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Conservation and management of peripheral populations: Spatial and temporal influences on the genetic structure of wood frog (*Rana sylvatica*) populations

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ABSTRACT

Peripheral populations of wide-ranging species are often of special conservation concern, and knowledge of genetic diversity, gene flow, and historical population dynamics are critical to informed management of these populations. In this study we evaluate patterns of genetic diversity and gene flow among a series of wood frog populations in east-central Missouri where it is a species of conservation concern. Because these populations are on the periphery of the species' range and are isolated from one another by fragmented landscapes, we expected to see population subdivision with signatures of range-edge effects and isolation by resistance. Our analyses reveal significant interpopulation differentiation and a distinct gradient in diversity where levels of allelic richness and heterozygosity decline as the species boundary is approached. These patterns likely stem from factors characteristic of peripheral populations, including reduced population size, reduced gene flow, and more frequent extinction–recolonization events. Population structure was evident, but isolation by resistance (i.e. fragmentation) was not a significant factor in our focal landscape. Isolation by distance was significant, but the level of population differentiation suggests that gene flow among populations is limited. We hypothesize that the lack of gene flow is due to the absence of available fishless breeding habitats outside of protected, managed lands, and recommend that future management efforts of the wood frog include increasing population connectivity at the landscape scale via the creation and maintenance of fishless aquatic breeding habitat.

1. Introduction

Many organisms inhabit broad geographic ranges spanning wide environmental gradients, which can directly translate to variable habitat quality across a species' distribution. This range-wide variation can affect the local abundance and demography of populations (Brown, 1984), which can influence gene flow, genetic drift, and intraspecific genetic variation (Slatkin, 1987). Notable differences can occur between populations that inhabit the more stable, well-connected, optimal habitat of the interior regions of a species' distribution as compared to populations near the range margin that are often patchily distributed, subjected to greater isolation, more limited resources, and greater habitat and environmental variability (Brown, 1984; Brussard, 1984; Eckert et al., 2008). These populations are often referred to as *core populations* and *peripheral populations*, respectively. Core populations tend to be characterized as having larger population sizes and greater amounts of gene flow, resulting in higher levels of genetic diversity which affords the population greater evolutionary potential and resilience to stochastic events (Frankham, 1996). In contrast, peripheral populations are expected to be smaller in size and receive few dispersers from more central populations, placing them at an increased risk of inbreeding, genetic drift, and extirpation due to insufficient gene flow and inability to adapt because of limited genetic variability (Kirkpatrick and Barton, 1997; Vucetich and Waite, 2003). Each of these genetic characteristics of peripheral populations, in isolation or in combination, makes them more likely to be imperiled than core populations (Lesica and Allendorf, 1995).

Genetic differences between central and peripheral populations have been empirically demonstrated in a number of organisms—including wide-ranging species as well as those with rather restricted distributions (reviewed by Eckert et al. (2008)). While some studies have concluded that genetic variation is highest within core populations (Dolan, 1994; Garner et al., 2003; Lammi et al., 1999), others have found peak diversities in populations occupying transitional habitat zones in the sub-peripheries (Kark et al., 2008; Rowe et al., 2006). Still others have observed a directional gradient in diversity that decreases away from a glacial refugium (Garner et al., 2004), suggesting patterns of population expansion. Because of a multitude of contemporary threats, populations lacking allelic diversity may be increasingly vulnerable to extirpation (Rogell et al., 2010). Global climate change and habitat loss/fragmentation are among the threats that have been

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implicated in observed population declines, and they are suspected to function as primary drivers of species extinction (Mantyka-Pringle et al., 2011; Thomas et al., 2004). It has been suggested that these factors in particular are especially pressing for amphibians, which are among the most threatened taxa worldwide (Cushman, 2006; Stuart et al., 2004; Wake and Vredenburg, 2008).

Conservation of species, whether currently common or rare, must be proactive as decline or extinction can be rapid and inexplicable (Lindenmayer et al., 2011). Even relatively common, wide-ranging species, such as the wood frog (*R. sylvatica*), have demonstrated susceptibility to anthropogenic pressures (Crosby et al., 2008; Zellmer and Knowles, 2009), but conservation and management of populations from a genetic perspective requires an understanding of the processes that have shaped genetic variation and population structure (Neuwald, 2010; Schoville et al., 2011). When populations of wood frogs have been found to be genetically subdivided, differentiation has been attributed to a combination of factors, including founder effects potentially linked to postglacial recolonization (Lee-Yaw et al., 2008), periodic changes in precipitation (Newman and Squire, 2001) and metapopulation dynamics (Newman and Squire, 2001; Zellmer and Knowles, 2009). Geographic barriers (Lee-Yaw et al., 2009), isolation by distance (Newman and Squire, 2001; Zellmer and Knowles, 2009) and habitat fragmentation induced by deforestation and/or recent development of transportation networks (Crosby et al., 2008; Zellmer and Knowles, 2009) have also been implicated as contributing factors.

Our study investigates the genetic structure of wood frogs in Missouri where the species is at the western periphery of its Midwest distribution (Fig. 1) and is a species of conservation concern. Breeding populations in this region are rare and occur at a fraction of the size of populations in more northerly and eastern parts of the range (Raithel et al., 2011; Rittenhouse and Semlitsch, 2007b). For our study, wood frogs were collected from geographically isolated conservation areas, and we seek to identify the functional status of these conservation areas in terms of wood frog population genetic diversity and structure. Specifically, we characterize the level of differentiation within and among these populations, and try to understand the role that past events (e.g., glaciation, population expansion) and/or contemporary factors (e.g., land use, extinction–colonization) have contributed to measured patterns of genetic differentiation.

2. Materials and methods

2.1. Study species

The wood frog (*R. sylvatica*) has one of the largest ranges of North American amphibians, which encompasses most of the northeastern United States, including the Appalachian Mountains, north into the Arctic Circle, and west across Canada into Alaska. Disjunct populations occur in Colorado, Wyoming, North Dakota, and the Ozark Plateau (Lannoo, 2005). In east-central Missouri, wood frogs are at their range limit. The species is found within forested habitats of the Ozark Plateau where they breed in fishless ponds, and depend on moist ravines for survival in terrestrial habitat (Rittenhouse et al., 2008; Rittenhouse and Semlitsch, 2007b).

2.2. Sampling sites

We sampled in Montgomery and Warren Counties in the Upper Ozark Plateau of east-central Missouri, USA. Three conservation areas managed by the Missouri Department of Conservation were sampled: Daniel Boone Conservation Area (DBCA), Danville Conservation Area (DANV), and Little Lost Creek Conservation Area (LLC). The conservation areas are part of the river hills physiographic region in east-central Missouri, which contains the largest tract of forest north of the Missouri River. The forested habitat is nonetheless fragmented to varying degrees by pasture land,

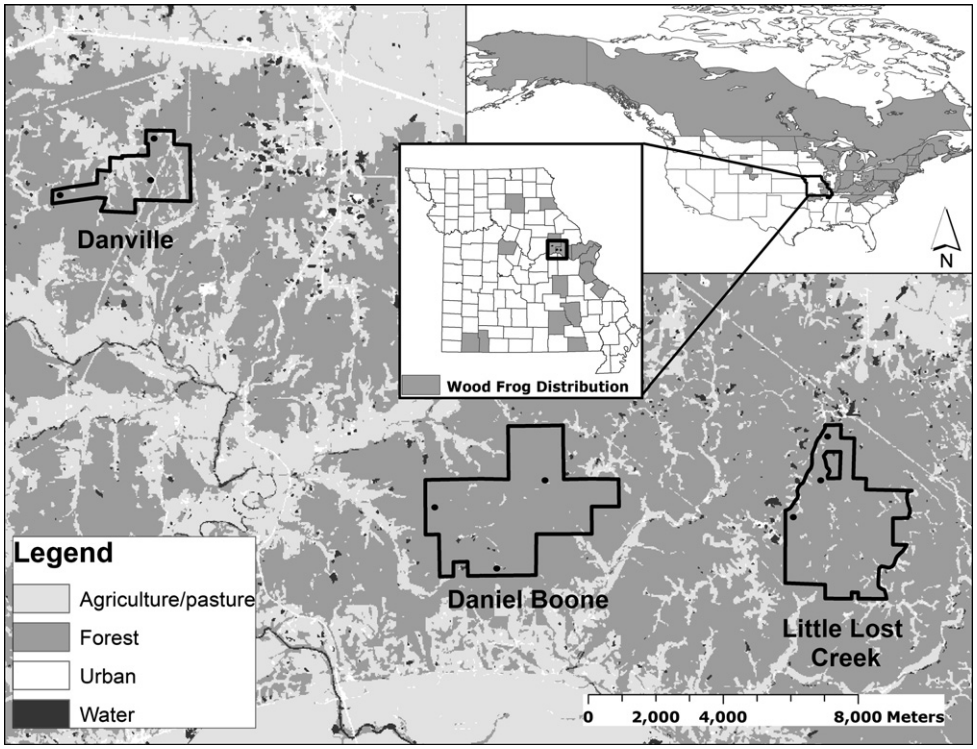

Fig. 1. Locations of the sampled conservation areas (CAs) where wood frog eggs were collected. The map details the habitat matrix between CAs and the inset shows Missouri wood frog distribution. Black points within conservation areas depict actual pond locations.

agriculture, and urban development (Fig. 1). Forest stands are characterized by mature, second-growth oak-hickory (*Quercus*/*Carya* spp.) and contain numerous fishless ponds that were created several decades ago for accommodating wildlife. Since then, they have been naturally colonized by 12 species of amphibians, including wood frogs (Hocking et al., 2008).

2.3. Collection techniques

Between 20 and 28 wood frog embryos ($n = 210$) were collected from three ponds at each of the three sampled conservation areas during the spring 2010 breeding season (Table A1). A single embryo was collected per clutch to avoid kinship related biases (Goldberg and Waits, 2010; Howard and Kluge, 1985). Upon collection, embryos were placed in 95% ethanol and stored at −20 °C until DNA extraction.

2.4. Laboratory procedures

Genomic DNA was extracted from embryos using the protocol outlined in the Wizard® SV 96 Genomic DNA Purification System (Promega, Madison, WI) with the following adjustments: the total elution volume was decreased from 500 μl to 300 μl using three 100 μl aliquots of nuclease-free water instead of two 250 μl aliquots. The extracted samples were genotyped using the polymerase chain reaction (PCR) in two multiplexes containing 11 total microsatellite primers (Table A2) developed by Julian and King (2003) for *R. sylvatica*. Primers for four loci (*Rsy*C23, *Rsy*C83, *Rsy*D77, *Rsy*D32) were redesigned using Primer 3 (v. 0.1.0; Rozen and Skaletsky, 2000) to accommodate multiplex arrangements. Forward primers were fluorescently 5′ labeled with FAM, NED, VIC, and PET. We used the manufacturers protocols for the Qiagen Multiplex PCR Kit (Qiagen, Valencia, CA) in 8 μl volume for each PCR reaction, with the addition of 0.8 μg/μl BSA (Ambion, Grand Island, NY). Amplification was performed on a Mastercycler ep (Eppendorf, Hauppauge, NY) with an initial denaturing cycle of 15 min at 95 °C followed by 35 cycles of 94 °C for 30 s, annealing at 58 °C for 90 s, and 72 °C for 60 s. A 30 min, 60 °C cycle concluded the PCR process. Positive and negative controls were utilized in all reactions. Seventy-two (34%) of the samples were randomly selected and re-genotyped to assess scoring consistency, and a genotyping error rate of 1.62% was observed.

2.5. Analyses

2.5.1. Genetic diversity

PCR products were separated in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and scored using GENEMARKER software version 1.95 (Softgenetics, State College, PA). We tested for deviations from expected genotype frequencies under Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using Fisher's exact test approximations and Markov chain algorithms (1000 batches, 10,000 iterations) using GENEPOP v. 4.0.10 (Rousset, 2008). Bonferroni corrections were applied to these computations, and all other multiple comparisons, to avoid chance occurrence of significance (Rice, 1989). Values for mean expected and observed heterozygosity were calculated in the MS toolkit Microsoft excel add-in for both population data and loci data (Parker, 2001). Allelic diversity was analyzed by calculating mean allelic richness and number of private alleles for each pond. These estimates were adjusted for sample size using rarefaction in HP-RARE (Kalinowski, 2005). We also compared our observed heterozygosity and number of alleles with those reported by Julian and King (2003) using two-sample *t*-tests. Values reported by Julian and King (2003) are from 113 samples collected from two disparate eastern populations in Tennessee and Maryland. As such, total number of alleles and heterozygosity estimates may be slightly inflated, but these comparisons were still made, in part, to underscore the limited genetic diversity present in Missouri populations.

2.5.2. Population differentiation and structure

Pairwise F_{ST} and population differentiation were evaluated in ARLEQUIN v3.5 (Excoffier and Lischer, 2010). We used a global analysis of molecular variance (AMOVA) based on genetic distances averaged across all 11 loci with 10,000 permutations. Variance was calculated at three different hierarchical levels: among conservation areas, among ponds within conservation areas, and within ponds. In addition, one-way analyses of variance (ANOVA) were conducted in SPSS (v. 17, IBM) to assess differences in average allelic richness, private alleles, and observed heterozygosities among conservation areas.

To infer population structure, we used STRUCTURE, version 2.3.3 (Pritchard et al., 2000), which groups samples into genetically distinct clusters, *K*, based upon similarities between the genotypes of individuals. Because our sampled populations have a historically common origin, the program was run using an admixture model with allele frequencies correlated among populations. Each sampled pond was considered an independent population ($n = 9$). We tested $K = 1$–18, with 10 replicates at each *K*-level, and an initial burn-in of 2.5×10^5 followed by 7.5×10^5 Monte Carlo Markov Chain iterations. We calculated ΔK following Evanno et al. (2005) to identify the most likely number of clusters.

2.5.3. Population expansion

To assess how genetic diversity in our peripheral Missouri populations changes with distance from the core of the Midwestern distribution, we used linear regression models with geographic distance from the Midwest range center as the independent variable, and allelic richness, heterozygosity, and private alleles as dependent variables. The core of the Midwest range was determined by finding the weighted center of the distribution encompassing Missouri, Illinois, Indiana, Kentucky, and Tennessee. Euclidean distance was measured in ArcGIS (v 9.3, ESRI, Redlands, CA) from each pond center to the core of the Midwestern distribution of wood frogs, which was determined to be due east of our study area in western Indiana, USA. This location coincides with the interior plains glacial refugium identified by Lee-Yaw et al. (2008). Detection of a linear diversity gradient could potentially be indicative of postglacial recolonization. To test for evidence of expansion, we analyzed the distribution of allele lengths using both intra- and inter-locus tests (Reich et al., 1999; Reich and Goldstein, 1998) implemented using an Excel macro (Kgtests; Bilgin, 2007). The intra-locus *k* test measures the peakedness and kurtosis of allele-length distributions. Following population expansion, the estimate of *k* is expected to decrease as kurtosis and peakedness increase. In contrast, the inter-locus *g* test assesses the variance in allele-length distributions at each locus and then across all loci. A low estimate of *g*, indicating minimal variance among loci, can be interpreted as an indication of population expansion.

2.5.4. Population bottlenecks and relatedness

To test for the presence of demographic bottlenecks we used two programs. To detect historical bottlenecks, M_P_VAL (Garza and Williamson, 2001; Williamson-Natesan, 2005) was used. This approach utilizes *M*, the ratio between the number of alleles within a population to the observed range in allele size. *M* is measured using the proportion of one-step mutations (p_s), the average size of multiple-step mutations (Δg), and theta ($\theta = 4N_e\mu$) which is population specific. Following recommendations provided in the software documentation, we set $p_s = 0.88$ and $\Delta g = 2.8$. θ was calculated using effective population sizes (N_e) and the expected microsatellite mutation rate ($\mu = 5 \times 10^{-4}$) obtained from

OneSAMP (Tallmon et al., 2008). We compared empirical values of M to 95% critical values (Mc) derived from 10,000 simulations using the program CRITICAL_M (Garza and Williamson, 2001). To detect more recent population bottlenecks, we used program BOTTLENECK (Cornuet and Luikart, 1996; Williamson-Natesan, 2005), which tests for significant heterozygosity excess or deficit. This approach is effective in detecting bottlenecks which have occurred relatively recently (within the past 0.2–4.0N_e generations: Luikart and Cornuet, 1998). Using the two phase model of microsatellite mutation (TPM; Di Rienzo et al., 1994), we set the variance to 12.00 and the probability of single-step mutations to 95% as recommended by Piry et al., (1999). We used the Wilcoxon one-tail test to determine significance. We also tested for relatedness among individuals both within ponds as well as within conservation areas (ponds pooled together) using the Queller and Goodnight relatedness metric (r; Queller and Goodnight, 1989) as calculated in GenAlEx 6.1 (Peakall and Smouse, 2006). Significance of mean relatedness values was calculated using 9999 bootstrap permutations. Relatedness estimates significantly greater than expectation may be indicative of reproductive skew, inbreeding, or drift.

2.5.5. Landscape resistance and isolation by distance

Because our conservation areas are relatively isolated from each other and the broader area has been subject to fragmentation, we explored the effects of distance, roads, topography, aspect, and intervening habitat matrix on population structure. Road, land cover, and digital elevation data were obtained from the Missouri Spatial Data Information Service (http://www.msdis.missouri.edu). We reclassified land cover into six classes and roads into four classes (Table A3). Using the digital elevation model, we derived topographic position using the slope position classification approach implemented in the ArcGIS Topography Tools extension (Dilts, 2010); aspect was also derived from the digital elevation model. For analysis the boundaries of each surface were clipped to the county level (Warren and Montgomery Counties), as these political boundaries contained natural biological boundaries (Fig. 1). Using these four surfaces, we assigned resistance values ranging from 1 to 10, based on field experience and previous studies conducted on Missouri wood frog populations (Rittenhouse et al., 2008, 2009; Rittenhouse and Semlitsch 2007b, 2009). We created low and high cost resistance layers for each of the four resistance layers (Table A3) to test alternative resistance values, as well as a null model (equivalent to isolation-by-distance, all levels equal to 1); each of these layers had a 30-m resolution. All combinations of these twelve resistance layers comprised the multivariate model set ($n = 81$ combinations). Because the assignment of resistance values is inherently flawed and unknowable, we conducted a secondary analysis following the methods outlined in Shirk et al. (2010). For this analysis we ranked-ordered values within each resistance layer (Table A3), then applied a response shape exponent of 0.25, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, or 5.0 and then calculated landscape resistances values using both least cost path and circuit-based methods (McRae, 2006). Following a Bonferroni correction of our p-values, we conducted simple and partial Mantel tests to correlate resistance values with genetic distance (see Table A3 for more detailed description of modeling methods).

Pairwise inter-pond resistances, accounting for multiple dispersal pathways, were calculated in Circuitscape (v 3.5.1; McRae, 2006) using an eight neighbor connection rule. We then conducted simple and partial Mantel tests (Mantel, 1967) to correlate genetic distance (F_{ST}) with landscape resistance estimates using the Ecodist package in R 2.12 (Goslee and Urban, 2007). To do so, we developed three organizational models (isolation by distance only, landscape only, landscape and distance), which we then tested in a causal modeling framework (Cushman et al., 2006). Significance of Mantel tests within each causal model was assessed using 10,000 permutations.

3. Results

3.1. Genetic diversity

No population or locus deviated significantly from expected genotype frequencies under Hardy–Weinberg equilibrium after Bonferroni corrections. Evaluations of linkage disequilibrium (LD) identified significant associations between locus D32a and D20, as well as locus D55 and C41. Significance levels, however, were inconsistent and LD was not detected in all populations (D32a and D20: DANV-2, DANV-3, DBCA-3 with $p < 0.001$; D55 and C41: LLC-3 with $p < 0.001$). Furthermore, the removal of locus D32a did not alter estimates of population differentiation, or structure as measured using AMOVA and STRUCTURE, so all 11 loci were included in the analyses.

All loci were polymorphic with substantial variation in number of alleles and observed heterozygosity (Table A1). The number of alleles per locus ranged from 3 to 10 (mean ± standard deviation; 7.55 ± 2.25)—a significant decrease (avg. 59.88%, $p < 0.001$) when compared to results published by Julian and King (2003; Table A4). The mean observed heterozygosity across loci was 0.66 (±0.10), which was almost 17% lower than the heterozygosity observed by Julian and King ($p = 0.0048$). Ponds contained, on average, between 4.00 and 5.88 alleles per locus (Table A1). Private alleles were generally rare, ranging from 0.00 to 0.27 alleles per pond. When averaged at the conservation area level, rarified allelic richness, private alleles, and observed heterozygosity followed a distinctive pattern with levels of each decreasing from east to west

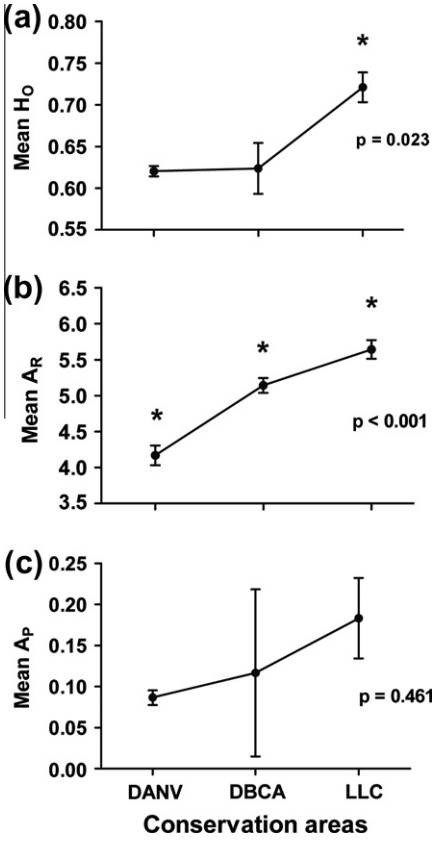

Fig. 2. Patterns observed in observed heterozygosity (A), rarified allelic richness (B), and private alleles (C) at the conservation area level. Error bars show standard error. Asterisks denote significant differences ($p < 0.05$).

(Fig. 2). Allelic richness varied significantly amongst all three conservation areas ($F_{2,8}$ = 36.619, $p \leqslant 0.001$). Specifically, the western most area (DANV) contained the fewest alleles, the central area (DBCA) had an intermediate number of alleles, and the eastern most area (LLC)—representing the conservation area closest to the core of Missouri's wood frog distribution—exhibited the greatest number of alleles. A similar pattern was evident in observed heterozygosity among conservation areas. LLC had a significantly higher mean observed heterozygosity (0.721) than either DANV (0.620) or DBCA (0.624; $F_{2,8}$ = 7.559, p = 0.023). Although not significant ($F_{2,8}$ = 0.885, p = 0.461), private alleles also decreased from east to west (Fig. 2). OneSAMP results also suggested that the mean effective population size increased from west to east (DANV = 43.31, DBCA = 53.10, LLC = 89.12, Fig. A3).

3.2. Population differentiation and structure

There was a significant amount of genetic structure across the nine populations sampled (mean pair-wise F_{ST} = 0.031, ±0.020), with 32 of 36 comparisons being significantly different from zero (Table 1). There was significant population structure at all three hierarchical levels (Table A5). While most of the variation resided within ponds (96.15%), some variation occurred among conservation areas (2.89%). Differentiation among conservation areas was supported by results from STRUCTURE, which provided evidence for two genetic clusters (ΔK = 2) with >75% assignment probability of individuals to each of these clusters (Fig. A1). Cluster one was comprised entirely of individuals from DANV while individuals from LLC made up cluster two. DBCA exhibited an admixture of cluster one and two, and individuals from DBCA had mean assignment probabilities 45% and 55% to cluster one and cluster two, respectively.

3.3. Landscape resistance and isolation by distance

Causal modeling provided unequivocal support for isolation-by-distance (IBD) as the driving mechanism for the genetic differentiation among our populations. The simple Mantel test of IBD had a correlation of 0.80 ($p < 0.001$). Further, seven of the 81 partial Mantel tests measuring the correlation between Euclidean distance and genetic distance, while partialling out landscape resistance, were also significant (r = 0.35–0.53, $p \leqslant 0.006$). In contrast, none of the Mantel tests assessing the correlation of landscape resistance with genetic distance, while partialling out Euclidean distance, were significant (Table A6a). This same trend was observed in analyses testing the magnitude of assigned resistance values; none of the tested models were significant after Bonferroni correction and partialling out the effects of distance (Fig. A2).

3.4. Population expansion, bottlenecks, and relatedness

The east–west decline in allelic diversity was significantly related to the distance of each conservation area to the core of the Midwest *R. sylvatica* distribution (adjusted R^2 = 0.856, $p < 0.001$ and R^2 = 0.492, p = 0.010 respectively). Despite this distinct linear gradient in diversity, no signature of expansion was detected (within locus [k] and interlocus [g] tests, p = 0.242 and 0.584, respectively). Signatures of historical bottlenecks were detected in all populations, but tests were only significant in DANV (average M = 0.4676, p = .0019) and only in two ponds (DANV-2: M = 0.4423, p = 0.0003 and DANV-3: M = 0.4309, p = 0.0002). There was no evidence for recent bottlenecks in our populations. Relatedness among individuals was significantly greater than random at four ponds (DBCA-1 and all DANV; r = 0.086–0.181, Table A7), but at the conservation area level, only DANV had a relatedness estimate that was significantly greater than random expectation (r = 0.151, $p < 0.001$).

4. Discussion

Conservation and management often require prioritization to achieve optimal gain from invested resources (Hughey et al., 2003). Priority may, in part, be determined by evolutionary significance, genetic variation, or location within a broader reserve network (Hedrick, 2001; Moritz, 1995; Neel, 2008). These attributes often make peripheral populations especially valuable from a conservation perspective (Lesica and Allendorf, 1995). Our study sought to measure genetic diversity and to characterize population structure of peripheral wood frog populations. Our sampling was focused on established conservation areas, allowing us to assess the role and function of these protected lands for Missouri wood frogs. Specifically, are conservation areas serving as isolated islands or are they integrated into the greater landscape, serving as stepping stones or conduits of connectivity?

Given the habitat specificity and high costs of dispersal and migration for amphibians generally (reviewed by Cushman (2006), Semlitsch (2008)), and wood frogs specifically (Rittenhouse and Semlitsch, 2007b; Rittenhouse et al., 2009), we predicted that genetic differentiation between conservation areas would be correlated with landscape attributes. This hypothesis was not fully supported in our causal modeling framework, and genetic distance was only significantly correlated with Euclidean distance in both the simple and partial Mantel tests (Table A6a, Fig. A2). Through our STRUCTURE analysis, we found evidence for 2 distinct genetic clusters and one admixed cluster, corresponding with DANV, LLC, and DBCA, respectively. We acknowledge that these STRUCTURE results should be interpreted cautiously given the strong isolation-by-distance relationship in our populations, and the known limitations of STRUCTURE to correctly classify individuals under such a model (Pritchard et al., 2000; Schwartz and McKelvey, 2009). The apparent admixture observed at DBCA could also be a result of shared ancestry with no contemporary gene flow, as has been suggested in other studies (Sousa et al., 2012). Further, our lack of finding a significant landscape-genetic relationship does not discount the importance of forested habitat for population connectivity of wood frogs, but suggests that overall, the land cover in

Table 1
Pairwise F_{ST} on the lower half and Euclidean distance (km) on the upper half. Significant pairwise comparisons are in bold text ($p \leqslant 0.05$).

	DBCA-1	DBCA-2	DBCA-3	DANV-1	DANV-2	DANV-3	LLC-1	LLC-2	LLC-3
DBCA-1	***	2.749	2.895	15.650	16.548	15.968	10.420	9.888	8.890
DBCA-2	**0.0222**	***	3.242	12.917	13.822	13.228	11.776	11.544	10.658
DBCA-3	**0.0092**	**0.0032**	***	15.204	15.461	15.021	8.538	8.367	7.535
DANV-1	**0.0394**	**0.0199**	**0.0265**	***	3.655	2.757	22.613	22.971	22.368
DANV-2	**0.0529**	**0.0373**	**0.0486**	0.0025	***	0.961	21.892	22.474	22.024
DANV-3	**0.057**	**0.0257**	**0.0366**	**0.0137**	0.004	***	21.725	22.248	21.758
LLC-1	**0.0349**	**0.0198**	**0.0136**	**0.0486**	**0.0584**	**0.049**	***	1.473	2.321
LLC-2	**0.057**	**0.019**	**0.0375**	**0.057**	**0.0705**	**0.0648**	**0.0273**	***	1.088
LLC-3	**0.026**	0.0049	**0.01**	**0.034**	**0.0436**	**0.0413**	0.0001	**0.0079**	***

our landscape was not variable to a degree that influenced the genetic structure among our populations (Short Bull et al., 2011). We know that forested ravines in Missouri are critical for wood frog movement and survival (Rittenhouse et al., 2008, 2009; Rittenhouse and Semlitsch, 2007b), but as the landscape is currently configured, these resources are not limiting factors such that patterns of gene flow are discernible from the effects of distance. The forest in our study region does become more heterogeneous as the western boundary is approached (Fig. 1), but the scale at which the forest is fragmented may not impose significant barriers to wood frogs (Rittenhouse and Semlitsch, 2009). Another potential limiting factor in our identification of landscape resistance as a mechanism for genetic differentiation is the predominantly linear arrangement of our study populations across the landscape (Schwartz and McKelvey, 2009). With a linear arrangement of ponds, we effectively reduced our interpopulation assessment of landscape resistance to one-dimension, and had only three points to make comparisons among. If our 9 sampled populations had been more uniformly distributed across the landscape in two-dimensions, more of the landscape would be encompassed, and potentially more meaningful relationships with the landscape could be determined. It is also possible we had low statistical power to detect the effects of landscape resistance. We feel that our overall sample sizes were not altogether poor (minimum of 20), but the lack of polymorphism in our 11 microsatellites may have reduced the strength of the relationship between genetic distance and landscape resistance (Landguth et al., 2012). Lastly, the relatively recent construction of many of our wetlands (50–100 yrs) means that only 15–30 generations are likely to have passed since colonization, which may further limit our ability to detect landscape effects on gene flow (Landguth et al., 2010).

A high degree of genetic structure was observed across our populations, with AMOVA results indicating significant differences occurring both within and among conservation areas. Additionally, we observed a distinct east–west gradient among our populations with the western-most populations having fewer alleles, lower heterozygosity, and fewer private alleles than populations closer to the core of the Midwest range to the east (Fig. 2). These patterns and structure are likely the result of both historic and contemporary processes. Suggestive of a common ancestry, there is substantial overlap in the alleles detected at each conservation area, but there are fewer private alleles present at DANV and DBCA than LLC. A likely process producing this pattern is drift in the absence of inter-conservation area migration, which is now eroding both heterozgysity and allelic richness at the western conservation areas. The observed linear gradients in genetic diversity, extending from the region of the proposed refugium of the last glacial maximum, might suggest that our observed genetic diversity is the result of population expansion, but we found no evidence for expansion using inter- and intra-locus tests (Reich and Goldstein, 1998).

Populations are dynamic in space and time, and peripheral populations are often predicted to experience local extinctions at a greater rate, and to have reduced recolonization due to smaller population sizes and more limited dispersal. It should be noted that these processes may have directly limited our ability to detect population expansion (Reich et al., 1999), but if occurring, these processes can also leave detectable signatures in the distribution of genetic diversity (Cornuet and Luikart, 1996; Garza and Williamson, 2001). We did find evidence for historical drift events in our populations, but only our western-most conservation area, DANV, was significant in this respect. The mechanism behind this event is unknown, and could stem from a single historical founder event or recurring local extinction–colonization processes. Regardless, a signature of these past population dynamics has persisted to present day in part due to the apparent absence of gene flow from more diverse easterly populations. Somewhat surprisingly, no patterns of recent bottlenecks were detected, bringing into question the extent and/or severity of local extinction–colonization processes. We did, however, find that relatedness of wood frogs at DANV was highest and deviated significantly from expectation. This increase in relatedness may be another contemporary manifestation of the observed historical founder event at DANV, and suggests a non-random selection of individuals. Philopatry to breeding ponds is likely to further compound the effects of genetic drift as a result of a founder event by increasing the rate of inbreeding. Overall, these conservation area-level differences in genetic diversity underscore the limited amount of gene flow that is occurring, despite the relatively short distances separating each conservation area (7.5–22 km; Table 1). Taken together, our wood frog populations exhibit many of the characteristics frequently associated with peripheral populations (Eckert et al., 2008). While our study is not an explicit test of the core-peripheral hypothesis, patterns in the distribution of genetic diversity fall in line with many of the tenets of this hypothesis.

If land cover and landscape structure are not limiting factors of dispersal among our conservation areas, why is there not more evidence for gene flow? Different lines of argument can be put forth in response to this question. First and foremost, our populations are located at the extreme periphery of the wood frog's Midwest distribution. In contrast to more northerly regions where adult and juvenile wood frogs can be found distributed widely across the forested landscape and dispersal opportunities are frequent (Patrick et al., 2008), Missouri wood frogs are largely confined to ravine habitats that remain moist and cool, and movement is almost exclusively restricted to nights during or immediately following substantial rainfall with subsequently high rates of mortality (Rittenhouse et al., 2009). As such, dispersal opportunities and corridors may be more limited, but again, we found no statistical support that ravine habitats related to genetic distance (Tables A6a, A6b, A7). Second, successful reproduction of wood frogs is dependent on fishless, seasonal water bodies as they actively avoid ponds inhabited by predatory fish (Hopey and Petranka, 1994). To facilitate connectivity, breeding habitat needs to be distributed in such a way as to provide stepping stones across the landscape. Wood frogs are not long-lived (2–5 yrs) and generally only reproduce once in their lifetime (Berven and Grudzien, 1990). Movement among populations is further restricted by the fact that wood frogs are highly philopatric to breeding ponds, although up to 20% of juveniles do disperse to new breeding ponds, and can travel up to 2.5 km during this dispersal phase (mean dispersal = 1.2 km; Berven and Grudzien, 1990). Throughout most of the wood frog's range aquatic breeding habitats are present in the form of ephemeral wetlands (Lannoo, 2005), but in Missouri these breeding habitats are almost exclusively man-made, relatively permanent, wildlife ponds (Hocking et al., 2008). Using leaf-off aerial imagery of our study landscape, we identified 232 small (<0.25 ha) wetlands located within forested habitat that could serve as breeding ponds for wood frogs (Peterman, unpublished data). The average minimum distance among ponds within conservation areas was 285 m, but the average minimum inter-pond distance outside of conservation areas was 463 m. While ponds are more diffuse outside of conservation areas, this network of ponds should be amenable to the dispersal capabilities of wood frogs. The apparent lack of connectivity, despite the presence of ponds, may be a result of fish stocking in many of these water bodies. In a broad survey of wetlands in northern Missouri, Shulse et al. (2010) found that 43% of constructed wetlands contained fish. If this trend is general throughout our study region, the effective distance between suitable breeding ponds may perhaps be closer to 1000 m and the upper limit of individual dispersal distance.

4.1. Conclusions

The predominant patterns observed in our study indicate that historical processes have been primary in shaping genetic diversity, and conservation and management efforts must be directed toward preserving, and ideally bolstering, current genetic diversity. Conservation of wood frogs in the River Hills region of Missouri requires a holistic landscape approach. First, the spatial arrangement of viable breeding habitat needs to be such that dispersing individuals can successfully reach other, potentially new fishless breeding habitats. Making this accommodation will foster metapopulation dynamics, to which wood frogs, like many pond-breeding amphibians, tend to conform (Gamble et al., 2007; Heard et al., 2012; Hecnar and M'Closkey, 1996; Marsh and Trenham, 2001). In our study, this would necessitate the creation and/or maintenance of fishless, seasonal breeding habitats. In order for these breeding ponds to serve as functional units within a metapopulation context, they must be placed within forested habitat (Lannoo, 2005), and in Missouri, be in proximity to forested drainages (Rittenhouse et al., 2008). Even though our genetic results suggest no influence of the current landscape configuration in the River Hills region, maintenance of contiguous forest adjacent to breeding ponds is essential for foraging and overwintering by wood frogs and other pond-breeding amphibians (Rittenhouse and Semlitsch, 2007a; Semlitsch and Bodie, 2003).

The wood frog populations in our study are located on the extreme western periphery of their Midwest distribution. The long-term viability of these populations is unknown in light of patterns of current and projected global climate change (Karl et al., 2009; Wuebbles and Hayhoe, 2004). One limiting factor of future population success, independent of habitat loss, is whether or not the reduced genetic variability within our populations affects individual fitness. Previous research relating genetic diversity to fitness in amphibians has produced mixed results. In a study of wood frogs in Connecticut, Halverson et al. (2006) found a significant negative relationship between survival to metamorphosis and relatedness in wild populations, but no relatedness-survival effects were observed under controlled lab settings. Further, Hitchings and Beebee (1998) found that toad populations with reduced genetic diversity had lower larval survival, while tree frog populations inhabiting a fragmented landscape were found to have lower genetic diversity and survival than those from a non-fragmented landscape (Luquet et al., 2011). Encouragingly, Luquet et al. (2011) found that the reduction in survival was a result of low genetic diversity and fixation load, which could be ameliorated through crosses with more outbred populations. Future research should seek to characterize the genetic diversity-fitness relationships of these populations (Angelone, 2010). Doing so may provide guidance for future management strategies that seek to increase fitness by increasing allelic diversity, and thereby fostering adaptability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2012.07.028.

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