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POPULATION AND CONSERVATION GENETICS OF CRAWFISH FROGS, *Lithobates areolatus,* AT THEIR NORTHEASTERN RANGE LIMITS

By

Schyler O. Nunziata

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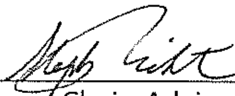

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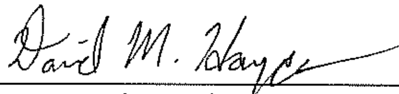

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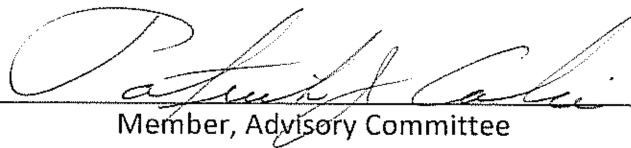

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POPULATION AND CONSERVATION GENETICS OF CRAWFISH FROGS, *Lithobates areolatus,* AT THEIR NORTHEASTERN RANGE LIMITS

By

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Bachelor of Science
Towson University
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ABSTRACT

Crawfish Frogs (*Lithobates areolatus*) are state endangered in Indiana and Iowa, with populations in decline throughout their range. I studied populations of Crawfish Frogs on local and regional scales at their northeastern range limits to (1) assess the level of genetic diversity within populations, (2) estimate fine-scale genetic structure, and (3) estimate genetic differentiation between populations at the regional level. Crawfish Frogs breed in temporary pools and wetlands, and have high breeding-pond fidelity; therefore I predicted to find genetic differences between ponds at small geographic scales. I used 10 microsatellite loci to genotype frogs collected from three primary populations in southeast and southwest Indiana, with distances between sites ranging from 0.29 km to 172 km. Heterozygosity estimates revealed high diversity in these populations (mean Ho: 0.54 to 0.67 per site), which is encouraging for future management. The degree of population subdivision was low at the regional level (F_{ST} = 0.071 for sites within 172 km), with little evidence for genetic structure at a fine scale (F_{ST} = 0.008 for ponds within 1 km). Genetic differentiation was explained by geographic distance between sampling sites, as predicted by an isolation-by-distance model. I observed no genetic differentiation between individuals sampled from ponds ca. 250 m apart, and slight divergence of individuals from a pond ca. 750 m away. This suggests ponds < 1 km from each other form a genetically distinct single breeding unit, made up of multiple subpopulations. Finally, I observed high genetic differentiation between southwest and southeast Indiana populations indicating historical (rather than recent) isolation of these populations. Further research is needed to determine the minimum

distance at which populations become distinct.

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CHAPTER 1

I. INTRODUCTION

One of the primary goals of conservation genetics is to estimate spatial patterns of intraspecific genetic diversity, which is a first step in designing successful management plans for a species (Allendorf and Luikart 2007). For endangered and threatened species, results of genetic surveys can be used to identify populations at risk for inbreeding and genetic erosion (Avise 1989; Frankel and Soulé 1981). Genetic surveys might also help to estimate the degree of connectivity between populations at different spatial scales (Chan and Zamudio 2009; Crowhurst et al. 2011). Estimating gene flow between populations is important in understanding how populations might be impacted by increasing fragmentation of landscapes (Zamudio and Wieczorek 2006). This information can then be applied in designing successful management plans, which may include designating wildlife management units, reconnecting isolated populations, and maintaining gene flow between populations (Crandall et al. 2000).

Contemporary population structure is shaped by current and historical processes on local and regional scales, and signatures of these processes are often evident in neutral genetic variation (Hutchinson and Templeton 1999). Across a landscape, species frequently have uneven distributions with populations linked by levels of gene flow that vary through time and space (Ibrahim 1996; Hewitt 2000). Pond-breeding amphibians often have patchy distributions due to their biphasic life cycle and habitat specificity (Stebbins and Cohen 1995; Pope et al. 2000). Individual ponds, or clusters of neighboring ponds, sometimes act as breeding aggregates with connectivity of these subpopulations

through low levels of dispersal (Breden 1987; Berven and Grudzien 1990). Amphibians generally have limited dispersal ability and strong adult fidelity to breeding sites (Blaustein et al. 1994; Gamble et al. 2007). However, a moderate amount of dispersal by juveniles (~10–20%) and some adults can maintain gene flow between subpopulations (Breden 1987). This connectivity is important for recolonization after local extinctions and maintenance of genetic diversity over the landscape (Marsh and Trenham 2001; Spear et al. 2006).

Studies on the genetic structure of amphibian populations demonstrate species-specific patterns of spatial dynamics; some species show genetic structure at small spatial scales (< 5 km), while others have little structure range wide (e.g. Kusano et al. 1999; Shaffer et al. 2000; Newman and Squire 2001; Petranka et al. 2004; Chan and Zamudio 2009; Blouin et al. 2004). Information on population structure is still absent for most amphibian species, making it difficult to establish general management plans for amphibian communities. Vagility, environmental tolerances, and historical processes will ultimately determine the pattern of population structure and connectivity across a landscape (Gibbs 1998; Guerry and Hunter 2002). By understanding the spatial dynamics of individual amphibian species, appropriate scales of management can be designed to maintain amphibian persistence in a landscape (Semlitsch and Rothermel 2003).

Understanding spatial dynamics of amphibians is especially important in light of ongoing amphibian declines (Alford and Richards 1999; Stuart et al. 2004). The loss of individual amphibian subpopulations may weaken, and ultimately destroy existing landscape connectivity by impacting dispersal and demographic characteristics of the

overall population (Gibbs 1998; Gonzalez et al. 1998). This disruption of genetic connectivity might be the result of habitat fragmentation, or loss of subpopulations from local extinctions (Marsh and Trenham 2001). As subpopulations are lost, the distance between remaining subpopulations increases, which reduces likelihood of dispersal. As individual subpopulations become isolated, they are at an increased risk for genetic erosion, inbreeding, and ultimately extinction (Spielman and Frankham 1992).

Crawfish Frogs, *Lithobates areolatus* [previously *Rana areolata*] (Baird and Girard 1852), are listed as near threatened on the IUCN Redlist (Hammerson and Parris 2004), and are state endangered in Indiana and Iowa (Engbrecht and Lannoo 2010). *Lithobates areolatus* is the sister species to the two gopher frog species, gopher frogs (*L. capito)* and dusky gopher frogs (*L. sevosus)* in the subgenus *Nenirana* (Young and Crother 2001; Hillis and Wilcox 2005). All of these species have declined throughout their respective ranges as a result of habitat loss, disease, and introduction of exotic species (Jensen and Richter 2005; Parris and Redmer 2005; Richter and Jensen 2005). However, all of these species are notoriously elusive, residing in underground burrows for much of the year, and are therefore difficult to monitor (Hoffman et al. 2010). Genetic studies of elusive species are especially important because of the difficulty in obtaining accurate demographic data.

Across Indiana, Crawfish Frogs have a patchy and somewhat clustered distribution in the southwest corner of the state, with one isolated population in the southeast (Engbrecht and Lannoo 2010). Declines of Crawfish Frogs in Indiana were documented beginning in 1970 (Minton 1998); the extinction of many local populations

in Indiana might have resulted in genetic isolation of populations throughout the state. A radio-telemetry study of *L. areolatus* at a wildlife management area in Indiana with three breeding ponds reported high movement ability by adults (> 1 km) and site-fidelity (Heeymeyer et al. in press). Although this species is highly mobile, it has very specific environmental requirements because it uses crayfish holes almost exclusively for upland retreats (Heeymeyer et al. in press). This specialization may limit dispersal and make impacts of fragmentation more severe than for habitat generalists (Marsh and Trenham 2001; Newman and Squire 2001; Zamudio and Wieczorek 2007).

In this study, I examined the genetic structure of Crawfish Frogs, *L. areolatus,* at their northeastern range limits. The goals of this study were to (1) assess the level of genetic diversity within populations, (2) estimate fine-scale genetic structure and connectivity between breeding ponds, and (3) estimate genetic differentiation between populations at the regional level. I sampled in a small network of breeding ponds (<1 km apart) and at the primary populations of Crawfish Frogs in Indiana (three sites separated by 50–172 km). Based on the population structure of other amphibians and observed site fidelity in *L. areolatus,* I predicted I would find genetic structure evident at localized scales, with high divergence over larger geographic scales (Marsh and Trenham 2001). An applied outcome of this research is providing information for conservation programs of Crawfish Frogs.

CHAPTER 2

II. MATERIALS AND METHODS

Study Sites and Population Sampling

From 2009–2011, a total of 189 Crawfish Frogs were sampled from nine breeding ponds at three sites in Indiana (Fig. 1[1]). Two sites were located in southwestern Indiana, the Hillenbrand Fish and Wildlife Area (HFWA) and Dave's Pond (DP), and a third was located in southeastern Indiana, the Big Oaks National Wildlife Refuge (BONWR). The HFWA site has three breeding ponds (Nate's Pond, Big Pond, and Cattail Pond), which were sampled separately (Fig. 2). The DP site is an isolated wetland, bisected by a paved road. The BONWR site contains a series of 23 breeding wetlands, and five were sampled (Bomb Pile, West Bomb Pile, Northwest Bomb Pile, Area 63, and South Perimeter). Because sample size was low at each wetland at BONWR, data were combined for analyses. These sites represent the primary breeding sites for Crawfish Frogs in Indiana, and ponds sampled at additional sites yielded too few individuals to be included in analyses (M.J. Lannoo personal communication).

Individual adult frogs were captured at drift fences, mesh wire traps, or by hand when encountered. Toe clips samples were taken and preserved in 95% ethanol for DNA isolation. Before release, some individuals at HFWA were fitted with radio-telemetry units, and at BONWR were pit-tagged for separate studies (Heemeyer et al. in press; J. Robb, unpublished data). All sampled individuals were reproductive adults sampled

[1] All figures are located in Appendix B.

during the breeding season to help ensure they were breeding at the sampled pond and were not transients.

DNA Extraction and Microsatellite Amplification

Total genomic DNA was extracted using Qiagen® DNEasy Blood and Tissue Kits, following protocols recommended by the manufacturers. Polymerase chain reaction (PCR) was used to amplify 10 microsatellite loci (Lica7, Lica8, Lica11, Lica14, Lica33, Lica37, Lica40, Lica41, Lica44, Lica25) originally developed for *L. capito* (Table 1[2]), following conditions in Nunziata et al. (2011). Genetic data were collected using an ABI 3130 Genetic Analyzer (Applied Biosystems, Inc.). Allele lengths were scored using GeneMapper version 3.7 (Applied Biosystems, Inc.).

Statistical Analyses

Microsatellite alleles were examined for the presence of null alleles and scoring errors with Microchecker v 2.2 (van Oosterhout et al. 2004). Tests for deviations from linkage disequilibrium between all pairs of loci and for deviations from Hardy-Weinberg equilibrium with Bonferroni corrections were performed using GenePop v 4.0.1 (Raymond and Rousset 1995). Allele frequencies, observed (H_O) and expected (H_E) heterozygosity, and standardized allelic richness (calculated using rarefaction based on the minimum number of samples per population) were estimated in FSTAT v 2.9.3

[2] All tables are located in Appendix B.

(Goudet 1995). Differences in genetic variation among sampling sites were evaluated using Independent-Samples Kruskal-Wallis Tests in SPSS v 18.0 (IBM Corporation).

To estimate degree of genetic subdivision among populations, F_{ST} was calculated across all sampling sites, and separately for HFWA sites in FSTAT v 2.9.3 (Goudet 1995). To test for genetic distance between sampling sites, pairwise F_{ST} (Weir and Cockerham 1984), was calculated in Arlequin v 3.5.1.2, and Goodman's estimate of R_{ST} (Goodman 1997) was calculated in RSTCALC v 2.2 (Goodman 1997), using permutation tests for significance with 1,000 permutations. To test for a relationship between genetic distance and geographical distance, $F_{ST} / (1– F_{ST})$ was compared to geographic distance across all sites and within HFWA using Mantel tests with 1,000 permutations in IBDWS v 3.21 (Jensen et al. 2005; Rousset 1997).

To examine data for population structure utilizing individual genotypes, a Bayesian assignment technique was implemented with Markov chain Monte Carlo (MCMC) algorithms to identify clusters (K) of genetically similar individuals using Structure v 3.2.3 (Pritchard et al. 2000; Hubisz et al. 2009). Ten replicate runs were performed consisting of 200,000 iterations for simulation burn in followed by 500,000 iterations for each K from 1 to 5, including no prior information about sampling location and again with sampling location included. Following these runs, means and standard deviations for each estimated K were calculated and the delta K statistic was used to determine the likely number of groups, using Structure Harvester v 0.6.8 (Evanno et al. 2005; Earl and vonHoldt 2011).

CHAPTER 3

III. RESULTS

Genetic Diversity

Microchecker detected the presence of null alleles at Lica8 in Nate's Pond. Because there was no evidence for null alleles at this locus in other populations or during primer development (Nunziata et al. 2011), it was retained for analyses. There was evidence for linkage disequilibrium at 5 of 45 loci combinations ($P < 0.0011$). Of the 50 tests for HWE, two deviated from expected after Bonferroni correction (Table 2). Observed and expected heterozygosity ranged from 0.00–0.91 and 0.00–0.84, respectively, and there were no differences among sample sites (Table 2). Total number of alleles across sampling sites ranged from 2 to 11 per locus (Table 2). After rarefaction, allelic richness was highest in the HFWA sample sites and Dave's Pond and lower at BONWR (Table 2); however allelic richness was only statistically different between Cattail Pond and BONWR ($P = 0.022$).

Population Structure

Overall F_{ST} was 0.071, and F_{ST} within HFWA was 0.008. Pairwise F_{ST} and R_{ST} values showed a similar pattern, with R_{ST} indicating slightly more population divergence (Table 3). F_{ST} values indicated that only Big Pond and Cattail Pond did not differ significantly from each other, while R_{ST} indicated that Big Pond, Cattail Pond, and Nate's Pond did not differ significantly from each other (Table 3). Genetic differences between all sampling

sites were explained by geographic distances between them as predicted by an isolation-by-distance model (Figure 3; Mantel test; P = 0.0190; R^2 = 0.897). Although a similar pattern was evident within HFWA (Figure 4), low sample size (n= 3) precluded statistical analysis.

Structure analyses identified two distinct population groups when evaluated with the delta K statistic (Table 4, Figure 5). Both models, with sampling location included as a prior and without, gave similar results and resolution did not increase greatly when sampling location was included as a prior (Table 4). BONWR samples were assigned to one cluster, and DP and HFWA samples were assigned to the second, with a small amount of admixture and strong support from the Evanno test (Figure 5).

CHAPTER 4

IV. DISCUSSION

Genetic Diversity

Diversity within populations was comparable to other frogs in the genus *Lithobates*, which commonly have H_E over 0.400 (reviewed in Blouin et al. 2010). A study of *L. areolatus* and its the sister species, revealed levels of heterozygosity ranging from 0.174 to 0.826 for *L. sevosus*, 0.595 to 0.946 for *L. capito*, and 0.625 to 0.875 for *L. areolatus* (Richter et al. 2009). Level of heterozygosity in *L. areolatus* was slightly decreased in this study compared to those in Richter et al. (2009), but it should be noted that different microsatellite markers were used. Richter et al. (2009) sampled Crawfish Frogs in Oklahoma in a contiguous landscape, with multiple breeding ponds, and no documented population reductions. The decreased heterozygosity observed in this study compared to those in Richter et al. (2009) may be the result of population reductions and isolation in Indiana. Another possible explanation is that the Indiana populations are on the periphery of the range of Crawfish Frogs, which may explain the decreased genetic diversity and lowered gene flow (Crowhurst et al. 2011).

However, diversity within Crawfish Frog populations in this study was still higher compared to critically endangered amphibian species. One species of the sister group to *L. areolatus, L. sevosus,* had levels of heterozygosity averaging 0.54 (Richter et al. 2009), and other endangered amphibian species often exhibit heterozygosity under 0.5 (Krvvijeveld-Smit et al. 2005; Ficetola et al. 2011). Genetic diversity id still high, which is

encouraging for future genetic management of Crawfish Frog populations in Indiana. My results serve as a baseline for future genetic monitoring of these populations.

Western Indiana sampling sites had greater genetic diversity than the BONWR site based on allelic richness, even though genetic diversity at BONWR may have been artificially inflated due to pooling of individuals from multiple, geographically distant ponds (1–10 km). Crawfish frogs in Indiana were historically only documented in the southwest region of the state, and the southeastern BONWR population was only recently documented in 2003 (Haswell 2004). This isolated population is 90 km from the nearest western-Indiana population and other Crawfish Frog populations, and it is unknown whether the population is naturally occurring or introduced. At this point, it is difficult to determine whether low diversity is the result of isolation or a founder effect (Frankham 1995; Cornuet and Luikart 1996; Johansson et al. 2007). Because data for individuals were combined from multiple sites, no bottleneck or founder effect tests could be conducted. These results suggest need for more intensive sampling at BONWR.

Genetic Structure

Overall genetic differentiation between sampling sites was low, but showed a positive correlation with geographic distance. At a fine scale, there was little genetic differentiation suggesting a high level of genetic connectivity between breeding ponds that decreased with geographic distance. Ponds within 250 m of each other showed no genetic divergence, and a pond 750 m away was weakly divergent. The genetic similarity at this site could be the result of two non-mutually exclusive hypotheses. The HFWA was only recently colonized by *L. areolatus,* because it was surface mined for coal until 1982

and then reclaimed to grasslands in 1983 (Lannoo et al. 2009). During mining all natural wetlands were destroyed (M.J. Lannoo, personal communication), so the surveyed ponds were only colonized within the past two decades from surrounding lands. There was potential for multiple founder events and sources, as multiple ponds surrounding the HFWA have had documented populations of *L. areolatus* (Fig. 6). Because this site was only recently colonized, it may have not reached genetic drift-gene flow equilibrium that is assumed when estimating genetic divergence (Lowe and Allendorf 2010). Therefore, the current level of gene flow may be over-estimated at the site.

Another possible explanation for the lack of genetic divergence at the HFWA is the movement of individuals to breed in non-natal ponds. In a radio-telemetry study at HFWA, one of eight radio-tracked adults shifted breeding ponds while others had fidelity over two successive years despite migrating past other potential breeding ponds (Heeymeyer et al. in press). Juveniles represent the majority of dispersers in many amphibian species, so it is likely this is the dispersal stage for Crawfish Frogs (Semlitsch 2007). Studies of other amphibians revealed high breeding site fidelity of adults with some juveniles dispersing to breed in non-natal ponds, causing small networks of ponds with little genetic divergence and higher divergence at larger scales (Berven and Grudzien 1990; Gamble et al. 2007).

Overall F_{ST} values were comparable to other ranid amphibians studied over similar geographic distances; *Rana arvalis* had overall F_{ST} of 0.065 over 0.3–150 km (Vos et al. 2001), and *L. sylvaticus* had an overall F_{ST} of 0.014 over 0.5–20 km (Newman and Squire 2001). These studies also showed weak divergence on a fine-scale, and evidence

for divergence over large distances. Even a small number of dispersers can maintain genetic similarity between ponds, based on the one-migrant per generation rule (Wright 1931). In non-ideal populations where effective population size (N_e) is often less than census size, more dispersers are needed to maintain genetic homogeneity between ponds, especially in species with fluctuating populations like amphibians (Vucetich and Waite 2000). Therefore, a moderate portion (>1 migrant per generation) of individuals likely disperse between neighboring ponds to maintain the genetic similarity observed at HFWA. At the next step up in scale, lower levels of differentiation between HFWA and DP suggest historic or a low level of recent gene flow through a stepping-stone pattern of dispersal (Kimura and Weiss 1964).

Gene flow between breeding populations at neighboring ponds might be sufficient to impact the population dynamics and long-term survival of individual populations (Hanski and Gilpin 1991). The disruption of networks of breeding ponds not only decreases overall population size, but also increases the distance a disperser must travel to the nearest neighboring ponds (Semlitsch and Bodie 1998). The periodic drying of ponds and other factors might cause localized extinction events, but connectivity between ponds also permits recolonization of ponds following these events (Marsh and Trenham 2001; Semlitsch 2002).

I found two statistically significant population clusters, dividing the eastern and western halves of the state. These sites are separated by ca. 150 km, and the closest (unsampled) western population is about 90 km from BONWR (Engbrecht and Lannoo 2010). There are physiographic barriers and anthropogenic land fragmentation that

cause resistance or complete barriers to dispersal between the eastern and western halves of the state. High divergence at BONWR indicates historical isolation (instead of recent) between the two sides of the state. Another possibility for high differentiation is that the BONWR population is introduced, and its origin may be from a different part of the range. More data is needed to estimate the cause of the divergence.

Conservation Implications

My results suggest two distinct management units in Indiana; one in the southwest and one in the southeast. These populations are highly divergent from each other, and may contain locally adapted alleles or allelic combinations. Management programs involving translocations and reintroductions of Crawfish Frogs in Indiana should consider this to avoid possible outbreeding depression (Lynch 1991). Additionally, translocations of Crawfish Frogs can be dangerous to wild populations because of potential disease transmission, e.g. the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) has been documented at ponds at HFWA (Kinney et al. 2011).

The high genetic diversity in remaining populations of Crawfish Frogs is encouraging for future management plans of the species. My results indicate that neighboring ponds within at least 1 km from each other form a genetically distinct single breeding unit, made up of multiple subpopulations. Loss of this gene flow between neighboring subpopulations may lead to loss of genetic diversity in local populations (Richter et al. 2009). To preserve population dynamics of the species, management should also focus on networks of ponds, instead of individual ponds. Establishing nearby breeding ponds has proven to be successful in other ranid species, and may be a

management option for this species (Chelgren et al. 2008). Therefore, maintenance of genetic connectivity between existing breeding ponds is an important management option for the persistence of *L. areolatus* populations in Indiana and should also be considered for the closely related and endangered species of gopher frogs.

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APPENDIX A:

Tables

Table 1. Primer information for microsatellite loci used in this study. (K = 186)

Locus	Primer Sequence 5' --> 3'	Repeat motif	Size (bp)	K
Lica7	F: GGGCTGAGAACTAACGTGC R:GTGTGCATCTACACAAGGGC	(AGAT)10	318-354	9
Lica8	F: TCTTCCACTACTCTGGAAAGC R: TGTGTGATACGCAGTTCCTTC	(AGAT)11	343-431	11
Lica11	F: TTTCAGGCCAGCATCAATGG R:ACTTGCAACGACTAGAGCC	(AGAT)14	144-156	4
Lica14	F: AGCAAACTGACACCTCCAG R: CAGAACTTTGGAAGTAGAAGCCC	(ATCT)14	257-313	9
Lica25	F:CGAATGAATTTGTCGCTCTACG R: GGCGACTACACACTGTTCTTATC	(ATCT)13	292-336	9
Lica33*	F:CGGATCTGCAGCGAATAATG R:TGGCAAGAAGAATATTGGGC	(AGAT)16	213–257	7
Lica37*	F:GTCACTATCCTCAAGGTG R:GTCCAAGATAGAAGGAGGAC	(CTTT)14	186–226	7
Lica40	F: CAGTGTGAACCAGGGCTTTG R: CCTGCCTAGAGAGTCTTCCG	(ATCT)14	300-360	10
Lica41	F: GGGTGGATAACACACTAGG R: CCACCCGGTAATATAAAGCTGTG	(AGAT)9	263-311	11
Lica44	F: TCTTGTAGCACAAAGCGGTG R: CCGTGCAATGTATCTCTGG	(AGAT)11	272-276	2

* Indicates unpublished primers

Table 2. Observed (H_O) and expected (H_E) heterozygosity for each locus and study area. Loci out of HWE are in bold.

	Nate's Pond		Big Pond		Cattail Pond		Dave's Pond		BONWR	
Sample size	47		44		51		11		36	
Mean allelic richness	4.77 (1.68)		4.84 (1.69)		5.22 (1.8)		3.8 (1.69)		2.93 (0.95)	
Locus	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E
Lica7	0.68	0.67	0.70	0.69	0.66	0.71	0.73	0.71	0.46	0.41
Lica8	0.68	0.81	0.86	0.75	0.75	0.81	0.73	0.77	0.68	0.62
Lica11	0.53	0.59	0.52	0.53	0.59	0.67	0.55	0.42	0.56	0.41
Lica14	0.80	0.81	0.86	0.81	0.82	0.78	0.27	0.57	0.47	0.48
Lica33	0.69	0.73	0.80	0.83	0.73	0.84	0.55	0.78	0.36	0.41
Lica37	0.52	0.54	0.57	0.52	0.43	0.51	0.18	0.18	**0.47**	**0.49**
Lica40	0.80	0.78	0.80	0.82	0.84	0.80	0.82	0.79	0.42	0.58
Lica41	**0.81**	**0.83**	0.91	0.83	0.90	0.83	0.82	0.68	0.78	0.74
Lica44	0.22	0.20	0.09	0.09	0.19	0.17	0	0	0	0
Lica25	0.76	0.65	0.55	0.67	0.71	0.71	0.64	0.71	0.71	0.75
Mean	0.65	0.66	0.67	0.65	0.66	0.68	0.59	0.62	0.54	0.54
s.d.	0.18	0.19	0.25	0.23	0.21	0.21	0.23	0.20	0.14	0.14

Table 3. Genetic distance values for Crawfish Frog populations in Indiana. Pairwise F_{ST} values are reported below the diagonal, R_{ST} values are above.

	Nate's Pond	Dave's Pond	Big Pond	Cattail Pond	BONWR
Nate's Pond	-	0.1350***	**-0.0003**	**0.0030**	0.1400***
Dave's Pond	0.10093***	-	0.0998**	0.1133***	0.3045***
Big Pond	0.01012**	0.09208***	-	**-0.0024**	0.1047**
Cattail Pond	0.01055***	0.07911***	**0.00183**	-	0.0946***
BONWR	0.13297***	0.17531***	0.14641***	0.12778***	-

*P<0.05,**P<0.01, and ***P<0.001 as determined by permutation test with 1,000 permutations

Table 4. Evanno statistics for the detection of Crawfish Frog populations using Structure v 2.3.2.

K	Avg ln P(K)	SD ln P(K)	Delta K
No Prior on Sampling Site			
1	-5258.72	0.2098	n/a
2	-4906.82	0.9378	285.39
3	-4822.57	1.8136	10.06
4	-4756.56	3.2308	38.77
5	-4815.80	31.9275	n/a
With Prior on Sampling Site			
1	-5258.63	0.1767	n/a
2	-4901.07	0.8314	309.83
3	-4801.1	2.0811	42.47
4	-4789.51	28.1396	7.06
5	-4976.56	366.7214	n/a

APPENDIX B:

Figures

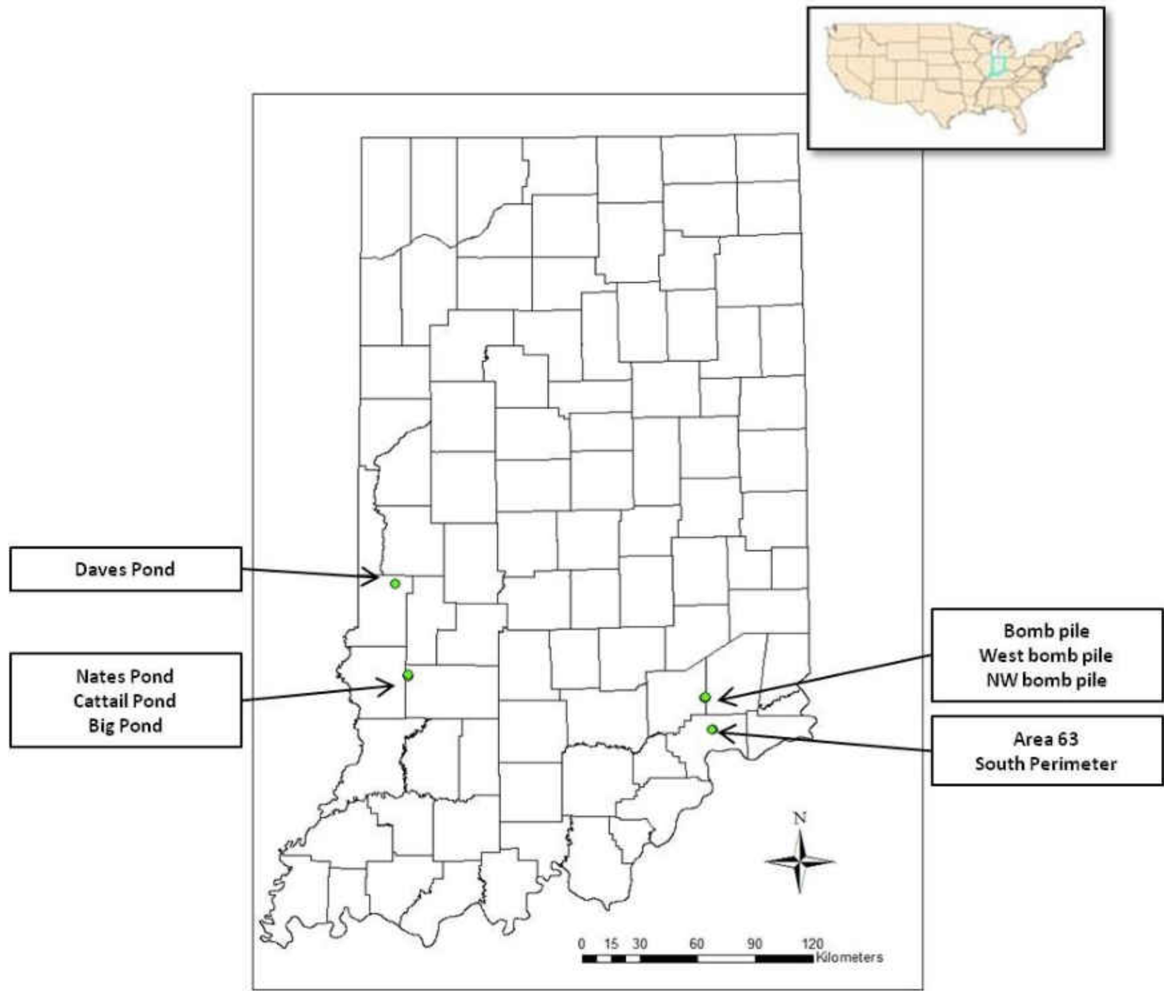

Figure 1. Map of Indiana with localities for four *Lithobates areolatus* populations sampled for this study. Ponds sampled at each site are labeled by name.

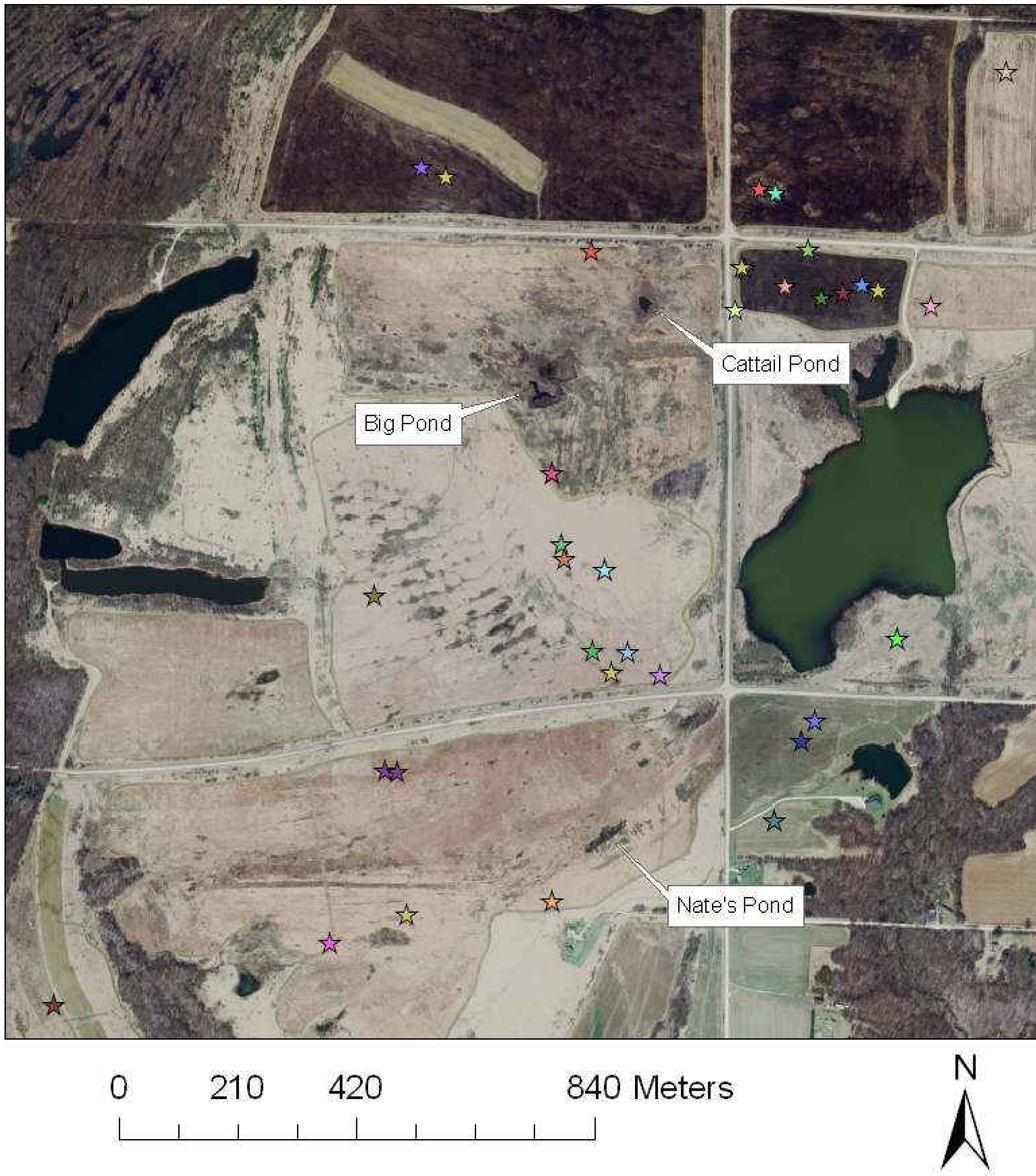

Figure 2. Aerial image of 3 ponds sampled for Lithobates areolatus at Hillenbrand Fish and Wildlife Area, Indiana. Source: Heemeyer J. L., P. J. Williamas, and M. J. Lannoo. (in press). Obligate crayfish burrow use and *core habitat requirements of crawfish frogs. Journal of Wildlife Management.*

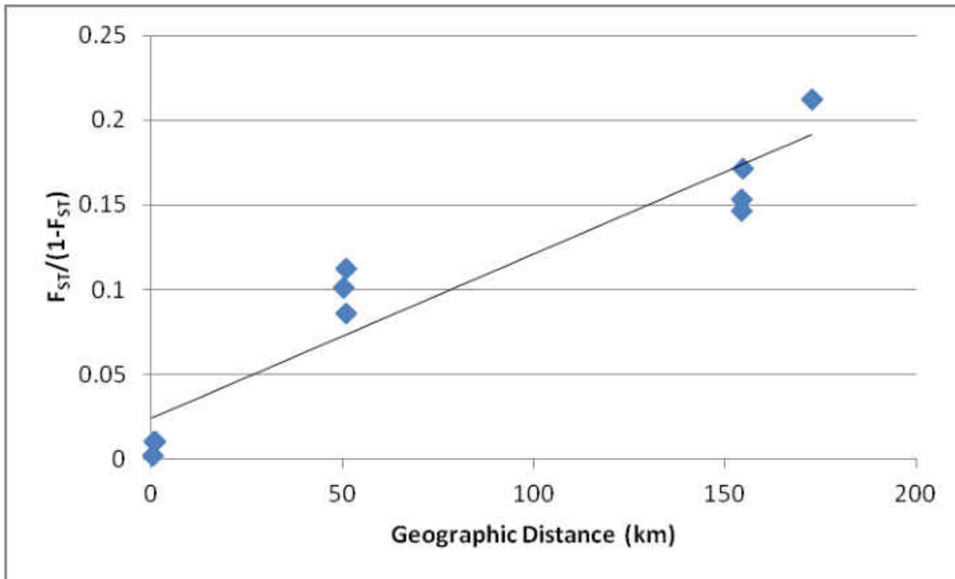

Figure 3. Genetic distance ($F_{ST}/(1–F_{ST})$) plotted against geographic distance (km) for all sampling sites. The solid line represents the best-fit linear regression.

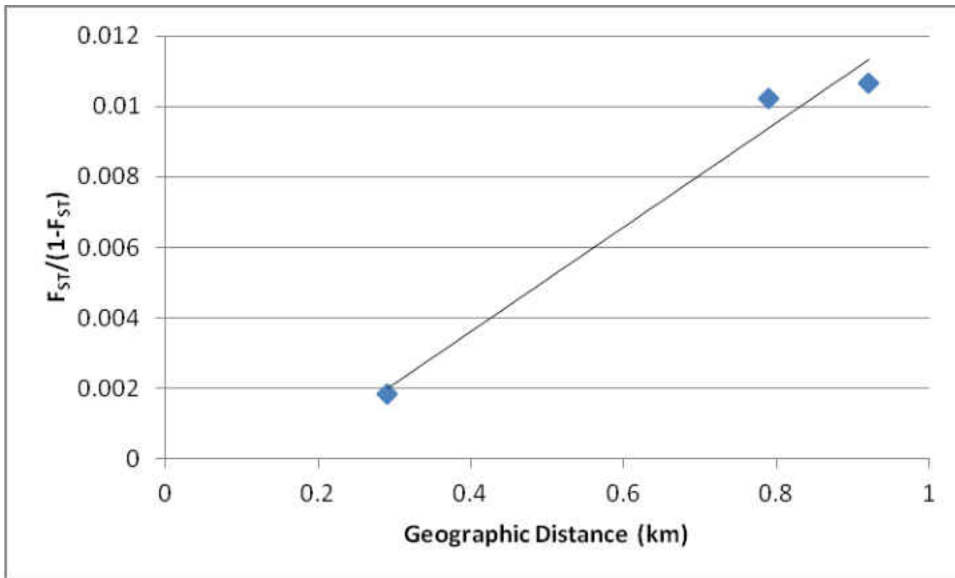

Figure 4. Genetic distance ($F_{ST}/(1–F_{ST})$) plotted against geographic distance (km) for Hillenbrand Fish and Wildlife Area sites only. The solid line represents the best-fit linear regression.

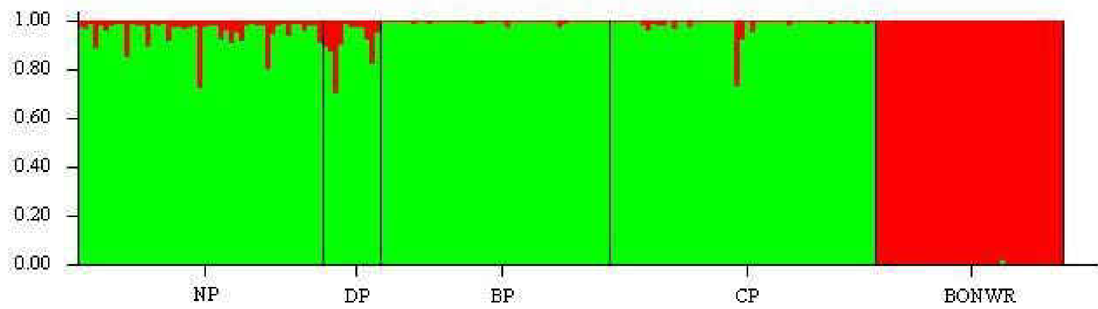

Figure 5. Results of analysis of population structure in Structure 2.3.2. Pond abbreviation codes are listed in Table 2.

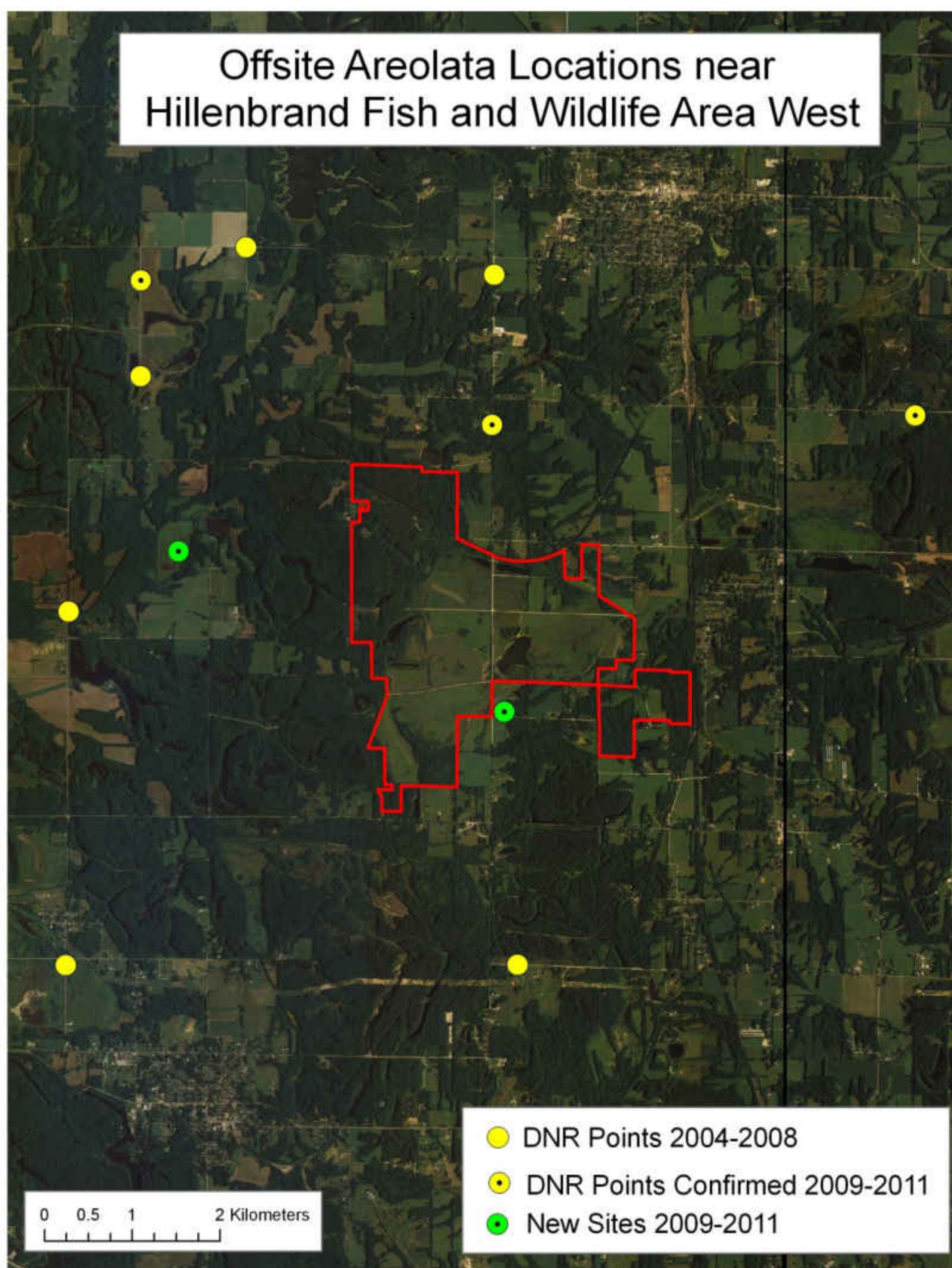

Figure 6. Documented breeding ponds for *Lithobates areolatus* surrounding Hillenbrand Fish and Wildlife Area. Hillenbrand Fish and Wildlife Area outlined in red, sampling sites from this study not represented. Source: Nathan Engbrecht, unpublished data

APPENDIX C:

List of Concepts

Bonferroni Correction: A correction of alpha level applied to offset the problem of increased probability of Type I error (incorrectly rejecting a false hypothesis) when performing multiple statistical tests. The significance level is changed to α/n to achieve your desired significance level (α), when testing n tests.

F_{ST}: A measure of population differentiation measured as differences in allele frequency among populations. Developed by Sewell Wright (1951), it is widely used to quantify genetic differentiation.

Genetic Connectivity: Gene flow between populations.

Genetic Differentiation: Differences in allelic and genotypic frequencies between sampled populations.

Genetic Diversity: Genetic variation in populations which can be measured as the level of heterozygosity or number of alleles.

Genetic Structure: Difference in allele frequency between sampled populations.

Hardy-Weinberg equilibrium: The proportions of homozygotes and heterozygotes expected in a large, closed, panmictic population when allele frequencies are known. Assumptions include no mutation or selection, so that allele frequencies are constant across generations.

Heterozygosity: The proportion of heterozygotes in a population. Can be expressed as either observed or expected (under Hardy-Weinberg equilibrium)

Rarefaction: A mathematical approach for estimating the number of alleles expected in a random sample of individuals taken from a population. In genetic studies, when comparing populations that differ in sample size, larger samples are more likely to contain more alleles than small samples. Rarefaction accounts for this difference in sample size, and allows for comparison of allelic richness between samples of different sizes.

R_{ST}: An analogue to F_{ST}, but takes into account the step-wise mutation rate that is unique to microsatellites.