GENETIC DIVERSITY OF SPOTTED TURTLE (*CLEMMYS GUTTATA*) POPULATIONS IN A FRAGMENTED LANDSCAPE

by

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To my wife MaryGrace, and my two loving parents Richard and Cindy, without your love and support I would never have reached this landmark in my life.

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ABSTRACT

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Turtle species are facing losses to genetic variation caused by habitat fragmentation and large-scale landscape changes caused by humans. These losses to genetic variation disproportionally effect species that have become rare due to human interaction. Spotted Turtle (*Clemmys guttata*) is one such species that has become rare across a large portion of its natural geographic range including the Midwest. I examined genetic variation in spotted turtles in Indiana and Ohio to delineate populations and assess levels of genetic variation within populations. Eight microsatellite loci were used to genotype 94 individuals, which originated from four sites in Indiana and two sites in Ohio. Bayesian and ordination based cluster analyses identified three genetic clusters within the sample. Individuals from one Ohio locality were largely clustered alone and had a significant degree of differentiation from all of the Indiana sites. A majority of the Indiana sites had individuals that were clustered into both of the two remaining clusters with the exception of IN-1 which only had individuals grouped into the first inferred cluster. The two sites geographically closest to one another IN-2 and IN-4, both had individuals grouped into the first and third inferred clusters. Differentiation statistics disagreed on the level of structure between these two populations. Two of the six sites had detectable bottlenecks, even though levels of genetic allelic diversity and heterozygosity were relatively high and comparable to populations investigated in other studies. Overall, population structure was identified among localities in Indiana and Ohio that may warrant independent management. Populations where the bottlenecks were detected may be of special concern in the future as they continue to be subjected to isolation.

INTRODUCTION

Challenges Facing Spotted Turtles

Turtles species worldwide are facing challenges related to the changes of natural landscape caused by humans (Buhlmann et al., 2009). One of the biggest challenges that turtle species face is habitat fragmentation. Habitat fragmentation is typically viewed as both the breaking apart of connective habitat as well as the loss of a portion of that habitat (Fahrig, 2003). The process of habitat fragmentation has the potential to effect the population structure of species as well as their genetic variation (Keyghobadi, 2007). Current research points to highly fragmented habitats having a disproportionate number of male and mature turtles. Fragmentation forces dispersing individuals to face more dangerous situations such as roads, and in turn suffer from higher rates of mortality (Baldwin, Marchand, & Litvaitis, 2004). This disproportionately affects female turtles who must travel greater distances than males while seeking out nesting habitat (Reid & Peery, 2014). This skewed sex ratio may also suggest that fragmented habitats provide less quality nesting habitat (Baldwin et al., 2004). When sex ratios are skewed by road effects, genetic variation and effective population size may also be reduced as has been observed in the endangered Blanding's Turtle (Reid & Peery, 2014).

The severity of habitat fragmentation has shown to have an effect on the loss of genetic diversity within a population. Rare alleles are especially likely to be lost in bottlenecks that result from reproductive populations being isolated and gene flow being ceased (Mhemmed, Kamel, & Chedly, 2008). The overall allelic diversity of a species is also easily impacted by habitat fragmentation. Even in populations that currently have a high degree of genetic diversity, the long term effects of habitat fragmentation may drive down the genetic variation in these populations (Mhemmed et al., 2008).

The Spotted Turtle (*Clemmys guttata*) is one such species that has suffered decline across its natural range (Lovich, 1989). This species is listed as state endangered in Indiana and is listed as threatened by the IUCN (Litzgus, 2004). Spotted turtles require a variety of connected habitats to sustain populations. These habitats include wetlands, swamp forest, early succession vegetation, and upland forest (Reeves & Litzgus, 2008). The requirement for multiple habitat types that are linked together makes this species vulnerable to habitat fragmentation since the

loss of any one or more types of habitat may reduce connectivity. Across its natural range, this species has seen a reduction in suitable habitat which is thought to contribute to the decline of this species (Davy & Murphy, 2014).

Remaining spotted turtle populations are often small and isolated from each other due to habitat fragmentation (Litzgus, 2004). Evidence suggests that historic populations were more interconnected than current populations. In the Canadian portion of this species' range, six genetically isolated populations have been identified (Davy & Murphy, 2014). Evidence points to these genetic divergences being influenced by habitat modifications that are anthropogenic in nature. This suggests that the changes in the environment made by humans are cutting these breeding populations off from one another and negatively impacting this species (Davy & Murphy, 2014). Interestingly, this species represents a unique case of having a threatened status while still maintaining its genetic diversity largely intact due to the long lifespan (Davy & Murphy, 2014).

It has been shown that populations of spotted turtles have varying levels of allelic richness which correlate to their population size (Davy & Murphy, 2014). This implies that small populations of spotted turtles may be especially vulnerable to losing genetic diversity in isolation, making them even more vulnerable to local extirpation. However, the long lived nature of turtles makes it more challenging to predict the genetic effects of population decline (Davy & Murphy, 2014). Many of the models used to detect bottlenecking in populations are ill-suited for use on long lived species with slow generation times (Willoughby, Sundaram, Lewis, & Swanson, 2013). This implies that many of the analyses used to detect bottlenecking in older studies may have used models that were unsuitable for turtles (Davy & Murphy, 2014). Turtles employ a survival strategy that results in high mortality of young and a long reproductive window for those individuals who survive to adulthood. This also results in overlapping generations being present and reproducing during the same period of time. This survival strategy makes it difficult to detect a recent bottleneck using popular models such as bottleneck and M-ratio (Davy & Murphy, 2014; Willoughby et al., 2013). Because of the limitations of bottleneck analyses it is necessary to examine measures of within locality genetic variation, and population structure in order to better understand the vulnerability of the populations examined.

Spotted turtle populations in Indiana are not well studied compared to other segments of their geographic range. It is not known how many distinct genetic clusters are present in Indiana

or the genetic stability of the populations that are present. Further complicating this is the fact that some of the most well-known spotted turtle populations are located in urbanized environments that have little connectivity that could allow gene flow between populations.

Microsatellites

Microsatellites were chosen over other genetic makers due the distinct advantages that they possess for this project. The long generation time of turtles limits the effectiveness of many other standard markers. Microsatellites on the other hand accumulate mutations at a much higher rate than the other regions of DNA. Microsatellites are a short series of repeated nucleotides that appear at a high incidence within a species genome (Ellegren, 2004). These repeating nucleotides typically number from one to six with no set number of repeats. These areas are very prone to mutations caused by proof reading errors and slippage during DNA replication. Mutations cause the number of repeats to change in overall length (Selkoe & Toonen, 2006). The region is then amplified by PCR. High-resolution electrophoresis then allows for the amplified region to be compared to amplifications of other individuals (Selkoe & Toonen, 2006). Microsatellites are one of the best available tools to examine recent changes in a populations genetic makeup due to the rapidly changing nature of microsatellite sequences (Selkoe & Toonen, 2006). Changes within a landscape may disrupt the connectivity of habitat and split up populations (Fahrig, 2003). Recent changes in genetic composition of spotted turtle populations suggests there is a recent change in the landscape that is separating populations that were previously genetically interconnected. Microsatellites are particularly well suited for this purpose since their high mutation rates make them optimal for examining recent genetic changes within a population (Selkoe & Toonen, 2006). Because they have been used in other studies of spotted turtles, they also provide an opportunity compare genetic variation to populations in other portions of the geographic range (Davy & Murphy, 2014).

Using microsatellites I aim to examine genetic variation in spotted turtles throughout the state of Indiana, as well as compare the populations in Indiana to populations of spotted turtles in Ohio. In doing so, populations of spotted turtles that are genetically isolated can be identified, and their vulnerability to decline based on their genetic variation within populations can be identified.

MATERIALS AND METHODS

Field Sampling

Field surveys took place 2017 to 2019 at locations across northern Indiana previously identified as having spotted turtles (Hinson, 2018). Sampling began in March to coincide with spring emergence and continued through July of each year. Collapsible 36 x12 inch minnow traps baited with sardines were used to capture spotted turtles. These traps were placed along predetermined transects at regular intervals. GPS coordinates and the depth of the water were recorded at each trap. Spotted turtles were also opportunistically captured by hand while checking traps. In sites where water depth did not allow for traps to be placed hand captures alone were used. Samples were obtained from six sites that represent five counties in Indiana. From these sites sufficient sample numbers were obtained from four sites that represent three counties. These sites are referred to as IN-1, IN-2, IN-3, and IN-4. The number of samples at each site can be seen in (Table 1; Figure 1). Other organizations were contacted to broaden sample size and provide comparison to Indiana populations. Two sites from Ohio, OH-1 and OH-2 had a sufficient number of samples to be included (Table 1; Figure 1).

Each capture was recorded using GPS. Captured individuals had their shell marked by filing a groove into their shell to notch the shell. This allowed us to assess what individuals were recaptured and prevent repeat sampling. Scale clippings from the tail region were taken from the captured individuals. Samples were placed in 95% ethanol while in the field, then frozen at -80 C until DNA extractions were performed. To prevent the spread of diseases among sample sites, all sampling gear was sanitized with a bleach solution. The number of surveys performed at each location was dependent on the number of captures at the site during the previous year, and how many more samples were needed. Samples that were received from groups in Ohio were in the form of blood samples stored in ethanol or on FTA cards (Gutiérrez-Corchero et al., 2002). These cards are convenient due to the fact that they do not require refrigeration after collection, and are effective for the long term preservation of blood samples (Gutiérrez-Corchero et al., 2002).

Microsatellite Genotyping

DNA extractions from scale clips and blood stored in ethanol were performed with the Qiagen, DNeasy Blood and Tissue extraction kit. DNA extractions from FTA cards were performed using the same kit, following the protocol put forward by Backeljau & De Meyer (2013). The presence and concentration of DNA eluted was examined through the use of both a Nanodrop spectrophotometer and a Qubit fluorometer. Samples that failed to elute useable amounts of DNA were either re-extracted if there was sufficient tissue remaining from the first extraction, or further concentrated through the use of the Zymo DNA Clean & Concentrator Kit. For several FTA card samples sufficient amounts of DNA were unable to be obtained using these methods and they were excluded from the study.

Due to previous microsatellite work with this species (Anthonysamy et al., 2017; Davy & Murphy, 2014), there were several loci available including: GmuD51, GmuD55, GmuA19, GmuD21, GmuD88, GmuB08, GmuD87, GmuD121, GmuA18, GmuD16 and, GmuD114 (Table 2). These loci were originally designed from bog turtles (King & Julian, 2004). These loci were chosen for their low null allele frequencies and their ability to be multiloaded. Of these 11 loci only eight were included for analysis due to inconsistent amplification in GmuB08, GmuD51 and GmuD21. On the 5' end of the forward sequence of each of these loci a universal primer specific sequence was added, allowing for the attachment of a florescent labeled universal primer during PCR. This follows the protocol set forth by (Blacket, Robin, Good, Lee, & Miller, 2012). The universal primer dyes used include PET, NED, and VIC.

PCR reactions were performed using the Qiagen Taq PCR master kit. Some primers required additional $MgCl_2$ be added to produce reliable amplification (Table 2). The PCR procedure followed a touchdown protocol described by Darren and John (2008). In the first phase of PCR, denaturation took place at 95 degrees C for 5 minutes, followed by a second denaturation at 95 degrees C for 30 seconds. Annealing took place at 65 degrees C for 45 seconds, and subsequently decreased one degree C for each cycle. Elongation took place at 75 degrees C for 45 seconds. Phase one consisted of a total of 10 cycles. Phase two started with a denaturation at 95 degrees C for 30 seconds. Annealing took place for 45 seconds and at a temperature of 55 degrees C. Elongation took place at 72 degrees C and lasted for 60 seconds. Phase two repeated for a total of 20 cycles. The termination phase of PCR started with elongation for 5 minutes at 72 degrees C. The final step halted the reaction by cooling off to 4

degrees C and held the product at this temperature until it was removed. PCR product was stored at -80.

PCR was initially performed on a small number of samples and the product visualized on a 2% agarose gel to check that bands were found in the expected size range. Following PCR, a small number of samples were diluted to varying degrees in formamide and sent to the Yale DNA Analysis Facility where fragment analysis was performed on the Applied Biosystems 3730xl 96-Capillary Genetic Analyzer. Electropherograms were visualized in Geneious v. 11.1.5 to assess signal strength and call alleles. Following the discovery of optimal dilutions, all samples were subjected to fragment analysis as described above. Observed allele calls were exported into an excel file formatted to be used with GenAlEx 6.5 (Peakall & Smouse, 2012). GenAlEx 6.5 was used to format the data into the different formats required by the analysis programs (Peakall & Smouse, 2012).

Statistical Analysis

Hardy Weinberg Equilibrium and Disequilibrium

Hardy Weinberg equilibrium and linkage equilibrium was assessed for each combination of locus and sample locality using GENEPOP version 4.2 (Rousset, 2008). Bonferroni correction was applied to as a threshold for determining statistical deviation from equilibrium (Rice, 1989). HWE was examined due to it being it a useful tool in initially evaluating populations. Examining HWE also prevents violating the assumptions of other statistical tests used. HWE exact test was run for 100 batches and 1000 iterations using the Markov chain method to determine *P*-values (Guo & Thompson, 1992). To assess the presence of linkage disequilibrium in the populations tested, Genotypic linkage disequilibrium was run in Genepop version 4.2 (Rousset, 2008). Genotypic linkage disequilibrium tests for genotypic disequilibrium at each pair of loci in all of the test populations by using the log likelihood ratio static. This test of linkage disequilibrium is also referred to as a composite linkage disequilibrium test. Bonferroni correction was applied to as a the threshold for determining statistical significance for linkage.

Descriptive statistics within localities

Observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated in GenAlEx 6.5 (Peakall & Smouse, 2012). GenAlEx6.5 was also used to detect the number of private alleles and the number of effective alleles in the populations. Private alleles are alleles that only occur in a single population, these can be used as a simple measure of population differentiation with a greater number of private alleles equaling a greater degree of differentiation between populations. Effective alleles are a useful measure when comparing populations that have different allele distributions but a similar number of alleles. This is because effective alleles are related to the expected heterozygosity, as the frequency of alleles become more even in the population, the heterozygosity and number of effective alleles increases. Allelic richness was evaluated for the populations through the use PopGenReport (Adamack & Gruber, 2014). PopGenReport was chosen due to its ability to compensate for differences in sample size when counting alleles. *F*is values were generated through the use of GENEPOP version 4.2 (Rousset, 2008).

Population Structure

Bayesian cluster analysis was performed using Structure v. 2.3.4, a model based approach that identifies groups of individuals independent of sample locality. This was accomplished by testing the fit of the data to different numbers of clusters (*K*). Structure was run 10 times for each value of *K* (1-10). Runs had a burn-in of 50,000 followed by100,000 steps. Structure was run both with and without user defined population identified using the LOCPRIOR function, with the results that include LOCPRIOR being shown. LOCPRIOR was implemented to identify if inputting user defined populations would influence the number of clusters produced. Historic admixture between the sampled populations was assumed in the model, and correlated allele frequencies were also assumed, thus allowing direct comparison with results from Ontario populations (Davy & Murphy, 2014). Identification of the most likely value for *K* and visualization of Structure output was performed using Structure Selector (Li & Liu, 2018). Structure Selector employs a method that accounts for uneven sample sizes when identifying *K* (Li & Liu, 2018; Puechmaille, 2016), an approach that has been shown to be more accurate than the commonly used Δ*K* method (Evanno, Regnaut, & Goudet, 2005). Structure selector employs

the Puechmaille method. The Puechmaille method uses four new estimators to identify the true *K* . These estimators identify and count clusters that contain one or more subpopulations to avoid underestimating the clusters that are present.

A Discriminant Analysis of Principal Components (DAPC) was used to infer genetic clusters without relying solely on model based Bayesian cluster analysis. DAPCs are capable of producing reliable clusters even when sample sizes are uneven or equilibrium assumptions are unmet (Jombart, Devillard, & Balloux, 2010), a characteristics of this dataset. Unlike typical Bayesian clustering methods, DAPCs do not rely on previously defined population genetic models to form clusters (Jombart et al., 2010), but rather use the geometry of multivariate allelic distances among individuals. Adegenet, a package of R, was used to perform this analysis. DAPCs are performed by first conducting a principal component analysis (PCA) on the allelic data (Jombart et al., 2010). PCA data are then input into a Linear Discriminant Analysis, to maximize variation between clusters while minimizing within cluster variation. Data is then visualized through the use of a scatterplot. For this project all PCs were retained both for finding K and during the discriminate analysis. This was done due to the small size of the data set being used (Jombart et al., 2010). All eigenvalues were also retained due to the low number of clusters. K values were chosen through running k-means sequentially with increasing values. Clusters are compared through the use of Bayesian Information Criterion.

Statistics related to population differentiation (*F*st and *D*) were calculated in Genalex6.5 (Peakall & Smouse, 2012). *F*st values range from 0 to 1, with number closer to one exhibiting a higher degree of genetic dissimilarity compared to other populations. *F*st essentially functions by relating the genetic variation present in a single population to the total possible genetic variation across all tested populations (Whitlock, 2011). Unlike Fst, *D* is not reliant on the measure of genetic variation across populations but is instead calculated based on the number of effective alleles at a set population size (Jost, 2009). It is less sensitive than *F*st to downward bias resulting from highly polymorphic loci, a feature characteristic of microsatellite polymorphism.

Bottleneck and Effective Population Size Estimation

I tested for genetic bottlenecks in the populations sampled using Bottleneck v.1.2.02 (Cornuet & Luikart, 1996; Piry, Luikart, & Cornuet, 1999). Under this approach, it is assumed that allelic diversity will decline faster than heterozygosity when a population declines leading to

heterozygosity excess (Luikart & Cornuet, 1998). Davy (2014) argues that this program is typically poor at detecting bottlenecks in turtles and other species with long generation times, but in select cases it has been successfully employed with turtles (Pearse et al., 2006; Vargas-Ramírez, Stuckas, Castano-Mora, & Fritz, 2012) and I use it here. Bottleneck was run under the same parameters used by Davy & Murphy (2014) in Ontario populations of *C. guttata* . The program was run for 1000 replications under the two phase model where single step mutation rates were set to 95% and multistep mutations were set to 5%. Variance was set to 12 for multiple steps. The statistical significance of the heterozygosity excess was determined by the Wilcoxon test, and allele frequency distributions were assessed for the presence of mode shift, within Bottleneck.

The ability to estimate population size is very useful for spotted turtles as they can be very difficult to capture even at locations they are known to be currently inhabiting. This makes the use of more traditional mark and recapture methods challenging to use without investing considerable field time at each site. NeEstimator V2.1 was used to approximate effective population sizes of each sample locality. NeEstimator uses the program LDNe to estimate the effective population size based on linkage disequilibrium data (Waples & Do, 2008). One reason this program was chosen over other population estimators is its ability produce confidence intervals using the typical parametric method as well as the jackknife method, the latter believed to be more precise (Waples & Do, 2008). LDNe has also been shown to perform well in single sample estimation when populations are small and relatively isolated (Gilbert & Whitlock, 2015). LDNe was run using the Linkage Disequilibrium method and employed a random LD mating model within the populations.

RESULTS

Hardy Weinberg and Linkage Equilibrium

Tests for HWE were run within each of the six sample localities and none exhibited any deviation from HWE (Figure 1). Linkage disequilibrium was not detected among any of the loci tested. Given that there was no systematic pattern of disequilibrium across sites or loci, all loci were retained for subsequent analysis.

Allele frequencies in the populations and changes

Data were missing for 2.13% of individuals tested. Observed heterozygosity was similar across all of the sites examined with a range of 0.601-0.781 and an overall mean of 0.678 (Table 2). These numbers generally agree with the heterozygosity that was observed by Davy and Murphy (2014) in spotted turtle populations. The lowest mean *H*o was detected at OH-1. OH-1 also possessed the lowest allelic richness of any of the sites after correction of sample size. Despite this, OH-1 possessed one of the larger counts of private alleles (n = 3), with each private allele occurring on a different locus. These general patterns point to OH-1 being isolated, and becoming differentiated from the other sites. OH-2 had the lowest number of effective alleles at 3.151, and possessed three private alleles (Table 3). IN-1 possessed a single private allele and a *Ho* of 0.679. This is surprising given the distance from this site to any of the other sites tested. IN-2 had the highest allelic richness of any site after correction for sample size. IN-2 and IN-4 were the two sites most geospatially close to one another, with IN-2 registering 4 private alleles and IN-4 possessing 3 private alleles, with both sites respectively having 4 and 3 loci where a private allele was located (Table 3). The number of private alleles between these sites is surprising given the proximity of these sites. IN-4 also possessed the highest *Ho* of any of the sites 0.781. IN-3 was the only site where no private alleles were detected. This was unexpected since there is considerable distance between IN-3 and any of the other locals.

Bayesian population genetics and DAPCs

Analysis of Structure results using the Puechmaille method suggested the best fit of K was three clusters (Figure 2). This indicated that in the populations sampled that three distinct genetic clusters were identified. This was in contrast to the results produced by Structure Harvester which instead found a K=2. The results from the Puechmaille Method are likely more representative of reality due to its ability to compensate for uneven sample size. However, both of these results show a clear split between the OH-1 population and the populations located in Indiana (Figure 3).

Three inferred clusters were formed using DAPC, confirming with the results from Structure (Figure 4). Individuals from OH-1 were largely sorted into the first inferred cluster and contained very few individuals from any other population. IN-2 was completely sorted into the second cluster while the other sites in Indiana had a proportion of their populations assigned to both the second and third clusters (Figure 4). OH-2 also had individuals assigned to all three clusters, but a majority of the population was assigned to the second cluster.

Diversity Indices

*F*st values ranged from 0.029 to 0.076 among sites (Table 4). The highest degree of genetic dissimilarity was between the OH-2 and IN-3. The Indiana populations showed less genetic dissimilarity between one another than between the Ohio sites. The Ohio sites also showed this trend with a lower degree of dissimilarity between OH-1 and OH-2 (*F*st = 0.03) than with any of the Indiana populations (Table 4).

D values showed a similar relationship with the Indiana sites with IN-2, IN-4, and IN-3 showing very little differentiation between one another (Table 4). IN-3 did not show significant differentiation with IN-1 or IN-2. IN-1 showed a nearly equal amount of differentiation between the other Indiana sites and the Ohio sites, with the exceptions of IN-3 and OH- 2 which were not statistically significant. The two Ohio sites did not have significant differentiation between each other (Table 4). *F*is results were estimated by Weir and Cockerham (1984). IN-3 and OH-1 had the highest average Fis values with 0.1330 and 0.099, respectively (Table 3). The lowest average *F*is values reported were at OH-2 and IN-4.

Bottleneck

In the six populations tested both IN-3 and IN-4 showed signs of a population bottleneck. IN-3 had six loci that exhibited heterozygosity excess (two-tail Wilcoxon p value = 0.01953). IN-4 had seven loci that exhibited heterozygosity excess (two tail Wilcoxon p value = 0.01953). No other populations exhibited significant p values for the presence of a bottleneck using the two tail Wilcoxon test. IN-3 also tested positive for a mode shift, while all the other populations including IN- 4 exhibited a normal L distribution.

LDNe

Estimated *N*e was reported at both the 0.05 lowest allele frequency as well as the 0.02 allele frequency with the associated 95% confidence interval (Table 5). IN-1 had a *N*e value of infinite, which is a result of not enough samples being present to create the necessary variation in the genetic information for the population size to be estimated (Do et al., 2014). IN-2 and IN-4 had the largest Ne at both the 0.05 and 0.02 lowest allele frequency, but both sites had a wide margin in their 95% confidence interval and increased into infinity (Table 5). OH-1 had a small NE value at both allele frequencies, but also had wide 95% confidence values from both frequencies (Table 5). This is surprising since this site had a more appropriate number of samples, and was the only location that exceeded the recommended 25 samples (Do et al., 2014). To help account for low sample size the two closest geographic populations in Indiana were combined and re-examined. Combining IN-2 and IN-4 resulted in increasing the minimum value for the 95% confidence interval and reducing the Ne value for the 0.02 lowest allele frequency. This did not resolve the upper limit for the 95% confidence interval, which was still infinite.

Table 1. Geographic location and sample size (N) for the study.

Site	N	State	County
OH-1	26	Ohio	Ashtabula
OH-2	8	Ohio	Portage
IN-1	7	Indiana	Steuben
IN-2	20	Indiana	Lake
IN-3	13	Indiana	Carroll
IN-4	20	Indiana	Lake

Table 2. Summary table for describing eight microsatellite loci used in the study.

Locus	Bp Size	Number of alleles	GenBank accession no.	Repeat	Primer sequence F	Primer sequence R	Annealing Temperature	$MgCl_2$
GmuD55	175-210	9	AF517240	(ATCT)10	GTG ATA CTC TGC AAC CCA TCC	TTG CAT TCA GAA TAT CCA TCAG	58 °C	No
GmuA19	125-135	3	AF517227	(GA)7(GT)14	TAA GAG ACA GAT GCT CAG CAA G	GTA CAT AAC ACG CAC CCA ATG	58 °C	No
GmuD88	115-130	8	AF517245	(ATCT)18	AAC AAT GCC TGA AA TGC AC	TAG GCT ACC TCT GAA AAT GCT G	58 °C	Yes
GmuD87	195-255	18	AF517244	(ATCT)22	AAA CCC TAA GAC ATC AGA CAG G	CAA ATC CAG TAC CCA GAA AGT C	58°C	No
GmuD121	130-165	8	AF517252	(ATCT)8	GGC AA TAT CCA ATA GAA ATC C	CAA CTT CCT CGT GG TTC AG	58°C	No
GmuA18	100-120	5	AF337648	(GT)14	TAT CAG GGA AAG CAA TGT AAG G	AGT GAA ACA AGC AGT TAT GGT G	58°C	No
GmuD16	135-190	10	AF517235	(ATCT)19	ATC CCT GAA ATT TTG TGT GTT C	TTT ACT CTA GAA GGG GCA ATC C	58°C	Yes
GmuD114	100-105	5	AF517251	(ATCT)13	ATA GAC ATA GTG CAT ATA GAC ATA GCC	ACG TTC TTG CAG GGT CAG AG	58°C	Yes

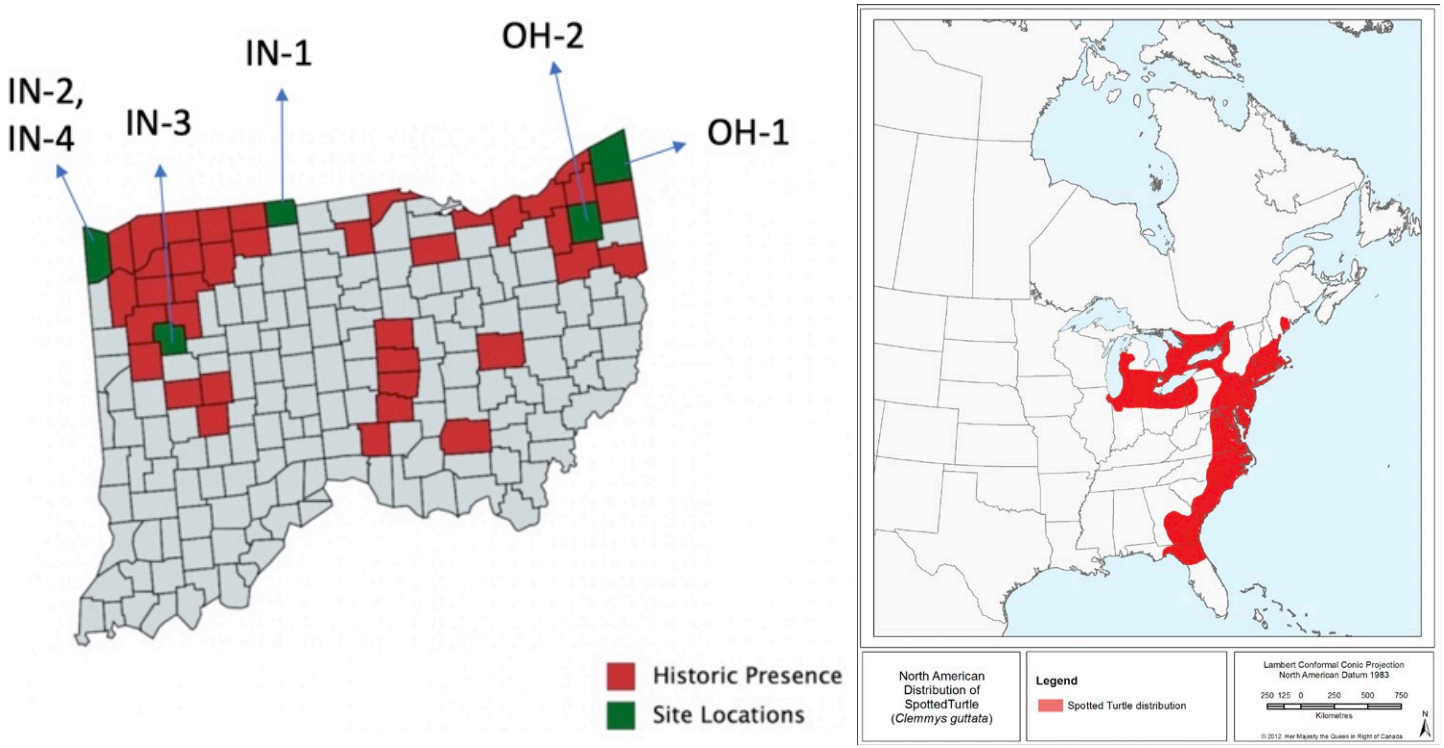

Figure 1. Site locations and Spotted Turtle distribution. The distribution in Ohio was mapped from data published by Lewis, Ullmer, & Mazza (2004). Indiana spotted turtle distribution was mapped from Hinson (2018). The North America distribution of Spotted Turtles (Clemmys guttata) is from Environment and Climate Change Canada (2018).

Table 3.Mean and standard error of genetic variation within each locality for allelic richness corrected for sample size (AR), private alleles per loci (PR), effective alleles (EA), observed heterozygosity (Ho), expected heterozygosity (He), and *F*is *(F*is W&C*)* using the calculation reported by Weir and Cockerham (1984)

Site	**AR**	**PR**	**EA**	**EA-SE**	**H_0**	**H_0-SE**	**H_E**	**H_E-SE**	***F*is W&C**
OH-1	3.71	0.375	3.340	0.522	0.601	0.065	0.662	0.038	0.099
OH-2	4.13	0.375	3.151	0.318	0.609	0.083	0.619	0.057	-0.0935
IN-1	4.03	0.125	3.275	0.523	0.679	0.084	0.643	0.048	0.0129
IN-2	4.69	0.500	4.054	0.393	0.736	0.059	0.735	0.029	0.0176
IN-3	4.39	0.000	3.798	0.404	0.663	0.097	0.716	0.029	0.1330
IN-4	4.27	0.375	3.776	0.483	0.781	0.063	0.701	0.042	-0.0813

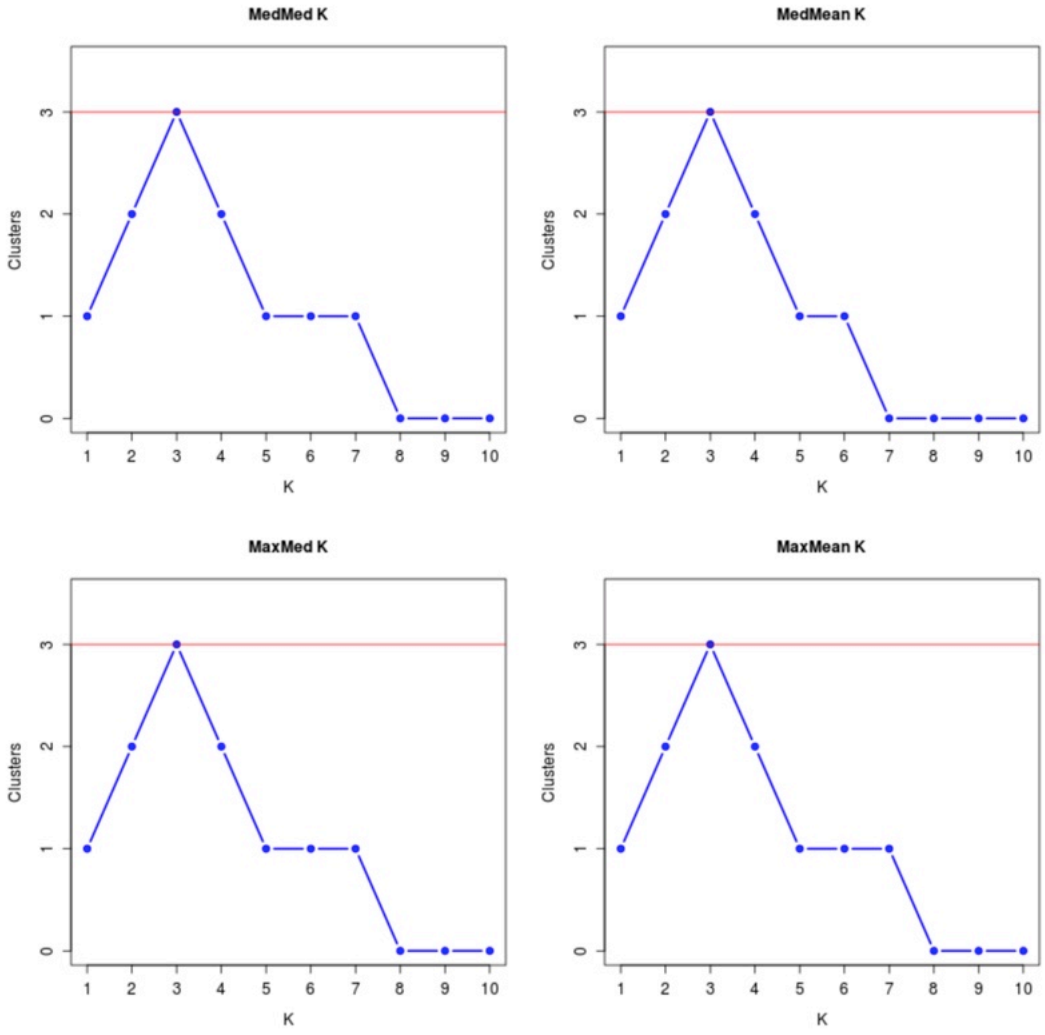

Figure 2. Estimates of the number of clusters (K) from Structure output using the Puechmaille method implemented in Structure Selector. MedMeaK and MedMedK look for the median number of clusters, while MaxMeaK and MaxMedK look for the maximum number of clusters that are represented by one or more subpopulations. The maximum value for clusters in each of these estimators represent the number of clusters estimated to be within the data (Li & Liu, 2018; Puechmaille, 2016; Willoughby et al., 2013)

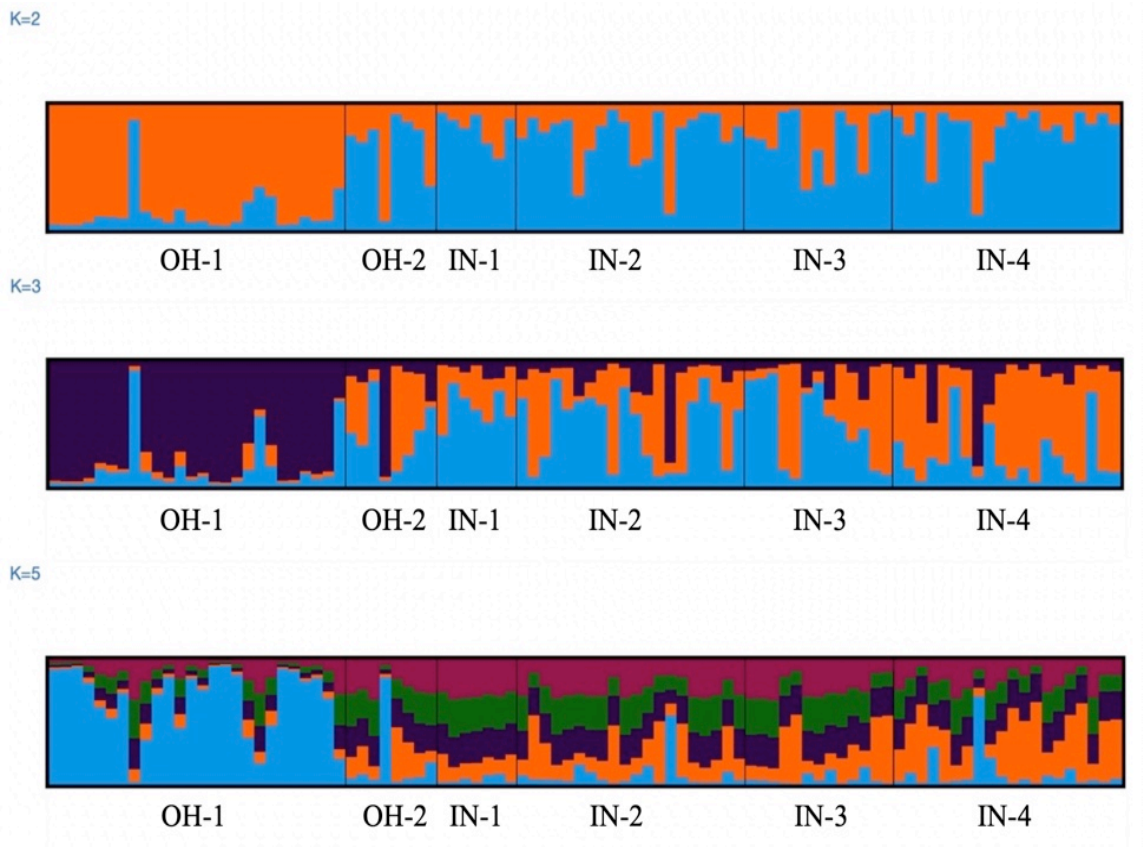

Figure 3. Structure bar plot of individual assignment probabilities to inferred clusters (K). Results for K=2, K=3, and K=5 are shown.

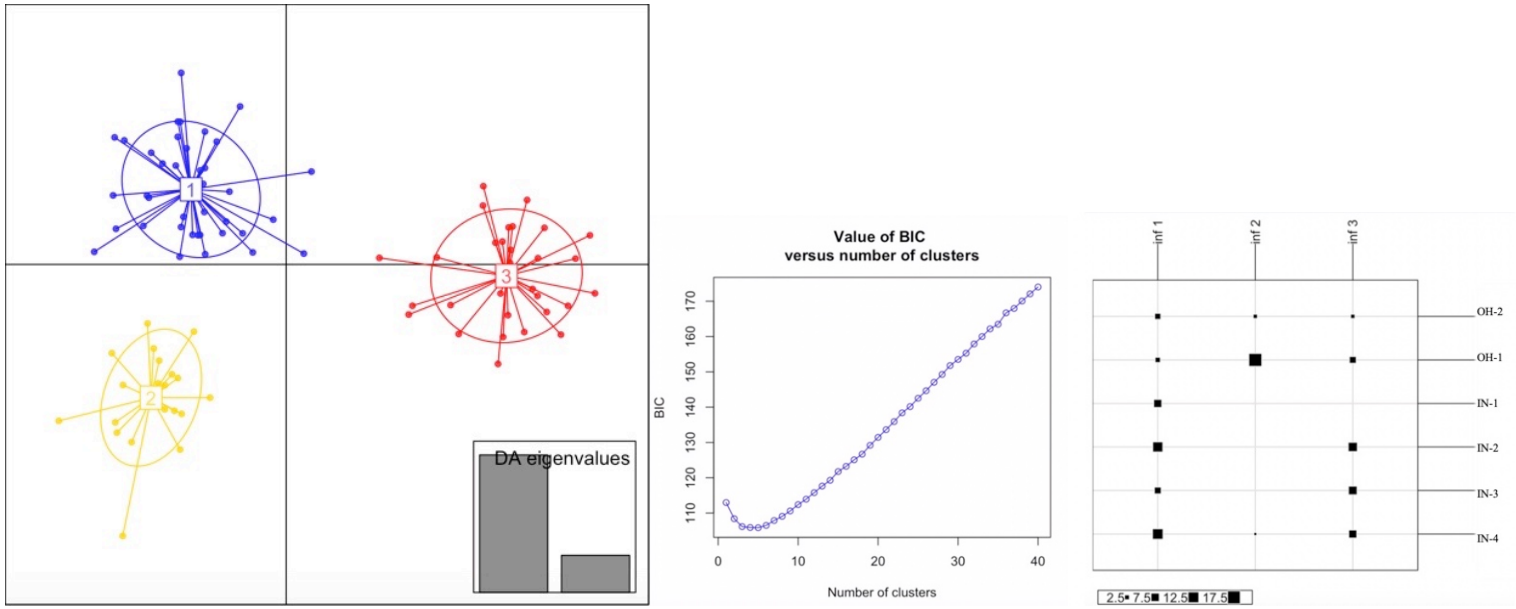

Figure 4. Scatterplot of inferred population clusters using DAPC. Inertia ellipses around each cluster demonstrate genetic variation within inferred clusters. Bar graph of eigenvalues represents the number of discriminate functions that are retained. BIC plot shows best number of possible clusters. The box plot demonstrates the number of individuals from each site that are grouped into the inferred clusters, with larger boxes representing a greater number of individuals.

Table 4. Pairwise diversity indices (*F*st, D) for sample sites. Fst values are below the diagonal, and D values are above the diagonal. Bolded numbers are statistically significant ($p < 0.05$) after Bonferroni correction.

	OH-1	**OH-2**	**IN-1**	**IN-2**	**IN-3**	**IN-4**
OH-1		0.027	**0.159**	**0.190**	**0.177**	**0.159**
OH-2	0.030		0.199	**0.163**	**0.230**	**0.198**
IN-1	**0.060**	0.081		**0.174**	0.097	**0.167**
IN-2	**0.049**	**0.056**	**0.057**		0.070	**0.071**
IN-3	**0.051**	**0.076**	0.049	0.029		**0.092**
IN-4	**0.045**	**0.065**	**0.058**	**0.025**	**0.033**	

Table 5. Effective population size estimates from LDNe. Ne 0.05 is the estimated population with a 0.05 lowest allele frequency, and Ne 0.02 is the estimated population with a 0.02 lowest allele frequency. 95% confidence interval for each measure are given.

Site	*N*e 0.05	95% CI	*N*e 0.02	95% CI
OH-1	33.3	8.7-∞	22.0	12.0-542.4
OH-2	11.6	2.3-∞	11.6	2.3-∞
IN-1	∞	7.4-∞	∞	7.4-∞
IN-2	116.2	27.1-∞	1089	40.6-∞
IN-3	18.4	7.1-366.6	24	8.9-∞
IN-4	120.7	24.9-∞	349.4	32.1-∞
IN-2+IN-4	102.7	35.7-∞	202.1	55.7-∞

DISCUSSION

Within Locality Genetic Variation

In this study I aimed to examine genetic variation in spotted turtles throughout the state of Indiana, as well as compare the populations in Indiana to populations of spotted turtles in Ohio. In doing so, I hoped to identify populations of spotted turtles that are genetically isolated, and access their vulnerability to decline based on their genetic variation within populations. It was previously unknown how many unique genetic clusters of spotted turtle existed in this region. The genetic variation present in the sites examined was also unknown.

The results of the Hardy Weinberg equilibrium test within the six geographic populations point to all of the populations being in HWE. This is reinforced by the fact that none of the loci tested were significant for linkage. These results give confidence to the other analysis's used. The OH-1 population possessed one of the higher number of private alleles of any of the populations tested (Table 2). The number of private alleles detected supports the outcome that OH-1 population is differentiated from the other sites (see below). The OH-1 site tested for the lowest level of heterozygosity of any of the populations tested at 0.601. Even so, this level of heterozygosity is not cause for immediate concern as it is still within the realm of what is typical for wild populations of spotted turtles (Davy & Murphy, 2014). The fact that 0.601 was the lowest heterozygosity value detected confirms that despite a fragmented habitat and small populations, spotted turtles in Indiana have retained their genetic diversity like other populations across their range (Anthonysamy et al., 2017; Davy & Murphy, 2014). This is also in line with the general findings that the long generation time of turtles favors the retention of allelic diversity even after population size declines that may have occurred hundreds of years ago (Kuo & Janzen, 2004). IN-2 was the site with the highest number of private alleles, indicating that there is some level differentiation at this site. This is surprising given its geographic closeness to IN-4, and the weak differentiation between these sites that was detected by *Fst* and *D*. Both IN-2 and IN-4 have retained high levels of heterozygosity and some of the highest levels of allelic richness detected in this study, 4.69 and 4.27 respectively. This further supports that a fragmented habitat and isolation has not majorly impacted the genetic diversity of these populations (Anthonysamy et al., 2017; Davy & Murphy, 2014).

Of the six sites tested two of the Indiana locations tested positive for evidence of a past genetic bottleneck occurring. Given the results of Davy and Murphy (2014) and their findings that genetic bottlenecks in this species are often very difficult to detect, this raises concerns on the possible severity of these bottleneck events that were detected. This may indicate that the bottlenecks that have occurred at these two sites is severe in nature. This could mean that these two populations may be especially at risk to future declines even though they are currently maintaining a reasonable amount of heterozygosity and allelic richness. Programs such as Bottleneck are just not optimally suited to work with turtles as they often underestimate the presence of bottlenecks in populations tested. Despite this, Bottleneck was still able to identify two localities that had evidence of a bottleneck occurring.

Of the populations tested IN-3 and OH-1 had the highest degree of inbreeding (Table 2). This higher degree of inbreeding in the OH-1 site could help explain the low heterozygosity detected at this site, and the number of private alleles detected. This greater degree of inbreeding may also be responsible for OH-1 being the site with the lowest allelic richness after adjustment. This result may suggest that OH-1 would benefit from outcrossing between it and other geographically close sites such as OH-2.

Population Structure

Cluster analyses identified three genetic groups within the sample. In Structure, the first cluster is clearly defined as the population found at OH-1 (Figure 2). The remaining two inferred populations are less clearly defined with many of the populations showing a pattern of admixture. The results of the DAPCs agree with Structure and show that the spotted turtles from Indiana and Ohio can be separated into three clusters (Figure 4). The first DAPC cluster is distinct to the population found at OH-1, with very few individuals from other populations being sorted into this cluster (Figure 4). OH-1 did have a smaller portion of individuals sorted into the other two inferred clusters, but they were much fewer in number by comparison. The remaining two clusters are less clearly defined, with many of the sites being split between them. IN-2 was completely sorted into the first inferred cluster unlike the other Indiana sites which were split between the first and third inferred clusters (Figure 4). This agrees with the *D* diversity indices which found significant differentiation between IN-2 and four of the other test sites. OH-2 was sorted most strongly into the second inferred cluster, but was one of the few sites that also

weakly sorted into the other two inferred populations. For clustering programs such as Structure and DAPCs a larger number of sample sites and a greater number of samples from the sites used would have likely resulted in a more robust and definitive clusters being formed. The use of samples from sites where I have no firsthand knowledge of habitat composition, or past human interactions with these sites limited the comparison between populations that could be performed.

D values were statistically significant in 11 of the 15 comparisons (Table 3). This generally agrees with the findings of Davy and Murphy (2014), with significant *D* values occurring between a majority of the sites tested in Ontario populations. My findings also agree with what has been documented in the Illinois populations, where Anthonysamy et al., (2017) detected within county structure among sites. This is in contrast to Buchanan, Kolbe, Wegener, Atutubo, and Karraker (2019), who found only a single significant *D* value between the spotted turtle populations they tested in Rhode Island. The results of *D* infer that the geographically close populations of IN-2 and IN-4 are differentiated from one another, even though the differentiation is small 0.071. The urbanization which separates these two sites is likely impeding any genetic flow between these two populations. The presence of major roads and the surrounding intensely urban environment, along with the lack of aquatic connectivity between these sites makes it unlikely that any recent genetic flow has recently occurred between these populations. These values demonstrate that a change between these localities is occurring. Further supporting this assertion is the presence of private alleles within both of these sites. Other notable *D* values are the lack of a difference between OH-1 and OH-2. OH-1 and OH-2 are geographically closest to one another, but they were not clearly clustered together by either of the other clustering programs. *Fst* values were significant in 11 of the 15 comparisons (Table 3). Overall results from *Fst* were similar to the patterns observed in *D*. The differences in the differentiation observed in my study for both *Fst* and *D,* and what has been observed in other studies for this species could be explained by the local history of human interaction, and to what degree and how long genetic flow between populations has been altered. Other factors such as pre settlement landscape changes and the order of dispersal into these current habitats could also influence these values.

Estimation of effective population sizes using LDNe proved difficult for all localities. The effectiveness of LDNe was likely limited by the number of loci that were used. For IN-2 and IN-4 it was possible to combine the populations to attempt to get a more accurate estimate of

population size. OH-1 also had sufficient samples to reasonably run Ne estimator. The results of Ne estimator for the other sites holds less value due to the small number of samples and the inability to combine them with one another due to the geographical distance from one another. Even the combination of IN-2 and IN-4 resulted in a 95% confidence intervals that extended into infinity.

Management Implications

This project helps to lay the groundwork for future captive breeding projects for spotted turtles in the Midwest. My findings show that geographically close populations of spotted turtles that are separated by habitat fragmentation are likely to have developed some degree of differentiation due to geographic isolation. The clustering of the geographically close sites IN-2 and IN-4 suggest that in the future these two populations could be good candidates to participate in a captive breeding project, with IN-4 being the recipient population. Both of these populations maintain high heterozygosity despite being separated from one another. Allelic richness in these two sites was also among the highest detected. The detection of a bottleneck at the IN-4 site further reinforces that this site may benefit from outcrossing between it and IN-2. The geographic closeness and the fact that these sites are showing very low differentiation from one another suggests that it would be unlikely for outcrossing between these populations to result in negative fitness for the offspring. This makes these populations good candidates to be used in captive breeding projects as parents could be collected from both populations with a minimum threat of outcrossing depression. Currently there are plans to develop a captive breeding project using the turtles from OH-1. The fact that this population seems to be highly differentiated from many of the other populations tested means that caution may need to be used in outcrossing members of this population with other populations. From what we see in this data OH-2 looks to be the most suitable site to collect individuals to breed with the turtles in OH-1. I recommend that clutch size and early hatchling success of these outcrosses be closely monitored to test for outcrossing depression as both measures can be used to assess the possible presence of inbreeding or outbreeding depression in turtles (Phillips, Jorgensen, Jolliffe, & Richardson, 2017).

Conclusions

The main goal of this study was to quantify genetic variation in spotted turtles throughout the state of Indiana, as well as compare the populations in Indiana to populations of spotted turtles in Ohio. This study indicates that spotted turtles in Indiana and Ohio have multiple distinct populations that have become differentiated. The structure detected among these populations may warrant independent management of these populations in the future. This is supported by the presence of private alleles in five of the six populations tested, and three distinct clusters among six populations tested. The second key objective of this study was to determine the vulnerability of the spotted turtle populations to current and future decline. Bottleneck tests indicate that two of the six localities have undergone a bottleneck event. This gives support that habitat fragmentation is reducing the genetic variability in turtle populations located in highly fragmented habitats. All of the populations tested did retain a high degree of observed heterozygosity which falls in line with the general consensus that turtle populations are capable or retaining a high degree of genetic variation even after a significant decline (Davy & Murphy, 2014; Rosenbaum, Robertson, & Zamudio, 2007). However, it is important to recognize that once a population is suffering from genetic decline, turtles are equally slow to recover (Kuo & Janzen, 2004; Rosenbaum et al., 2007). It is imperative for preservation of long-lived species such as the spotted turtle to be proactive rather than reactive in order to insure the future success of the species.

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