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RESEARCH ARTICLE

Population genetic structure and history of fragmented remnant populations of the New England cottontail (*Sylvilagus transitionalis*)

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Abstract The New England cottontail (*Sylvilagus transitionalis*) has suffered from extensive loss and fragmentation of its habitat and is now a species of conservation priority in the northeastern United States. Remnant New England cottontail populations currently occur in five geographically disjunct locations: southern Maine and southeastern New Hampshire (MENH); the Merrimack Valley in central New Hampshire (NH-MV); Cape Cod, Massachusetts (CC); parts of eastern Connecticut and Rhode Island (CTRI); and western Connecticut, southeastern New York and southwestern Massachusetts (CTNY). We used microsatellite genotyping to discern patterns of population structure, genetic variability, and demographic history across the species' range and to assess whether the observed patterns are a consequence of recent habitat loss and fragmentation. Our findings show that the geographic populations are highly differentiated (overall $F_{ST} = 0.145$; $P < 0.001$). Using Bayesian clustering analyses, we identified five genetic clusters, which corresponded closely to the geographic populations, but grouped MENH & NH-MV together (ME/NH) and identified an isolated population in eastern Connecticut (Bluff Point). The genetic clusters showed little evidence of recent gene flow and are highly influenced by genetic drift. The CC and Bluff Point populations show signs they experienced a genetic bottleneck, whereas the ME/NH population shows evidence of ongoing decline. Populations in Bluff Point, CC, and ME/NH also show significantly reduced genetic variation relative to the other clusters (CTNY and CTRI without Bluff Point). Without immediate human intervention, the short-term persistence of New England cottontail populations in Maine, New Hampshire and Cape Cod is at great risk. Conservation efforts at this time should focus on within-population sustainability and eventually restoring connectivity among these isolated populations.

Keywords Fragmentation · Population genetic structure · New England cottontail · Genetic bottleneck · Microsatellite · Conservation · Genetic drift · *Sylvilagus transitionalis*

Introduction

The extent to which landscape effects promote or prevent genetic exchange is becoming increasingly important to consider in conservation efforts of wild plant and animal species. Habitat loss and fragmentation reduce connectivity among wildlife populations and can ultimately drive populations to extinction (Reed 2004). Isolated populations have smaller effective sizes and incur a greater risk of extinction due to stochastic effects (Frankham et al. 2002). As evidenced in many recent studies (e.g., White and Searle 2007; Dixo et al. 2009), when populations become isolated, they lose genetic diversity. The loss of genetic diversity limits evolutionary potential (Johansson et al. 2007) and populations are more likely to experience inbreeding depression (Keller and Waller 2002; Willi et al.

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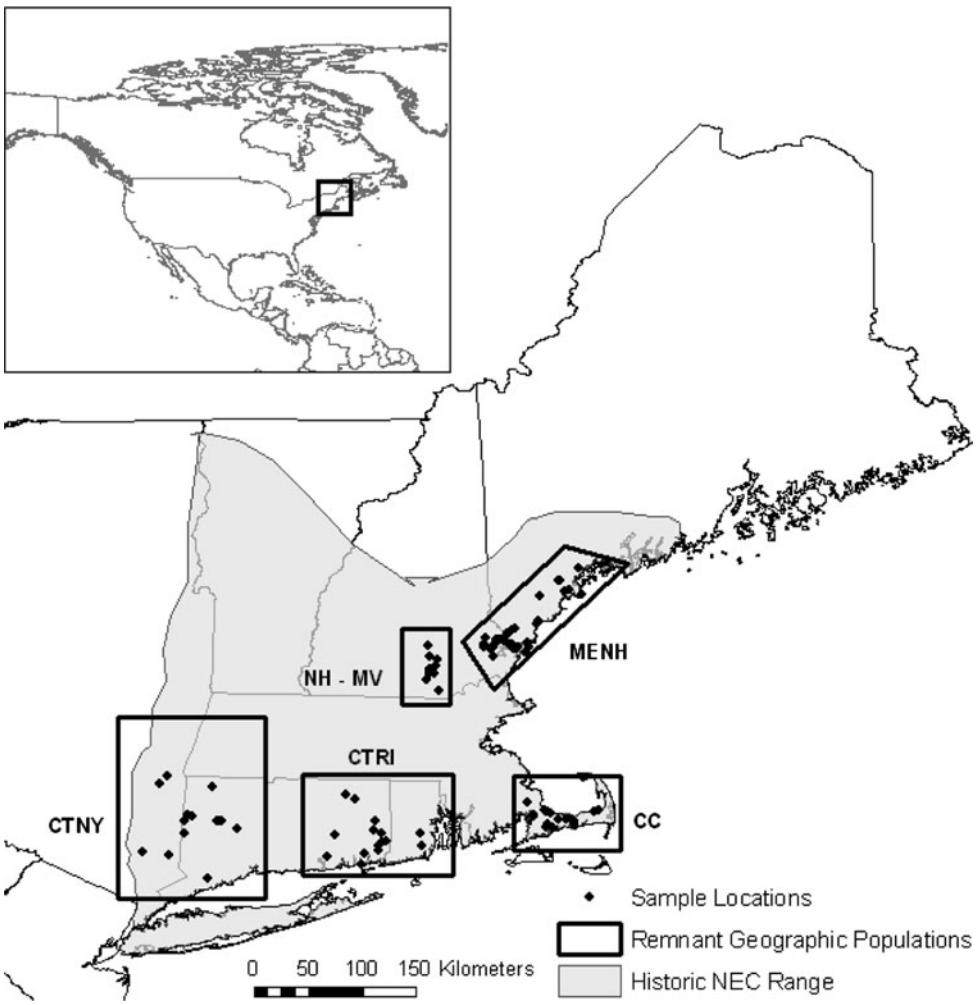

Fig. 1 Historic range (ca. 1960) and current distribution of the New England cottontail (NEC) in the northeastern United States. Remnant populations are found in five geographic locations: seacoast region of southern Maine and New Hampshire (*MENH*); Merrimack River valley of New Hampshire (*NH-MV*); Cape Cod, Massachusetts (*CC*); eastern Connecticut and Rhode Island (*CTRI*); western Connecticut, southeastern New York, and southwestern Massachusetts (*CTNY*). Historic range GIS data obtained from Patterson et al. (2007) and modified based on Tash and Litvaitis (2007)

2006; Wright et al. 2008). Without habitat connectivity, populations cannot exchange individuals and maintain genetic diversity. Consequently, understanding the genetic structure of threatened and endangered species is important in management and conservation efforts.

Population genetic studies, especially in combination with other ecological research, can aid our understanding of population history (Hansen and Taylor 2008) and provide necessary information for the designation of management units (González-Suárez et al. 2009). Such data also provide information about population connectivity and the genetic diversity of populations, which are beneficial when making translocation or captive breeding decisions (Johnson 2000) and for successful reintroductions to increase the genetic exchange of individuals (Maudet et al. 2002). Whether the goal is to increase genetic diversity or to conserve locally adapted populations, by recognizing the degree of genetic divergence among populations, wildlife professionals can make more informed management decisions.

One species that is threatened with extinction as a result of habitat loss and fragmentation is the New England cottontail (*Sylvilagus transitionalis*). This habitat specialist requires densely vegetated areas, such as coastal thickets or early-successional habitat with extensive understory vegetation (Litvaitis et al. 2003). Its historic range extended throughout most of New England and eastern New York (Hall and Kelson 1959; Godin 1977; Chapman et al. 1992, Fig. 1), a landscape that has undergone vast transformations since European settlement. Beginning in the early 1600s, forests were gradually cleared for agriculture, peaking in deforestation around 1850 (Foster et al. 2002). Wide-spread farm abandonment throughout New England in the latter half of the nineteenth and early twentieth centuries initially resulted in an increase of early-successional habitat, with a concurrent increase in New England cottontail populations (Litvaitis 1993). However, this type of habitat is ephemeral and is only ideal for cottontails for a span of approximately 15 years. About 25 years after abandonment, idle fields become reforested, causing much

of the understory vegetation to die out and hence become unsuitable for cottontails (Litvaitis et al. 2008). By about 1960, most abandoned agricultural lands had become reforested and New England cottontail habitat drastically diminished, resulting in New England cottontail population decline (Litvaitis 2003). Increased development in New England in the last 50 years has resulted in further fragmentation of the remaining suitable habitat. Consequently, New England cottontails have not simply returned to pre-settlement population levels, but instead are suffering continuous population decline (Litvaitis 2003; Litvaitis et al. 2006; Tash and Litvaitis 2007).

As a consequence of forest maturation and land-use changes, current habitat of New England cottontails is largely characterized as small, isolated patches of native shrublands or young forests. Remnant patches span approximately 14% (12,180 km^2) of the 90,000 km^2 historically occupied range (ca. 1960; Fig. 1; Litvaitis et al. 2006). A recent range-wide inventory found that the species currently exists in five geographically disjunct locations (hereafter referred to as geographic populations): southern Maine and southeastern New Hampshire (MENH); the Merrimack Valley in central New Hampshire (NH-MV); Cape Cod, Massachusetts (CC); parts of eastern Connecticut and Rhode Island (CTRI); and western Connecticut, southeastern New York and southwestern Massachusetts (CTNY). The approximate area occupied by these populations ranges from 1,260 km^2 for NH-MV to 4,760 km^2 for CTNY (Litvaitis et al. 2006). Cottontail abundance within these areas is unknown, with the exception of a rough estimate of approximately 300 rabbits in Maine (the majority of the area occupied by the MENH population; Litvaitis and Jakubas 2004); recent evidence shows this population has declined further since that estimate (Fenderson and Kovach, unpublished data). Range-wide, Litvaitis et al. (2006) only detected New England cottontails in 7% of the 2,301 patches of suitable habitat searched. Further, many of these patches were small (<3 ha) and likely only supported 3–4 rabbits within a highly fragmented landscape. The long-term viability of remnant New England cottontail populations is at risk (Litvaitis and Villafuerte 1996; Litvaitis 2001; Litvaitis et al. 2006). For these reasons, the New England cottontail is currently a candidate for federal listing under the Endangered Species Act and is considered to be, along with several other early-successional obligate species, one of the species of greatest conservation need in all New England states and New York (USFWS 2006, 2009).

As a result of the severe habitat loss, range contraction, and population decline, the New England cottontail may be at risk for deleterious population effects such as loss of genetic diversity and extinction (e.g., Ciofi and Bruford 1999; Bijlsma et al. 2000). Its protection and recovery will require an understanding of not only its ecology but also its population genetic structure. The latter will yield insight into the genetic connectivity of remnant populations and indicate which populations are the most genetically isolated and most in need of restoration management.

To address these needs, we investigated the genetic structure of the remaining New England cottontail populations. Our objectives were to assess levels of genetic variation within, as well as genetic differentiation and gene flow among, the five geographically isolated populations. We also infer patterns of demographic history, including tests to assess the relative influence of genetic drift versus drift-migration equilibrium and to determine if there are genetic signatures of recent population bottlenecks. Our results are aimed to inform resource managers about the processes that have shaped the genetic diversity of these remnant populations and what they imply for the conservation of the species given the current landscape structure.

Methods

Sample collection and DNA extraction

New England cottontail tissue ($n = 153$) and fecal pellet ($n = 81$) samples were obtained from range-wide live-trapping or noninvasive surveys conducted between 1990 and 2009. Two opportunistically collected road-kill samples were also used. Both pellet and tissue sample locations were distributed throughout the range of each of the five geographic populations, to the extent occupied patches were present (Fig. 1). Pellets comprised 3% (in CTRI) to 73% (in CC) of the samples from each geographic population. All samples were stored at −20°C prior to extraction.

DNA was extracted from the tissue samples using a standard phenol–chloroform–isopropanol extraction (Litvaitis and Litvaitis 1996) or by using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, Calif.), following the manufacturer's instructions. DNA was extracted from fecal pellets using the QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, Calif.), following the manufacturer's instructions with minor modifications, as described in Kovach et al. (2003). Due to the possible presence of other sympatric lagomorph species (snowshoe hare, *Lepus americanus*; eastern cottontail, *Sylvilagus floridanus*) in the surveyed sites, all pellets were first identified to species using a diagnostic RFLP analysis (Litvaitis and Litvaitis 1996; Kovach et al. 2003).

Microsatellite genotyping

DNA samples were amplified at 16 microsatellite markers using multiplexed PCR and published protocols optimized

for this study (Electronic supplementary Table S1). These loci were developed for the European wild rabbit (*Oryctolagus cuniculus*—Sol03—Rico et al. 1994; Sol44—Surridge et al. 1997; Sat3, Sat7, Sat 12, Sat13—Mougel et al. 1997; INRACCDDV016, INRACCDDV021, INRACCDDV0100, INRACCDDV0106, INRACCDDV0241, INRACCDDV0259, INRACCDDV0326 (SRY marker)—Chantry-Darmon et al. 2005 (hereafter, all INRA primer names have been abbreviated to 'INRA' followed by the last three digits of the primer name); D6Utr4—Korstanje et al. 2003) and two South African hares (*Lepus saxatilis* and *L. capensis*—Lsa1, Lsa8—Kryger et al. 2002). Samples were genotyped using fluorescent dye-labeled primers and an automated DNA sequencer (ABI 3130, Applied Biosystems, Foster City, CA). Alleles were manually scored and genotypes determined for each individual using Peak Scanner 1.0 (Applied Biosystems, Foster City, CA).

We used a multiple-tubes approach of at least three amplifications to detect and eliminate genotyping errors for the pellet samples, since they yielded lower quality and quantity of DNA than the tissue samples (Taberlet et al. 1996). If the DNA sample was exhausted before three replicate genotypes could be obtained, we retained a genotype at a given locus if it successfully amplified twice and an identical genotype was obtained each time. While we may have slightly overestimated homozygosity with this method, pellets comprised only a quarter of the samples in our dataset, and of those, only 12% of the single locus pellet genotypes were determined to be homozygous from only two replicates. Raw genotypes were binned using FlexiBin V2 (Amos et al. 2007) and a consensus multilocus genotype for each sample was manually created. Samples missing data at four or more loci were excluded from analyses.

To quantify pellet sample genotyping error rates, we manually compared all replicate genotypes to the consensus genotype and calculated per allele and per locus error rates as the number of mismatches between the consensus and replicate genotypes, following Eqs. 1 and 2 of Pompanon et al. (2005). Mean genotyping error was 0.04 per allele and 0.08 per locus (see Electronic Supplementary Table S2 for per locus error rates). Because we were unable to use species-specific primers, the possible presence of null alleles was a concern. The program INEST (Chybicki and Burczyk 2009) was used to simultaneously estimate the presence of null alleles and inbreeding coefficients, using the individual inbreeding model and 1,000,000 iterations of the Gibbs sampler. We also estimated null allele frequencies using the method of Dempster et al. (1977) and generated a null allele (NA) corrected dataset in FreeNA (Chapuis and Estoup 2007) using 10,000 replicates. We used this dataset in addition to the original dataset for determining the degree of population differentiation.

Descriptive statistics

To ensure that resampled individuals were not used in these analyses, we identified unique genotypes using the computer program DROPOUT (McKelvey and Schwartz 2005). We evaluated the discriminatory power of our loci using the more conservative probability of identity statistic for related individuals ($P_{ID\text{-}SIB}$), as it is appropriate for wildlife populations that may be comprised of close relatives or for small populations that may be inbred (Waits et al. 2001).

We tested for deviations from Hardy–Weinberg and linkage equilibrium with GENEPOP 3.4 (Raymond and Rousset 1995) and significance was evaluated using an adjusted *P*-value corresponding to $\alpha = 0.05$ after false discovery rate (FDR) control (Benjamini and Hochberg 2000) as implemented in Excel Spreadsheet Tabulator (Verhoeven et al. 2005). Observed and expected heterozygosities and the number and frequency of private alleles were also calculated in GENEPOP. The inbreeding coefficient F_{IS} was calculated in FSTAT (Goudet 1995) and significance determined using the FDR control. Number of alleles, allelic richness, and private allelic richness were calculated in HP-RARE (Kalinowski 2005), which uses the rarefaction method to correct for sample size differences.

Population differentiation and structure

Genetic differentiation among geographic populations was measured using pairwise F_{ST} calculated in FSTAT, with significance determined using the FDR control. A Mantel test was performed in the R statistical software (R Development Core Team 2006) with the vegan package (Oksanen et al. 2010) to test for isolation by distance among populations. We used the natural log of the shortest estimated overland distance between the approximate centers of the geographical populations and $F_{ST}/(1 - F_{ST})$ as the genetic distance.

Population structure was also evaluated using two individual-based, Bayesian clustering methods: STRUCTURE 2.3.3 (Pritchard et al. 2000) and TESS 2.3 (Chen et al. 2007). We conducted 20 runs of STRUCTURE at each K (the number of putative genetically-defined populations) from 1 to 10 with a burn-in of 100,000 iterations and run-length of 500,000 iterations. Because the five geographic populations are spatially disjunct, we used the no admixture model and assumed independent allele frequencies. The optimal K was determined both by examining the plateau of the ln Pr(X|K) as suggested by Pritchard et al. (2000) and by calculating the second order rate of change in the likelihood of K (ΔK) (Evanno et al. 2005). Although STRUCTURE has a model to incorporate data with null alleles (Falush et al. 2007), it was not

utilized due to its reported lack of improved performance over the other models and because the influence of null alleles on these types of analyses is most likely minimal (Carlsson 2008).

For comparison, we also used TESS 2.3 (Chen et al. 2007) to estimate the number and locations of genetically similar clusters. Unlike STRUCTURE, TESS incorporates the spatial sampling locations into the analyses to assess genetic cluster membership. We used the no admixture model and conducted twenty runs at each K from 2 to 10 with 600,000 total sweeps including a burn-in of 100,000 sweeps. The interaction parameter was set to 0.6 and the deviance information criterion (DIC) was averaged across runs for each K. The average DIC was then plotted against K and the optimal K was determined from the beginning of the plateau as well as stabilization of the barplots, as recommended by Durand et al. (2009).

Detection of migrants and recent gene flow

Using the results from the cluster analyses described above, samples from the geographic populations were regrouped according to their genetic cluster assignment (hereafter referred to as genetic populations or genetic clusters) and this prior knowledge was incorporated in STRUCTURE to detect migrants and individuals with migrant ancestry. We tested a range of migration values (0.001–0.1) and assessed whether individuals or their immediate ancestors (up to two generations back) had migrant ancestry by setting GENSBACK = 2 (Pritchard et al. 2000).

Self-assignment tests and population simulations to test for first generation migrants were also conducted in GENECLASS 2.0 (Piry et al. 2004). The assignment test was performed using the Rannala and Mountain (1997) Bayesian method. Detection of migrants was conducted using the L_home/L_home_max criterion with Monte Carlo resampling (Paetkau et al. 2004) and an alpha level of 0.01.

Population history and genetic diversity

We performed several analyses to examine genetic variability and recent population processes. To assess genetic variability among the genetic clusters, we compared allelic richness using a one-way analysis of variance (ANOVA), blocked by locus in JMP8 (SAS Institute, Cary, NC). Effective population sizes (*Ne*) of each genetic cluster were estimated using two methods—a linkage disequilibrium method as performed in LDNe (Waples 2006), using 0.05 as the lowest allele frequency, and a Bayesian method implemented in ONeSAMP (Tallmon et al. 2008). To test for recent genetic bottlenecks, we used two approaches known to differ in their sensitivity for detecting bottlenecks of different time scales and durations (Williamson-Natesan 2005): BOTTLENECK 1.2.02 (Piry et al. 1999) and the M-ratio method (Garza and Williamson 2001). We ran 1000 replications in BOTTLENECK with all mutation models, assuming 88% stepwise mutation and 12% infinite allele mutation to coincide with the model parameters used in the M-ratio test (see below), and set the variance among multiple steps to 12. We assessed the results with the Wilcoxon signed-rank test of heterozygosity excess and the allele frequency mode-shift test (Luikart et al. 1998). To calculate the M-ratio, since historic effective population sizes are unknown, we compared results using both an approximation of a maximum, historic N_e of 5,000 per population and the mean of our estimates of the current N_e of each cluster obtained using LDNe and ONeSAMP. We considered these to be the two extremes of effective population sizes; the upper estimate of 5,000 is based on the large population sizes that would have had to exist to support historic reports of large cottontail harvests (e.g., 33,000 cottontails hunted annually in Vermont in 1944; Foote, as cited in Eabry 1983). We then calculated Θ assuming $\mu = 5 \times 10^{-4}$. The softwares M_P_Val.exe and Critical_M.exe were used for the simulations (available at http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298). The mean size for non-single step mutations was set to 2.8 and the percent of mutations larger than single step was 0.12, as these were the average parameter values found in a literature survey by Garza and Williamson (2001).

We further evaluated how long populations have been isolated by examining whether populations have been more affected by genetic drift or are in migration-drift equilibrium. We tested this with the program 2MOD, using 100,000 iterations (Ciofi and Bruford 1999). This analysis compares the relative likelihood that a population's gene frequencies are the result of a balance between drift and immigration or if the gene frequencies are the result of the population diverging in isolation. We evaluated the relative importance of drift to gene flow in each population by modeling the posterior distribution of F. We used LOCFIT (Loader 1999) implemented in the R statistical package to graph the posterior distributions and summarized the distribution by the mode and 95% highest posterior density interval (HPDI), which were calculated using the hdrcde (Hyndman 2010) and boa (Smith 2005) packages, respectively.

Results

Descriptive statistics

Two hundred samples successfully amplified (tissue: $n = 149$; pellet: $n = 51$) at 13 or more loci and were examined for duplicate genotypes. $P_{ID\text{-}SIBS}$ for females at

Table 1 Genetic diversity of the five geographic populations of the New England cottontail

Population[a] (*N*)	Alleles	Allelic Richness	Ho	UHe	Private alleles	F_{IS} FSTAT/INEST
MENH (58)	3.2	2.6	0.223	0.288	1 (0.17)	0.229*/0.017
NH-MV (14)	2.9	2.9	0.287	0.338	0 (0.12)	0.157/0.017
CC (25)	3.7	3.1	0.277	0.346	9 (0.62)	0.203*/0.013
CTRI (57)	4.6	3.4	0.371	0.442	2 (0.20)	0.162*/0.036
CTNY (38)	4.7	4.0	0.492	0.555	8 (0.77)	0.115*/0.012

[a] Population abbreviations as in Fig. 1; sample size in *parentheses*. Alleles, allelic richness, observed heterozygosity (Ho), Nei's unbiased heterozygosity (UHe) and F_{IS} (calculated both in FSTAT using the original dataset and in INEST using a null-allele corrected dataset—see text) are averaged across loci. Private alleles are the total number of private alleles (and private allelic richness, averaged across loci) for all loci in each population. F_{IS} significance at the $P < 0.05$ level after FDR control is indicated by an *asterisk*

the fifteen autosomal loci was 5.340×10^{-4}, meaning for every 1873 (closely-related) females, each individual should have a unique multilocus genotype. Males could be distinguished with even greater certainty, with a $P_{ID\text{-}SIBS}$ of 2.892×10^{-4}. This was due to the polymorphism of the SRY marker, as well as a characteristic of this marker (probably a gene duplication event—see Geraldes and Ferrand 2006) that resulted in many male individuals having heterozygous genotypes at this locus. These probabilities of identity indicate sufficient power for detecting unique individuals. Eight pairs of duplicated samples were found. For each pair, a randomly selected sample was removed from the dataset, unless one duplicate was a tissue sample and the other a pellet sample, in which case the tissue sample was retained. The sex ratio was slightly male-biased overall, with 0.9 females identified for every male. The sex ratio within most populations was nearly equal, although there were twice as many male samples analyzed from CC (1 M:0.47 F), while more females were sampled in MENH (1 M:1.23 F).

Two loci (INRA100 and INRA021) exhibited very low polymorphism (H_o: 0.014 and 0.016, respectively) and were excluded from further analyses. Null allele frequency estimates from FreeNA ranged from essentially 0 to 20% and INEST estimates, while comparable in most cases, were slightly higher for all loci in all populations (range 3.7–27.6%; Electronic Supplementary Table S3). Deviations from HWE at a number of loci in each population were detected (Electronic Supplementary Table S4). Overall, only the NH-MV population did not show significant deviation from HWE ($P = 0.0881$). Several locus pairs also showed evidence of linkage disequilibrium, primarily within the CTNY population (Sat12/Lsa1, D6Utr4/INRA241, Sol44/INRA259) and one pair was significant for the CTRI population (Sat3/INRA16). Given the population-specific nature of the linkage disequilibrium, it is not likely a result of physical chromosomal linkage nor a concern for further analyses. Linkage disequilibrium is often found in small populations and may be a result of subdivisions within the population sample or recent fragmentation (Frankham et al. 2002; Zartman et al. 2006).

The number of alleles in each population, averaged across loci, ranged from 2.9 to 4.7 (Table 1). Average allelic richness was lowest in the MENH population (2.6) and highest in the CTNY population (4.0). Observed heterozygosity ranged from 0.223 in MENH to 0.492 in CTNY and was lower in all cases than the unbiased expected heterozygosity (range 0.288–0.555). Numerous private alleles were found in the CC and CTNY population and only the NH-MV population lacked any private alleles (see Electronic Supplementary Table S5 for per locus allele frequency distributions in each geographic population). Estimated F_{IS} values calculated in INEST were much smaller than those calculated in FSTAT. All inbreeding coefficients calculated in FSTAT with the original dataset were significantly positive; however none were significantly different from zero when null alleles were acknowledged.

Population differentiation and structure

All geographic populations were significantly differentiated from each other, using both the original (overall $F_{ST} = 0.145$) and the null allele corrected datasets (overall $F_{ST} = 0.121$; Table 2). In general, the MENH population was most differentiated from the remaining populations, but had the lowest F_{ST} when compared with the NH-MV population. Cape Cod was also highly differentiated from the other populations. There was a significant signal of isolation by distance among the five populations (Mantel test, $y = 0.1132x - 0.4766$; $r^2 = 0.5649$; $P = 0.03$).

Analyses in STRUCTURE indicated the presence of five genetic clusters (Fig. 2), however not exactly corresponding to the five geographically delimited populations. The MENH and NH-MV populations clustered together (hereafter referred to as ME/NH) and the eastern CTRI population differentiated into two clusters (Fig. 3). Closer examination of the eastern CTRI populations revealed that

Table 2 Pairwise F_{ST} values for the five geographic New England cottontail populations, calculated using original (*above diagonal*) and null allele-corrected FreeNa (*below diagonal*) datasets

	MENH	NH-MV	CC	CTRI	CTNY
MENH		0.0343	0.2002	0.1434	0.1753
NH-MV	0.0317		0.1305	0.1045	0.1122
CC	0.1808	0.1249		0.1281	0.1565
CTRI	0.1274	0.0930	0.1267		0.0649
CTNY	0.1609	0.1018	0.1378	0.0622	

All F_{ST} values were significant after FDR control ($\alpha = 0.05$)

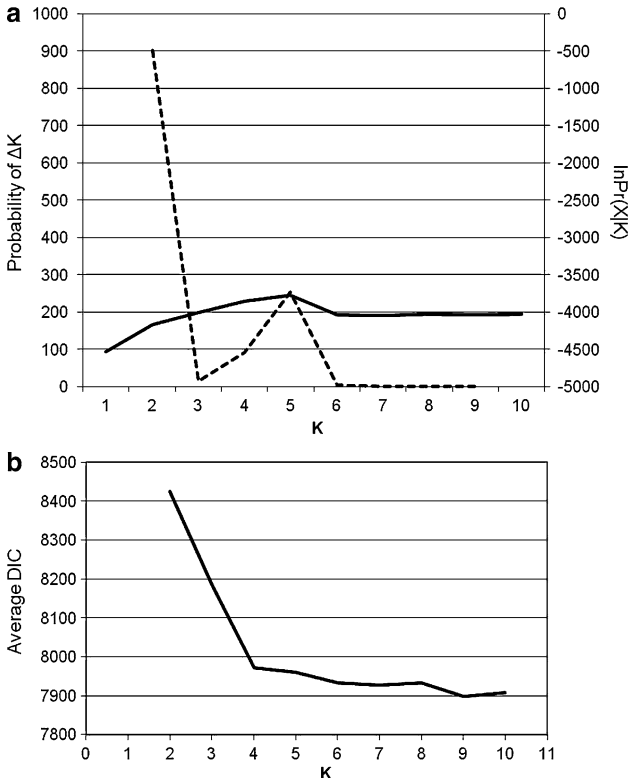

Fig. 2 Determination of K, the number of genetic clusters of New England cottontails from: **a** STRUCTURE analyses: solid line indicates ln Pr(X|K) (*right y*-axis); *dashed line* indicates ΔK (*left y*-axis). **b** TESS analyses

one cluster corresponded primarily to a group of individuals found in Bluff Point State Park, a designated coastal reserve on a peninsula in Groton, CT (Fig. 3; hereafter the Bluff Point cluster is referred to as 'Bluff Point' and the remaining eastern CT and RI cluster is referred to as 'CT/RI').

Results of analyses in TESS were similar to those of STRUCTURE, as it also grouped MENH and NH-MV and differentiated the remaining geographic populations. However, TESS only detected four clusters, as it did not identify the Bluff Point individuals as a separate cluster (Figs. 2, 4).

Detection of migrants and recent gene flow

Increasing the migration rate used in the STRUCTURE assignment test only slightly increased the number of individuals detected as putative migrants and reduced the individual resident probabilities by a small amount. As the differences were minimal and did not affect overall conclusions, we only report the results for MIGPRIOR = 0.05. Given the significant genetic divergence between MENH and NH-MV, as well as the extensive geographic distance between the two populations (at least 44 km, which likely exceeds cottontail dispersal ability), we tested for migrants using K = 6, keeping MENH and NH-MV as separate populations and considering Bluff Point as a separate cluster in both STRUCTURE and GENECLASS assignment tests.

Assignment tests revealed that most individuals were residents of their sampled populations (82.8% correctly assigned in GENECLASS, also see Fig. 4). Six individuals were identified as putative migrants in STRUCTURE and four individuals may have recent migrant ancestry (Electronic Supplementary Table S6). GENECLASS identified the same six individuals as possible migrants, but also three others, which were considered to have possible migrant ancestry. Thirteen individuals that were not detected as migrants in either program but were assigned by both programs to populations other than the one in which they were sampled, or had nearly equal Q values in two clusters, were also considered to be of admixed ancestry. The majority (17 of 26) of migrants and admixed individuals were identified as migrating into or out of the CT/RI population.

Population history and genetic diversity

Comparisons of allelic richness using a nested ANOVA showed significant differences among the five clusters identified by STRUCTURE ($F = 17.05$, df = 4, 44, $P < 0.0001$, Fig. 5). The Bluff Point population has significantly lower allelic richness than any of the other populations. The allelic richness of ME/NH was significantly reduced compared to the CT/RI and CT/NY populations, and allelic richness of CC was significantly reduced relative to CT/NY.

With the exception of CT/RI, estimates of current *Ne* from both LDNe and ONeSAMP were similar and had overlapping confidence intervals (Table 3). N_e estimates in all populations were relatively low and ranged from 13 in Bluff Point to 233 in CT/RI.

The two methods we used to test for population bottlenecks produced slightly different results. BOTTLENECK tests showed evidence of a recent bottleneck for the Bluff Point population, which had a shifted-mode distribution as

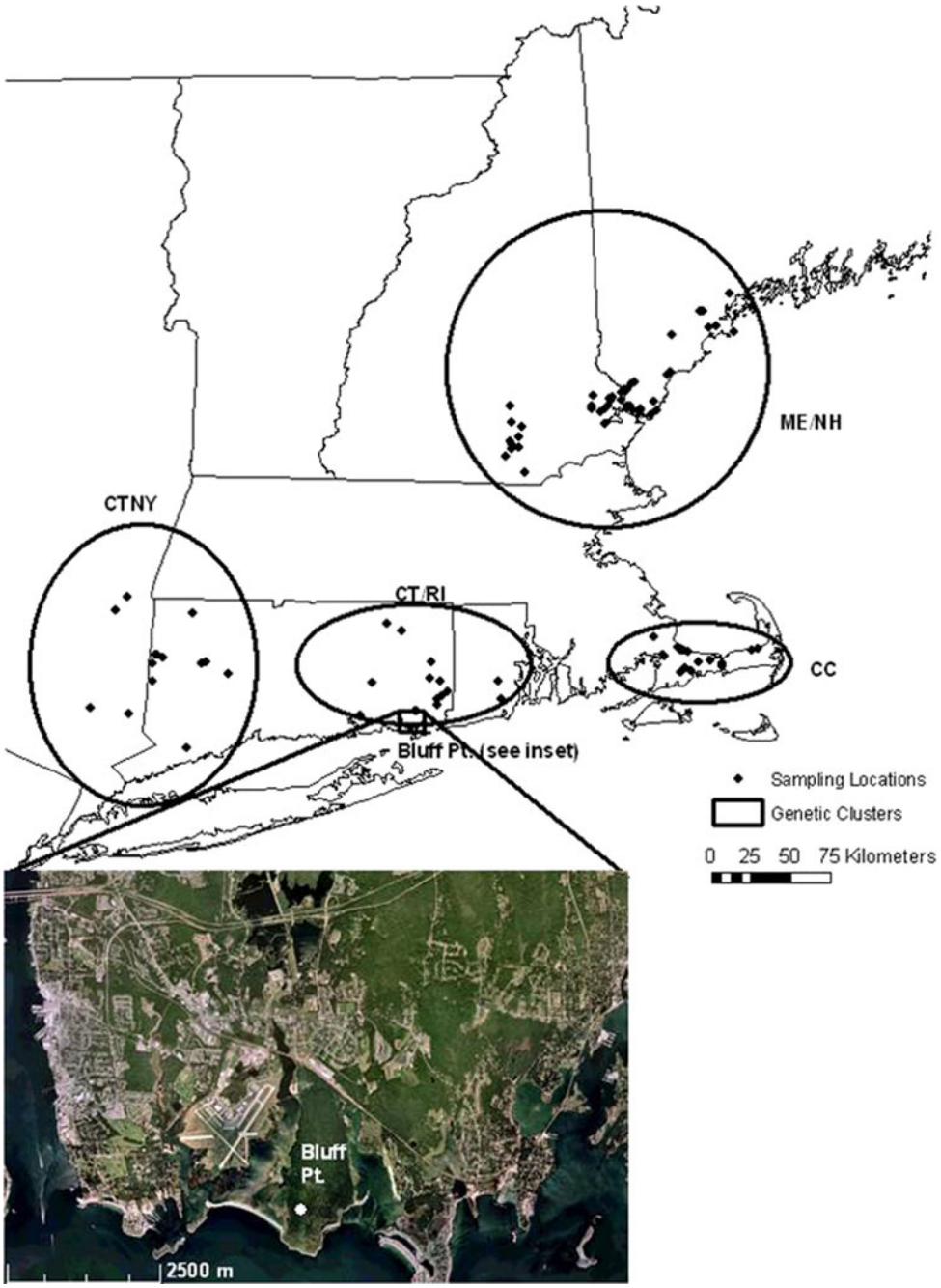

Fig. 3 Genetic clusters of New England cottontails identified by STRUCTURE analyses

well as significant heterozygosity excess under all mutation models ($P < 0.004$; Table 3). It should be noted, however, that there are only 12 samples in the Bluff Point population, and only eight loci were polymorphic in these individuals. All remaining populations demonstrated normal L-shaped allele frequency distributions. With the exception of CTNY, the remaining populations had significant heterozygosity deficiencies under the two-phase and stepwise mutation models. CTNY exhibited a significant heterozygosity excess under the infinite allele model ($P = 0.01$).

Results of the M-ratio test also showed that most populations have not experienced a bottleneck (Table 3). The M-ratio test for CC, on the other hand, was highly significant, with an M-value typical of bottlenecked populations (0.6751; $P < 0.001$). None of the other populations showed significant signs of experiencing a bottleneck, although the M-ratio of the CTNY population approached the critical M-ratio value estimated using the current N_e ($P = 0.056$).

Results from 2MOD indicated that NEC populations are much more likely to be experiencing genetic drift in isolation

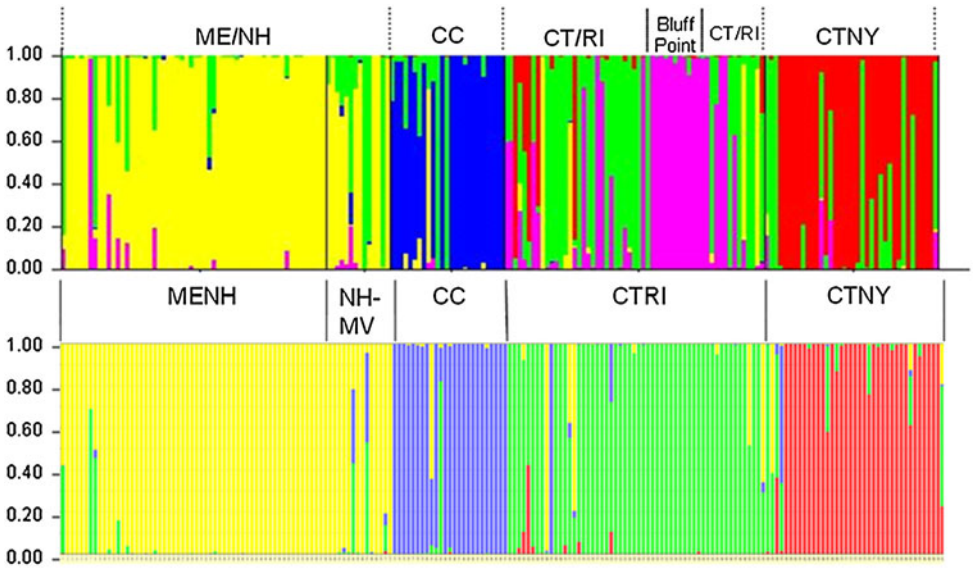

Fig. 4 Individual assignment probabilities of New England cottontails to genetic clusters. Results from **a** a STRUCTURE run of K = 5; **b** a TESS run of K = 4. Genetic cluster indicated above figure, geographic sampling locations are indicated in *center* of figure

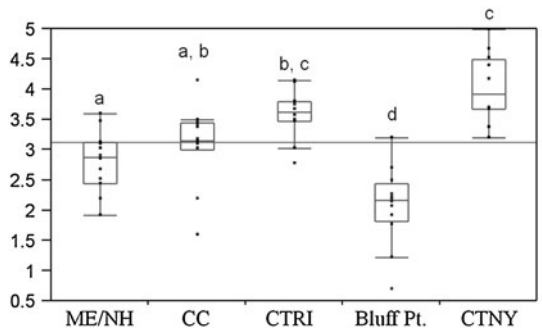

Fig. 5 One-way ANOVA of mean allelic richness of the five New England cottontail genetic clusters identified in STRUCTURE. *Shared letters* indicate populations that are not significantly different

than to be in drift/migration equilibrium (Probability of drift model = 0.997, Bayes factor = 284), and if the first 10% of the results were excluded as a burn-in period, only the genetic drift model was possible. Density plots of F revealed that Bluff Point is experiencing the greatest amount of drift and is the most isolated ($F = 0.467$; 95% HPDI = 0.360–0.532), while CT/RI ($F = 0.123$; 95% HPDI = 0.060–0.123) and CT/NY ($F = 0.088$; 95% HPDI = 0.059–0.106) have higher levels of gene flow (Fig. 6). ME/NH ($F = 0.190$; 95% HPDI = 0.167–0.260) and CC ($F = 0.258$; 95% HPDI = 0.149–0.285) showed intermediate levels of isolation relative to the other populations.

Discussion

Habitat loss and fragmentation are serious threats to the viability of animal and plant populations, especially for species of conservation concern (Fahrig 2003; Lindenmeyer and Fischer 2006). Numerous studies have demonstrated the negative genetic and demographic consequences that result from loss of connectivity among populations and reductions in population sizes (e.g., Segelbacher et al. 2003; Walker et al. 2008; Wu et al. 2010). In this study, we have shown that habitat loss and fragmentation have shaped the genetic structure of remaining New England cottontail populations. Remnant cottontail populations exhibit limited gene flow and low effective population sizes, with several populations exhibiting comparatively reduced genetic diversity. As a result of these consequences, human intervention will be required to mitigate and reverse continued population declines. Our findings should assist management efforts for this imperiled species.

Population structure

We found genetic distinctiveness of the five geographically separated cottontail populations and evidence for a lack of ongoing gene flow. The results of population-level F_{ST} analysis and the two Bayesian clustering methods were largely similar, with some slight discrepancies. Neither of the Bayesian clustering methods recognized the distinctiveness of the NH-MV population, despite significant differentiation by F_{ST} analysis. The F_{ST} value between the NH-MV and MENH populations was the smallest in the study ($\sim$0.03), and was approaching the lower limit of performance of these methods (Latch et al. 2006; Chen et al. 2007). The genetic grouping of these two population is likely reflective of historical connectivity between the two currently disjunct, but geographically proximate, locations and not indicative of current gene flow, the latter of which has likely not occurred for several decades due to loss of suitable habitat in the intervening landscape.

Table 3 Estimated effective population sizes (N_e) and results of genetic bottleneck tests of New England cottontail genetic clusters

Population	N_e LDNe[a]	N_e ONeSAMP[b]	BOTTLENECK[c] Wilcoxon test probability			BOTTLENECK mode-shift	M—ratio[d]	Mc ($N_e = 5{,}000$)	Mc (N_e current)
			I.A.M.	T.P.M.	S.M.M.				
ME/NH	152.6 (53.3–infinite)	78.2 (47.1–316.0)	0.867	0.999	1.000	Normal L-shaped	0.865	0.785	0.856
CC	39.2 (11.5–infinite)	34.9 (26.5–55.6)	0.867	0.974	0.995	Normal L-shaped	0.675*	0.731	0.865
CT/RI	47.7 (28.8–99.3)	232.6 (126.4–922.7)	0.368	0.980	0.999	Normal L-shaped	0.886	0.762	0.851
Bluff Point	17.0 (2.2–infinite)	13.4 (11.1–18.8)	0.002*	0.002*	0.004*	Shifted mode	0.901	0.801[e]	0.869
CTNY	35.7 (23.6–61.3)	76.7 (44.3–193.0)	0.011*	0.812	0.980	Normal L-shaped	0.865	0.755	0.864

* $P < 0.05$

[a] N_e values for LDNe method are based on the lowest allele frequency used of 0.05 and the parametric 95% confidence interval is reported in parentheses

[b] The mean N_e is reported for the ONeSAMP method with 95% confidence intervals

[c] For the tests performed in BOTTLENECK, the Wilcoxon one-tail probability of heterozygosity excess for the three mutation models are given, as well as results of the allelic mode shift test

[d] The M-ratio for each genetic cluster is specified; critical M values (Mc) were calculated both using the mean estimated current N_e for each population and with $N_e = 5{,}000$

[e] Due to the small size of the peninsula, the historic Θ for Bluff Point was calculated with an estimated N_e of 500

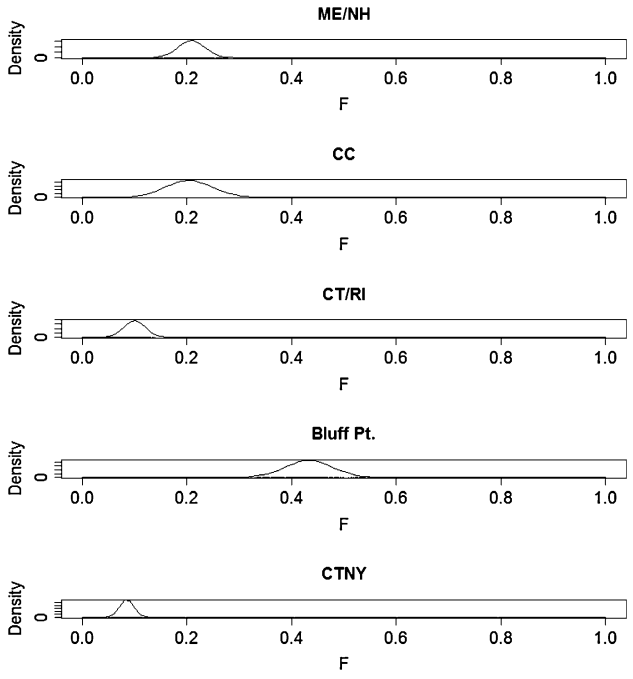

Fig. 6 Posterior density distribution plots of *F*, the probability of any two genes sharing a common ancestry within a population, under the genetic drift in isolation model for each New England cottontail genetic cluster

The genetic clustering method of STRUCTURE (but not TESS) recognized a subdivision within the CTRI population, consisting of individuals in Bluff Point State Park. This coastal reserve is located on a peninsula in southeastern Connecticut and is geographically proximate to nearby cottontail populations (<20 km). However, it is surrounded by extensive development, including the Groton-New London Airport, major highways, and the Northeast Corridor, the busiest passenger rail line in the U.S. Despite strong evidence of genetic differentiation ($F_{ST} = 0.09$ between Bluff Point and CT/RI), it is likely that TESS did not differentiate the Bluff Point individuals from the rest of CTRI due to the interaction parameter between the spatial coordinates and genetic data used in the TESS algorithm. The genetic distinctiveness of the Bluff Point individuals suggests that they have been isolated from CT/RI for a relatively long time (for comparison, $F_{ST} = 0.03$ for MENH vs NH-MV populations, which are currently separated by a minimum of 44 km, but were connected several decades ago). Alternately, this may be the result of a founder effect with little to no recent contact between groups. The limited genetic variability of this population and results from tests of population history (see below) further support the isolation of the Bluff Point population. Due to the highly fragmented nature of remaining New England cottontail habitat, small, isolated patches or clusters of patches, such as Bluff Point, exist across the species' current range. These "populations" may become differentiated due to lack of genetic exchange and the rapid effects of genetic drift. It is likely that we were able to identify the genetic distinctiveness of Bluff Point

due to a relatively large number of samples collected from this location, relative to the broader scale sampling effort across the remainder of CTRI and the other geographic populations.

Sampling scheme is known to have a significant impact on the outcome of genetic clustering analyses (Schwartz and McKelvey 2009; Segelbacher et al. 2010). The broad-scale sampling effort employed in our study did not enable us to address substructuring within each geographic population. Given the extent of habitat fragmentation, such substructuring is likely to occur, especially among occupied patches separated by relatively large geographic distances. Additional fine-scale sampling of cottontails within each of the geographic populations would allow detection of other isolated and genetically distinct patches, such as in Maine, where a recent fine-scale study detected four distinct genetic clusters within that population (Fenderson 2010). This finding suggests that numerous genetically distinct populations might also occur within CTNY and CTRI, the two geographic populations with the largest occupied areas.

Although assignment tests in STRUCTURE and GENECLASS assigned the majority of individuals to their population of origin, they also identified several individuals with migrant ancestry and a few individuals as potential migrants from distant source populations. Of all of the putative migrants and admixed individuals, the majority were found to migrate into or out of the CT/RI population. Assignment test results are typically interpreted as direct genetic evidence of individual dispersal events (Paetkau et al. 2004; Bergl and Vigilant 2007). Given the extensive distances between geographic populations, however, we interpret these findings as reflective of past connectivity consistent with the CT/RI population's central location and not as evidence for ongoing dispersal between the populations. Extensive habitat loss throughout the range, especially between geographic populations, must inhibit, if not completely prevent, current gene flow across this broad scale. While there may be unsampled stepping-stone patches between populations, it is doubtful there are enough persistently occupied patches to allow for connectivity between populations (Barbour and Litvaitis 1993). Further, although long-distance dispersal is not unheard of, it is uncommon and is unlikely to exceed 10–20 km, based on maximum dispersal distances of other lagomorphs (e.g., Gillis and Krebs 1999; Estes-Zumpf and Rachlow 2009). Thus, with the exception of perhaps the Bluff Point and CT/RI genetic clusters, which are less than 20 km apart, the extensive distances between remnant populations make it highly improbable that they are currently exchanging individuals. More likely, we are detecting historic genetic signatures of connectivity. This interpretation is further substantiated by the results of population history analyses in 2MOD, which indicate that the current genetic structure has been shaped by genetic drift in isolation and that dispersal among populations is negligible.

Population history and genetic diversity

The two tests for recent population bottlenecks showed different results, likely indicative of differences in the time-scales of population reductions. Only the Bluff Point population showed a significant bottleneck effect according to all BOTTLENECK analyses, while the M-ratio method only detected a significant bottleneck in the CC population. Based on known differences in the performance of these methods (BOTTLENECK tests are more likely to detect recent bottlenecks, e.g., within 40 generations, whereas the M-ratio tests tend to detect bottlenecks that occurred longer ago and persisted for a comparatively longer duration; Williamson-Natesan 2005), the Bluff Point population likely experienced a more recent population bottleneck than the CC population, which may have experienced a more historic reduction that lasted several generations.

The Cape Cod Canal, which opened in 1914, was widened significantly in the late 1930s. With a current width of 146 meters at depth, it is the widest sea-level canal in the world. As such, it is likely a significant dispersal barrier for the cottontail, and its isolating effect in combination with habitat loss in this highly developed landscape may have led to the earlier bottleneck observed in this population. Estimates of an effective population size below 50 individuals and reduced allelic richness both point toward a severe and long-lasting bottleneck of the Cape Cod population.

Isolation or founding of the Bluff point population, on the other hand, appears to have occurred more recently than the isolation of the CC cottontails, as evidenced by detection of a bottleneck effect by the BOTTLENECK but not the M-ratio test. Bluff Point State Park, acquired in 1963 and designated a coastal reserve in 1975, is the last significant piece of undeveloped land along the Connecticut coast. The surrounding development likely functions to limit or prevent exchange between these individuals and the closest nearby patches. Arrival of cottontails in Bluff Point was not the result of wildlife management activities as there have been no known translocations of cottontails to the park (H. Kilpatrick, personal communication). Estimates of effective population size indicate that this is a very small population of less than 20 breeding adults. The high F-value estimated by 2MOD ($F = 0.47$) and the lowest allelic diversity (mean allelic richness = 2.08) observed in our study further confirm that genetic drift is acting rapidly in this small, isolated population and influencing its genetic differentiation from the nearest cottontails in CT/RI.

There was a significant indication of a genetic bottleneck in the CTNY population with the heterozygosity excess test when the I.A.M. model was used and the M-Ratio method approached significance when Θ was calculated with the current effective population size ($P = 0.056$). This may indicate that this population has experienced a recent demographic and genetic bottleneck as well.

While we did not find evidence for bottleneck effects in the remaining New England cottontail populations, our results merit some caveats because of the methods used. First, both approaches are suited for detecting relatively rapid losses of genetic diversity and may not be able to recognize slow and steady or very recent declines, situations that may be representative of the remaining New England cottontail populations. Further, the performance of the tests may have been limited by the presence of null alleles, which may have influenced the allele distributions and heterozygosity estimates used in BOTTLENECK (Cornuet and Luikart 1996).

Results from 2MOD and tests of allelic richness are consistent with the bottleneck results in showing that the Bluff Point and CC populations have reduced allelic diversity and elevated *F*-values, and thus have been most strongly influenced by genetic drift. The ME/NH population cluster also shows reduced allelic richness relative to the CT/RI and CTNY populations and is similar to the CC population in terms of allelic diversity and *F*-values. Additionally, the MENH geographic population and the Bluff Point population are monomorphic at 33% of the loci examined. These results confirm recent survey efforts (Fenderson 2010), which indicate the ME/NH population cluster is suffering an ongoing decline. The genetic data indicate that the CTNY and CT/RI populations, while also experiencing appreciable genetic drift, have suffered the least in terms of reductions in population size and genetic diversity.

Null alleles and deviations from Hardy–Weinberg equilibrium

The primers used in this study were developed for distantly related lagomorph species, which likely contributed to the relatively high frequency of null alleles observed. Most methods of null allele estimation assume populations are in HWE (e.g., van Oosterhout et al. 2004). However, we did not consider this a valid assumption for the New England cottontail. Given the geographical segregation of currently occupied patches (Litvaitis et al. 2006) and short expected dispersal distances typical of lagomorphs ($\sim$3 km on average, Gillis and Krebs 1999; Estes-Zumpf and Rachlow 2009), we considered inbreeding to be possible. Therefore, we utilized INEST to simultaneously estimate null allele frequencies and inbreeding within populations. This method determined relatively low inbreeding coefficients for each population, indicating that the observed homozygosity excess was driven primarily by null alleles and not inbreeding.

Deviations from HWE may also be a result of the Wahlund effect. The linkage disequilibrium found among three pairs of loci in CTNY and one locus pair in CTRI point toward this possibility. While it is quite probable that subdivisions exist within each of the geographic populations, our sampling scheme was not on a fine enough scale to detect them (Fenderson 2010). Further, the Bayesian clustering methods we used are designed to group individuals so as to minimize deviations from HWE that would be caused by the Wahlund effect. F_{IS} values and Hardy–Weinberg probabilities were not significantly different before and after genetic clustering (data not shown), suggesting that the Wahlund effect was not the primary cause of the deviations from HWE.

Conservation implications

All remnant New England cottontail populations have relatively low genetic diversity and small effective population sizes. Small effective population sizes are cause for concern because they are indicative of increased susceptibility to genetic stochasticity and are correlated with reduced genetic diversity and increased inbreeding, all of which heighten the probability of population extinction (Palstra and Ruzzante 2008). The estimated effective population sizes for New England cottontails are insufficient for long-term, and in some cases short-term, population persistence (Franklin 1980; Soulé 1980; Franklin and Frankham 1998; Lynch and Lande 1998). Given the observed genetic consequences, and the lack of current gene flow among remnant populations, human intervention is warranted to mitigate further declines.

Conservation measures should focus on maintaining and expanding current populations, as well as promoting connectivity within and among populations (Tash and Litvaitis 2007). These efforts should include augmenting population sizes in all portions of the species' range, but especially in the ME/NH and CC populations, as well as stabilizing the Bluff Point population. These three populations show the greatest reduction in genetic diversity and the strongest effects of genetic drift. The CC and Bluff Point populations show signs of significant past population size reductions, while a reduction in ME/NH appears to be ongoing. From a conservation standpoint, efforts to maintain all geographically distinct populations are advisable, to decrease the risk of stochastic extinction (Frankham et al. 2002). In addition, the strong differentiation among these populations indicates that each population could represent a potential

reservoir of genetic diversity available to the others. From a practical standpoint, conservation of the CC and ME/NH populations warrants prioritization, as these comprise the last remaining New England cottontails from a large geographic area—Maine, New Hampshire and Massachusetts, whereas the cottontails in Bluff Point comprise a small (both geographically and in terms of population size), recently isolated subpopulation of the larger CT/RI geographic population.

Given their small population sizes and reduced genetic diversity, it may be prudent to consider genetic rescue of the ME/NH, CC (and Bluff Point) populations via translocations from the more diverse CT/RI & CTNY populations. Although translocations may alleviate the risk of inbreeding depression, the risk of outbreeding depression is also a serious concern, as it has been shown to diminish reproductive success, decrease viral resistance, and negatively impact survival (e.g., Marr et al. 2002; Goldberg et al. 2005). Conservative measures propose minimizing the risk of outbreeding depression by only initiating translocation plans when inbreeding depression is apparent (Edmands 2007; Hedrick and Fredrickson 2010). Our results do not provide conclusive evidence that New England cottontails are currently inbred. While all populations exhibited relatively low mean heterozygosity, which is often correlated with inbreeding (e.g., Slate et al. 2000; Shikano and Taniguchi 2002; Reed and Frankham 2003; but see Coltman and Slate 2003; Balloux et al. 2004; Alho et al. 2009), this may have been confounded by the presence of null alleles; the inbreeding coefficient was very low once null alleles were taken into account. Further research is needed to assess whether there are manifestations of inbreeding depression in individual rabbits. Additionally, non-native eastern cottontails (*S. floridanus*), while now sympatric throughout most of the New England cottontail range, have not yet expanded into Maine. Thus translocation of cottontails from other geographic populations into Maine also risks accidental introduction of eastern cottontails into a state where they have not previously existed. Such an introduction may exacerbate New England cottontail decline and hinder management and conservation efforts in Maine. These concerns should be given serious consideration prior to initiation of inter-population translocations. Therefore, we suggest that it may be more beneficial in the short-term to promote genetic exchange *within* populations via translocation and reintroduction methods, while habitat reconstruction projects are pursued.

Species recovery for the New England cottontail will require habitat reconstruction and restoration of connectivity within and among remnant populations, while further research is conducted to assess if there is evidence of inbreeding depression and reduced fitness. Extensive research has been conducted on the habitat requirements of New England cottontails (e.g., Barbour and Litvaitis 1993; Litvaitis et al. 2003). Litvaitis (2001) described several recommendations on how to maintain and establish early successional habitat, and Tash and Litvaitis (2007) identified habitats across the species' range that are highly suitable for restoration. Remaining populations are so fragmented that they will first require intensive management to restore connectivity among patches within the populations, and within population translocations to simulate gene flow in the short-term may be necessary. Once geographic populations are sustainable, reestablishing connectivity among populations and eventually reintroducing cottontails to historically occupied parts of the range (e.g., Vermont) will help ensure the persistence of this species.

In conclusion, we make the following recommendations for future research and immediate conservation actions on behalf of the New England cottontail.

- Implement habitat restoration efforts immediately to increase available habitat and connectivity among patches within each geographic population.
- Continue monitoring occupancy and population status of all range-wide populations to ensure that goals of population sustainability are being met.
- Initiate intensive surveys and fine-scale sampling efforts within each geographic region to identify additional population subdivisions and to better estimate effective population sizes range-wide; these efforts will also allow for the identification of potentially isolated populations that may require focused resource management to restore connectivity.
- Conduct studies of reproductive fitness to assess whether there is evidence of inbreeding depression; knowledge of reproductive and demographic parameters will also be useful in constructing population viability models for the species.
- Based on occupancy monitoring, employ reintroductions to restored habitat, if necessary, to promote genetic exchange and population augmentation *within* geographic populations.
- Avoid translocations between geographic populations unless it becomes justified by future findings of inbreeding depression; if translocations become warranted, use geographically proximate source populations.

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