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Population and Conservation Genetics of Crawfish Frogs, *Lithobates areolatus*, at Their Northeastern Range Limit

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ABSTRACT.—**Crawfish Frogs (*Lithobates areolatus*) are a North American ranid, considered near threatened globally with populations in decline throughout their range. We studied populations of Crawfish Frogs on local and regional scales at their northeastern range limit 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. We used 10 microsatellite loci to genotype frogs collected from three regional sites in Indiana separated by 50–172 km and at one of these sites within a network of three breeding ponds <1 km apart. Heterozygosity estimates revealed high levels of diversity within these populations (mean H_O: 0.54–0.67 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). Genetic differentiation was related to geographic distance between sampling sites, as predicted by an isolation-by-distance model. We observed no genetic differentiation between individuals sampled from ponds approximately 250 m apart and slight divergence of individuals from a pond approximately 750 m away. This suggests ponds within 1 km form a genetically distinct single breeding unit composed of multiple subpopulations. Finally, we observed high genetic differentiation between southwest and southeast Indiana sites indicating historical (rather than recent) isolation of these sites. These data will be applied to a regional management plan in an attempt to recover Crawfish Frogs along the northeastern extreme of their range.**

For endangered and threatened species, results of genetic surveys can be used to identify populations at risk for inbreeding depression and genetic erosion, both of which reduce fitness (Frankel and Soulé, 1981; Avise, 1989) and to estimate scale of connectivity among populations (Chan and Zamudio, 2009; Crowhurst et al., 2011). This information can then be applied in designing management plans, which might 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 acting at local and regional scales, with signatures of these processes often evident in neutral genetic variation (Gibbs, 1998; Hutchinson and Templeton, 1999; Guerry and Hunter, 2002). Across a landscape, species frequently have uneven distributions with populations linked by levels of gene flow that vary through time and space (Ibrahim et al., 1996; Hewitt, 2000). Pond-breeding amphibians often have patchy distributions caused by 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; Marsh and Trenham 2001).

Studies on the genetic structure of amphibian populations have shown species-specific patterns of spatial dynamics; some species demonstrate genetic structure at small spatial scales (<5 km), whereas others have little structure rangewide (e.g., Kusano et al., 1999; Shaffer et al., 2000; Newman and Squire, 2001; Petranka et al., 2004; Chan and Zamudio, 2009). 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 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 regional and ultimately species-level diversity of amphibians (Semlitsch and Rothermel, 2003).

Crawfish Frogs, *Lithobates areolatus* (previously *Rana areolata*), are members of the subgenus *Nenirana* (Hillis and Wilcox, 2005) and are of conservation concern. Crawfish Frogs are listed as near threatened globally (IUCN, 2011) and are state endangered in Indiana and Iowa (Engbrecht and Lannoo, 2010). Minton (1998) documented severe Crawfish Frog declines in Indiana beginning in 1970, with local extinctions producing the geographic isolation of many populations. Today, Indiana Crawfish Frogs have a patchy distribution centered in the southwest, with one isolated population in the southeast (Engbrecht and Lannoo, 2010). For such endangered and threatened species, genetic surveys can estimate the scale of connectivity among populations (Chan and Zamudio, 2009; Crowhurst et al., 2011) and identify populations at risk for inbreeding depression and genetic erosion, both of which reduce fitness (Frankel and Soulé, 1981; Avise, 1989).

Here, we examine the genetic structure of Crawfish Frogs in Indiana, at their northeastern range limit. Our goals were to (1) assess the level of genetic diversity within populations, (2) estimate fine-scale genetic structure between breeding ponds, and (3) estimate genetic differentiation between populations at the regional level. We sampled adults from three regional sites separated by 50–172 km and at one of these sites within a network of three breeding ponds <1 km apart. Based on the population structure and site fidelity of Crawfish Frogs (Kinney, 2011; Heemeyer and Lannoo, 2012; Heemeyer et al., 2012), we expected to find genetic structure evident at small scales (<1 km apart), with high divergence over larger geographic scales

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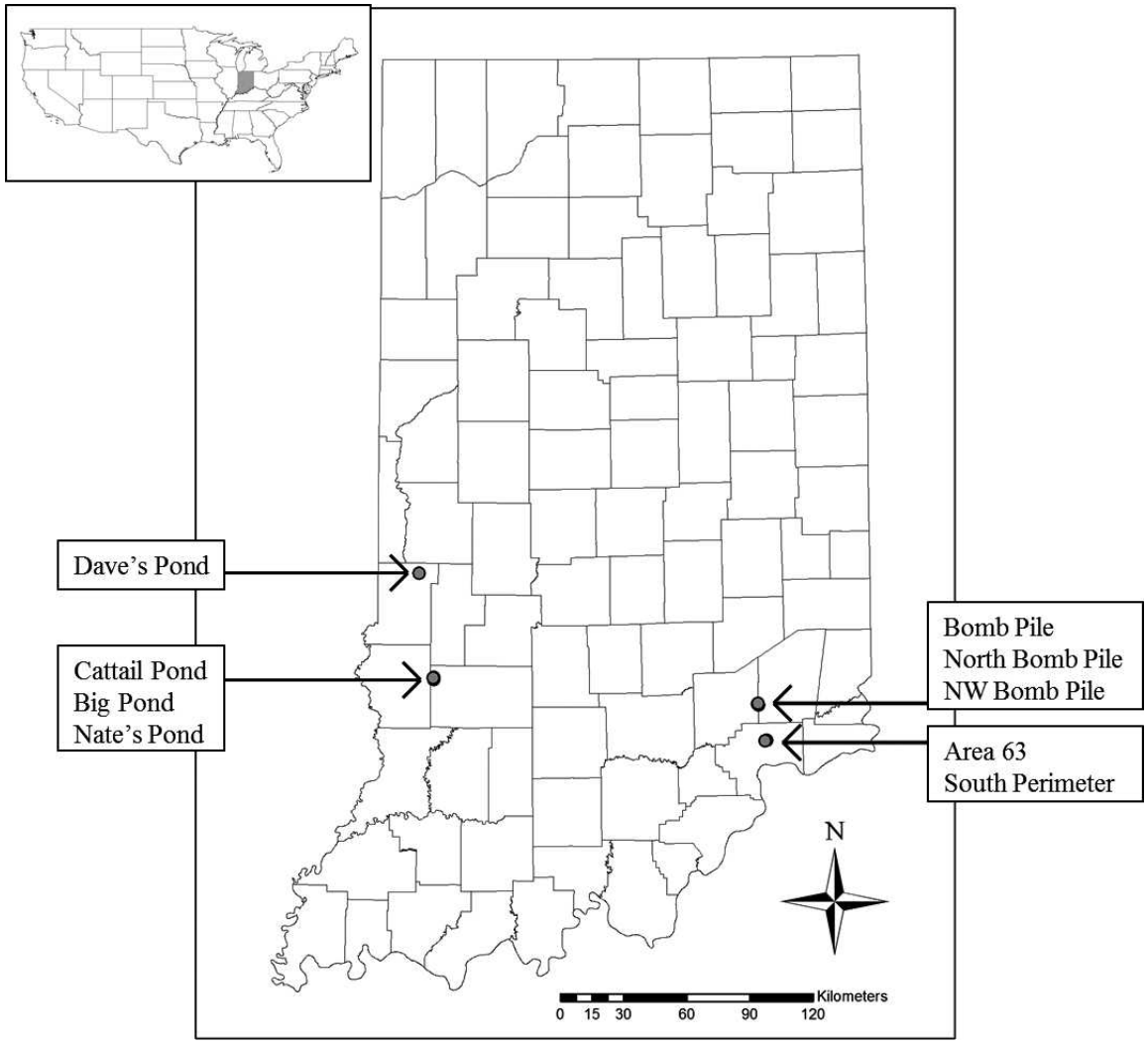

FIG. 1. Map of Indiana with localities of our three study sites: Hillenbrand Wildlife Management Area (Nate's Pond, Big Pond, and Cattail Pond), Dave's Pond, and Big Oaks National Wildlife Refuge (BONWR). Ponds sampled at each site are labeled by name.

(Marsh and Trenham, 2001). This research will assist the conservation and management planning of Crawfish Frogs by providing baseline data on population genetic variability and information on geographic scale and genetic extent of population structure.

MATERIALS AND METHODS

Study Sites and Population Sampling.—From 2009–11, we sampled 189 Crawfish Frogs from three regional sites (Fig. 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 pond, bisected by a paved road. The BONWR site contains a series of at least 25 breeding ponds, and five were sampled (Bomb Pile, West Bomb Pile, Northwest Bomb Pile, Area 63, and South Perimeter). Because sample size was low ($\leq$9) at each pond at BONWR, data were combined for analyses. Our three regional sites represent the primary breeding areas for Crawfish Frogs in Indiana; ponds sampled at additional sites yielded too few individuals to be included in analyses.

Individual adult frogs were captured at drift fences, with mesh wire traps, or by hand when encountered. Toe clips were taken and preserved in 95% ethanol for DNA isolation. Before release, some individuals at HFWA and BONWR were fitted with radiotelemetry units, and were marked using PIT (passive integrated transponder) tags for separate studies (Heemeyer et al., 2012; Williams et al., 2012). All sampled individuals were reproductive adults captured during the breeding season to help maximize likelihood these individuals 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, eight of which were developed for *Lithobates capito* (Nunziata et al., 2012; Lica7, Lica8, Lica11, Lica14, Lica25, Lica40, Lica41, and Lica44) and two of which were optimized for this study (Table 1; Lica33 and Lica37). PCR conditions for each locus followed Nunziata et al. (2012). Fragment analysis data were collected using an ABI 3130xl Genetic Analyzer (Applied

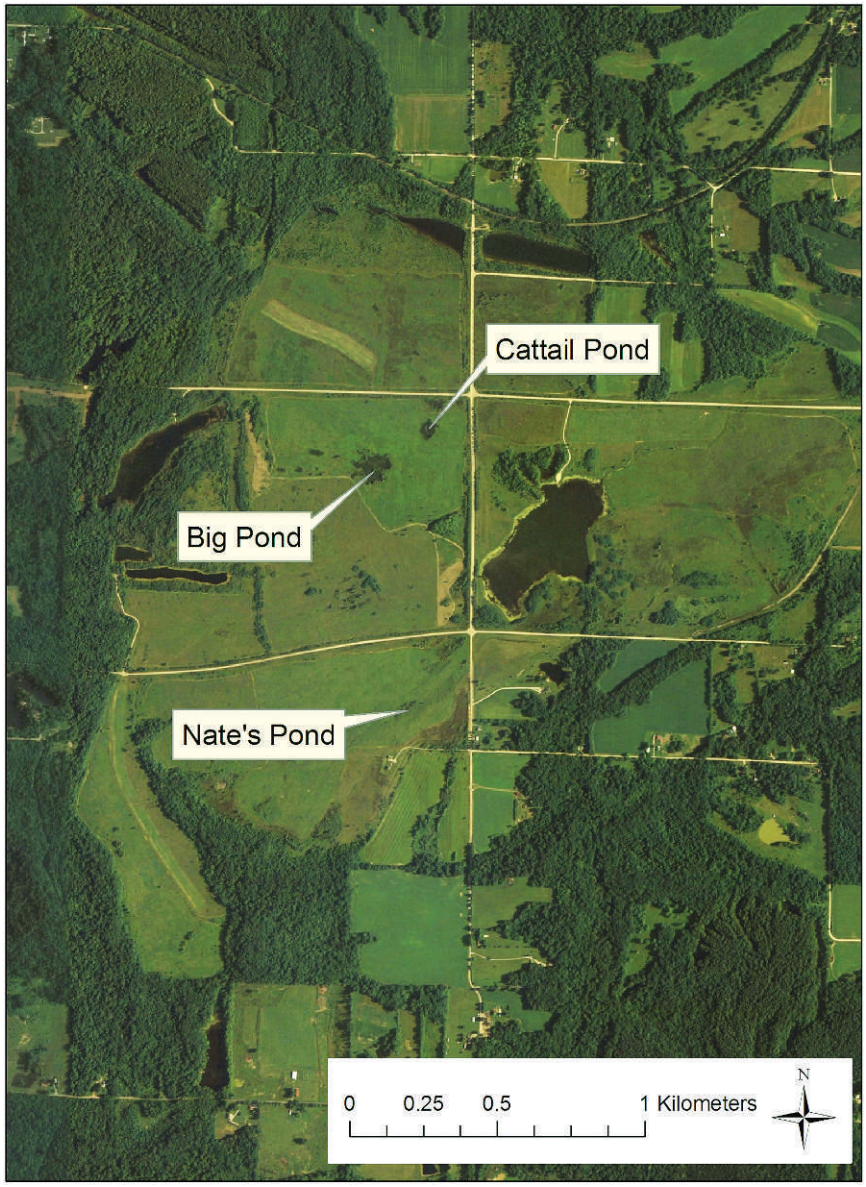

FIG. 2. Aerial image of three ponds sampled for *Lithobates areolatus* at Hillenbrand Fish and Wildlife Area, Indiana (Heemeyer et al., 2012).

Biosystems, Inc.). Allele lengths were scored using GeneMapper version 3.7 (Applied Biosystems, Inc.).

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

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 (Excoffier

TABLE 1. Primer information for two new microsatellite loci used in this study. For information about other primers, see Nunziata et al. (2012). K = 186.

Locus	Primer sequence 5′ to 3′	Repeat motif	Size (bp)	K
Lica33	F:CGGATCTGCAGCGAATAATG R:TGGCAAGAAGAATATTGGGC	(AGAT)16	213–257	7
Lica37	F:GTCACTATCCTCAAGGTG R:GTCCAAGATAGAAGGAGGAC	(CTTT)14	186–226	7

TABLE 2. Observed (H_O) and expected (H_E) heterozygosity for each locus across our three study sites: Hillenbrand Wildlife Management Area (Nate's Pond, Big Pond, and Cattail Pond), Dave's Pond, and Big Oaks National Wildlife Refuge (BONWR). Loci out of HWE are in bold.

	Nate's Pond (NP)		Big Pond (BP)		Cattail Pond (CP)		Dave's Pond (DP)		BONWR	
Sample size	47		44		51		11		36	
Mean allelic richness (SD)	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
SD	0.18	0.19	0.25	0.23	0.21	0.21	0.23	0.20	0.14	0.14

and Lischer, 2010), and Goodman's estimate of R_{ST} 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 (Rousset, 1997; Jensen et al., 2005). Hierarchical partitioning of genetic variance was examined using an analysis of molecular variance (AMOVA) to examine the distribution of genetic variation at two hierarchal levels: among individuals within populations, and among populations within sites using GenAlEx (Peakall and Smouse, 2006).

To examine data for population structure using 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, followed by 500,000 iterations for each K from 1–5, 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).

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., 2012), it was retained for analyses. There was evidence for linkage disequilibrium at 5 of 45 paired locus 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 2–11 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 (within HFWA) 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}-values 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, whereas R_{ST}-values 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 (Fig. 3a; Mantel test; $P = 0.0190$; $R^2 = 0.897$). Although a similar pattern was evident within HFWA (Fig. 3b), low number of ponds at HFWA ($N = 3$) precluded statistical analysis. The hierarchal AMOVA revealed that 88% of genetic variation is within populations, and 12% is distributed among populations ($P = 0.01$).

Structure analyses identified two distinct population groups when evaluated with the delta K-statistic (Table 4, Fig. 4). Both models, with sampling location included as a prior and without,

TABLE 3. Genetic distance values for Crawfish Frog populations at ponds across our three study sites: Hillenbrand Wildlife Management Area (Nate's Pond, Big Pond, and Cattail Pond), Dave's Pond, and Big Oaks National Wildlife Refuge (BONWR). 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

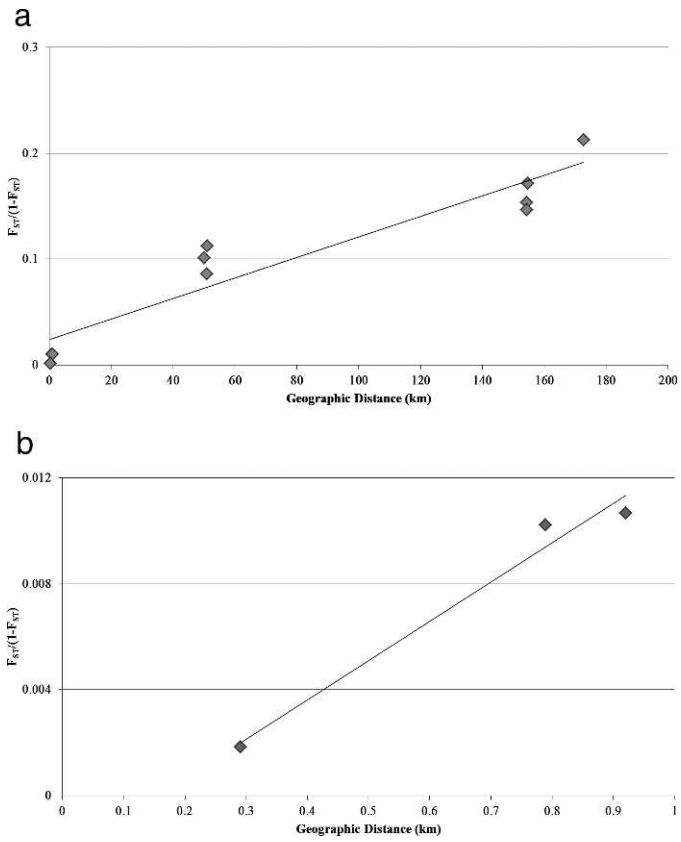

FIG. 3. (a) Genetic distance ($F_{ST}/[1–F_{ST}]$) plotted against geographic distance (km) for populations at ponds across all sampling sites. (b) Genetic distance ($F_{ST}/[1–F_{ST}]$) plotted against geographic distance (km) for Hillenbrand Fish and Wildlife Area ponds only. The solid line represents the best-fit linear regressions.

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 (Fig. 4).

DISCUSSION

Genetic Diversity.—Despite documented declines and an increasingly patchy distribution (Engbrecht and Lannoo, 2010), genetic diversity within the Crawfish Frog populations studied here (0.540–0.680) was comparable to other frogs in the genus *Lithobates*, which commonly have H_E over 0.500 (Newman and Squire, 2001; Zeisset and Beebee, 2003; Funk et al., 2005). A study of *L. areolatus* and its sister clade (Gopher Frogs, *L. capito* and *Lithobates sevosus*) revealed levels of heterozygosity ranging from 0.174–0.826 for *L. sevosus*, 0.595–0.946 for *L. capito*, and 0.625–0.875 for *L. areolatus* (Richter et al., 2009). Level of heterozygosity in *L. areolatus* was lower in our study compared to nonisolated populations of both *L. areolatus* and *L. capito* in Richter et al. (2009), but it should be noted that different microsatellite loci were used. However, diversity within our Crawfish Frog populations was still higher compared to a geographically isolated population of endangered *L. sevosus* (Richter et al., 2009) and to other endangered amphibian species, which often exhibit heterozygosity under 0.500 (Kraaijeveld-Smit et al., 2005; Blouin et al., 2010; Ficetola et al., 2011).

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

Comparison of our results to studies of other North American ranids is also encouraging. For example, a threatened amphibian in the Pacific Northwest, *Rana pretiosa*, with a similar history of population fragmentation to our study system showed lower genetic diversity and higher population differentiation compared to our results (Blouin et al., 2010). Additionally, our results were similar to those of a non-threatened congener, *Rana cascadae*, in genetic diversity and lower differentiation among populations. Current genetic diversity of these Crawfish Frog populations is encouraging for future genetic management, to prevent future loss of genetic diversity as seen in similar species.

The decreased heterozygosity observed in our study compared to those in Richter et al. (2009) might be the result of population reductions and isolation in Indiana. Richter et al. (2009) sampled Crawfish Frogs in Oklahoma in a contiguous landscape, with multiple breeding ponds and no documented population reductions. Related Gopher Frogs are typically distributed across landscapes where individual breeding populations act as subpopulations within a larger population (Semlitsch et al., 1995; Palis, 1998, Greenburg, 2001; Richter et al., 2009). Crawfish Frogs appear to be distributed similarly; thus, the loss of multiple subpopulations across Indiana and the fragmentation of remaining subpopulations might have contributed to decreased genetic diversity within the remaining populations.

Western Indiana sampling sites had greater genetic diversity than the BONWR site based on allelic richness, even though genetic diversity at BONWR might have been artificially inflated because of pooling of individuals from multiple, geographically distant ponds (1–10 km). Crawfish Frogs in Indiana were historically documented only in the southwest region of the state, and the southeastern BONWR population was documented recently in 2003 (D. Karns and J. Robb, unpubl. data). 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). Although our data demonstrate low genetic diversity for BONWR, further research with intensive sampling at multiple ponds is required to address how genetic variability is distributed across the site and whether a signature of population bottleneck or expansion exists.

Population 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

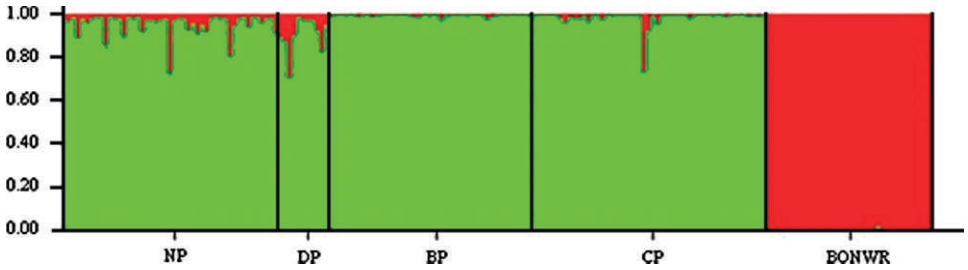

FIG. 4. Results of analysis of population structure in Structure 2.3.2. Pond abbreviation codes are listed in Table 2.

differentiation suggesting a high level of connectivity between breeding ponds that decreased with geographic distance. Ponds within 250 m of each other showed no genetic divergence, whereas a pond 750 m away was weakly divergent. AMOVA results loosely support these trends, revealing that there was little difference between ponds and that most genetic variation was at the pond level. Overall F_{ST}-values were comparable to other ranid amphibians studied over similar geographic distances; for example *Rana arvalis* had overall F_{ST} of 0.065 over 0.3–150 km (Vos et al., 2001), whereas *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.

The genetic similarity at HFWA could be the result of a combination of two nonmutually exclusive hypotheses. HFWA has been colonized by *L. areolatus* only within the past 30 yr; it was surface-mined for coal until 1982 and then reclaimed to grasslands beginning in 1983 (Lannoo et al., 2009). During mining, all natural ponds were destroyed (M. J. Lannoo, pers. comm.); therefore, the surveyed ponds were only colonized within the past two decades from surrounding lands. Recolonization offered potential for multiple founder events and sources, because multiple ponds within 5 km of HFWA have had documented populations of *L. areolatus* (Engbrecht and Lannoo, 2010). Because of this recent history, Crawfish Frogs at HFWA might not have yet reached genetic drift-gene flow equilibrium that is assumed when estimating genetic divergence (Lowe and Allendorf, 2010). Therefore, the current level of gene flow might be overestimated at the site.

The second explanation for the lack of genetic divergence at the HFWA is the movement of individuals to breed in nonnatal ponds (Kinney, 2011; Heemeyer and Lannoo, 2012; Heemeyer et al., 2012). Little is known about dispersal frequency and distance in pond-breeding amphibians, and known observations are highly variable (Kusano et al., 1999; Shaffer et al., 2000; Newman and Squire, 2001; Petranka et al., 2004; Chan and Zamudio, 2009). In a radiotelemetry study at HFWA, one of eight radio-tracked adults shifted breeding ponds, whereas others had fidelity over two successive years despite migrating past other potential breeding ponds (Heeymeyer et al., 2012). Juveniles represent the majority of dispersers in many amphibian species; hence, it is likely that this is the dispersal stage for Crawfish Frogs (Funk et al., 2005; Semlitsch, 2007). Studies of other amphibians revealed high breeding site fidelity of adults with some juveniles dispersing to breed in nonnatal ponds, causing small networks of ponds with little genetic divergence and higher divergence at larger scales (Berven and Grudzien, 1990; Gamble et al., 2007).

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 for extensive periods and other factors might cause localized extinction events (Semlitsch, 1987; Blaustein et al. 1994; Richter et al., 2003), but connectivity among ponds permits recolonization following such events (Marsh and Trenham, 2001; Semlitsch, 2002).

At the next step up in scale, lower levels of differentiation between HFWA and DP suggest historic or low-level, recent gene flow through a stepping-stone pattern of dispersal (Kimura and Weiss, 1964). Regionally, we found two statistically significant population clusters, dividing the eastern and western halves of the state. These sites are separated by approximately 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 of BONWR relative to the other two sites indicates historical isolation (instead of recent) between these two sides of the state. Another possibility for high differentiation is that the BONWR population is introduced, and its origin might be from a different part of the range. More data are needed to address the cause of this differentiation.

Conservation Implications.—The moderate to high genetic diversity in remaining populations of Indiana Crawfish Frogs is encouraging for future management plans for the species at their northeastern range limit. Our results indicate that neighboring ponds within at least 1 km from each other form a genetically distinct single breeding unit consisting of multiple subpopulations. Loss of this gene flow between neighboring subpopulations might lead to loss of genetic diversity in local populations (Richter et al., 2009). To preserve the population dynamics in Indiana Crawfish Frogs, management should focus on networks of ponds, instead of individual ponds. Establishing nearby breeding ponds has proven to be successful in other ranid species and might be a management option for this species (Chelgren et al., 2008). Therefore, maintenance of genetic connectivity between existing breeding ponds via upland habitat management and construction of breeding ponds in areas where populations consist of a single pond are important management options for the persistence of *L. areolatus* populations.

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