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Origin and genetic structure of a recovering bobcat (*Lynx rufus*) population

C.S. Anderson, S. Prange, and H.L. Gibbs

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Abstract: Genetic analyses can provide important insights into the demographic processes that underlie recovering populations of mammals of conservation concern such as felid species. To better understand the recent and rapid recovery of bobcats (*Lynx rufus* (Schreber, 1777)) in Ohio, we analyzed samples from four states in the lower Great Lakes Region using 12 microsatellite DNA loci and a portion of the mtDNA control region. Our results showed that a newly established population of bobcats in the eastern part of Ohio was genetically distinct from a multistate population distributed across Kentucky, southern Ohio, West Virginia, and western Pennsylvania. There was no direct genetic evidence of a bottleneck or inbreeding in this population. A lack of private alleles and only slightly lower levels of allelic richness and heterozygosity compared with its neighbors suggest that the eastern Ohio population likely originated from the migration of relatively large numbers of individuals from a source population rather than re-emerging from an undetected residual population. We recommend that a management plan should define the areas occupied by the two populations in Ohio as separate management units at least for the near future.

Key words: Lynx rufus, bobcat, mtDNA, microsatellites, population bottleneck, conservation genetics, management units.

Résumé : Les analyses génétiques peuvent fournir d'importants renseignements sur les processus démographiques qui sous-tendent les populations en cours de rétablissement de mammifères dont la conservation est préoccupante, comme des espèces de félidés. Afin de mieux comprendre le rétablissement récent et rapide des lynx roux (*Lynx rufus* (Schreber, 1777)) en Ohio, nous avons analysé des échantillons provenant de quatre États de la partie inférieure de la région des Grands Lacs en utilisant 12 sites d'ADN microsatellites et une portion de la région témoin d'ADNmt. Nos résultats montrent qu'une population de lynx roux nouvellement établie dans la partie orientale de l'Ohio est différente sur le plan génétique d'une population répartie sur plusieurs États, dont le Kentucky, le sud de l'Ohio, la Virginie occidentale et l'ouest de la Pennsylvanie. Aucun indice génétique direct de la présence d'un goulot d'étranglement ou de consanguinité dans cette population n'a été observé. L'absence d'allèles privés et des degrés de richesse allélique et d'hétérozygotie juste un peu plus faibles que ceux de ses voisins donnent à penser que la population de l'est de l'Ohio est vraisemblablement issue de la migration d'un assez grand nombre d'individus à partir d'une population source, plutôt que de la réémergence d'une population résiduelle non détectée. Nous recommandons qu'un plan d'aménagement devrait définir les aires occupées par les deux populations de l'Ohio comme représentant deux unités d'aménagement distinctes, à tout le moins pour l'avenir rapproché. [Traduit par la Rédaction]

Mots-clés : Lynx rufus, lynx roux, ADNmt, microsatellites, goulot d'étranglement génétique, génétique de la conservation, unités d'aménagement.

Introduction

Conservation biologists use analyses of genetic data to gain demographic information about endangered species or species of concern that are hard to study using conventional census techniques (Nowell and Jackson 1996; Palomares et al. 2002). For example, if populations are demographically isolated, then limited migration can lead to the development of significant population differences in allele frequencies (Moritz 1994). Identifying genetically distinct populations can be used to identify putative management units (Moritz 1994). In recovering populations, genetic comparisons between new and existing populations can also identify the demographic processes that have resulted in recolonization. If a newly occupied area is sufficiently isolated, then a recent colonization by a small number of individuals will leave a genetic signature of divergence of the newly formed population from its source through genetic drift (Ibrahim et al. 1996; Haanes et al. 2010). In contrast, sustained colonization by large numbers of immigrants will result in little or no divergence in the newly established population.

Different patterns of within population levels of genetic variation can result when recolonization occurs via alternative processes. If a small number of individuals are the source for population reestablishment in reintroduction programs, then founder effects or genetic drift can occur (Walker et al. 2001; Clark et al. 2002). In American black bears (*Ursus americanus* Pallas, 1780), there is evidence that recent natural recolonizations have likely been initiated by a single, dispersing female which has resulted in low levels of genetic variation in extant populations (Onorato et al. 2004*b*). Low overall genetic variation is also a common pattern in animals colonizing new habitats and has been documented in a recently recovered population of European otters (*Lutra lutra* (L., 1758)) (Janssens et al. 2008). However, if the number

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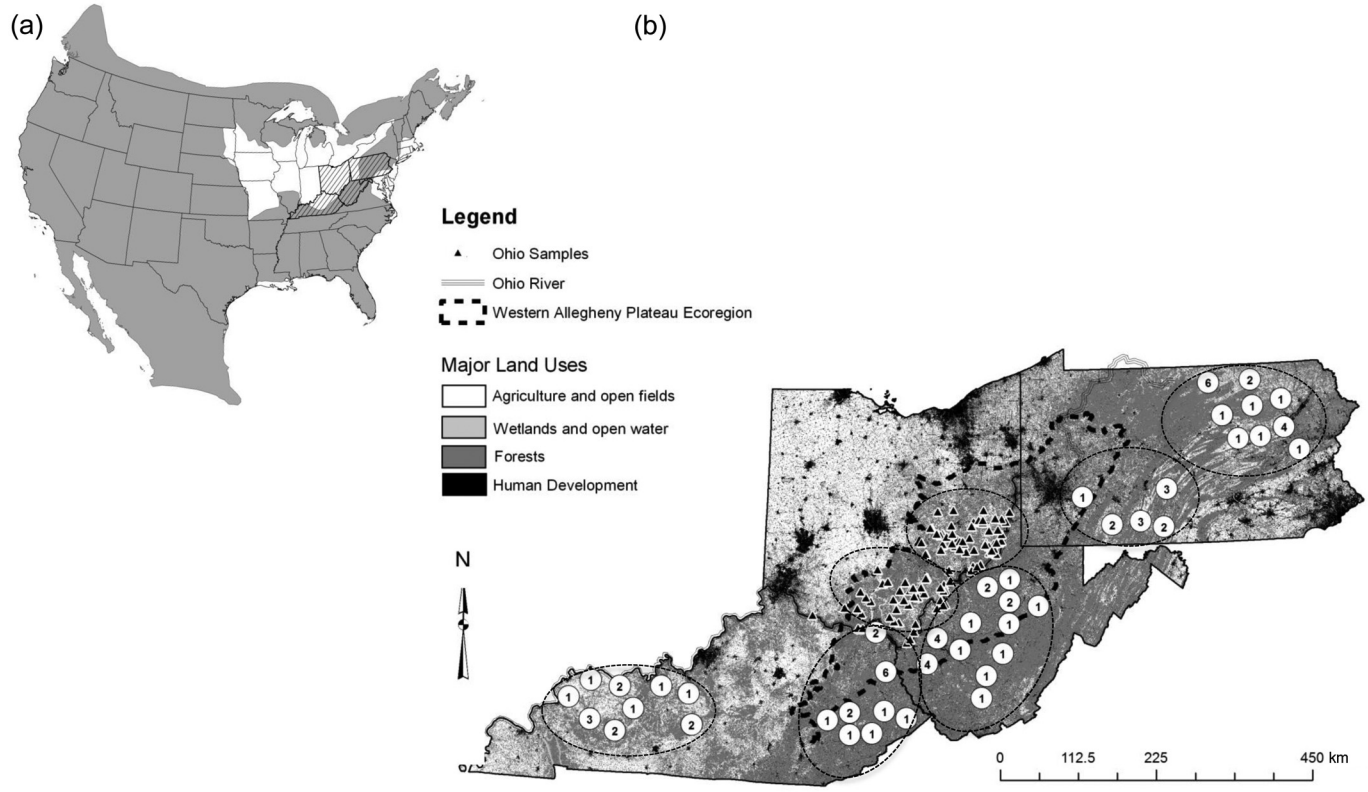
Fig. 1. (*a*) Map showing the study area within North America with hash marks and the range of bobcats (*Lynx rufus*) modified from spatial data in IUCN (2008) shaded in gray. (*b*) Map showing where bobcat samples were obtained. The samples for Ohio are mapped by actual GIS coordinates with triangles and samples from the surrounding states are mapped by county with a circle denoting the number of samples. The Ohio River, the Western Alleghany Plateau Ecoregion, and other major land-use features are marked. Ellipses with broken outlines represent the seven a priori sample locations used in subsequent STRUCTURE analyses.



of colonizing individuals is large and (or) there is ongoing gene flow between source and newly established populations, then there will be no difference in levels of variation between the original and the colonizing populations (Onorato et al. 2004*b*; Kendall et al. 2009).

Bobcats (*Lynx rufus* (Schreber, 1777)) are currently distributed from Canada south into Mexico, and within the continental United States occur from coast to coast. There is a conspicuous absence in the upper Midwest, presumably due to the bobcat's avoidance of intensive agriculture (McDonald et al. 2008; Fig. 1*a*). However, the majority of states including those in the Midwest have reported an increasing trend in bobcat numbers, as well as expanding distributions (Roberts and Crimmins 2010). This trend has been attributed to increased habitat availability due to changing land-use practices (e.g., reversion of agricultural land and changes in farming practices) and more intensive harvest management at the state level (Roberts and Crimmins 2010). In Ohio, bobcats were once found throughout the state, but were extirpated by the 1850s as forests were cleared for settlement and agriculture (Trautman 1977). Since 1946, there have been 464 verified reports of bobcats in Ohio, of which 94% have occurred since 2000 (S. Prange, unpublished data). Bobcat recovery in Ohio has occurred in conjunction with the reversion of the Ohio portion of the Western Allegheny Plateau ecoregion from farmland back to woodland (Hutchinson et al. 2003; Fig. 1*b*). In 2012, the bobcat was reclassified from endangered to threatened; in 2014, it was removed from Ohio's threatened and endangered species list.

Previous genetic studies on bobcats have found evidence for and against genetic structuring among bobcat populations at different spatial scales. For example, at regional to local geographic scales, Williams (2006) and Millions and Swanson (2007) found evidence for genetic differentiation between bobcats found in the lower and upper peninsulas in Michigan. Reding (2011) found structure between populations in the Midwestern USA in habitats subdivided by intensive row-crop agriculture, and Riley et al. (2006) and Lee et al. (2012) found genetic differences between bobcat populations on either side of a major highway in southern California. In contrast, Croteau et al. (2010) found the bobcat population in southern Illinois to be genetically panmictic, as did Reid (2006) for bobcats sampled in southern Georgia and northern Florida. At a larger spatial scale, with samples from 14 states and 2 Canadian provinces, Croteau (2009) identified an isolation-by-distance effect and potential historical subdivision between western bobcats (California, Wyoming, Nevada, and North Dakota) and those from the rest of the sampled range. Reding et al. (2012) analyzed 1700 samples throughout the majority of the bobcat range and found that the data distinguished bobcats in the eastern USA from those in the western half, with no obvious physical barrier to gene flow.

One limitation of these studies is that many have focused on long-standing populations of these animals (e.g., Croteau 2009; Millions and Swanson 2007). However, over much of their USA range, bobcats are showing increases in abundance and the recolonization of areas where they were previously extirpated (Woolf and Hubert 1998; Roberts and Crimmins 2010). Natural recolonizations are rarely documented in large terrestrial mammals (Onorato et al. 2004*a*). Therefore, genetic studies that estimate levels of inbreeding and the likelihood of bottlenecks as bobcats recover and reclaim parts of their former range could provide valuable insights as to the demographic processes that underlie these recolonization events and elucidate the genetic characteris-

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Table 1. Characteristics of 12 microsatellite loci in bobcats (*Lynx rufus*) from seven sampling locations in the southern Great Lakes region, USA.

Locus	*N*	T_a (°C)	Size range (bp)	N_a	Allelic richness	H_O	H_E	P_{HWE}	Null allele frequency
BCE5T[a]	192	TD 59–55	235–287	11	6.54	0.77	0.76	0.37	0.04
FCA35[a]	186	TD 59–55	124–160	14	8.84	0.79	0.86	0.02	0.09
FCA90[a]	193	TD 59–55	101–113	8	6.24	0.76	0.80	0.38	0.08
FCA96[a]	183	TD 59–55	175–201	11	7.06	0.80	0.80	0.69	0.04
FCA132[a]	194	TD 59–55	165–179	8	6.27	0.78	0.80	0.44	0.04
BCG8T[b]	188	TD 55–50	258–284	14	9.47	0.89	0.88	0.28	0.02
FCA23[b]	190	TD 55–50	132–144	6	4.93	0.77	0.75	0.33	0.09
FCA26[b]	188	TD 55–50	124–142	10	5.07	0.68	0.71	0.74	0.03
Lc109[b]	189	TD 55–50	164–186	10	6.98	0.80	0.82	0.40	0.04
BC1AT[c]	188	TD 55–50	284–312	8	5.21	0.73	0.74	0.53	0.01
FCA43[c]	190	TD 55–50	114–124	6	3.94	0.56	0.57	0.94	0.01
FCA126[c]	190	TD 55–50	114–140	11	7.02	0.87	0.81	0.98	0.00

Note: Sample size (*N*), annealing temperature for amplification (T_a, where TD is touchdown), size range (bp) of alleles, number of alleles (N_a), allelic richness, observed (H_O) and expected (H_E) heterozygosities, *P* value for the exact test of Hardy–Weinberg equilibrium (P_{HWE}), and estimated null allele frequency are given above. Loci multiplexed together are denoted by superscript letters.

tics of these newly re-established populations (also see Lee et al. 2012; Reding et al. 2012).

The recent and rapid recovery of bobcats in Ohio provides an opportunity to examine the demographic and genetic characteristics of a recolonization event involving a terrestrial mammal of conservation concern. To carry out such a study, we tested the predictions that (*i*) Ohio bobcats would consist of multiple genetic distinct populations that originated from different source populations in nearby states, (*ii*) the genetic characteristics of Ohio bobcat populations would reflect a recent colonization event with a small number of founders with low levels of genetic variation and evidence of inbreeding, and (*iii*) there would be genetic signatures of a bottleneck consistent with recent founder events.

Materials and methods

Sample collection

We obtained tissue samples for genetic analyses from the Ohio Department of Natural Resources (*N* = 111), the West Virginia Division of Natural Resources (*N* = 25), the Kentucky Department of Fish and Wildlife Resources (*N* = 31), and the Pennsylvania Game Commission (*N* = 29) (Fig. 1*b*). Bobcats were obtained through road mortality and incidental captures from 2002 to 2012. Samples of skin, tongue, or other tissue were collected from specimens and stored at –20 °C. Any county within these states where no samples were collected was due to a lack of available carcasses and was not the result of purposely sampling in spatially discrete groups (Fig. 1*b*).

Laboratory methods

We extracted DNA from tissue samples using the DNeasy Blood and Tissue Kit (Qiagen) with the addition of 2 μL Rnase A (10 mg/mL). We then genotyped samples at 12 microsatellite loci: *BC1AT*, *BCE5T*, *BCG8T* (Faircloth et al. 2005), *Lc109* (Carmichael et al. 2000), *FCA23*, *FCA26*, *FCA35*, *FCA43*, *FCA90*, *FCA96*, *FCA126*, and *FCA132* (Menotti-Raymond et al. 1999). These primers have previously been used to investigate population structure, genetic diversity, and sex-biased dispersal in bobcats (e.g., Millions and Swanson 2007; Croteau et al. 2010). Samples were genotyped at 3–5 loci in each of three multiplex polymerase chain reactions (PCR) (Table 1). We performed PCR in 10 μL reactions containing 5 μL of Multiplex PCR Master Mix (Qiagen), 1 μL of primer mix (containing 2 μmol/L of each primer), and 3.5 μL of RNase-free H_2O. Thermal cycling followed a touchdown program with an initial activation step at 95 °C for 15 min followed by eight cycles of 94 °C for 30 s, annealing temperature for 90 s decreasing 0.5 °C per cycle, 72 °C for 60 s, followed by 21 cycles at the lowest annealing temperature, and a final extension at 60 °C for 30 min (Table 1). Fragments were sized with the Naurox size standard described in DeWoody et al. (2004) and products were run on a 3100 Genetic Analyzer (ABI). Alleles were identified and binned using GENEMAPPER version 3.7 software (ABI).

To generate additional genetic information from these samples, we also amplified and sequenced approximately 600 bp of the left domain of the mtDNA control region of all samples using primers CR1 and CR2R (Palomares et al. 2002). Structure and variation in this region has been previously used to examine genetic variation and spatial structure in Eurasian lynx (*Lynx lynx* (L., 1758)) (Hellborg et al. 2002; Rueness et al. 2003) and bobcats (Croteau 2009). This region contains an 80 bp repeat (Lopez et al. 1996) in the repetitive segment (RS-2) that varied in the number of repeats across samples (Jae-Heup et al. 2001; Hellborg et al. 2002). Due to the difficulty of aligning the sequences (Croteau 2009) and potential issues with heteroplasmy (Hoelzel et al. 1994), we included only 138 bp of nonrepetitive sequence upstream of RS-2 in the analysis.

To generate sequence, we amplified this region using PCR in 10 μL reaction volumes consisting of 5.5 μL ddH_2O, 1 μL $MgCl_2$-free 10× PCR buffer, 1 μL BSA (10 mg/mL), 0.2 μL dNTPs (10 μmol/L), 0.3 μL each of forward and reverse primer (10 μmol/L), 0.6 μL $MgCl_2$ (50 mmol/L), and 0.05 μL Platinum *Taq* DNA polymerase (5 U/μL). Amplifications included an initial incubation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 20 s, 56 °C for 20 s, 72 °C for 60 s, and a final extension at 72 °C for 5 min. We precipitated PCR products using a polyethylene glycol – ethanol procedure and resuspended them with ddH_2O. We sequenced in the forward and reverse direction using primers CR1 and CR2R with the Big Dye version 3.1 Cycle Sequencing Kit (ABI). We cleaned sequencing products with Sephadex and ran them on a 3100 Genetic Analyzer (ABI). We edited sequences using ALIGNER (CodonCode) and aligned them in BIOEDIT (Hall 1999) using CLUSTAL W (Thompson et al. 1994).

Assessing genetic variation

The samples were divided into seven a priori sample locations based on spatial clustering as follows: E OH (eastern Ohio), S OH (southern Ohio), W KY (western Kentucky), E KY (eastern Kentucky), WV (West Virginia), W PA (western Pennsylvania), and E PA (eastern Pennsylvania) (Fig. 1*b*). Departures from Hardy–Weinberg equilibrium for each microsatellite loci and sample location were assessed using the Markov chain method in GENEPOP version 4.2 (Raymond and Rousset 1995) and critical *P* values were corrected for multiple tests using the Benjamini–Yekutieli method (Narum

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2006). The frequency of potential null alleles at each locus in each sample location was estimated using the EM algorithm of Dempster et al. (1977) in GENEPOP version 4.2 (Raymond and Rousset 1995). To calculate an error rate for the microsatellite and mtDNA data in our study, we randomly chose a subset of 18 samples for microsatellite and 6 samples for mtDNA to be amplified and genotyped blindly a second time. The genotypes obtained were compared with the original runs and the number of mismatches was counted (Bonin et al. 2004; Hoffman and Amos 2005).

For a preliminary assessment of levels of genetic variation at each of the seven a priori sample locations, we used FSTAT version 2.9.3.2 (Goudet 2001) to determine the number of alleles, observed and expected heterozygosities, and allelic richness (corrected for sample size) for each microsatellite locus. To assess genetic variation within genetically distinct populations as defined by STRUCTURE (see below), we again used FSTAT (Goudet 2001) to calculate expected heterozygosity and allelic richness (corrected for sample size) and GENEPOP (Raymond and Rousset 1995) to calculate observed heterozygosity. CONVERT (Glaubitz 2004) was used to indicate the number of private alleles per genetically distinct population and ADZE version 1.0 (Szpiech et al. 2008) was used to estimate private allelic richness using a standardized sample size of 17 individuals. In ADZE, the estimated private allelic richness is the number of private alleles expected in a population based on the rarefaction method when sample sizes differ across populations (Szpiech et al. 2008). For mtDNA data, we calculated the number of haplotypes, haplotypic (gene) diversity, and nucleotide diversity per genetically distinct population in DnaSP (Rozas et al. 2003).

Defining genetically distinct populations

To identify possible genetically distinct populations (e.g., Moritz 1994), we assessed genetic differentiation in three ways. First, we used the Bayesian clustering method implemented in STRUCTURE version 2.3.4 to infer the number of distinct genetic groups observed in our microsatellite data (Pritchard et al. 2000). Each STRUCTURE run consisted of a burn-in of 100 000 Markov chain Monte Carlo (MCMC) iterations followed by 300 000 iterations using the admixture model with sample locations (E OH, S OH, W KY, E KY, WV, W PA, E PA) as priors and correlated allele frequencies (Falush et al. 2003) as recommended in Gilbert et al. (2012). In addition, runs of different lengths were also performed to check for consistency among runs. We performed 20 runs for each value of K ranging from 1 to 7 after initial results suggested that Ohio samples should be further subdivided into two separate populations (i.e., eastern and southern; Fig. 1*b*). To confirm that burn-in was adequate, we checked for convergence in time-series data plots of values of summary statistics estimated by the program. To determine the most likely value of K suggesting the number of populations, we used the Evanno et al. (2005) method implemented in the program STRUCTURE HARVESTER (Earl 2009). We provide both the bar plot showing individual assignments for the given K and the ΔK graph as recommended by Gilbert et al. (2012). The estimated membership of individuals (using mean values of q) assigned to different clusters based on a priori sample locations was calculated as mean ± SE.

To complement the STRUCTURE analysis, we identified an optimal number of genetic clusters and probabilistically assigned samples to groups with adegenet, as implemented in R version 2.12 (R Development Core Team 2013). Adegenet performs model-free K-means clustering, which, in contrast to STRUCTURE, does not rely on assumptions such as Hardy–Weinberg equilibrium and linkage disequilibrium within groups (Jombart et al. 2010). Specifically, we first used the find.clusters function in adegenet to identify the optimal clustering solution based on Bayesian information criterion (BIC) for possible K values 1–10. We then used the optimal clustering solution to perform discriminant analysis of principal components (DAPC), which is a multivariate analysis that minimizes within-group variance while maximizing among-group variance (Jombart et al. 2010). We plotted the identified clusters along the first two discriminant functions to visualize how variation is partitioned among the identified groups and we also obtained posterior probabilities of group membership for each sample based on the DAPC analysis.

We analyzed levels of genetic differentiation between the same seven a priori sample locations based on F_{ST} values generated using FSTAT (Goudet 2001). We assessed whether there was evidence for isolation by distance using F_{ST} values between the seven individual sample locations generated using microsatellite data. This analysis was performed using IBD Web Service (Jensen et al. 2005). For mtDNA data, we used ARLEQUIN (Schneider et al. 2000) to calculate pairwise F_{ST} based on haplotype frequencies and used DnaSP (Rozas et al. 2003) to calculate overall F_{ST}. To compare the overall F_{ST} values from each type of marker, we applied the correction described in Crochet (2000) of $F_{ST(mitochondrial)} = 4 \cdot F_{ST(nuclear)}/(1 + 3 \cdot F_{ST(nuclear)})$.

Estimates of contemporary migration

To identify recent immigrants within genetically distinct populations, we used assignment tests (e.g., Waser and Strobeck 1998) implemented in the program GENECLASS version 2 (Piry et al. 2004). Each individual's probability of genetic assignment to the population from which it was collected was estimated using Bayesian probabilities based on the similarity of its multilocus genotype to genotypes found in each population (Rannala and Mountain 1997). We used a threshold of ≥90% for the likelihood scores in assigning individuals to a population.

We also used a Bayesian method implemented in BAYESASS version 1.3 to estimate rates of recent immigration (i.e., within the last 1–3 generations) with microsatellite genotypes in BAYESASS (Wilson and Rannala 2003). This program does not assume Hardy–Weinberg equilibrium within populations and provides an estimate of the mean posterior distribution of m for all population pairs, which is the proportion of individuals in location i that have location j as their ancestral location. This provides both the proportion of residents and the proportion of immigrants in each population. The program was run for 3×10^6 iterations, 2000 sampling frequency, and the first 1×10^6 iterations were discarded as burn-in. We report the proportion of residents in both Ohio sampling locations to compare with the results reported with GENECLASS.

Inbreeding and bottlenecks

We estimated levels of inbreeding by calculating F_{IS} (the inbreeding coefficient) for samples from genetically distinct bobcat populations using microsatellite data in FSTAT (Goudet 2001). Because the recent recolonization of Ohio suggests the possibility that populations have undergone a bottleneck, we used two methods implemented in BOTTLENECK version 1.2.02 to detect whether there was a genetic signature of such a phenomenon (Cornuet and Luikart 1996; Piry et al. 1999). First, we used Wilcoxon's test, which examines whether populations exhibit a greater level of heterozygosity than predicted in a population at drift–migration equilibrium. This test is most sensitive at detecting bottlenecks within the last 2–4 N_e generations (where N_e is the effective population size). We performed 10 000 simulations under the stepwise mutation model (SMM) and the two-phase model (TPM). Second, we examined whether the allele frequency followed a normal L-shaped distribution because a mode shift discriminates recently bottlenecked populations from stable populations. This test is based on the idea that nonbottlenecked populations at mutation–drift equilibrium are expected to have a large proportion of alleles at low frequency and a smaller proportion of alleles at intermediate frequencies (L-shaped distribution; Luikart et al. 1998). Due to the relatively recent time frame of bobcat recolonization in Ohio, the mode-shift test should be more

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appropriate in detecting recent bottlenecks than analyses based on heterozygote excess.

Results

Genetic variation and population differentiation

We genotyped 194 bobcats from seven sample locations (circled with ellipses in Fig. 1*b*) at 12 microsatellite loci. The number of alleles per locus ranged from six (*FCA23* and *FCA43*) to 14 (*FCA35* and *BCG8T*; Table 1). Allelic richness varied from 3.94 to 9.47 and observed and expected heterozygosities for each locus were moderate to high and ranged from 0.56 to 0.89 (Table 1). No microsatellite loci deviated from Hardy–Weinberg equilibrium and estimates of the frequency of possible null alleles were ≤9% (Table 1). The genotyping error rate was 0% for the 16 samples amplified and genotyped a second time.

STRUCTURE, which was used to estimate the number of genetic clusters of populations among the seven sample locations (E OH, S OH, W KY, E KY, WV, W PA, E PA; circled with ellipses in Fig. 1*b*), showed that the optimal number of clusters was K = 3 (mean ln $P(K)$ = –7944.1; Fig. 2*a*). We found that 84% (48/57 total) of individuals from the a priori population in E OH were assigned to one cluster with a mean estimated membership of 0.914 (SE = 0.077), 100% (18/18) of E PA bobcats were assigned to a second cluster with a mean estimated membership of 0.957 (SE = 0.044), and 89% (48/54) of the S OH bobcats were assigned to a separate multistate cluster with a mean estimated membership of 0.885 (SE = 0.107) that included samples from the remaining a priori sample locations (Fig. 2*b*).

The results from the adegenet cluster analyses broadly support the STRUCTURE results. Based on K values evaluated using BIC scores, there were four clusters of genetically distinct samples in the data: all contain a mixture of samples from different locations, but like the STRUCTURE results, are dominated by samples from specific locations (Supplementary Fig. S1*a*).[1] E OH and S OH samples are largely found in different clusters, supporting the conclusion that they are genetically distinct at least to a limited extent: 54% (31/57 total) of E OH samples but only 13% (7/54) of S OH are assigned to cluster 1, whereas 48% (26/54) of S OH samples but only 11% (6/57) of E OH are assigned to cluster 4 (Supplementary Fig. S1*b*).[1]

Finally, as an alternative way of assessing structure, for microsatellite data, we calculated pairwise values of F_{ST} and found that even though values were low, all 21 comparisons were significantly different from zero after correction (Table 2) with an overall F_{ST} = 0.037 (95% confidence interval (CI) = 0.029–0.046, $P < 0.001$). This means that all seven a priori sample locations show some level of genetic distinctiveness from each other. A significant isolation-by-distance effect was found among the seven a priori sample locations ($R = 0.71$, $P = 0.005$; Fig. 3). For mtDNA data, we found that nine pairwise comparisons of F_{ST} were significant after Benjamini–Yekutieli correction (Table 2). The overall level of population differentiation in mtDNA (F_{ST} = 0.240) was an order of magnitude larger than that based on microsatellite data alone. After correction following Crochet (2000), the overall F_{ST} value observed for mtDNA was 0.133, which is still roughly an order of magnitude larger than that observed for microsatellites.

Within-population genetic variation for populations based on the three clusters from STRUCTURE was relatively high with measures slightly lower in E OH compared with the multistate population (S OH, WV, W KY, E KY, and W PA; Table 3*a*). Expected heterozygosity ranged from 0.73 in E OH to 0.80 in the multistate population with a value of 0.74 in E PA. Allelic richness averaged across loci varied from 6.0 for E PA to 7.5 for the multistate population that includes S OH but was 6.4 for E OH (Table 3*a*). The number of private alleles without accounting for differences in sample size across populations was 0 in E OH, 14 in the multistate population including S OH, and 1 in E PA. When sample size was standardized at 17 individuals, the number of private alleles was still the lowest in E OH compared with the other two populations (Fig. 4).

mtDNA haplotype designations were identical for the six samples that provided informative sequence in two independent runs, resulting in an error rate of 0%. We identified 6 unique mtDNA haplotypes and the number of haplotypes ranged from 2 to 6 per genetically distinct population (Table 3*b*). The haplotypes in the E OH population are a subset of those found in the multistate population that includes S OH. Haplotype diversity varied between 0.111 in E PA to 0.568 in the multistate population (S OH, WV, W KY, E KY, and W PA) and nucleotide diversity was low across populations (range 0.001–0.005).

Estimates of contemporary migration

We found that GENECLASS could assign 150 of 194 individuals to one of the three populations with ≥90% likelihood. Not surprisingly, most of these individuals were classified as residents in that they were assigned to their population of collection (≥80% for all three populations; Table 4). Nine bobcats (20.5%) likely moved into E OH from the multistate population to the south (S OH, WV, W KY, E KY, and W PA), compared with eight migrants (8.8%) in the opposite direction (Table 4). Although it was estimated that there was no migration between E OH and E PA likely due to the large distance between them, results suggest that 1–2 bobcats migrated between the multistate population and the E PA (Table 4). Multiple runs of BAYESASS provided similar results, with the proportion of residents in each of the three populations ranging from 0.88 to 0.96.

Inbreeding and bottlenecks

The coefficient F_{IS} suggested that inbreeding levels within each population were low, with 0.000 for E OH and 0.043 for the multistate population containing S OH (Table 3*a*). The populations of bobcats in both E OH and E PA showed no genetic evidence of a bottleneck based on results from both Wilcoxon's test and the mode-shift test (Table 5; Fig. 5). For the multistate population of bobcats from S OH, WV, KY, and W PA, the Wilcoxon's test under the TPM was significant for heterozygote excess, whereas the Wilcoxon's test under the SMM and the mode-shift test suggested no recent genetic bottleneck (Table 5; Fig. 5).

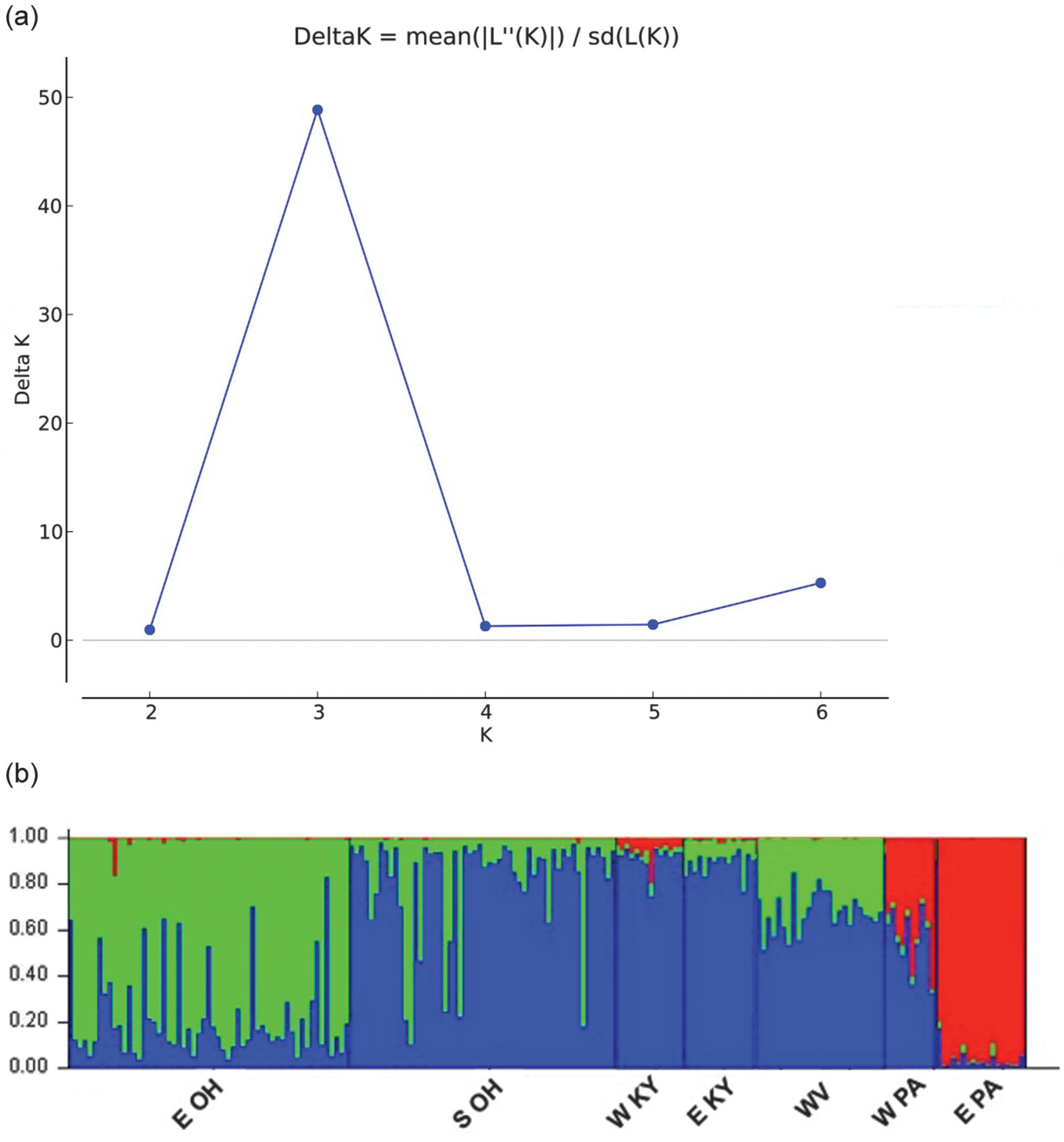
Discussion

This study was focused on using genetics to make inferences about the dynamic processes associated with the range expansion and natural recolonization of a large mammal. Our major results are that (*i*) based on analyses of population structure and genetic differentiation, we found evidence for a separate population of bobcats in eastern Ohio, but (*ii*) given that overall levels of genetic variation were moderate to high and there was no evidence for a bottleneck, the numbers of founders was likely relatively large and (or) there was recent migration of individuals between source and recolonized populations. We discuss the implications of these results below.

A number of results support the scenario that the genetic distinctiveness of the eastern Ohio population developed through a founder effect affecting a limited number of recolonizing animals (e.g., Walker et al. 2001; Randi et al. 2003; Haanes et al. 2010) rather than the re-emergence of a small undetected residual population in the area that had experienced high levels of genetic drift (Szpiech et al. 2008). First, the eastern Ohio population contains no private alleles or haplotypes as are predicted to be present

[1]Supplementary Figs. S1*a* and S1*b* are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2015-0038.

Fig. 2. STRUCTURE results for bobcats (*Lynx rufus*) showing (*a*) the Δ*K* graph from STRUCTURE HARVESTER and (*b*) the bar plot with individual assignments from STRUCTURE for *K* = 3 (ln *P*(*D*) = −7944.1). Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); W KY (western Kentucky); E KY (eastern Kentucky); WV (West Virginia); W PA (western Pennsylvania); E PA (eastern Pennsylvania). Figure appears in colour on the Web.



under the re-emergence hypothesis (Szpiech et al. 2008). The variation observed is a limited subset of that presence in potential source populations. Second, our assignment test and immigration results show direct evidence for movement of individuals from a potential source population (the multistate cluster) consistent with a colonization scenario. Other studies of recolonizing populations of mammals have shown similar patterns suggesting similar colonization dynamics. For example, a study on natural recolonization of river otters (*Lontra canadensis* (Schreber, 1777)) in Missouri reported significant genetic differentiation among some newly founded populations, little to no loss of genetic variation, and the presence of the most common mtDNA haplotypes range-wide in recolonized populations (Mowry et al. 2015). In Scandinavian wolverines (*Gulo gulo* (L., 1758)), founding effects were also

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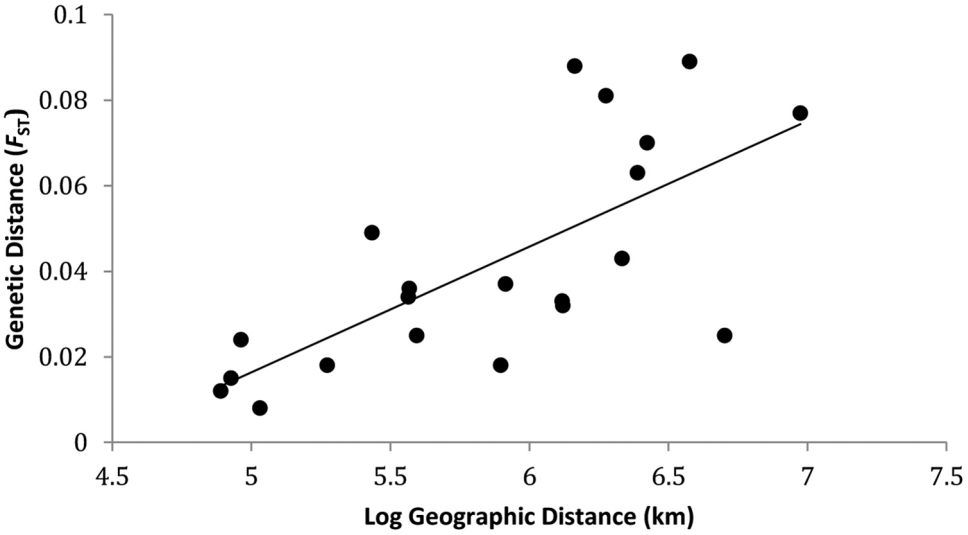
Table 2. Pairwise F_{ST} values for seven sample locations of bobcats (*Lynx rufus*) based on microsatellite loci are given above the diagonal and mtDNA sequences are given below the diagonal.

	N								
	Microsatellite	mtDNA	E OH	S OH	W KY	E KY	WV	W PA	E PA
E OH	57	49	—	0.024*	0.063*	0.034*	0.015*	0.049*	0.088*
S OH	54	51	0.217*	—	0.033*	0.012*	0.008*	0.018*	0.070*
W KY	14	15	0.163	0.000	—	0.037*	0.043*	0.025*	0.077*
E KY	15	14	0.034	0.264*	0.198	—	0.018*	0.032*	0.089*
WV	25	23	0.122	0.000	0.000	0.169*	—	0.025*	0.081*
W PA	11	9	0.157	0.499*	0.510*	0.158	0.439*	—	0.036*
E PA	18	18	0.124	0.480*	0.503*	0.147	0.426*	0.000	—

Note: Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); W KY (western Kentucky); E KY (eastern Kentucky); WV (West Virginia); W PA (western Pennsylvania); E PA (eastern Pennsylvania).

*Denotes significance after a Benjamini–Yekutieli adjustment for multiple tests.

Fig. 3. Genetic isolation by distance of the seven a priori sample locations of bobcats (*Lynx rufus*) as inferred using multilocus estimates of F_{ST} values and the logarithm of the geographical distance ($R^2 = 0.50$, $P = 0.005$).



inferred in significant subdivision and a loss of genetic variation, and no private alleles were found in the recolonizing populations (Walker et al. 2001). Thus, this pattern of recolonization seems to be common among populations of terrestrial mammals recolonizing areas where species were previously found until extirpation by humans.

Only a limited loss of genetic variation in the recovering bobcat population in eastern Ohio has occurred. Other studies on bobcats in the USA and Canada have found very similar levels of heterozygosity and allelic richness using the same loci (Millions and Swanson 2007; Croteau 2009; Reding et al. 2012). We also found no evidence of inbreeding in any of the populations, including the recently recolonized eastern Ohio population. Rapid inbreeding in small populations produces increased homozygosity and can reduce fitness (Lande 1988). While the overall inbreeding statistic F_{IS} was significant across the 10 groups covering the USA in Reding et al. (2012), levels were not significant in the Pennsylvania groups that were close to our study populations. Another study found no evidence of inbreeding in bobcat populations (Lee et al. 2012). While levels of genetic variation are slightly lower in eastern Ohio, at this time there is little concern about the genetic "health" of bobcats in the newly recolonized areas.

Why is it that only a limited loss of genetic variation has occurred during the recolonization of bobcats in Ohio and no evidence of a bottleneck? One possibility is likely that these recolonization events involved relatively large numbers of founding individuals. We also found genetic evidence of substantial recent migration of bobcats in both directions connecting the eastern Ohio population to the large population to the south (Table 4). High recent gene flow has also been documented in other large carnivores like the wolverine (Walker et al. 2001). Our evidence for a significant isolation-by-distance relationship suggests that bobcat populations do not generally consist of discrete genetically isolated populations, but that migration between populations is constrained by distance. At large scales, isolation by distance can explain genetic differentiation in wolf (*Canis lupus* L., 1758) (Geffen et al. 2004), puma (*Puma concolor* (L., 1771)) (McRae et al. 2005), and river otters (Blundell et al. 2002). We view the genetic signature of differentiation in bobcats as likely a transitory nonequilibrium feature that will erode over time as populations come into migration–drift equilibrium. The statistically significant but low levels of genetic differentiation may already be evidence for this. In work on other recolonizing species, Missouri river otters have retained genetic diversity levels similar to those of the source populations, but genetic structure also has not reached an equilibrium between migration and genetic drift 30 years after the start of reintroduction efforts (Mowry et al. 2015). Another study on the genetic structure of recovering European otters also found significant genetic differentiation and moderately high heterozygosity, and showed that some populations were partially admixed with no recent bottlenecks observed (Randi et al. 2003). Still, evidence for present-day genetic differentiation among bobcat populations implies some level of demographic independence, and so based on Moritz's (1994)

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Table 3. Genetic variation of populations of bobcats (*Lynx rufus*) based on (*a*) microsatellites and (*b*) mtDNA.

(*a*) Microsatellites.

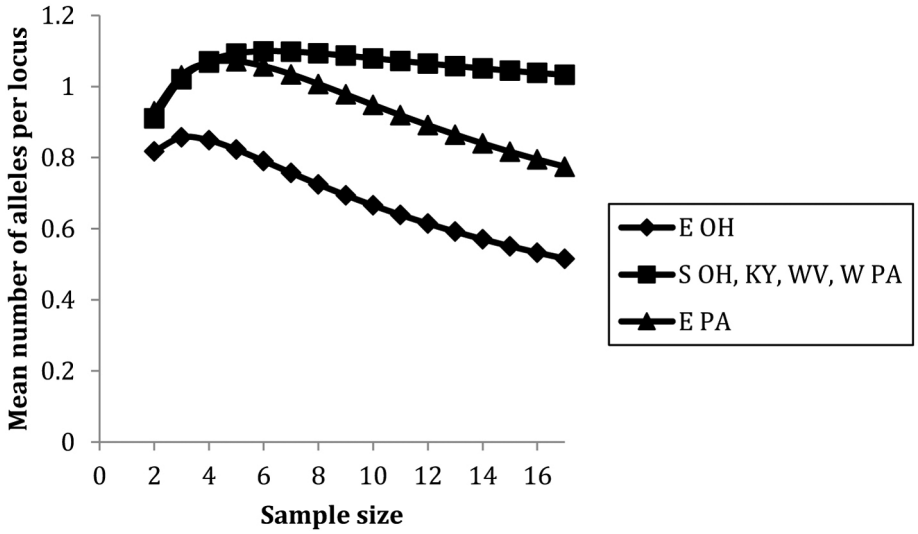
Population	*N*	Mean allelic richness	H_O Mean	H_O SD	H_E Mean	H_E SD	F_{IS}
E OH	57	6.4	0.73	0.09	0.73	0.11	0.000
S OH, WV, W KY, E KY, W PA	119	7.5	0.77	0.10	0.80	0.08	0.043
E PA	18	6.0	0.76	0.12	0.74	0.09	−0.042

(*b*) mtDNA.

			Percentage of mtDNA control region variants						
Population	*N*	No. of haplotypes	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Haplotypic (gene) diversity
E OH	49	2	69.39	30.61	—	—	—	—	0.434
S OH, WV, W KY, E KY, W PA	112	6	41.96	50.89	3.57	1.79	0.89	0.89	0.568
E PA	18	2	94.44	5.56	—	—	—	—	0.111

Note: H_O, observed heterozygosity; H_E, expected heterozygosity. Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); WV (West Virginia); W KY (western Kentucky); E KY (eastern Kentucky); W PA (western Pennsylvania); E PA (eastern Pennsylvania).

Fig. 4. Mean number of private alleles per locus for bobcats (*Lynx rufus*) as a function of standardized sample size for three populations of bobcats calculated in ADZE. Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); KY (Kentucky); WV (West Virginia); W PA (western Pennsylvania); E PA (eastern Pennsylvania).



management-unit criteria, we recommend that for the near future the two Ohio populations be managed as separate management units, which will require the coordination of agencies in Ohio, Kentucky, West Virginia, and Pennsylvania. Other recent work by Croteau et al. (2012) proposed that multistate consortia might be a more appropriate way to manage bobcats because this scenario will conserve both historical and current levels of genetic diversity.

Another reason for the minimal loss of genetic variation during the recolonization of bobcats in Ohio is that populations grew rapidly, avoiding the effects of genetic drift due to small population size. All of the Western Alleghany Plateau ecoregion in Ohio (approximately 30 750 km^2 in southeastern Ohio) appears to be suitable bobcat habitat with no apparent barriers to movement (see Fig. 1*b*). Based on camera surveys during 2008, bobcat occupancy (MacKenzie et al. 2002) of this area was only about 35% (95% CI = 13%–64%; S. Prange, unpublished data). In the absence of dispersal barriers, a higher population growth rate of the eastern Ohio population and the spatial clustering of bobcats could be due to higher food availability. This area differs from areas to the south in that it contains some of the most heavily mined counties; at the center of the eastern Ohio population is Noble County with approximately 45% of its land consisting of reclaimed surface mines (ODNR 2014). The relationship between reclaimed mine land and bobcat population growth is unknown; however, red foxes (*Vulpes vulpes* (L., 1758)) and gray foxes (*Urocyon cinereoargenteus* (Schreber, 1775)) likely use reclaimed surface mines in West Virginia because of the presence of seasonally important food items such as small mammals (Yearsley and Samuel 1980).

Rapid growth of a small number of recolonizing animals containing a subset of the genetic make-up of the source population may account for why the eastern and southern Ohio populations are genetically differentiated even though they appear spatially contiguous. It is possible that the eastern population has potentially greater habitat quality and food availability, which has allowed the original founders to quickly build up the population, whereas southern Ohio may be more dependent on continuous dispersal from neighboring states (i.e., sink habitat). Eventually, we predict that all suitable habitat will be used and the species should become panmictic within the southeastern portion of the state. For large carnivores, such as American black bears (Pelletier et al. 2011) and Canada lynx (*Lynx* canadensis Kerr, 1792) (Schwartz

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Table 4. Estimates of contemporary migration of bobcats (*Lynx rufus*) based on assignment test results with a ≥90% likelihood value from GENECLASS.

		Population of genetic assignment		
Population of collection	Total *N*	E OH	S OH, WV, W KY, E KY, W PA	E PA
E OH	44	**35 (79.5)**	9 (20.5)	0 (0)
S OH, WV, W KY, E KY, W PA	91	8 (8.8)	**81 (89.0)**	2 (2.2)
E PA	15	0 (0)	1 (6.7)	**14 (93.3)**

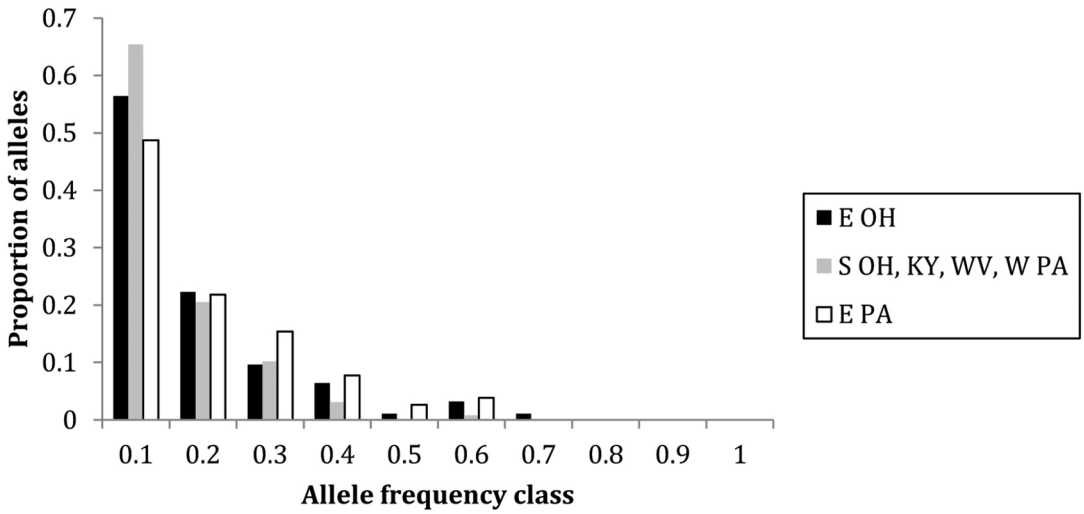
Note: Values shown are the number of bobcats with percentages in parentheses and residents are shown in boldface type. Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); WV (West Virginia); W KY (western Kentucky); E KY (eastern Kentucky); W PA (western Pennsylvania); E PA (eastern Pennsylvania).

Table 5. Summary of results from BOTTLENECK for three populations of bobcats (*Lynx rufus*) using both the two-phase model (TPM) and the stepwise mutation model (SMM) for the Wilcoxon's test along with results from the mode-shift test.

	Wilcoxon's test (TPM)		Wilcoxon's test (SMM)		
Population	Two-tailed	One-tailed for heterozygote excess	Two-tailed	One-tailed for heterozygote excess	Mode-shift test
E OH	0.42	0.21	0.11	0.95	L-shaped
S OH, WV, W KY, E KY, W PA	0.003*	0.002*	0.42	0.81	L-shaped
E PA	0.13	0.06	0.27	0.88	L-shaped

Note: Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); WV (West Virginia); W KY (western Kentucky); E KY (eastern Kentucky); W PA (western Pennsylvania); E PA (eastern Pennsylvania).
*Denotes significance after a Benjamini–Yekutieli adjustment for multiple tests.

Fig. 5. Distribution of allele frequencies from BOTTLENECK for three populations of bobcats (*Lynx rufus*) showing an L-shaped distribution. Abbreviations for sample locations are as follows: E OH (eastern Ohio); S OH (southern Ohio); KY (Kentucky); WV (West Virginia); W PA (western Pennsylvania); E PA (eastern Pennsylvania).



et al. 2002), microsatellite markers have shown panmictic population structure where topographic barriers to dispersal are absent. As a result of these processes, distinct population structure should be assessed on a regular basis until bobcats expand farther into unoccupied habitat, the eastern population is no longer spatially distinguishable, and the need to account for two management units is no longer required for conservation actions.

Range expansions into both eastern and southern Ohio likely came from the south and east. These states contain healthy bobcat populations and a higher proportion of patchy forested landscapes that are likely correlated with bobcat presence (Lovallo and Anderson 1996; Woolf et al. 2002). Additionally, primary bobcat habitat in Ohio exists in the forested southeastern hill country, whereas the western portion of Ohio consists largely of agricultural lands that are typically avoided by bobcats (Woolf et al. 2002; Tucker et al. 2008). Consequently, bobcat sightings in western Ohio are practically nonexistent. Bobcats are considered rare in Indiana, which borders Ohio to the west, with most sightings occurring in the southern portion of Indiana near its border with Kentucky. Similarly, although bobcats are common in northern Michigan and the Upper Peninsula, they are uncommon in the southern half of the state where bobcat trapping is prohibited.

The recent recolonization of bobcats in both eastern and southern Ohio requires animals to cross the Ohio River (see Fig. 1*b*). Some studies have reported that landscape elements may limit dispersal (Riley et al. 2006; Lee et al. 2012). However, range-wide studies by both Reding et al. (2012) and Croteau (2009) indicated that the Mississippi River was not a major barrier to gene flow for bobcats. Bobcats are considered good swimmers (Young 1958; Van Wormer 1964; Rue 1981; Merritt 1987) and it is possible that summer reductions in river flow or ice cover during winter provide opportunities for dispersal (Croteau 2009). In contrast, populations of bobcats were found to be genetically isolated by the Straits of Mackinac between the upper and lower peninsulas in Michigan (Millions and Swanson 2007). The extent to which

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bridges and other structures are used to cross major rivers is unknown, but may represent another method of movement.

We also found that the northeast Pennsylvania population in our study was genetically isolated from all of the other sampling localities, including the population in nearby southwest Pennsylvania. Since much of Pennsylvania is forested, it is possible that interstate highways such as I-80 act as barriers to gene flow. However, we found a significant isolation-by-distance effect, implying that this is related to overall geographic distance and not anthropogenic barriers to dispersal. Southeastern Ohio was unglaciated during the last ice age and has extensive patches of deciduous forest habitat, but also major highways. In fact, both interstate highways I-70 and I-77 run through the eastern population and I-77 largely bisects it north to south, suggesting that they are not major barriers to movement. Millions and Swanson (2007) investigated the impact of natural and artificial barriers to dispersal on population structure of bobcats and found no evidence that a greater density of roads in the lower peninsula of Michigan resulted in population structure. In contrast, Lee et al.'s (2012) recent work suggests that urban development, including freeways, was a physical barrier that has reduced bobcat movement and gene flow between some isolated groups of individuals but not others.

Based on the known number of vehicle-related mortalities of bobcats in Ohio, we project that there are a minimum of approximately 450 bobcats in Ohio (S. Prange, unpublished data). Our genetic analyses suggest that these are historically divided into two relatively independent management units that are growing and regularly exchange individuals. We recommend that population structure should be assessed on a regular basis in this landscape until bobcats expand farther into unoccupied habitat, the eastern population is no longer spatially distinguishable, and the need to define two units for management purposes no longer exists.

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