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Genetics and the successful reintroduction of the Missouri river otter

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Abstract

Reintroduction is an effective tool for restoring endangered populations. There is increasing concern, however, that demographic restoration may not equate with genetic restoration. We examine the demographic-genetic contrast in the context of one of the world's most successful carnivore population restorations. Beginning in 1982, a total of 835 river otters *Lontra canadensis* were reintroduced to Missouri, USA, more than 50 years after extirpation. Most otters were translocated from Louisiana, USA, and released at 43 sites across the state. An estimated population of 11 000–18 000 otters existed by 2000, and density estimates for Missouri otters are now similar to those reported for populations across the continent, indicating demographic recovery. We used microsatellite genotyping and mitochondrial sequence analysis of DNA extracted from fecal samples from eight southern Missouri rivers, in conjunction with mitochondrial DNA (mtDNA) analyses from several native Louisiana otter populations, to evaluate the genetic diversity and population structure of otters within Missouri as compared with Louisiana. The Missouri population showed moderate to high heterozygosity and allelic diversity, similar to that of the source populations, but low mtDNA haplotype diversity. We detected five distinct genetic clusters distributed throughout the eight rivers, with no evidence of isolation by distance. These data collectively suggest that 30 years after restoration efforts commenced, Missouri river otters have retained genetic diversity levels similar to those of the source populations, but that genetic structure has not reached an equilibrium between migration and genetic drift. Thus, the Missouri otter population has made a robust recovery despite retaining the genetic signature of the reintroduction.

Introduction

Reintroduction and translocation are effective tools for augmenting or reestablishing endangered wildlife populations. The goal of such efforts is usually demographic: to return a population to some target size or density. Less commonly the goal is to restore genetic diversity (Hostetler *et al*., 2013; Reynolds, Waycott & McGlathery, 2013) or ecological functionality (Griffiths *et al*., 2013). Demographic, ecological and genetic endpoints are unlikely to occur simultaneously, and the focus on one endpoint without regard to the others can result in contention as to whether a population is deemed restored (Pyare & Berger, 2003; Menges, 2008; Li *et al*., 2012). Armstrong & Seddon (2007) argue that reintroduced populations progress from the establishment phase characterized by founder survival and reproduction, to the persistence phase, which is strongly influenced not only by the ability of the habitat to sustain a healthy population but also by the genetic makeup of the reintroduced population.

The restoration of genetic diversity can be especially problematic because it depends on the number and genetic diversity levels of the founding individuals as well as their reproductive success (Groombridge *et al*., 2012; Hufbauer *et al*., 2013). During the early generations, the subset of genetic diversity represented in the founders may be reduced due to genetic drift, and the effects will be most severe in isolated populations (Rhodes & Latch, 2010; Reynolds *et al*., 2013; Tollinger *et al*., 2013). Because such diversity can be a predictor of short-term and long-term adaptive potential (Caballero & Garcia-Dorado, 2013), managers may implement genetic monitoring to maximize success

(Latch & Rhodes, 2005; Wisely *et al*., 2008; Hedrick & Fredrickson, 2010). Although the need for genetic monitoring of reintroduced populations has long been recognized (Haig, Ballou & Derrickson, 1990; Jamieson & Lacy, 2012), many reintroductions were implemented without preassessment of the diversity of the founders, such that questions remain about the overall restoration success (Seddon, Armstrong & Maloney, 2007).

The restoration of the North American river otter *Lontra canadensis* in Missouri, USA, has been regarded as one of the most successful carnivore recovery programs in history (Breitenmoser *et al*., 2001). Beginning *c*. 50 years after extirpation from overharvesting (Bennitt & Nagel, 1937), and running for more than a decade (1982–1992), the Missouri Department of Conservation (MDC) released 835 river otters at 43 sites across 35 counties (Hamilton, 2007; Table 1). Most otters were obtained from the Houma area of Louisiana, USA (D. Erickson, MDC, pers. comm.). A few otters were also obtained from Arkansas, USA, although the numbers, sources and release sites of those animals are not well known, and from Ontario, Canada, where 12 animals were trapped and translocated to the Chariton River in Northern Missouri (Penak & Code, 1987; Raesly, 2001). Furthermore, a small remnant population (35–70 otters) may have existed in the southeastern corner of Missouri prior to the reintroductions (Shelton, 1987; Hamilton, 2007). Unfortunately, no samples were taken from the reintroduced otters, nor do records exist describing the exact source populations associated with each translocation event.

The establishment phase of the otter reintroduction in Missouri was an unqualified success with population estimates ranging from 3000 in 1995 (Hamilton, 1998) to a projected 18 000 by 2000 (Gallagher, 1999). Crimmins, Roberts & Hamilton (2011) reported high proportions of animals exhibiting signs of having bred, including 59.1% of yearling otters. Harvesting resumed in 1996 (Hamilton, 2007), and data obtained from harvested animals suggested that by 2009, densities of otters in southern Missouri were similar to or higher than densities reported in healthy populations across the country. The movement of otters from rivers that were stocked during the reintroductions into those that were not stocked suggests that water and soil conservation efforts had improved habitat quality to the point that many rivers, lakes, ponds and wetlands statewide were able to support populations of otters (Missouri Department of Conservation, 2002).

Whether the successful establishment of the Missouri otter population has resulted in a genetically healthy population, reflected in a level of genetic diversity and population structure similar to that of the source populations, is not known. Here we address this issue 30 years after the start of reintroduction efforts and 20 years after the last translocations. Using DNA from fecal samples, we genotyped otters from transects along four rivers in southern and south-central Missouri that were stocked during the reintroduction and four that were presumably colonized by dispersing otters. We examined microsatellite genotypes and mitochondrial DNA (mtDNA) haplotypes to evaluate the levels and distribution of genetic diversity and population structure. In addition, we tested the hypotheses that rivers that were not stocked with otters during the reintroduction would exhibit lower genetic diversity than stocked rivers, and that otters in rivers nearer to each other, linked by common waterways, would show greater genetic similarity than more distant populations.

Methods

Study area and sample collection

Details on survey efforts are given in Mowry (2010) and Mowry *et al*. (2011). In brief, we conducted surveys for otter scats on 22–34 km transects in eight rivers spanning six primary watersheds in south and south-central Missouri between 6 January and 23 April 2009 (Table 2; Fig. 1a). Rivers were chosen based on public reports of otter activity, similar size and fish species composition, and accessibility for winter canoe routes (J. Beringer, unpubl. data). The transects sampled represented 8–77% of the total length of each river. Four of the rivers (Big Piney, Current, Niangua and Osage Fork) were directly stocked with otters during the reintroductions, while four rivers (Courtois, Maries, Roubidoux and West Piney) were presumably recolonized by dispersing otters. Upon locating a latrine (a site where otters regularly defecate or mark with anal sac secretions), field crews recorded the Global Positioning System coordinates and cleared all scats, then returned after 5 days to collect all new scat and anal sac samples. We collected 1421 samples including 104 anal sac secretions and 1317 scat samples (Mowry *et al*., 2011).

Genetic analyses using nuclear microsatellite loci

Techniques for fecal DNA extraction, polymerase chain reaction (PCR), microsatellite genotyping and molecular sexing are described in Mowry *et al*. (2011). Briefly, we used a panel of 10 microsatellite loci that had high levels of diversity and low rates of allelic dropout and false alleles when tested on matched scat and tissue samples. We used positive and negative controls throughout to monitor contamination and consistency of allele scoring, and assigned genotypes using the comparative method (Frantz *et al*., 2003; Hansen, Ben-David & McDonald, 2008). We successfully genotyped 24% ($n = 343$) of our samples and identified individuals based on unique multilocus genotypes ($n = 63$, each detected 4.5 ± 3.7 SD times, range 1–24). We tested for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium, calculated observed and expected heterozygosity values and inbreeding coefficients, corrected allele frequencies and private allele frequencies for unequal sample sizes, and inbreeding coefficients (F_{IS}) using Genepop (Raymond & Rousset, 1995), Arlequin 3.11 (Excoffier & Lischer, 2010), HP-Rare (Kalinowski, 2005)

Table 1 Years and locations of river otter *Lontra canadensis* reintroductions across the state of Missouri, USA[a]. Rivers in bold were sampled in this study

Year	Location	County	No. of otters
1982	Swan Lake National Wildlife Refuge	Chariton	12
1983	Fountain Grove Wildlife Area	Linn	10
	Lamine River	Cooper	20
1984	Fountain Grove Wildlife Area	Linn	7
	Ted Shanks Wildlife Area	Pike	24
	Four Rivers Wildlife Area	Vernon	18
	Rebel's Cove Wildlife Area	Putnam	6
	Little Chariton River	Chariton	7
1985	Big Creek	Daviess	20
	Blackwater-Perry Wildlife Area	Pettis	18
	Little Chariton River	Chariton	12[b]
	Rebel's Cove Wildlife Area	Putnam	6[b]
	Schell-Osage Wildlife Area	Vernon	20
	Shoal Creek	Caldwell	20
1986	Cuivre River-Argent Slough	Lincoln	23
	Cuivre River-West Fork	Lincoln	22
	Moreau River-Burris Fork	Moniteau	21
	Platte River-Castile Creek	Platte	18
	Rebel's Cove Wildlife Area	Putnam	1[b]
	South Fabius River	Marion	21
1987	One Hundred Two River	Andrew	22
1988	Bourbeuse River	Gasconade	20
	Meramec River	Dent	20
	Middle Fabius River	Knox	20
	Middle Salt River	Macon	10
	Niangua River	Dallas	20
1989	Middle Bourbeuse River	Franklin	24
	Middle Meramec River	Crawford	21
	Middle Salt River	Macon	10
	Upper Gasconade	Wright	20
1990	**Big Piney River**	Texas	20
	Big Piney River	Pulaski	21
	Current River	Dent	17
	Dry Wood Creek	Burton/Vernon	22
	Gasconade/Hazelgreen	Pulaski	20
	Horse Creek	Barton	22
	Jacks Fork River	Texas	17
	Niangua River	Laclede	17
	Osage Fork River	Webster	21
	Pomm-Tin Town	Polk	20
	South Grand/Big Creek	Henry	18
1991	Eleven Point River	Oregon	21
	Bryant Creek	Douglas	20
	Gasconade/Bell Chute	Maries	20
	Jacks Fork River	Texas	2
	James River	Christian	20
	Loutre River	Montgomery	4
	North Fork, White River	Douglas	20
1992	Perche Creek	Boone	20

[a]Total number of otters reintroduced to known localities is 835. Hamilton (1998) reported a total of 845 reintroduced otters, but records for the additional otters are not available and the higher value may be a reporting error.

[b]Some of these reintroduced otters were derived from Ontario source populations (Penak & Code, 1987).

and FSTAT 2.9.3.2 (Goudet, 1995), respectively. We sampled the Big Piney, Roubidoux and West Piney rivers in both the winter and spring (Table 2). Because otters detected in winter were largely a subset of otters detected in spring in these rivers (Big Piney: 4 of 6; Roubidoux: 5 of 6; West Piney: 1 of 3), and no otter was observed moving between rivers, we pooled genotypes collected in different seasons within a river.

We compared levels of genetic diversity in Missouri river otters with those of two studies (Latch *et al.*, 2008; Brandt *et al.*, 2014) that analyzed samples from the presumed Louisiana source region. In Latch *et al.*'s (2008) study, the Houma region is best represented by the Atchafalaya River sample ($n = 32$ samples, 64 genes). In Brandt *et al.'s* (2014) study, otters came from both the coastal and inland regions of Louisiana ($n = 12$ samples, 24 genes). We corrected allelic values for Missouri otters to those expected in sample sizes comparable to these studies in HP-RARE. Because heterozygosity values are not strongly affected by sample sizes, we compared them directly.

We tested for the signal of a genetic bottleneck in the Missouri population as a whole and in each of the four populations for which we had sufficient samples (BP, Big Piney River; RO, Courtois River; OF, Osage Fork River; CR, Current River) using BOTTLENECK (Piry, Luikart & Cornuet, 1999). Allelic diversity is predicted to decline before heterozygosity in a bottlenecked population, resulting in a significant number of loci that exhibit heterozygosity excess. We used BOTTLENECK to test for deviations from expected heterozygosity values under mutation-drift equilibrium using the infinite alleles model (IAM), the stepwise mutation model (SMM) and the two-phase model (TPM) with the proportion of SMM at 70%. Results were evaluated using a Wilcoxon signed-rank test.

We calculated genetic distances (F_{ST}) for each Missouri population pair based on microsatellite genotypes in ARLEQUIN, and evaluated their significance using permutation tests (1000 permutations) after applying a Bonferroni correction. We tested for isolation by distance (IBD) across river populations using the Isolde option in GENEPOP, with default parameters for the Mantel test. To calculate geographic distances, we used the straight-line distance (km) separating the midpoints of each river measured in ARCGIS 9.3 (ESRI, Redlands, CA, USA). We did not evaluate IBD between individuals. Although many otters were captured multiple times and it might be possible to use those locations to determine a 'central' position, one in five otters ($n = 13$) was captured only once. With this level of variance in the precision of both spatial and temporal locations, estimating IBD between individuals would likely produce incorrect results.

We evaluated the genetic structure of Missouri otters by analyzing microsatellite genotypes in STRUCTURE 2.3.4 (Pritchard, Stephens & Donnelly, 2000), which estimates the likelihood of a variable number of genetic clusters (K) based on allele frequencies. We used an admixture model with a burn-in period of 100 000 iterations followed by runs of 1 000 000 iterations, with allele frequencies correlated

Table 2 Number of unique microsatellite genotypes, sex ratios, observed (H_o) and expected (H_e) heterozygosity values, mean uncorrected number of alleles per locus (A), mean number of alleles corrected for a sample size of two otters (A_r), mean number of private alleles corrected for a sample size of two otters (A_{rp}), and inbreeding coefficients (F_{IS}) for eight rivers in Missouri, USA, based on fecal genotyping. River abbreviations follow Fig. 1. For rivers that were sampled more than once (BP, RO, WP), the total numbers of genotypes are given in bold above the counts per season (accounting for otters that appeared in both seasons)

River	Watershed	Unique genotypes (M:F)	Transect length (km)	H_o	H_e	A	A_r	A_{rp}	F_{IS}
BP	Big Piney	**14 (8:6)**	23.5	0.591	0.584	4.7	2.3	0.15	–0.018
Winter		6 (2:4)							
Spring		12 (6:6)							
CO	Meramec	3 (3:0)	22.4	0.767	0.720	3.1	2.6	0.28	–0.112
CR	Current	11 (8:3)	27.3	0.684	0.631	3.9	2.4	0.21	–0.077
MA	Osage	3 (3:0)	27.2	0.815	0.719	2.9	2.5	0.15	–0.230
NI	Niangua	2 (2:0)	29.0	0.875	0.854	2.7	2.7	0.08	–0.125
OF	Gasconade	14 (8:6)	31.7	0.682	0.632	4.4	2.4	0.19	–0.136
RO	Gasconade	**11 (8:3)**	34.4	0.645	0.488	3.2	2.0	0.09	–0.289*
Winter		6 (4:2)							
Spring		10 (8:2)							
WP	Big Piney	**5 (2:3)**	24.8	0.547	0.719	3.6	2.6	0.35	0.233
Winter		3 (1:2)							
Spring		3 (2:1)							
Average				0.701	0.668	3.6	2.4	0.19	–0.094
Standard deviation				0.112	0.110	0.7	0.2	0.09	0.157

* $P = 0.0006$.

BP = Big Piney River, CO = Courtois River, CR = Current River, MA = Maries River, NI = Niangua River, OF = Osage Fork River, RO = Roubidoux River, WP = West Piney River.

between populations. We performed 10 independent runs for each K from 1 to 9, and inferred the most likely number of genetic clusters by calculating ΔK (Evanno, Regnaut & Goudet, 2005) in Structure Harvester (Earl & vonHoldt, 2012).

Genetic analyses using mtDNA

To amplify and sequence a fragment of the mtDNA control region, we used published sequence (Accession No. AF418979) to design primers that flank a 222-bp fragment [LcanF3 (5′-TGTATATCGTGCATTAATGGTTTG-3′) and LcanR2 (5′-GATGTCCCATTTGCAAGGTT-3′)]. For each of the 63 Missouri otters, we amplified this fragment in 25 μL volumes containing 1X PCR Gold buffer (Applied Biosystems, Foster City, CA, USA), 2 mM deoxyribonucleotide triphosphates, 0.4 μM forward and reverse primers, 0.8X bovine serum albumin, 2 mM $MgCl_2$ solution, 0.5 units of AmpliTaq Gold© DNA polymerase (Applied Biosystems) and 3 μL (15–50 ng) fecal extract. The PCR profile included an initial cycle of 96°C for 10 min; followed by 45 cycles of denaturation at 96°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min; followed by a final extension cycle at 72°C for 10 min. Each reaction included a negative control to detect contamination. We purified PCR products using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and sequenced them in both directions on a 3730 × l DNA Analyzer (Applied Biosystems). We repeated sequencing of poorer quality samples as needed, visually checking each alignment and identifying unique haplotypes using Geneious Pro 5.5.3 (Biomatters Ltd, Auckland, New Zealand).

We compared the mtDNA haplotypes found in Missouri to those derived from otters harvested in several areas of Louisiana. In a separate study (Latch *et al.*, 2008), tissues were collected from 111 harvested otters from two coastal areas and one inland habitat in Louisiana, and microsatellites were used to infer barriers to gene flow. We amplified and sequenced the mtDNA fragment in those 111 samples and compared the haplotypes to Missouri haplotypes in Geneious. We used permutation tests to calculate pairwise F_{ST} values between the eight Missouri rivers and the three Louisiana groups in Arlequin, applying a Bonferroni correction to evaluate significance. Haplotype and nucleotide diversity indices (h and π, respectively) were calculated in DnaSP (Rozas & Rozas, 1999). We compared haplotype frequencies in the combined Missouri data with those found in the Louisiana group that contains otters from the Houma region (LA1) using a chi-square contingency test. To visualize relationships among haplotypes, we used the median-joining option in Network 4.5.1.0 (Bandelt, Forster & Röhl, 1999).

Populations that have undergone a recent expansion are expected to display weak genetic structure, one or a few common mtDNA haplotypes, and possibly several new haplotypes that differ by only a few mutations from the common haplotypes (Avise, 2000). We tested for patterns of sequence differences within and between populations, and evaluated their significance using the mismatch distribution

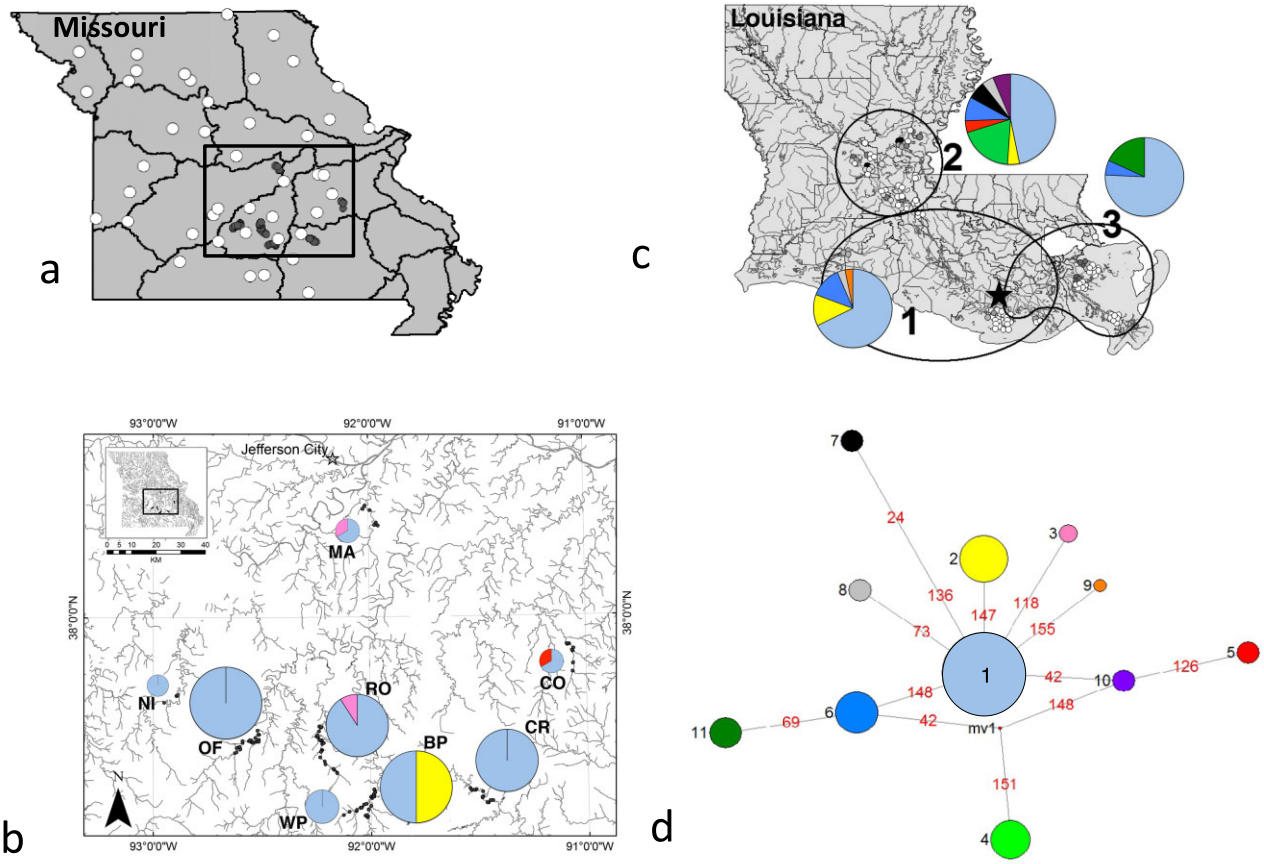

Figure 1 Geographic distributions of (a) sampled latrine sites in Missouri (dark gray circles) and approximate locations of reintroduction events (white circles); (b) mitochondrial DNA haplotypes in the eight sampled rivers in Missouri (BP = Big Piney River, CO = Courtois River, CR = Current River, MA = Maries River, NI = Niangua River, OF = Osage Fork River, RO = Roubidoux River, WP = West Piney River; size of each pie chart corresponds to the number of otters identified in each river); (c) haplotype distributions in the three Louisiana clusters identified in Latch *et al.* (2008); star indicates the location of Houma, the possible source of otters translocated to Missouri; (d) haplotype network; numerical haplotype designations are in black text, base-pair substitution sites in red text, and median vector 1 (mv1) is a hypothesized sequence required to connect existing sequences within the network.

(Rogers & Harpending, 1992) and Tajima's *D* statistic (Tajima, 1989). We conducted these tests in Arlequin 3.11 using the frequencies of mtDNA haplotypes in Missouri populations.

Results

Genetic analyses using nuclear microsatellite loci

We identified 63 unique genotypes, with numbers varying from 2 otters in the Niangua River to 14 in the Big Piney River and Osage Fork of the Gasconade River (Table 2). For the eight loci common to our study and Latch *et al.* (2008), allelic richness in the combined Missouri dataset averaged 6.1 alleles/locus (Table 3), and was not significantly different from the 6.1 alleles/locus detected for the Atchafalaya region (E. K. Latch, unpubl. data). For the six loci common to our study and Brandt *et al.* (2014), allelic

Table 3 Comparisons of allelic richness [number of alleles corrected for sample size (A_r) in HP-RARE between Missouri (MO) river otters *Lontra canadensis* from all rivers with the presumed Louisiana (LA) source populations using loci common to the studies; light shading indicates comparison with data from Latch *et al.* (2008), dark shading indicates comparison with data from Brandt *et al.* (2014)

	MO = All $n = 32$	LA $n = 32$	MO-All $n = 12$	LA $n = 12$
Locus	A_r	A_r	A_r	A_r
Rio01			4.9	7.0
Rio02	8.6	9.0	6.9	7.0
Rio04			3.9	5.0
Rio06	4.8	5.0	4.0	4.0
Rio07	7.5	8.0		
Rio08	4.9	6.0	4.4	5.0
Rio11	4.0	5.0		
Rio13	10.0	10.0	7.8	10.0
Rio15	3.0	3.0		
Rio16	6.2	3.0		
Average	6.1	6.1	5.3	6.3
Standard deviation	2.4	2.6	1.6	2.2

Table 4 F_{ST} values (top half of matrix, with significance levels) derived from microsatellite genotypes and geographic distances (km, bottom half of matrix) between all Missouri population pairs. Values in bold are significant after Bonferroni correction ($P < 0.001$)

	BP	RO	CO	OF	CR	NI	WP	MA
BP	–	**0.116**	0.137	**0.133**	**0.082**	–0.035	0.077	0.088
RO	29.5	–	**0.160**	**0.147**	**0.157**	0.018	0.096	0.106
CO	102.9	110.1	–	0.040	**0.133**	0.015	0.061	0.005
OF	54.0	28.0	134.8	–	**0.151**	0.005	**0.112**	0.014
CR	46.4	68.5	67.9	97.4	–	0.024	0.081	0.095
NI	87.3	61.0	160.9	32.8	129.0	–	0.000	–0.079
WP	4.5	27.4	107.0	52.1	51.4	85.4	–	0.044
MA	118.1	98.4	100.2	104.5	123.4	110.0	119.3	–

BP = Big Piney River, CO = Courtois River, CR = Current River, MA = Maries River, NI = Niangua River, OF = Osage Fork River, RO = Roubidoux River, WP = West Piney River.

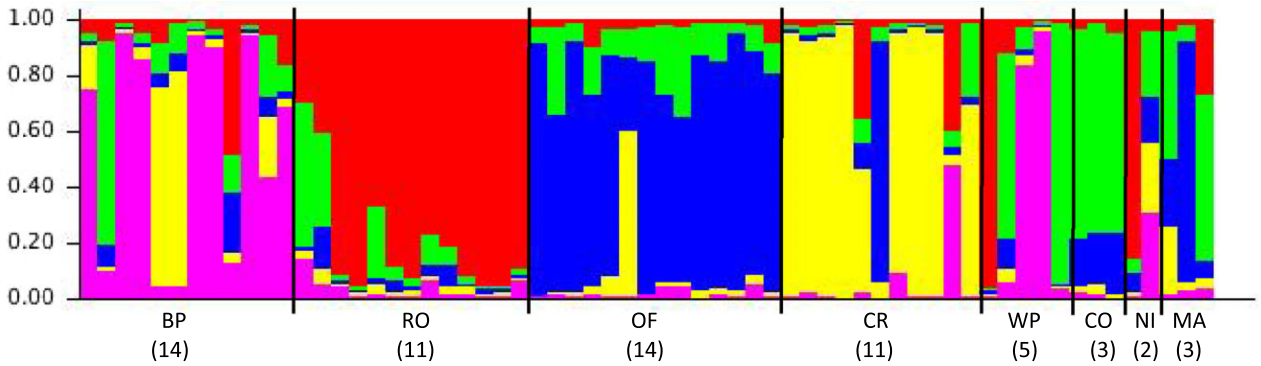

Figure 2 STRUCTURE cluster probabilities for each river at $K = 5$; clusters corresponded most closely with the Big Piney, Roubidoux, Osage Fork of the Gasconade, Current, and Courtois Rivers (numbers below the river abbreviations denote sample sizes).

richness in the combined Missouri dataset averaged 5.3 alleles/locus, and was not significantly different ($t = 0.918$, d.f. = 10, $P = 0.380$) from the average of 6.3 alleles/locus detected in Brandt *et al.'s* (2014) study (Table 3). Observed heterozygosity (H_o) in the combined dataset averaged 0.701 (± 0.112 SD, range 0.547–0.875) and expected heterozygosity (H_e) averaged 0.668 (± 0.110 SD, range 0.488–0.720), similar to H_o and H_e values reported by Latch *et al.* (2008) and Brandt *et al.* (2014) for the Louisiana source populations.

No river population showed significant deviations from Hardy–Weinberg equilibrium or evidence of linkage disequilibrium. Inbreeding coefficients ranged from –0.289 to 0.233, averaging –0.094 (Table 2). Allelic diversity differed among Missouri rivers due to sample size variation; corrected for sample size ($n = 2$ samples, 4 alleles), allelic richness averaged 2.4 alleles/locus and did not differ significantly among rivers [analysis of variance (ANOVA), $F_{0.05,7} = 1.387$, $P = 0.224$]. We found no evidence for a genetic bottleneck in the Missouri population as a whole, but found evidence of heterozygosity excess in two of the populations for which we had sufficient samples for testing in BOTTLENECK. The Osage Fork River showed heterozygosity excess under both the IAM ($P = 0.002$) and the TPM ($P = 0.005$), while the Current River showed heterozygosity excess under the IAM ($P = 0.002$), the TPM ($P = 0.006$) and the SMM ($P = 0.019$).

Pairwise values of F_{ST} for Missouri rivers based on microsatellite genotypes ranged from –0.079 to 0.160 (Table 4) with a mean of 0.071. Although nine comparisons showed significant differentiation after Bonferroni correction, no significant relationship was found between genetic and geographic distances (Mantel test, $F_{1,26} = 1.716$, $P = 0.202$, Supporting Information Figure S1).

STRUCTURE simulations indicated that the 63 river otter genotypes clustered into five groups [$K = 5$, mean $L(K) = -1580.91$, $\Delta K = 80.09$, Supporting Information Figure S2], corresponding most prominently with the Big Piney, Roubidoux, Osage Fork, Current and Courtois Rivers (Fig. 2). The Big Piney and West Piney showed strong cluster similarity, suggesting migration between these rivers. However, geographically distant pairs of rivers, such as Niangua and Big Piney, showed unexpected cluster similarities.

Genetic analyses using mtDNA

Mitochondrial haplotype diversity (h) among all samples (Missouri and Louisiana combined) was 0.505 with a nucleotide diversity (π) of 0.006. For Louisiana samples, haplotype diversity was 0.607 and nucleotide diversity was 0.006, and for Missouri samples haplotype diversity was 0.283 and nucleotide diversity was 0.002. We identified four

Table 5 Nucleotide differences and sampling locations for the 11 observed mitochondrial haplotypes in Missouri (n = 63) and Louisiana (n = 111) river otters *Lontra canadensis*

	Nucleotide position											Location										
Haplotype	24	42	69	73	118	126	136	147	148	151	155	BP	CO	CR	MA	NI	OF	RO	WP	LA1	LA2	LA3
1	T	A	C	T	T	C	T	T	C	T	A	7	2	11	2	2	14	10	5	21	22	25
2	–	–	–	–	–	–	–	C	–	–	–	7								4	2	
3	–	–	–	–	C	–	–	–	–	–	–				1			1				
4	–	G	–	–	–	T	–	–			–										9	
5	–	G	–	–	–		–	–	–	–	–		1								2	
6	–	G	–	–	–	–	–	–	T	C	–									4	4	2
7		–	–	–	–	–		–	T	–	–										3	
8	–	–	T		–	–	–	–	T	–	–									1	2	
9	C	–	–	–	–	–	C	–	–	–										1		
10	–		–	C	–	–	–	–	–	–	–										3	
11	–	–		–	–	–	–	–		–	G											6

BP = Big Piney River, CO = Courtois River, CR = Current River, MA = Maries River, NI = Niangua River, OF = Osage Fork River, RO = Roubidoux River, WP = West Piney River.

Table 6 F_{ST} values derived from mitochondrial haplotypes and significance levels between the eight Missouri populations and three Louisiana clusters. Values in bold are significant after Bonferroni correction ($P < 0.0009$)

	BP	RO	WP	OF	MA	NI	CR	CO	LA1	LA2	LA3
BP	–										
RO	**0.373**	–									
WP	0.320	–0.089	–								
OF	0.462	0.023	0.000	–							
MA	0.313	0.012	0.189	0.511	–						
NI	0.176	–0.325	0.000	0.000	–0.200	–					
CR	**0.425**	0.000	0.000	0.000	0.441	0.000	–				
CO	0.309	0.195	0.189	0.511	–0.000	–0.200	0.441	–			
LA1	0.134	0.031	–0.043	0.041	–0.015	–0.247	0.025	–0.103	–		
LA2	**0.206**	0.088	0.021	**0.099**	0.025	–0.157	0.085	–0.136	0.036	–	
LA3	**0.296**	0.034	–0.042	0.041	0.051	–0.245	0.026	–0.001	0.045	0.010	–

BP = Big Piney River, CO = Courtois River, CR = Current River, MA = Maries River, NI = Niangua River, OF = Osage Fork River, RO = Roubidoux River, WP = West Piney River.

haplotypes in Missouri rivers and 10 in Louisiana, with haplotype 1 occurring most frequently in both states (Table 5, Fig. 1), in all groups and rivers. Haplotype 2 was found in both states, but only in Big Piney in Missouri, where it was found in 7 of the 14 individuals detected. Haplotype 4 was common in Louisiana group 2, but was also found in a single otter in the Courtois River. Haplotype 3 was unique to Missouri and was represented by two male otters, one in the Maries River and one in the Roubidoux River. Haplotype diversity differed between the combined Missouri dataset and the presumed source population as represented in the Atchafalaya sample (group LA1). Although the numbers of haplotypes were similar (Table 5), there were significant differences in frequencies ($\chi^2 = 14.4$, d.f. = 6, $P = 0.025$).

Pairwise F_{ST} values based on mtDNA (Table 6) indicated that the Big Piney River showed significant differentiation from the Roubidoux and Current Rivers as well as from Louisiana groups 2 and 3, while among the other Missouri locations, only Osage Fork showed significant differentiation (from Louisiana group 2). The three Louisiana groups, originally classified using microsatellite data, did not differ with respect to mtDNA haplotype frequencies. We were only able to conduct mismatch distribution analyses (Rogers & Harpending, 1992) on the Big Piney, Roubidoux, Maries and Courtois River populations, but did not find evidence of recent population expansion ($P = 0.080$–0.920). Testing using Tajima's D statistic (Tajima, 1989) could only be conducted in the Big Piney and Roubidoux Rivers, but neither test revealed evidence of population expansion ($P = 0.969$ and $P = 0.171$, respectively).

Discussion

The Missouri otter reintroduction was clearly a success during the establishment phase (Armstrong & Seddon, 2007), with the population as a whole more than tripling in size by 1995, only 3 years after the end of the translocations (Hamilton, 1998). Reintroduced otters established multiple populations in habitats within their native range, and robust population growth and dispersal resulted in the colonization of rivers that were not stocked. The large-scale and diffuse

nature of the reintroductions (43 events across 35 counties over 11 years) was likely a key factor in this success (Derdedec & Courchamp, 2007). In their review of studies of invasive nonindigenous species, Kolar & Lodge (2001) found that the number of individuals released and the frequency of releases, collectively known as propagule pressure, was the strongest predictor of establishment, and it appears to have played an important role in the reintroduction of river otters in Missouri.

While propagule pressure was a key factor in the establishment phase, it has also likely played a role in the persistence phase. The early years (1982–1983) involved translocations of a small number of otters into only a few rivers, factors that would likely result in the loss of genetic diversity during the first few generations. However, the early reintroductions were followed by releases of a large number of individuals into multiple rivers over an 11-year period, increasing the probability that the diversity of the source populations would be transferred to and retained in the reintroduced populations. Our results indicate that allelic diversity and heterozygosity levels in the combined Missouri population do not differ significantly from those in the Louisiana source population.

In contrast to nuclear microsatellite diversity, mtDNA haplotype diversity in Missouri otters is low (0.283), whether compared with Louisiana as a whole (0.607) or to the Atchafalaya population (0.523). The lower haplotype diversity supports the evidence that most otters inhabiting the sampled region of Missouri are descendants of founders from a single region in Louisiana. Two of the four haplotypes found in Missouri were detected in the Atchafalaya region and three of the four were detected in Louisiana, and there was little differentiation between Missouri and Louisiana locations. The presence of haplotype 5 in the Courtois River and haplotype 3 in the Maries and Roubidoux Rivers was unexpected, as we did not detect either of these haplotypes in the samples from the Atchafalaya region. The haplotype network (Fig. 1d) suggests haplotype 5 is more distinct than others; thus, it is unlikely that it arose in the reintroduced population. It is possible that this haplotype may be found at low frequency in the Atchafalaya region, or that some of the translocated otters believed to have come from the Houma area actually came from the inland Louisiana region, where haplotype 5 was found at low frequency. It is also possible that haplotype 5 was introduced in otters translocated from Arkansas or Ontario, although the Ontario animals were released into northeastern localities in Missouri (Penak & Code, 1987) and we would not expect to detect haplotypes from those animals in the sampled rivers. Haplotype 3 differs from the most common haplotype by only one substitution, and was found only in Missouri. Although this haplotype may have also been present in Louisiana or in a remnant Missouri population (Shelton, 1987; Hamilton, 2007), it may have arisen after the translocations in Missouri.

Our results did not support the hypothesis that rivers that were not stocked with otters during the reintroduction would exhibit lower genetic diversity than stocked rivers. Assuming a generation time of 6.4 years for North American river otters (Boyle, 2006), reintroduced Missouri otter populations have persisted for up to five generations with reproductive levels sufficient to maintain healthy population sizes in the stocked rivers and produce dispersers. If dispersal to each of the unstocked rivers involved only a few individuals from a single source, naturally colonized rivers would be expected to display the characteristics of bottlenecked populations, including lower allelic diversity, heterozygosity excess, and differentiation from other populations due to the effects of genetic drift. However, although small sample sizes limited the power of our analyses, we found evidence of heterozygosity excess only in the Osage Fork and Current Rivers, both of which were stocked during the reintroductions. Each of these populations displayed higher overall levels of genetic differentiation (F_{ST}) at nuclear loci and only the most common mitochondrial haplotype. Thus, despite evidence of the retention of the diversity of the founders, we were able to detect the effects of genetic drift.

Overall, our genetic results indicate that the general reintroduction strategy of translocating a large number of individuals over an extended period was successful in both the establishment and the persistence phases. The high level of propagule pressure was followed by rapid population growth and colonization of suitable habitats outside the reintroduction areas, resulting in a robust demographic recovery. It avoided the losses of nuclear genetic diversity and higher inbreeding levels seen in the early generations after translocations that involve a small number of individuals (Groombridge *et al.*, 2012). Although allelic diversity and heterozygosity at neutral microsatellite loci themselves are unlikely to affect the fitness of individuals, they are often used as a proxy for levels of genetic variability at adaptive loci (Väli *et al.*, 2008; Ljungqvist, Åkesson & Hansson, 2010; Ruiz-Lopez *et al.*, 2012). Populations with low allelic diversity may lack the diversity that would allow them to respond to environmental changes that represent stressors (Keller *et al.*, 1994; Reed, Briscoe & Frankham, 2002; Fox & Reed, 2011), and theoretical studies have shown that long-term responses of populations to environmental change are strongly affected by allelic diversity (Caballero & Garcia-Dorado, 2013). Although reduced mtDNA diversity itself may not be as directly linked to reduced fitness, haplotype diversity in Missouri otters should be monitored, as previous studies have shown linkages between some mtDNA mutations and reduced fitness (Gemmell, Metcalf & Allendorf, 2004).

Previous studies have shown that river otters are able to disperse long distances, including over terrestrial areas (Melquist & Hornocker, 1983), using available small lakes and ponds (e.g. Schwartz & Schwartz, 1981; Hamilton, 1999) to facilitate their movements. In the absence of barriers to movement, over time we would expect that a balance between migration and genetic drift would result in a pattern of IBD among Missouri otter populations. Despite evidence of dispersal, however, the pairwise F_{ST} and the

geographic patterns observed among STRUCTURE clusters indicate that Missouri river populations do not conform to an IBD model. In Louisiana, Latch *et al.* (2008) observed an IBD pattern between Atchafalaya River and Mississippi River populations but not between the Atchafalaya River and Inland populations. They proposed that differences in diet and in the availability of freshwater between coastal and inland river otter populations might explain differences in dispersal rates and habitat utilization (Latch *et al.*, 2008). However, in Missouri there are no obvious barriers to dispersal and no clear ecological differences between rivers inhabited by otters. Simulations by Landguth *et al.* (2010) suggest that for the lag time for detecting the signal of barriers using genetic measures may be as short as 1–15 generations or as long as 200 generations, depending on dispersal distances of the species and the metric used. If barriers exist, we were not able to detect them. Thirty years, approximately five generations, after the beginning and 20 years after the end of the translocation program, the population genetic structure we detected, which has little relationship with geographic location, remains an artifact of the reintroduction, during which genes from a small number of source populations were thoroughly mixed after otters were translocated into different areas multiple times.

Conservation implications

The reintroduction of river otters in Missouri has succeeded at both the establishment and persistence phases, and may be a model for the reintroduction of other small carnivores. Our results lend support to the theoretical work of Hufbauer *et al.* (2013), whose models disentangled the effects of propagule size and genetic background. Their results suggested that populations founded by multiple introductions of genetically diverse individuals immediately avoid the effects of inbreeding but may also develop long-term benefits from outbreeding. After five generations, we found no significant loss of genetic diversity and very little evidence of inbreeding. Developing the patterns of genetic structure we might expect under a balance between migration and genetic drift will take longer, as seen in populations of wild turkeys *Meleagris gallopavo* (Latch & Rhodes, 2005), swift foxes *Vulpes velox* (Cullingham & Moehrenschlager, 2013) and Alpine ibex *Capra ibex ibex* (Biebach & Keller, 2009). With management to avoid local extinctions and maintain connectivity between rivers, we predict that over time the Missouri otter population will develop patterns of population structure that are characteristic of native populations.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Analysis of isolation by distance between river transects using F_{ST} and geographic distance data from Table 3. Negative F_{ST} values were set to 0. Geographic distance measures were made from the midpoint of the transect in each river to the midpoint of the transect in each other river. No significant relationship was found between genetic and geographic distances (Mantel test using Isolde in GENEPOP, $F_{1,26} = 1.716$, $P = 0.202$).

Figure S2. Results of Evanno, Regnaut & Goudet's (2005) test of STRUCTURE analyses for otters in eight river transects in Missouri, USA.