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RESEARCH ARTICLE

Limited effects of suburbanization on the genetic structure of an abundant vernal pool-breeding amphibian

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Abstract Habitat fragmentation and degradation associated with suburbanization can have negative consequences on population persistence through the reduction of dispersal and concomitant gene flow. Using eight polymorphic microsatellite loci, we assessed the effects of forest fragmentation, water quality and hydroperiod on the genetic structure of a vernal pool-breeding amphibian, the wood frog (*Lithobates sylvaticus*), across 20 ponds in an unfragmented, forested landscape and 45 ponds in a landscape fragmented by moderate suburban development. Analyses were performed at the broad scale of the study area and at a fine scale, with spatially independent clusters of ponds selected within each landscape. Bayesian clustering approaches and AMOVA identified little population structure at the scale of the study area. At the fine scale, genetic structure was correlated with geographic distance and the presence of roads in two of the three fragmented clusters. Spatial autocorrelation analyses detected positive spatial genetic structure and restricted dispersal in one of the clusters in the fragmented landscape. We identified barriers associated with roads and suburban development in the fragmented landscape and with large bodies of water and elevation in the unfragmented landscape. Lastly, we found no biologically meaningful effects of water quality or hydroperiod on genetic variation. The results of this study indicate that wood frog populations are well connected, with high gene flow, across the landscape of southeastern New Hampshire, and that fragmenting features of suburbanization to date have a small but detectable impact on fine-scale genetic structure. The potential exists for greater impacts with higher levels of development or longer time scales. Our findings also highlight the importance of replication in landscape genetic studies, as the genetic response we detected varied with a gradient of fragmentation.

Keywords Habitat fragmentation · Landscape genetics · Dispersal · *Lithobates sylvaticus* · Roads · Water quality · Wood frog

Introduction

Habitat fragmentation due to human modification of the landscape is one of the greatest current problems in conservation biology. Increasing residential and urban development, agricultural land use, forestry management, and road development are all limiting the extent of once continuous habitats and ecosystems, resulting in the patchy distribution of habitats across the landscape. Among these factors, suburbanization is a pervasive land use pattern that has the potential to disrupt landscape connectivity. Understanding the effects of this decreased landscape connectivity on natural populations is essential if we are to minimize or mitigate human impacts on ecosystems (Lindenmayer et al. 2008; Storfer et al. 2010).

Among the many negative consequences of habitat fragmentation is the potential to limit animal dispersal and thereby reduce gene flow (*reviewed in* Andrén 1994; Fahrig 2003; Cushman 2006; Keyghobadi 2007). Reduced

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dispersal results in increased spatial genetic structure and differentiation among populations, as well as reduced within-population diversity (Keyghobadi 2007). Such changes in genetic structure and diversity may have negative consequences for population persistence, resulting from inbreeding depression, loss of local adaptation, isolation, and ultimately local extinctions (Frankham et al. 2002). Landscape genetics provides a useful framework for studying the fragmentation effects of suburban development on natural populations, by offering tools to quantify population structure, identify barriers to gene flow, and relate landscape and environmental variables to dispersal patterns of individual species (Holderegger and Wagner 2008; Balkenhol et al. 2009b).

Vernal pool-breeding amphibians may be particularly vulnerable to landscape alternations, due to their patchy distributions, generally low vagility, and dependence on both aquatic and terrestrial habitat for different stages of their life cycles (Sinsch 1990; Alford and Richards 1999; Gallant et al. 2007). Pool occupancy and population persistence are strongly influenced by landscape features of the terrestrial habitat surrounding and between pools (Guerry and Hunter 2002; Herrmann et al. 2005; Veysey et al. 2011). Additionally, many amphibian species exist as metapopulations (Alford and Richards 1999; Smith and Green 2005), where frequent local population extinctions are offset by successive recolonization events (Levins 1969; Hanski and Simberloff 1997). These species rely on inter-pond migration and dispersal to maintain metapopulation processes (Marsh and Trenham 2001), and they are therefore negatively impacted by habitat fragmentation. If connectivity among breeding pools is interrupted, gene flow, recolonization and rescue effects will be impeded, with negative consequences for population persistence (Hitchings and Beebee 1997; Rothermal 2004; Compton et al. 2007).

In addition to the obvious barrier effects of fragmentation, suburbanization may result in a variety of landscape and environmental modifications with negative consequences for vernal pool-breeding amphibians. For example, in addition to dispersal impairment, roads contribute a suite of ecological effects, including road-kill and road chemical run-off (*reviewed in* Balkenhol and Waits 2009). All of these effects may reduce connectivity as spatially-critical habitat patches (e.g. "stepping stones") become unoccupied due to increased local mortality or reduced recolonization (Trombulak and Frissell 2000). Functional connectivity of amphibian populations has been shown to be influenced by local environmental variables (Goldberg and Waits 2010; Murphy et al. 2010a, b).

For vernal pool amphibians, hydroperiod and water quality are critical local pond parameters. Population size, breeding success and survival of amphibians is influenced by the hydroperiod (Babbitt et al. 2006) and water quality (Karraker et al. 2008) of the pools in which they live. Pond hydroperiod may impact the growth, survival, recruitment, and timing and size at metamorphosis of several amphibians (Pechmann et al. 1989; Semlitsch and Reyer 1992). Degraded aquatic habitat limits breeding success and survival (Sanzo and Hecnar 2006; Karraker et al. 2008; Snodgrass et al. 2008), thereby reducing the number of dispersers that are able to immigrate into or colonize adjacent populations. The resulting reduction in gene flow may lead to increasingly structured populations (Hanski and Gilpin 1997). If breeding habitat degradation or pond drying increases rates of local extinction, the resulting effects on genetic structure will depend on dispersal rates and founder effects (Wade and McCauley 1988; Pannell and Charlesworth 1999).

In this study, we investigated the effects of suburbanization on wood frog (*Lithobates sylvaticus*) population structure using a landscape genetics approach. We compared patterns of genetic connectivity between replicate clusters of vernal pools in a continuously forested, unfragmented landscape and a fragmented landscape characterized by moderate levels of suburban development and roads. Our study is one of only a few to date (e.g., Smouse et al. 2008; De-Lucas et al. 2009) to utilize a comparative landscape approach with replication within each landscape. Our objectives were to (1) characterize the genetic structure of wood frog populations in the two landscapes and (2) determine the effect of roads on genetic connectivity of wood frog populations. We hypothesized that: (a) habitat fragmentation increases fine-scale spatial genetic structure of wood frogs in the fragmented landscape relative to that in the unfragmented landscape and (b) roads act as barriers to dispersal to wood frogs, resulting in elevated genetic divergence for populations separated by roads. Lastly, we tested the effect of environmental factors, including water quality and hydroperiod, on the population genetic structuring of wood frogs. We hypothesized that, through their influence on population recruitment and persistence, these factors would impact metapopulation structure. Specifically, we predicted that ponds with reduced water quality and hydroperiod length would be characterized by elevated genetic differentiation.

Methods

Study Area

This study was conducted in public and privately owned wetlands in southeastern New Hampshire that were located in an area of approximately 375 km^2 (Fig. 1). Historically, the region has been heavily forested with second-growth

mixed hardwoods, white pine (*Pinusstrobus*), and eastern hemlock (*Tsugacanadensis*),and it contains abundant wetlands (Veysey et al. 2011). Since the 1950s, pressures from human population growth and development have resulted in increased fragmentation (SPNHF 2005).

We developed a study design that enabled us to address issues of scale and replication, both of which are important considerations in studies of landscape genetics (Balkenhol et al. 2009a; Anderson et al. 2010). We selected small (<1 ha) wetlands with National Wetland Inventory (NWI) classifications of PUBF, PFO1/4E, or PSS1E (i.e., vernal pools) in southeastern New Hampshire using NWI maps and aerial photographs, and locations were verified in the field. We aimed to sample multiple, spatially-independent clusters of vernal pools in each of two landscapes: one unfragmented, forested landscape, and one fragmented landscape, characterized by moderate levels of suburban development. We used the maximum, known, dispersal distance for wood frogs (2.5 km; Berven and Grudzien 1990) as a minimum intervening distance between three clusters of pools in the suburban landscape (actual intervening distances 3.5–4.0 km). These clusters were located in the towns of Lee, Nottingham, and Barrington, NH, in which fragmenting features included New Hampshire State divided highways Rte 4, Rte 125, and Rte 9, local roads, agricultural fields, and housing developments. The unfragmented landscape was located in Pawtuckaway State Park, Raymond, NH and lacked development, cleared land and roads, excepting access roads. The two clusters of ponds in this landscape were on opposite sides of a 2 km tract containing a mountain (269 m above sea level) and swampy marshland. No wood frog egg masses were found during a survey of this intervening region. The two landscapes were separated by 8 km.

Within the spatial extent of each cluster, we sampled pools continuously as we encountered them in the landscape, resulting in pair-wise distances of 100 m to 6 km. This fine-scale, continuous sampling was appropriate for the vagility of our study organism, and it was designed to capture relationships among adjacent ponds, without missing stepping stone ponds, which could facilitate wood frog dispersal. Due to the importance of continuous sampling, we sampled wood frogs from any wetlands that we encountered with egg masses; this resulted in the inclusion of two roadside ditches and three man-made ponds. This sampling procedure yielded 65 ponds in 5 clusters comprised of 9–18 ponds each (Fig. 1). Twenty ponds were distributed across two clusters in the unfragmented landscape (Cluster 36: $N = 11$; Cluster 39: $N = 9$). Forty-five ponds were distributed throughout three clusters in the fragmented landscape (Cluster 16: $N = 11$; Cluster 18: $N = 18$; and Cluster 29: $N = 16$). The ponds in this study are a subset of those used in the study of Veysey et al. (2011); pond and cluster names are derived from that study. Land use characteristics for each cluster of ponds are summarized in Table 1.

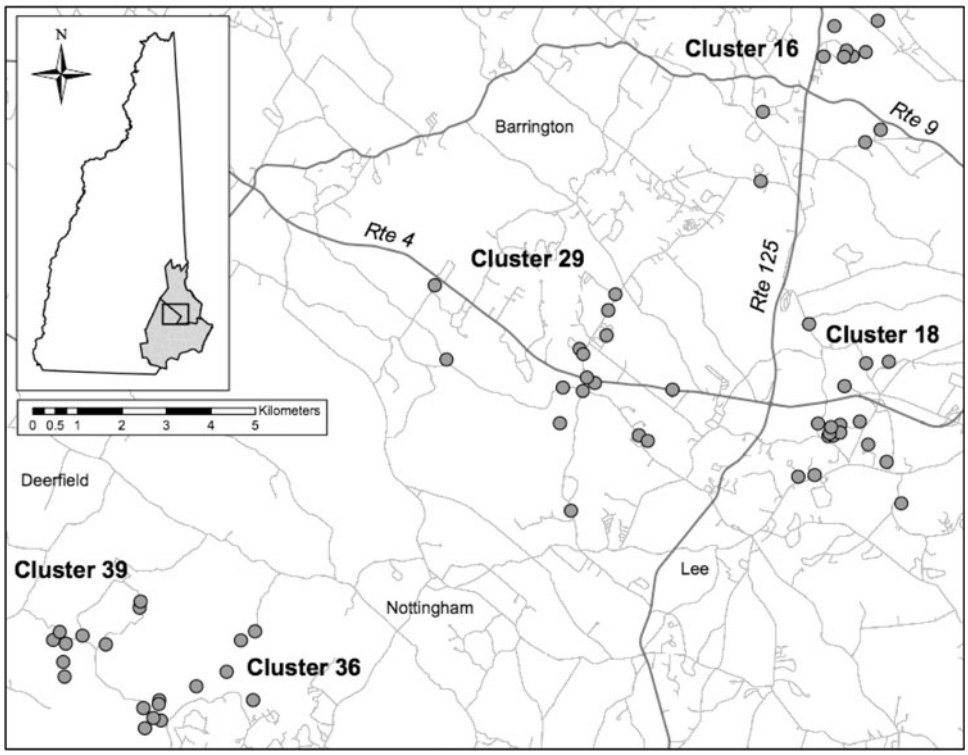

Fig. 1 Locations of five clusters of 65 vernal pools within Rockingham and Strafford counties in southeastern New Hampshire where wood frogs were sampled. Clusters 16, 18 and 29 are within a suburban, developed landscape and clusters 36 and 39 are in an unfragmented landscape

Table 1 Land cover characteristics from NH Public Roads and New Hampshire Land Cover Assessment (2001) layers from NH GRANIT for five vernal pool clusters in southern New Hampshire. The proportion of each cluster comprised by each landcover type is indicated for the two landscapes

Proportion of coverage							
Landscape	Cluster	Roads	Forest cover	Open water	Development	Agriculture	Power lines and quarries
Fragmented	16	0.05	0.74	0.00	0.18	0.01	0.02
	18	0.03	0.66	0.00	0.14	0.07	0.10
	29	0.04	0.78	0.03	0.09	0.01	0.05
Unfragmented	36	0.01	0.84	0.11	0.04	0.00	0.00
	39	0.01	0.98	0.00	0.01	0.00	0.00

Sampling Methods

In April and May of 2005 and 2008–2010, we surveyed vernal pools for wood frog egg masses. We collected one embryo per each of 15–30 egg masses per pond, for a total of 1,489 wood frog embryos from the 65 ponds. Samples were preserved in individual vials in 95 % ethanol for future genetic analysis.

We conducted egg mass counts subsequent to wood frog breeding events to estimate the number of breeding females (Crouch and Paton 2000). We visited ponds twice to account for differences in egg-deposition time, and used the maximum observed egg-mass count from each pool. Assuming one egg mass per female wood frog and a 1:1 sex ratio provides an estimate of breeding population density (Brede and Beebee 2004).

We visited ponds biweekly from April to October to measure a suite of environmental parameters related to water quality and hydroperiod. We measured temperature, pH, conductivity, and dissolved oxygen using a YSI 556 MPS water meter (YSI Incorporated, Yellow Springs, OH). We monitored water levels from the first date in which wood frog eggs were observed (approximately at ice out) until the drying date. We assigned a hydroperiod score to each pond based on 2-week increments, such that every unit increase in the hydroperiod score represented a 2-week increase in hydroperiod. A score of 20 was given to ponds that retained water for the duration of the season. In addition, grab samples were collected at the time of sampling and were analyzed for major nutrients, anions, cations, and organic matter using standard analytical methods (automated colorimetry, ion chromatography, and high-temperature combustion, respectively).

DNA Extraction and Microsatellite Analysis

We extracted DNA from whole wood frog embryos using the DNeasy®Tissue Kit (Qiagen, Valencia, CA). DNA was amplified by polymerase chain reaction (PCR) at nine polymorphic, species-specific, tetranucleotide loci in two multiplexes: one multiplex consisted of *RsyC52, RsyC83, RsyD32*, and *RsyD55*, and the other multiplex consisted of *RsyC11*, *RsyC41*, *RsyC63*, *RsyD20*, and *RsyD77* (Julian and King 2003). Microsatellite loci were amplified in 15 µl polymerase chain reactions consisting of 3 µl of eluted genomic DNA, 0.2–0.3 µM of each primer (fluorescently-labeled with HEX, NED, or FAM), 2.1 µM $MgCl_2$, 1XGoTaq Flexi PCR buffer (Promega Corp., Madison, WI, US), 0.2 mg/ml bovine serum albumin (BSA), 0.2 mM of deoxyribonucleotides (dNTPs), and 0.75 units of GoTaq Flexi DNA polymerase (Promega). Cycling conditions were as follows: initial denaturing step of 4 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 58 °C, and 72 °C for 1 min; and a final extension for 5 min at 72 °C.

Amplified products were electrophoresed using an automated DNA sequencer (ABI 3130 genetic analyzer, Applied Biosystems, Foster City, CA), from which genotype data were collected and genotypes scored using PEAKSCANNER software (Applied Biosystems). Positive controls were used in conjunction with the software program Allelogram (Morin et al. 2009) to standardize allele calls across electrophoretic runs. Alleles were binned manually based on the normalized raw scores generated by Allelogram.

We checked the data set for scoring errors and large-allele dropout using MICROCHECKER (van Oosterhout et al. 2004). We used the program FreeNA (Chapuis and Estoup 2007) to estimate null allele frequencies for each locus for each population. We dropped locus *RsyC63* from the dataset, as its null allele frequency exceeded 10 % in 42 of 65 populations, our a priori determined threshold for null allele inclusion. All other loci had a frequency of null alleles of less than 10 % in less than 10 % of the populations.

We calculated measures of genetic diversity, including mean number of alleles, allelic richness, F_{IS} and observed

and expected heterozygosities (H_O and H_E) for each locus and population with the program GDA (Lewis and Zaykin 2001). We calculated locus-specific F_{ST} values in GENEPOP version 4.0.10 (Raymond and Rousset 1995). Using GENEPOP, we tested for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) within each population at each locus. Tests employed the Markov chain Monte Carlo (MCMC) method and significance testing was performed using 10,000 iterations and 10,000 batches prior to analysis. We adjusted for the number of simultaneous tests using Bonferroni corrections, to maintain an overall alpha of 0.05.

Population Genetic Structure

To characterize genetic differentiation among ponds, we calculated pair-wise F_{ST} values for all pairs of ponds in FSTAT (Goudet 1995). Significance testing was performed using 1,000 permutations, with a Bonferroni adjustment for multiple tests ($\alpha = 0.05$). To test for genetic differences among populations that were sampled in two separate years (ponds 16, 16C, 16E, 16H, 16I, 18O, 29 N, 36 K, and 39E), we compared pair-wise F_{ST} values for the same pond across years. As we observed no significant differences in F_{ST} when comparing the same sites across years, we combined multi-year data for these ponds. As F_{ST}, which is heterozygosity-based, is known to be limited in its sensitivity to change and may reflect cumulative, including past, effects, we also conducted analyses with chord distance, *Dc*. *Dc* is an allele frequency-based metric, which assumes genetic differences are due to drift and not mutation (Cavalli-Sforza and Edwards 1967). *Dc* was calculated for all pairs of ponds using the program FreeNA (Chapuis and Estoup 2007).

We used the Bayesian clustering method implemented in STRUCTURE 2.3 (Pritchard et al. 2000) to characterize the genetic structure across the entire study area, without defining populations a priori. This approach partitions individuals from a population into a number of subpopulations (K) based on their genetic similarity. We conducted five independent runs for each K between one and 50 using the LocPrior model (Hubisz et al. 2009) with admixture and correlated allele frequencies, with a burn-in period of 500,000 replications followed by 100,000 Markov chain Monte Carlo steps.

We tested for hierarchical population structure with an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using ARLEQUIN (Excoffier et al. 2005). To test for regional structure at the landscape level, we placed ponds into five different hypothesized population groupings, including the two landscapes, the five pre-defined cluster assignments, and three configurations of ponds around New Hampshire state highways Rte 4, Rte 125, and Rte 9, the largest and most significant roads in our study area.

Effect of Geographic Distance and Roads on Genetic Structure

To determine the effect of geographic distance on fine-scale genetic structure within each cluster, we tested for isolation by distance (IBD) effects by comparing matrices of geographic distance and genetic distance (linearized F_{ST}) using a Mantel test with 1,000 permutations in GenAlEx 6.41 (Peakall and Smouse 1995). To test our hypothesis that roads are barriers to dispersal and increase the genetic divergence between ponds, we used t-tests to compare the mean F_{ST} and *Dc* values for all pairs of ponds within a 1 km distance separated by at least 1 road to those not separated by roads. We also used Mantel tests to test for correlations of genetic distance with road barriers in the three fragmented clusters, to determine if the presence of roads explained more variation than geographic distance. To quantify the barrier effect of roads, we mapped the NH Public Roads layer from NH GRANIT within the study area in GIS. We assigned roads values of 1–6 to reflect their hypothesized barrier effect (resistance to dispersal): 1—no roads, was assumed to present the least resistance to frog movement; 2—dirt, non-public, and private roads; 3—local dirt roads; 4—local paved roads; 5—major and minor collector, a low to moderate-capacity road; 6—principle arterial, a high-capacity urban road, assumed to present the highest resistance to frog movement. Using a least-cost path (LCP) model (Adriaensen 2003), we constructed a matrix of additive road barrier effects for all pair-wise comparisons of ponds. We performed partial Mantel tests in R version 2.5.0 (R Development Core Team 2007) using the Vegan package (Oksanen et al. 2006), to determine the relationship of F_{ST} and roads, while controlling for the effect of distance, and assessing significance with 1,000 permutations.

We used spatial autocorrelation to evaluate spatial genetic structure and potential fragmentation effects on wood frog dispersal. Spatial autocorrelation tests the significance of the correlation between geographic and genetic distance for pairs of individuals at defined distance classes, thereby evaluating the degree to which related individuals tend to cluster together spatially (Smouse and Peakall 1999). This fine-scale approach permits inference of dispersal processes and detection of finer scale patterns than might not be evident through broad-scale analyses (e.g., Peakall et al. 2003). We performed spatial autocorrelation for each cluster of ponds in each landscape using GenAlEx 6.41. We used eight even distance classes of 250-m increments, from 0 to 2,000 m, and evaluated significance with 999 permutations for each test. We compared patterns of spatial autocorrelation among the fragmented and unfragmented clusters by evaluating the significance of the correlogram at similar distance classes and comparison of

the x-intercepts. The distanceat which r intercepts the x-axis provides an estimate of the spatial extent of genetic structure, beyond which gene flow is no longer effective in connecting populations (Peakall et al. 2003).

To further characterize dispersal patterns in the two landscapes, we estimated mean parent-offspring dispersal distances (gene dispersal, σ; Hardy et al. 2006) within each cluster of ponds, using Wright's (1943) genetic neighborhood approach. We used SPAGeDi 1.3 (Hardy and Vekemans 2002) to fit a model of IBD, by regressing pair-wise linearized genetic distances (Rousset's "a;" Rousset 2000) onto log-transformed geographic distances among populations. The slope of this regression (b) allows estimation of the standard deviation of the distribution of dispersal distance (σ) using the relationship $b = 1/4D\pi\sigma^2$, where $D$is the effective density of reproducing individuals in the population (Broquet and Petit 2009). We calculated the effective density of wood frogs in each cluster from our estimates of population size (number of egg masses multiplied by two, assuming a 1:1 sex ratio; Brede and Beebee 2004, summed across all ponds within the cluster) divided by the area of the polygon that enclosed all ponds within the cluster. With SPAGeDi, we also computed the Sp statistic, which quantifies the strength of spatial genetic structure (Vekemans and Hardy 2004), using Wang's (2002) relationship coefficient. We compared Sp values across clusters in the two landscapes.

Detection of Barriers to Dispersal

To evaluate potential effects of landscape features on genetic variation, we used the single species genetic divergence tool employed in the Landscape Genetics Toolbox in ArcGIS (Vandergast et al. 2010) and the program BARRIER (Manni et al. 2004). The genetic divergence tool creates maps of genetic landscapes, which allow visualization of the genetic diversity across geographic space. Pair-wise F_{ST}s from multiple collection points are interpolated across space, such that nearby points have greater influence than more distant points. BARRIER identifies barriers to gene flow by identifying the largest genetic discontinuities in comparisons among adjacent ponds only. A combination of both approaches, when mapped in relation to landscape features, should capture the most significant influences on gene flow. BARRIER analyses were conducted with eight single locus pair-wise F_{ST} matrices and one multi-locus matrix. To avoid spurious results from one or a few loci, we only considered barriers supported by more than half of the loci. We mapped genetic divergence and barriers for each cluster and evaluated results in relation to underlying landscape features including roads, agriculture, development, and elevation. The latter was evaluated only in the unfragmented landscape, as no substantial elevation gradients exist in the fragmented landscape of our study area.

Effect of Water Quality and Hydroperiod on Genetic Structure

Means of the water quality parameters and hydroperiod scores were compared between ponds in the fragmented and unfragmented landscapes using two-sample t tests with unequal variance.We used GESTE (Foll and Gaggiotti 2006) to evaluate the effects of environmental parameters on population genetic structure. This approach uses a hierarchical Bayesian analysis to estimate pond-specific F_{ST} values, which measure the genetic differentiation between each pond and the ancestral common population (Gaggiotti and Foll 2010), and generalized linear models to identify environmental factors influencing this genetic differentiation. Posterior probabilities were estimated from different alternative models, each including a different set of environmental variables. GESTE analyses were conducted by landscape type to test the hypothesis that ponds in the fragmented landscape are affected differently by alterations to their surrounding environment. We first ran the models with only the water quality parameters (pH, conductivity, and dissolved organic carbon [DOC]) and hydroperiod as the non-genetic factors. We then ran another analysis that included the environmental parameter(s) from the highest probability model, in addition to parameters used to represent road effects, geographic distance, and population size. To obtain a population-specific parameter for road effects, we calculated the mean least cost path (LCP) road cost for each pond as the average of pair-wise LCP road costs from each pond to every other pond within each cluster (Lada 2008). The same method was used to calculate mean pond-specific geographic distance. Pond-specific population size was estimated by egg mass counts conducted at the time of sampling.

Results

Descriptive Statistics

The eight loci lacking null alleles were highly polymorphic and individual loci exhibited similar patterns of polymorphism ($H_e = 0.632$–0.942) and differentiation ($F_{ST} = 0.0050$–0.0135), with a total of 182 alleles and a mean of 22.8 alleles per locus (range 10–35; Online Resource 1). Multilocus genotypes were compiled for the 1,489 individuals at these eight loci. Six individuals (0.4 %) had missing data for no more than 2 loci. Mean number of alleles per locus per pond ranged from 9.8 to 13.4, while allelic richness ranged from 9.3 to 11.1 (Online

Resource 2). Observed and expected heterozygosities ranged from 0.716 to 0.880 per pond, F_{IS} ranged from −0.038 to 0.161, and pond-specific F_{ST}s ranged from 0.007 to 0.028 (Online Resource 2). There were deviations from Hardy–Weinberg in 6.7 % of the tests performed, but none of these deviations were significant after Bonferonni correction (adjusted p value < 0.000096). We observed linked loci in 4.2 % of the tests, and only 0.16 % of these tests were significant following Bonferonnicorrection (adjusted p value < 0.000027). Given a lack of significant pattern by locus or population, we assumed these minor deviations had no impact on the remainder of our analyses.

Population Genetic Structure

Across the entire study area, values of pair-wise F_{ST} across all pond pairs ranged from 0 to 0.037 (F_{ST}s available upon request). Fifty-six of 2,025 comparisons were significant following Bonferonni correction. Within each cluster, several pairs of ponds were significantly differentiated, suggesting that not all ponds were equally connected throughout the landscape. STRUCTURE detected no significant population structure across the study area, with the logarithm probability of the data, lnPr (X|K), maximized for $K = 1$ as the most probable number of populations.

We conducted multiple AMOVAs to test for potential road effects on population genetic structure and cluster differentiation. In all population groupings performed, the percentage of variation among the groups constituted less than a fraction of a percent (values range from 0.07 to 0.18 %; Online Resource 3). Though the values are all statistically significant ($p < 0.001$), the minimal among-group variation suggests that these might not be biologically meaningful partitions of genetic variation.

Effect of Geographic Distance and Roads on Genetic Structure

Mantel tests indicated no effect of geographic distance on genetic distance (linearized F_{ST}) over the entire study area ($R = 0.007$). When the analyses were performed by cluster, two of the three fragmented clusters (16 and 18) showed patterns of IBD, though this correlation was only significant for cluster 16 for F_{ST} and was significant for both clusters with Dc (Table 2). No IBD effect was observed using either F_{ST} or Dc in fragmented cluster 29 or in either unfragmented cluster (Table 2).

Mean F_{ST} and Dc were higher for ponds separated by roads than for those without roads in the intervening landscape (F_{ST}: 0.009 ± 0.0009 roads, 0.006 ± 0.008 no

Table 2 Mantel correlation coefficients (and p-values) for the relationships between genetic and geographical distance and genetic distance (F_{ST} and Dc) and road cost distance, and partial Mantel correlation coefficients for road cost distance while controlling for geographic distance, for each cluster of wood frog ponds. Significant correlations are indicated in bold. Road effects were only tested on the fragmented clusters

Mantel test					Partial mantel
Landscape	Cluster	Genetic distance	IBD	LCP road cost	LCP road cost
Fragmented	16	F_{ST}	**0.60**	**0.74**	0.55
			(0.008)	(0.001)	(0.012)
		Dc	**0.51**	**0.55**	0.26
			(0.026)	(0.032)	(0.175)
	18	F_{ST}	0.25	0.25	0.07
			(0.107)	(0.093)	(0.326)
		Dc	**0.38**	**0.44**	0.23
			(0.025)	(0.011)	(0.098)
	29	F_{ST}	−0.16	−0.14	−0.09
			(0.810)	(0.936)	(0.819)
		Dc	−0.11	−0.01	0.03
			(0.716)	(0.550)	(0.406)
	36	F_{ST}	−0.07	–	–
			(0.656)		
		Dc	0.01	–	–
			(0.457)		
Unfragmented	39	F_{ST}	0.00	–	–
			(0.482)		
		Dc	−0.26	–	–
			(0.917)		

roads; $p = 0.02$; *Dc*: 0.343 ± 0.004 roads; 0.332 ± 0.003 no roads; $p = 0.02$; *t*-test). Mantel tests showed a significant correlation of F_{ST} and roads for one of the fragmented clusters, Cluster 16 ($R = 0.74$; $p < 0.01$; Table 2). This correlation was significant after controlling for distance with a partial Mantel test ($R = 0.55$; $p < 0.01$; Table 2). A small positive, but nonsignificant, effect was also apparent in Cluster 18. For analyses with *Dc*, significant Mantel's correlations were found with roads for both Clusters 16 and 18, but neither partial Mantel's test was significant when controlling for distance (Table 2). No significant road effects were detected for either F_{ST} or *Dc* in Cluster 29.

Four of the clusters, 16 and 29 in the fragmented landscape and 36 and 39 in the unfragmented landscape, exhibited similar patterns of spatial autocorrelation. For these clusters, positive spatial genetic structure was only detected at the zero distance class (Fig. 2), which compares relatedness of frogs within the same pond. The extent of spatial genetic structure in these clusters, as measured by the x-intercept, ranged from 168 to 246 m (Fig. 2). In contrast, cluster 18 in the fragmented landscape exhibited positive spatial structure up to 500 m and a higher x-intercept (837 m; Fig 2).

Dispersal distances estimated in SPAGeDi ranged from 166 m to 453 m (Table 3). Wood frogs in cluster 18 had a smaller dispersal distance than frogs in the other two fragmented clusters, but similar to that of the unfragmented clusters. The value of *Sp* was an order of magnitude higher in cluster 18 than in the other fragmented clusters, which coincides with the pattern of spatial autocorrelation

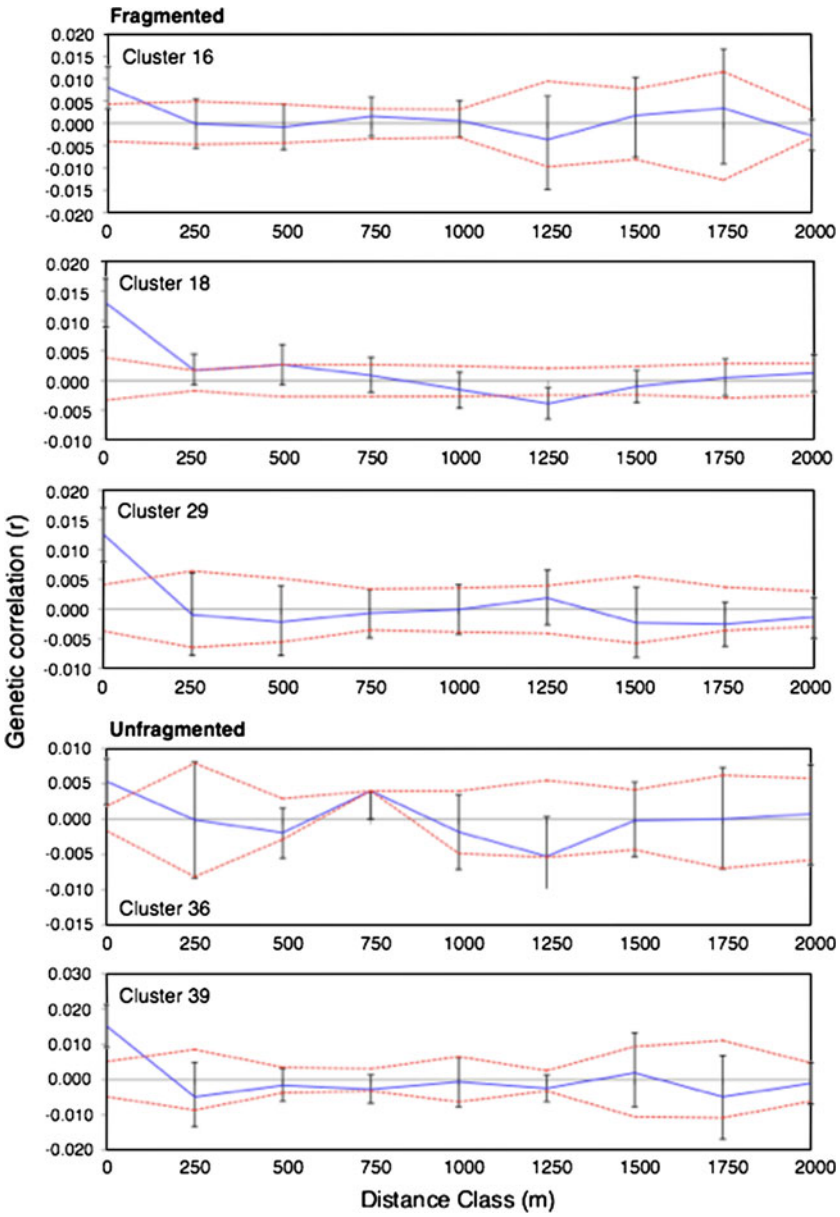

Fig. 2 Correlograms of the genetic correlation coefficient (*r*) as a function of distance for wood frogs in the three clusters in the fragmented landscape and two clusters in the unfragmented landscape. Even distance classes of 250 m were used. The extent of the spatial genetic structure, as measured by the *x* intercept, is 246, 837, and 168 m for fragmented clusters 16, 18, and 29, respectively and 245 and 110 m for unfragmented clusters 36 and 39. Null hypothesis of a random distribution of individuals is bounded by the 95 % confidence intervals (*dashed lines*). *Error bars* for mean *r* at each distance class were determined by bootstrapping

Table 3 Mean parent-offspring dispersal distances and values of *Sp* statistic, indicative of the level of spatial genetic structure, calculated with the program SPAGeDI for wood frogs in each of the five clusters of ponds in the two landscapes

Landscape	Cluster	Dispersal distance (m)	*Sp*
Fragmented	16	421	0.0003
	18	177	0.0054
	29	453	0.0007
Unfragmented	36	166	0.0027
	39	187	0.0060

observed. *Sp* for the unfragmented clusters 36 and 39 were similarly high, however, even though no spatial structure was observed in the correlograms.

Detection of Barriers to Dispersal

For each cluster, we generated maps of the genetic landscape showing hot spots of genetic divergence and superimposed on them the strongest three genetic barriers identified in BARRIER (Fig. 3 shows an example from each landscape). In the fragmented landscape, barriers and areas of high genetic divergence were associated primarily with roads, development and agriculture. For example, barriers in cluster 18 were correlated with Rtes 4 and 155 and agricultural fields (Fig. 3). In the unfragmented landscape, barriers and areas of high divergence coincided primarily with physical landscape features such as large bodies of water and high elevation. For example, barriers in cluster 39 correspond to a ridge and a lake (Fig. 3).

Effect of Water Quality and Hydroperiod on Genetic Structure

Conductivity and specific conductivity were marginally higher ($p = 0.09$; t test) in the fragmented (conductivity: mean = 118 µS/cm; range: 8–894 µS/cm; specific conductivity: mean = 156 µS/cm^c; range: 10–1231 µS/cm^c) relative to the unfragmented landscape (conductivity: mean = 45 µS/cm; range: 13–610 µS/cm; specific conductivity: mean = 57 µS/cm^c; range: 17–761 µS/cm^c). There were no differences in mean pH (range 3.4–4.9 across ponds), DOC (3.0–19.9 mg/ml) or hydroperiod scores (5.4–20) between the two landscapes. GESTE analyses for the unfragmented landscape yielded the constant model as the highest probability model, suggesting that the environmental factors are not correlated with any pattern in genetic structure. However, in the fragmented landscape, the highest probability model contained the constant and pH, with a posterior probability of 0.73 (Table 4). The positive value of alpha (α) indicates that higher pH is related to higher F_{ST}, or greater genetic differentiation. The mean σ^2 value (0.36) associated with the pH model represents a moderately good fit with the F_{ST} values and the low posterior probability (0.12) for the null model signified the validity of the pH model. Even after

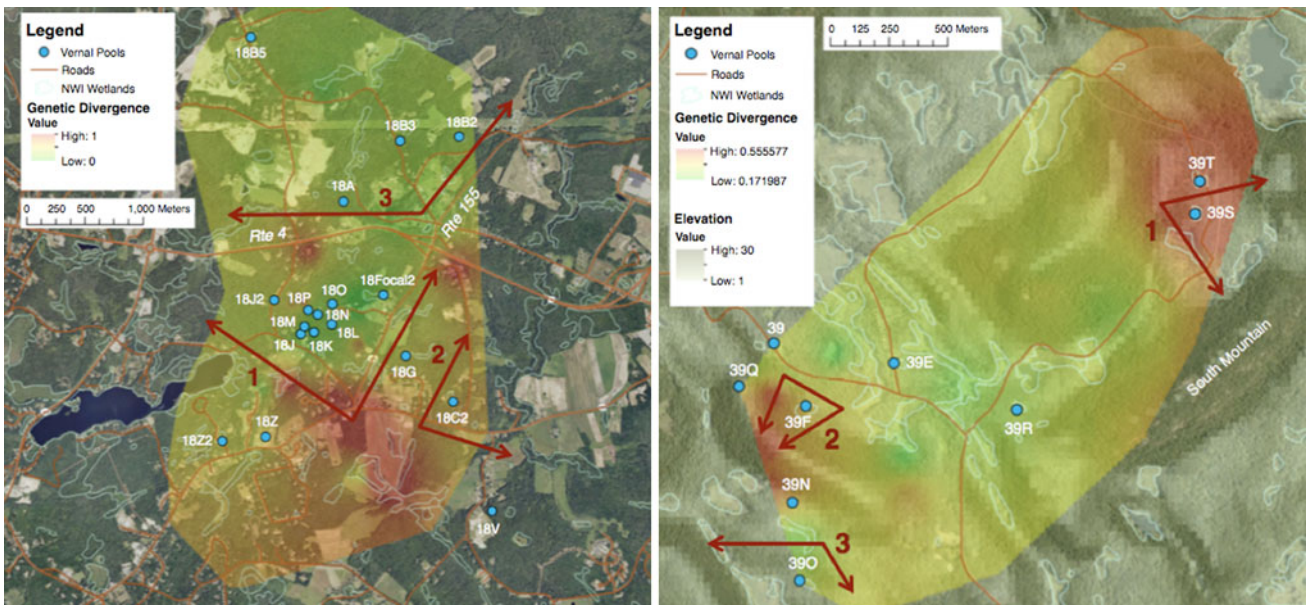

Fig. 3 Map of the genetic landscape for wood frogs in fragmented Cluster 18 and unfragmented cluster 39. Genetic divergence is represented by the *grey scale* depicted in the legend. Elevation is also depicted by a *shade gradient* of dark to light, with *darker shades* indicative of higher elevation. Overlaid are first, second, and third order genetic barriers (labeled 1–3)

Table 4 Summary of results of GESTE analyses testing influence of environmental and landscape variables on population genetic structure of wood frogs, showing the highest probability model selected for each analysis and the associated posterior probabilities

Landscape type	Analysis	Highest probability model	Posterior probability
Fragmented	Water factors [hydroperiod, pH, conductivity, DOC]	Constant, pH	0.656
	pH + road effect + geographic distance + population size	Constant, pH	0.727
Unfragmented	Water factors [hydroperiod, pH, conductivity, DOC]	Constant	0.601
	Geographic distance + population size	Constant	0.531

geographic distance and road effects were introduced into the highest probability water model, there was no influence of either factor on the outcome of the Bayesian analysis.

Discussion

Population structure

We found high connectivity and little genetic structuring among wood frog breeding ponds across the scope of the study area. This was evidenced by low pair-wise F_{ST} values (0.00–0.037), a lack of detectable structure using Bayesian clustering, and little variation in the among-groups component of AMOVA. Previous studies found similarly high levels of gene flow among wood frog breeding ponds at comparable scales (Newman and Squire 2001; Squire and Newman 2002; Crosby et al. 2009). These findings contribute to a growing body of work showing that dispersal rates of some vagile amphibians are sufficiently high to preclude metapopulation structuring. Smith and Green (2005) suggested that genetic structuring of anurans should only be expected for pond networks separated by distances of 11–13 km or more. The lack of genetic structuring observed for wood frogs at the scale of our study area may be due to the abundance of vernal pools and high forest cover in the landscape (Veysey et al. 2011), which facilitates high rates of gene flow via stepping stone dispersal.

Despite the high connectivity across the study area, fine-scale analyses revealed evidence of landscape and pond-specific influences on spatial genetic structure on a local scale. These findings suggest that the effects of suburbanization are not yet measurable on the broad scale of our study area, but that habitat fragmentation may be affecting local population dynamics. Genetic effects may not be observed for many generations following landscape change, and therefore populations may still appear connected at the broad scale due to a time lag (Keyghobadi et al. 2005; Holzhauer et al. 2006). This lag in response time is most apparent for organisms with relatively low dispersal ability (<10 km; Landguth et al. 2010), such as the wood frog, and for analyses conducted with F_{ST} as a distance metric in population-based approaches (Landguth et al. 2010; Murphy et al. 2008, 2010b). Time for 20 wood frog generations has passed since major road construction and development in the study area; theoretically, this should be sufficient time for a genetic signal to develop, although its detection requires appropriate analytical approaches (Landguth et al. 2010). Although roads have been found to have rapid effects on population structure (Keller et al. 2004; Riley et al. 2006; Clark et al. 2010), their barrier effects are more readily detected with individual-based methods and with allele frequency-based genetic distance metrics (Landguth et al. 2010; Murphy et al. 2008, 2010b). Accordingly, our fine-scale analyses and our barrier analyses were more sensitive at detecting genetic influences of the landscape than were our broad-scale approaches, and analyses with *Dc* were slightly more sensitive than analyses with F_{ST}. Lastly, while analyses with additional loci may have yielded greater sensitivity, the high polymorphism of our eight loci and the large sample sizes (almost 1,500 frogs sampled from 65 ponds) indicated that the marker set and study design were powerful for detecting fine-scale genetic differences.

Effects of habitat fragmentation and other landscape influences

Fine-scale genetic analyses revealed differences in the local dynamics in the fragmented and unfragmented landscapes. As predicted, we found higher spatial genetic structure in the fragmented than the unfragmented landscape, although patterns varied across replicate pond clusters. We found no patterns of isolation by distance (IBD) in either of the unfragmented clusters, while two of three clusters in the fragmented landscape (16 and 18) exhibited patterns of IBD. These IBD effects are consistent with findings for amphibians in other landscapes fragmented by roads (Hitchings and Beebee 1997; Rowe et al. 2000; Vos et al. 2001). For example, Hitchings and Beebee (1997) found IBD in the common frog (*Ranatemporaria*) across a scale of 0.7–4.4 km in urban landscapes and no IBD across 41.3 km in rural landscapes. Other researchers have found a lack of IBD in anurans in natural landscapes (e.g. Seppä and Laurila 1999), including wood frogs

(Newman and Squire 2001; Squire and Newman 2002; Crosby et al. 2009). By restricting dispersal, fragmentation accelerates genetic divergence, which can result in a pattern of local IBD (Rowe et al. 2000; Jump and Peñuelas 2006; Alcaide et al. 2009).

We also found some evidence that roads have impacted the genetic structure within the fragmented landscape. Across the whole study area, pairs of ponds within 1 km distances separated by one or more road had higher genetic divergence, as measured by both F_{ST} and *Dc*, than ponds without roads in the intervening landscape. Further, for both clusters 16 and 18, genetic differentiation was correlated with the additive effect of crossing roads, although the effects of roads and geographic distance could not always be separated, as suggested by nonsignificant partial Mantel's tests. We also found pronounced genetic divergence between many ponds on opposing sides of divided highway Rte 4, the largest road in our study area, and BARRIER analysis identified several genetic discontinuities consistent with roads. These findings support our prediction, as well as earlier work, showing the barrier effect of roads (Shepard et al. 2008; Balkenhol and Waits 2009).

Contrary to our predictions, fragmented cluster 29 did not exhibit IBD or road effects, which suggests that gene flow in this cluster is adequate to prevent genetic differentiation over the sampled distance, despite the presence of major roads. Some characteristics of cluster 29 may explain this finding, as it has the greatest proportion of forest cover and the smallest proportion of development among the fragmented clusters (Table 1). As such, it might be more appropriate to view the focal clusters as points along a continuum of fragmentation, rather than classifying them as either fragmented or unfragmented. Accordingly, correlations of genetic distance with geographic distance are stronger as the proportion of development increases and forest cover decreases. Genetic effects of landscape features may only be detectable when the pattern in the landscape is sufficiently variable to have an impact on gene flow (Short Bull et al. 2011).

The lack of IBD and road effects in cluster 29 might also be attributed to the presence of a culvert under Rte 4. The location of this culvert likely facilitates connectivity between two genetically similar ponds separated by only 170 m but located on opposite sides of Rte 4. Culverts are known to be used by a range of vertebrates (Hunt et al. 1987) and can function as effective dispersal corridors for amphibians (Dodd et al. 2004; Lesbarres et al. 2004; Woltz et al. 2008), since the moist conduit mitigates the risks ordinarily associated with crossing roads (Yanes et al. 1995; Jackson and Griffin 2000; Dodd et al. 2004; Patrick et al. 2010). As long as some individuals utilize the culvert, reduced mortality from road effects may be sufficient to promote connectivity.

In the unfragmented clusters, we found that genetic differentiation was relatively low, with fewer hot spots of divergence than in the fragmented clusters. Genetic divergence and barriers in the unfragmented clusters coincided primarily with natural landscape features, such as large bodies of water and elevation. Previous studies have found rivers and large bodies of water to present high resistance to amphibian dispersal (Lampert et al. 2003; Compton et al. 2007), and elevation and mountain ridges to serve as barriers to gene flow, notwithstanding geographic proximity (Funk et al. 2005; Spear et al. 2005; Giordano et al. 2007).

Our final approach for evaluating fine-scale spatial genetic structure and fragmentation effects was by spatial autocorrelation and subsequent estimation of dispersal distance from the regression of individual relatedness and geographic distance (Broquet and Petit 2009). Mean parent-offspring dispersal distances estimated from the genetic data were appreciably lower than the 1.1 km mean natal dispersal distance reported from a mark-recapture study (Berven and Grudzien 1990). Our dispersal estimates ranged from 166 to 453 m for the five clusters and did not vary predictably with landscape type. We expected that wood frog dispersal distance would be lower in the fragmented clusters, as we predicted the barrier effect of roads to restrict dispersal. We found restricted dispersal in cluster 18 relative to clusters 16 and 29, and a pattern of significant spatial genetic structure with an elevated *Sp* statistic. This pattern is consistent with the land cover characteristics of cluster 18, which had the lowest proportion of forest cover and the highest amount of fragmentation from a combination of development, agriculture and powerline corridors.

While the unfragmented clusters lacked significant spatial genetic structure, as predicted, they had small estimated dispersal distances, similar to that of cluster 18, which did not meet our expectations. This contradictory finding could be a result of the limitations of our methods. Genetic estimates of dispersal rely on several assumptions and require accurate estimates of population density. Our density estimates may have been impacted by a failure to sample all ponds in the intervening landscape or by a faulty assumption of the wood frogs' sex ratio. While studies of anurans typically assume a sex ratio of 1:1 to estimate population size, researchers have suggested that substantial variability may exist among wood frog populations (Howard and Kluge 1985; Berven and Grudzien 1990; Crouch and Paton 2000; Stevens and Paszkowski 2004). Thus our estimates might not be accurate estimates of the actual dispersal distances of wood frogs in these clusters. Additional efforts should be made with intensive sampling to evaluate the effectiveness of quantifying wood frog dispersal using genetic data.

Effects of water quality and hydroperiod on genetic structure

Negative effects on wood frog survival and abundance have been associated with water quality changes resulting from suburbanization, including acidification (low pH) and increased conductivity from road-salt runoff (Pough and Wilson 1977; Sadinski and Dunson 1992; Horne and Dunson 1994; Sanzo and Hecnar 2006; Karraker et al. 2008; Collins and Russell 2009). Additionally, landscape changes in a fragmented environment may alter the hydrology of vernal pools (Richter and Azous 1995), which may negatively affect wood frog populations if they are unable to produce metamorphs for dispersal (Pechmann et al. 1989; Babbitt et al. 2003). We predicted that poor water quality and low hydroperiod would increase genetic divergence through reduced dispersal resulting from decreased recruitment and population persistence. We found no support for our hypothesis, as with the exception of pH, no environmental factors were associated with genetic structure in the GESTE analyses. The positive relationship identified between pH and genetic differentiation contradicted our expectation that low pH would increase genetic differentiation, due to its reportedly negative effects on survival and development in several amphibian species (Horne and Dunson 1995). Although pH values ranged from 3.4 to 6.2 among ponds in the fragmented landscape, the majority of ponds had pH values below 5.0 and only three ponds had values above 6.0. We suggest, therefore, that the effect of pH detected by our GESTE models may be a spurious result that is not biologically meaningful or perhaps reflects the influence of an unmeasured variable.

The lack of correlation between genetic differentiation and conductivity and DOC may be attributed to the relatively moderate values of both in the ponds within the current study area. Karraker et al. (2008) demonstrated significant effects of conductivity on wood frog development and survival at levels of 3,000 μS, a value far exceeding that found in any of the ponds in our study (mean = 117 μS/cm; maximum = 1,231 μS/cm). Similarly, levels of DOC were not within the range found to have a negative effect on wood frogs. Horne and Dunson (1995) reported lower survival of wood frogs in water with 30 mg/l DOC, while average DOC in ponds in the current study was 8.5 mg/l and the maximum DOC recorded was 26.0 mg/l. Thus, the moderate level of suburbanization in our study area did not appear to have a negative impact on the water quality in our study ponds, and the observed values of these parameters were apparently sufficiently moderate that they did not influence local population persistence and genetic variation of wood frogs. Further, given the abundance of both wood frogs and vernal pools in this study area, hydroperiod was also not an important factor influencing genetic structure. Lastly, while we predicted greater genetic differentiation from founder effects during recolonization, the genetic effects of extinction and recolonization depend on the relative magnitude of dispersers and colonizers. If dispersal is high and colonization occurs by a large number of founders, as seems characteristics of wood frogs in our study area, then genetic differentiation will be low (Wade and McCauley 1988, Pannell and Charlesworth 1999); under such scenarios environmental impacts on metapopulation processes will be difficult to evaluate from patterns of genetic variation.

Conclusions

We found high genetic connectivity among wood frog breeding ponds across the scope of the entire study area, but evidence of fine-scale genetic structuring within independent clusters of vernal pools. This may be explained by several factors, including the abundance of both wood frogs and vernal pools in the study area, as well as the relatively high proportion of forest cover in the landscape, despite moderate levels of suburbanization. As a result, immigration and concomitant gene flow may remain high, despite the localized effect of roads. We also did not find impacts of water quality or hydroperiod on genetic structure. The degree and duration of fragmentation in the study area may still be insufficient to create genetic structuring at the broad scale. Genetic responses to fragmentation are difficult to detect by population-based analyses, and the rate of landscape change can be greater than the rate of the genetic response (Holzhauer et al. 2006; Landguth et al. 2010). This scenario is likely in southern New Hampshire, which has been experiencing a rapid, ongoing increase in population and suburbanization for the last several decades (Thorne and Sundquist 2001).

High gene flow notwithstanding, fine-scale analyses with a comparative approach indicated a response to fragmentation, including barrier effects of roads. The response to fragmentation was not equivalent across three replicated clusters of ponds, but was consistent with the degree of fragmentation observed at each. Our study highlights the importance of replication in landscape genetics research (Holderegger and Wagner 2008; Short Bull et al. 2011), by showing that different gradients of fragmentation may impact the genetic structure differently even within a similar landscape. Given the abundance and dispersal ability of wood frogs, the presence of even slight fine-scale genetic structure and barrier effects of roads, as observed in our study, could indicate the beginning of fragmentation effects on the population. It will be important to retain connectivity across the landscape, as the

expected further increases in development of this region (Thorne and Sundquist 2001) could heighten fragmentation and result in greater genetic consequences.

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