, more population genetic studies of amphibian species in decline due to habitat loss, degradation, and fragmentation are needed to aid in their conservation.

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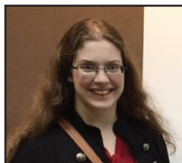
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