ESTIMATING ABUNDANCE OF NEW ENGLAND COTTONTAIL POPULATIONS USING FECAL DNA COLLECTED FROM WINTER PELLET SURVEYS

A Product of RCN Project – "Development of Noninvasive Monitoring Tools for New England Cottontail Populations: Implications for Tracking Early Successional Ecosystem Health"

Project Director
Adrienne Kovach, Research Assistant Professor of Natural Resources, University of New Hampshire, Rudman Hall, 46 College Rd, Durham NH 03824
603-862-1603 (phone); 603-862-2621 (fax), akovach@unh.edu

Other Principal Investigators
Kathleen O'Brien, Refuge Biologist, USFWS - Rachel Carson National Wildlife Refuge, 321 Port Road, Wells, ME 04090, Kate_OBrien@fws.gov.
Anthony Tur, Endangered Species Biologist, USFWS, New England Field Office, 70 Commercial St., Suite 300, Concord, NH 03301, Anthony_Tur@fws.gov

Collaborators
Daniel Brubaker, Masters student, University of New Hampshire, Durham, NH 03824
Mark Ducey, Professor of Forest Biometrics, University of New Hampshire, Durham, NH 03824

Supporting Partners
Walter Jakubas, Maine Inland Fisheries & Wildlife; Walter.Jakubas@Maine.gov
Steven Fuller, Wildlife Management Institute; Steven.Fuller@wildlife.nh.gov
Heidi Holman, New Hampshire Fish & Game; Heidi.Holman@wildlife.nh.gov
Emma Carcagno, UNH Cooperative Extension; emma.carcagno@unh.edu
Howard Kilpatrick, Connecticut Dept. of Environmental Protection; howard.kilpatrick@ct.gov
Paul Novak, New York Dept. of Environmental Conservation; pgnovak@gw.dec.state.ny.us

Funding
NEAFWA RCN Grant #2009-04
Wildlife Management Institute
National Fish & Wildlife Foundation
Maine Division of Inland Fish & Wildlife
U.S. Fish & Wildlife Service

Report prepared by: Adrienne Kovach & Daniel Brubaker

May, 2012

Purpose

In response to the input of federal and state agency biologists in the Northeast, a range-wide collaborative project was developed in 2009 to address monitoring needs for the New England cottontail, a Species of Greatest Conservation Need in the Northeast and a candidate for federal listing under the Endangered Species Act. The overarching goal of this project was to develop noninvasive genetic monitoring tools for measuring the effectiveness of conservation actions for the New England cottontail. The objectives were two-fold, including the development of optimal methods for estimation of patch-specific occupancy and abundance. In a previous report (Kovach and Brubaker 2012), we provided the results of a study addressing detection rates in occupancy monitoring. Herein, we describe our efforts in developing a genetic population estimation protocol and provide the first baseline estimates of New England cottontails on a select group of range-wide sites. Integrating this monitoring protocol into the conservation initiative will increase our knowledge of the current status of New England cottontail populations, enable tracking population responses to management action, and provide a means for detecting changes in status. The stated objective of this project was to

- ***Develop a noninvasive genetic monitoring technique for estimating the numbers of individuals occupying a habitat patch; apply this tool to measure baseline population levels on multiple sites throughout the range of NEC.***

Addressing this goal required evaluation of several aspects of the methodology, for which we developed the following specific objectives:

1. *Develop the laboratory protocols and evaluate the suitability and power of the genetic markers for discriminating individual cottontails.*
2. *Identify the most appropriate sampling scheme with respect to the number of independent surveys and distance between collected pellets.*
3. *Determine the most appropriate mark-recapture algorithms and study design, by comparing rarefaction approaches with single and multi-session capture-mark-recapture approaches.*
4. *Make recommendations for the optimal approach and protocol for range-wide population estimation.*
5. *Apply this protocol to obtain baseline abundance estimates for range-wide sites.*

Problem Statement & Context

Occupancy data alone are insufficient to assess population status or to measure the success of management efforts, and effective adaptive management requires accurate and reliable population estimates. Noninvasive genetic sampling tools (often referred to as "genetic tagging" approaches) have proven very effective in estimating abundances of rare, secretive and difficult to monitor species (reviewed in Marucco et al. 2011). These methods have numerous benefits over traditional, field-based population estimation methods and the resulting genetic data can yield additional information on gene flow, dispersal, relatedness, mating behavior, and genetic diversity. In the case of the New England cottontail, information on population abundance is lacking and no method currently exists for population estimation. A noninvasive genetic population estimation protocol, therefore, will provide critical information for adaptive

management. Further, the resulting "real time" genetic data can be used to address additional research priorities, such as measuring dispersal and movement patterns, which may aid implementation of management actions.

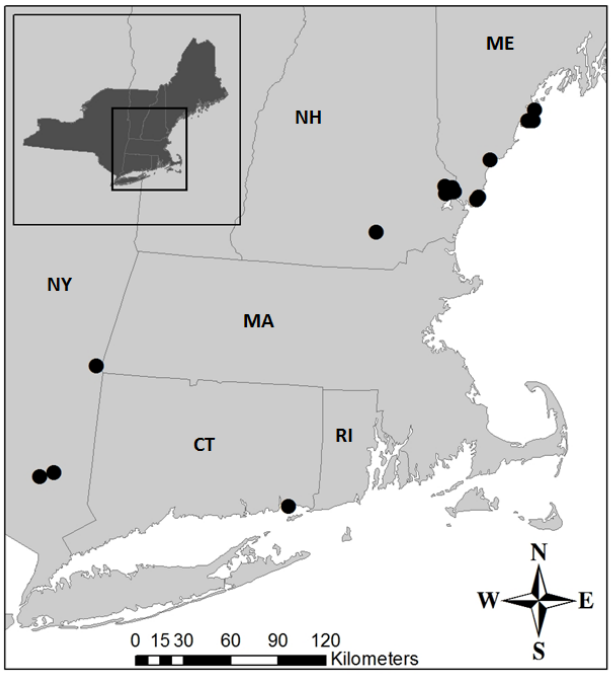
Noninvasive genetic monitoring approaches rely on genetic analysis of tissue samples (typically hair, feathers or feces) deposited by the target populations. For New England cottontails, winter pellet surveys have been used for over a decade to determine patch-specific occupancy (Kovach et al. 2003, Litvaitis et al. 2006); these surveys can be modified to obtain appropriate samples for abundance estimation. For occupancy surveys, a few pellets (3-5) are typically collected from distinct locations within a patch. Species identification of fecal pellets is made using a diagnostic assay of the mitochondrial DNA to distinguish three sympatric lagomorph species – New England cottontails, eastern cottontails and snowshoe hares. In the case of abundance estimation, the goal is to identify individual rabbits by their unique genetic signatures obtained from genotyping with microsatellite DNA markers. Each time pellets are collected, genotypes are obtained from the extracted DNA and compared to previous genotypes to determine if they are newly sampled (unique genotype) or resampled (previously sampled genotype) individuals. Population estimation algorithms are applied, just as with traditional capture-mark-recapture, to estimate the number of individuals from the ratio of newly captured to recaptured genotypes. To this end, a thorough survey of the patch is required to generate sufficient samples from multiple unique individuals, as well as sufficient "recaptures" or resampled genotypes.

Besides sampling issues, a number of methodological considerations are important in the laboratory. First, the genetic markers must have sufficient power to discriminate individuals with high confidence (i.e. no two rabbits should have identical genotypes). Further, because noninvasive DNA samples are of low quality and quantity, they might not all yield successful genotypes, and appropriate laboratory quality control procedures must be used to address the potential for genotyping error. Sample quality is likely to be an important constraint in the case of New England cottontail fecal DNA, which is known to degrade rapidly upon environmental exposure (Kovach et al. 2003).

A few different analytical approaches can be used for estimating abundance from the genetic data. In the rarefaction approach, the cumulative number of unique genotypes is expressed as a function of the number of samples collected; population size is then estimated from the asymptote of the curve (Kohn and Wayne 1997). This method is highly suitable to sampling individuals through transect-based schemes. Another approach utilizes collection of samples across multiple independent visits to the same sites. Individual captures are considered only once in each sampling session and closed session mark-recapture models can be used to estimate abundance from capture and recapture rates across the multiple sessions (Otis et al. 1978). This approach has proven successful in genetic tagging of wide-ranging carnivores with systematic placement of hair-snare traps (Woods et al. 1999). Alternately, samples can be pooled from single or multiple, site visits and utilized in a single session mark-recapture estimation model (Miller et al. 2005). This approach is advantageous in that it utilizes all samples and does not require a large number of site visits; it has also been shown to generate reliable estimates for small populations. Algorithms based on single surveys (rarefaction and single-session mark-recapture) are appropriate for cottontails, as they meet assumptions of population closure and are logistically advantageous by only requiring one visit during ideal snow conditions.

Approach

Study Sites – We focused on 20 range-wide sites, which were a subset of those used in a simultaneous detection study (Kovach and Brubaker 2012; Figure 1 - right). Ten sites were in Maine, four in New Hampshire, five in New York, and one in Connecticut. Sites ranged in size from 0.7 to 26.3 hectares. With the help of partners, these 20 sites were surveyed once thoroughly for population estimation, using methods outlined below. Three sites were surveyed a second time, to investigate the potential variation in capture rates across independent visits. Two of the New York sites (Doodletown and Shuman Road) and the one site in Connecticut (Bluff Point) were found to only contain eastern cottontails and are therefore not considered further in this report.



Surveys – Survey sites were delimited by patches of continuous suitable habitat that a cottontail may utilize without venturing into a risky open area (>30 feet wide), such as highly unsuitable vegetation, a high traffic road, or a water body. Sites were surveyed in the wintertime, with snow on the ground, and at least 3 days after a snowfall event to allow ample time for pellets to accumulate. Patches were surveyed systematically using loose, continuous transects, winding back and forth across the patch with approximately 30-meter spacing (Figure 2). Searches focused on suitable habitat within a patch and continued until all suitable habitat had been exhaustively searched. Pellet samples were collected systematically throughout the entire patch, whenever encountered, given a minimum distance of 30 – 50 meters from any other sample. On a few small sites or sites with high pellet densities, samples were collected with a minimum distance less than 30 meters. To maximize the likelihood that each sample of pellets originated from a single rabbit, individual vials of samples were collected from an area of no more than 5 x 5 ft. The species identity of collected pellets was confirmed using two diagnostic mitochondrial DNA assays (Litvaitis and Litvaitis 1996; Kovach et al. 2003). Only New England cottontail samples were used for population estimation. Samples collected from select sites in Maine and New Hampshire did not require species identification because the sites were known from extensive prior survey data to only contain New England cottontails.

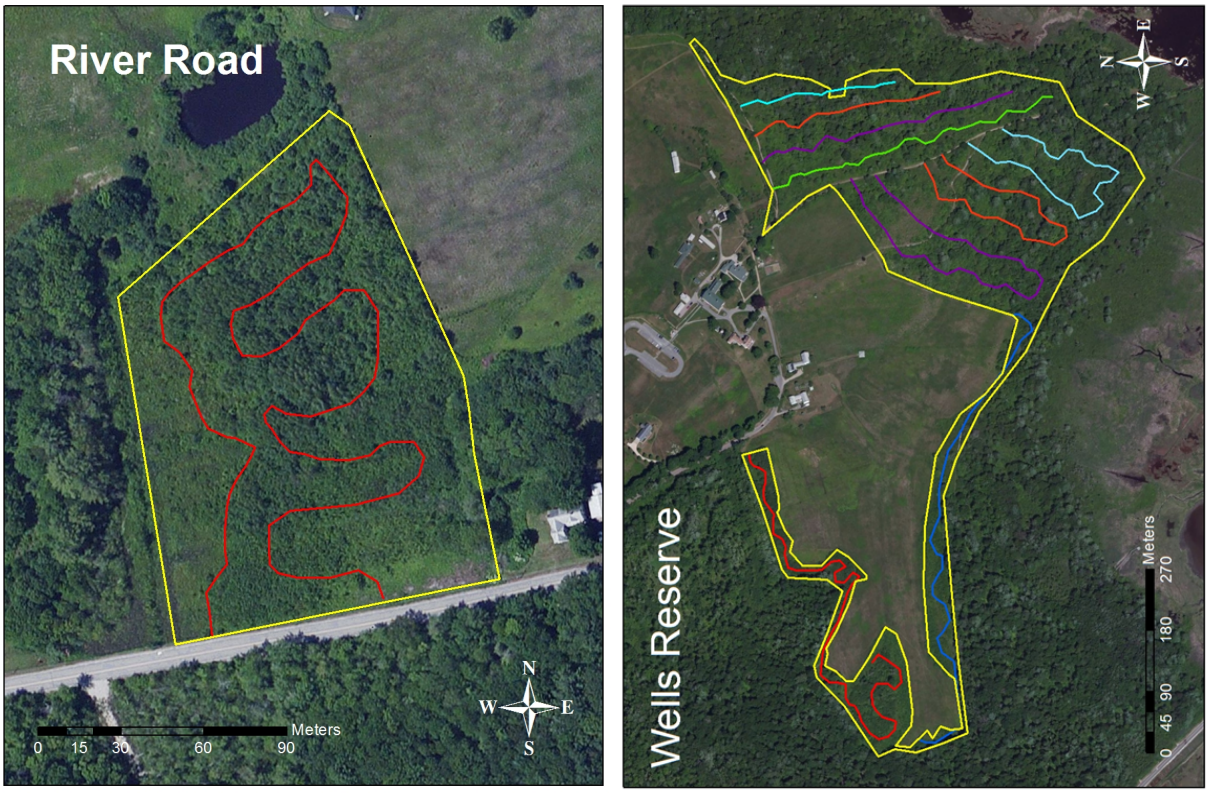


Figure 2 – Example exhaustive survey search paths for a small site (River Road – 2 ha; left) and a large site (Wells Reserve – 19 ha; right).

Subsampling – To identify the most appropriate sampling scheme, we evaluated the effects of varying sampling intensity and survey visits on the consistency and precision of the population estimates. To evaluate the effect of sampling intensity, we conducted analyses for three different sampling densities: 1) the most intensive sampling effort, either exhaustive or with the 30-m sampling interval (30-m minimum distance between separate samples); 2) subsampling of the full set of samples to obtain samples with a 50-m sampling interval; and 3) subsampling with a 75-m sampling interval. Subsampling was performed in the GIS software ArcMap (ESRI 2010) on moderate and large patches with sufficient sample sizes (n= 5). To evaluate the potential for temporal variation in capture rates on small patches, we sampled three sites on two separate occasions and compared the specific individuals identified on each visit and the resulting population estimates generated from each visit and the combined sample from both visits.

Genotyping – DNA was extracted from pellet samples using QIAamp® DNA Stool Mini Kits (Qiagen, Valencia, California) following the methods of Kovach et al. (2003). Samples were genotyped with a suite of 10 microsatellite markers, including a gender marker (a microsatellite marker only found on the Y chromosome and thereby only amplifying in males), following the protocols of Fenderson (2010) with slight modification. To address issues of low quantity and quality of fecal DNA, we used a systematic quality-control protocol based on multiple, replicated amplifications of each sample (Taberlet et al. 1996). We initially amplified each sample four times and used these replicates to create a consensus genotype. For samples that amplified poorly, an additional 2-4 replicates were performed in order to achieve four positive

amplifications. For these samples, DNA was reextracted, from two pellets this time instead of just one, in an attempt to obtain a greater concentration of DNA. With this reanalysis, some additional genotypes were generated, but some continued to fail to produce reliable genotypes and were discarded. We accepted heterozygote genotypes if each allele amplified at least twice (Gervasi et al. 2010) and both alleles amplified together in at least one replicate. Homozygous genotypes were only accepted if they amplified consistently four times, and, for samples with >4 replicates, if a second allele co-amplified no more than once across all replicates (i.e. it was considered a false allele). Using these criteria, consensus genotypes were obtained for 86% of the samples analyzed. For 3.6% of these samples we accepted a consensus homozygote after only three replicates, but only if all three replicates were identical, and only after attempting additional amplifications. Samples with >3 loci that failed to produce a consensus genotype were removed from the dataset.

We calculated genotyping error rates by manually comparing replicate genotypes to the consensus genotype for each sample. Errors were classified as either allelic dropout, failure of an allele to amplify in one or more replicates, or false allele, amplification of an allele in a single replicate different from either allele in the consensus (Taberlet et al. 1996). We calculated per alleles and per locus error rates for both classes of error, as ratios of alleles and genotypes containing mismatched alleles relative to the total number of alleles or genotypes across all replicates, following Pompanon et al. (2005).

Individual Identification – We developed criteria for discriminating samples from unique individuals and identifying samples with identical genotypes, indicating that they were deposited by the same individual (resampled genotypes). For the latter, we considered not only completely identical genotypes, but also genotypes that were similar enough that their differences could be explained by genotyping error. To this end, we used the program DROPOUT (McKelvey & Schwartz 2005) to identify unique samples with identical genotypes, as well as those mismatched at one, two, or three loci. We then manually compared each of these samples to determine if they were recaptures or unique individuals. Initially, we explored different criteria for individual discrimination, in order to account for uncertainties associated with genotyping error. We evaluated a minimum and maximum number of unique individuals on each site: the minimum number was inclusionary with respect to mismatched genotypes (conservative), while the maximum was exclusionary (minimizing the contributions of genotyping error). Using these criteria, the minimum number generated conservative, but reasonably precise estimates, while the maximum yielded estimates that appeared to be biased high, with low recapture rates and wide confidence intervals (data not shown). Low recapture rates and estimates with low precision indicate that genotyping error has resulted in over-estimation of the number of unique individuals (Marucco et a. 2011). Using the insight from these exploratory analyses, we developed a single set of criteria designed to minimize spurious identification of individuals resulting from genotyping error. These criteria are as follows: Samples that differed by four or more mismatches were considered unique individuals. If three mismatches occurred, the two samples were considered identical if all three mismatches could be explained by allelic dropout and if there was no differentiation by gender marker. If one of the mismatches could not be explained by allelic dropout (i.e. three different alleles were found across putative individuals), only one additional mismatch, of any type, was needed to differentiate the two samples. To determine the discriminatory power of the loci, we calculated the probability of identity for

siblings (PI_{Sib}; Waits et al. 2001). This statistic quantifies the probability that any two siblings in a population have identical multi-locus genotypes for the suite of markers used, and therefore be misclassified as the same individual. PI_{sib} is considered an appropriate forensic statistic for natural populations that are small and/or consist of related individuals (Waits et al. 2001).

Population estimation – Our sampling approach, which used transect sampling in a single site survey, was suitable for analysis by rarefaction and single-session mark recapture approaches. We therefore evaluated both of these methods. For the rarefaction index, we used the program GIMLET (Valiere 2002) to generate capture histories for each site and an R script for accumulation curve analysis, using the Kohn et al. (1999) method. By this method, population size is estimated as the asymptote of the curve defined by the equation $y = (ax)/(b+x)$, where a is the asymptote, x is the number of pellets sampled, y is the number of unique genotypes, and b is the rate of decline in the value of the slope. Because the order in which the pellets are sampled has an influence on the curve generated, we simulated 500 iterations of the sampling order and plotted rarefaction curves in R software (http://www.r-proejct.org). Rarefaction analyses were conducted for the data collected by the most intensive sample scheme on each of the seven sites with sufficient sample size for this method.

For single session mark-recapture estimation, we used the maximum likelihood approach implemented in the program CAPWIRE. The program incorporates two models, the even capture model (ECM), which assumes equal capture rates for all individuals, and the two innate rates model (TIRM), which allows for capture heterogeneity by classifying individuals as having one of two distinct capture probabilities within the population. We allowed the program to use a likelihood ratio test to select ECM or TIRM model, rather than imposing one or the other on the data set. CAPWPIRE estimates were used to evaluate effects of subsampling and visitation frequency. On the five sites where subsampling was performed, we generated separate abundance estimates for datasets comprised of the 30-m, 50-m, and 75-m subsampling intervals. For the three sites with two independent survey visits, we generated estimates for each survey separately, as well as for the combined data set. Abundance could not be estimated for four sites for which an insufficient number of samples or unique individuals were available, either due to small size of the site (Frieze), low density (Bellamy and Weed Mine), or poor sample quality (Radio Station).

Results & Interpretation

A total of 427 individual pellet samples were collected from 20 visits to 17 sites. All samples from Maine and New Hampshire were from New England cottontails. For the New York sites, only New England cottontails were sampled at TSP-301, and both species were sampled at the other two sites, CFSP (34 of 42 samples were New England cottontails) and Weed Mine (5 of 46 samples were New England cottontails). 290 of the 427 samples were selected for genotyping, based on the subsampling scheme. 250 of the 290 samples (86%) amplified successfully and yielded usable genotypes. Samples that failed to provide quality genotypes were primarily from the Radio Station, Coast Bus, CFSP and TSP-301 sites.

Evaluation of Genetic Methods

Genotyping – Genotyping error rates varied across loci, but on average were typical for fecal DNA studies. Average false allele error rates across loci were 0.020 per allele and 0.036 per genotype, while average allelic drop out error rates were 0.043 per allele and 0.086 per genotype. These are raw genotyping error rates, and their effects on individual discrimination were ameliorated, at least to some extent, by the use of multiple replicates in consensus genotype determination and consideration of closely mismatching genotypes. Nonetheless, as with all noninvasive studies, genotyping error was a consideration in this study. The use of microsatellite markers adapted from other lagomorph species (i.e. not developed specifically for New England cottontails), likely contributed to the error rates we observed.

Individual Discrimination – The discriminatory power of the loci was another important factor, which provided challenges in discriminating individuals on some sites. The PI_{sib} values for the majority of the sites were below the 0.05-criterion recommended for genetic tagging studies (Waits et al. 2001). This criterion means that 5 out of every 100 siblings might by chance have the same multi-locus genotype at these markers. In small populations, such as for New England cottontails, these are sufficiently stringent odds. The average PI_{sib} across all sites was 0.02 for males and slightly higher (poorer discriminatory power) at 0.026 for females. Because the Y marker is only found in males, it provides an additional locus for distinguishing male individuals, yielding slightly higher discriminatory power. For two sites in Maine, Wells Reserve and WPRE, the PI_{sib} values for both males and females were above 0.05, and for two additional sites in Maine, Fort Williams and River Road, female PI_{sib} values were above 0.05, indicating lower discriminatory power on these sites (See Table 1). The differences in the PI_{sib} values across sites might result from differences in their genetic diversity. Maine and seacoast New Hampshire populations have been shown to have reduced genetic diversity relative to other range-wide populations (Fenderson et al. 2011), and the relatively low PI_{sib} values for some of the sites in this study are consistent with this finding. Accordingly, patch-specific allelic richness (sample-size adjusted number of alleles) was typically lowest on sites in Maine and higher on New York sites and Stonyfield (central NH). Based on these findings, vigilant monitoring of the fitness of the cottontails on sites in Maine and seacoast New Hampshire may be warranted. A rigorous assessment of genetic diversity is limited, however, by the use of markers that were adapted from other species. Null alleles (alleles that are present in a population, but fail to amplify due to a mutation in the primer sequence) and low polymorphism of the markers may have contributed to observations of reduced diversity. Nonetheless, the consistently lower allelic diversity in Maine and seacoast NH relative to NY and central NH across loci in this study and that of Fenderson et al. (2011) is not likely to be solely an artifact of the markers used.

Sample quality – Sample quality was another potentially limiting factor that we encountered. Overall our genotyping success rate was relatively high (86%) and compared favorably with other fecal DNA studies. The majority of samples that failed to amplify came from the same four sites, suggesting that sample quality was a site-specific rather than universal issue. Surveys on these sites occurred with suboptimal timing, as many as 7-9 days after a snowfall event. Kovach et al. (2003) showed that fecal DNA of New England cottontails degrades rapidly, with mitochondrial DNA amplification decreasing substantially after a week. Kovach and Brubaker (2012) suggest that sample collection is ideal 3-5 days after a snowfall. DNA degradation is a

greater concern for population monitoring than for species identification, because nuclear DNA (such as the microsatellite markers used in this study) is present in smaller amounts (1 copy per cell) in the pellet samples than mitochondrial DNA (many copies per cell).

Table 1 – Patch-specific genetic diversity measures. Statistics were not evaluated for sites with <4 rabbits.

Population	Patch Size	Sample Size (n)	# Unique	PIsib Male	PIsib Female	Allelic Richness (Avg per Locus)
Bellamy	13.2	3	1	—	—	—
CFSP	10.3	20	4	6.65E-03	2.66E-02	13 (1.4)
Coast	3.3	8	5	9.05E-03	9.05E-03	13.5 (1.4)
Coast Bus	5.4	23	7	2.71E-04	6.45E-04	15.7 (1.6)
Crescent Beach	12.4	49	21	5.99E-03	9.61E-03	13.2 (1.3)
Fort Williams	1.5	9	4	4.39E-02	6.97E-02	12.7 (1.3)
Frieze	0.7	4	1	—	—	—
Kettle Cove	20.4	21	7	1.54E-02	2.55E-02	12.7 (1.3)
Orchard	3.8	7	6	9.83E-03	1.31E-02	13.3 (1.3)
Radio Station	2.5	1	1	—	—	—
River Road	1.8	22	4	3.50E-02	5.89E-02	12.1 (1.2)
Sliver	1.0	16	4	4.57E-02	4.57E-02	12.3 (1.2)
Stonyfield	4.9	18	10	3.49E-04	7.83E-04	15.5 (1.6)
TSP-301	26.3	9	7	5.27E-03	5.27E-03	14.0 (1.6)
Weed Mine	10.2	3	3	—	—	14.4 (1.6)
Wells Reserve	18.9	4	2	—	—	13.2 (1.3)
WPRE	1.1	33	3	2.04E-01	2.04E-01	10.2 (1.0)

Conclusions about Genetic Methods: Overall, we conclude that the genetic approach used in this study, while suitable for individual discrimination and subsequent abundance estimation, had substantial limitations stemming from the use of microsatellite markers that were adapted from other species. Markers that are not specific to the target species may have null alleles, low polymorphism, and be difficult to score for their genotypes. We encountered all of these issues with the markers in this study. It is likely that species-specific markers would perform better with respect to these aspects and also yield higher discriminatory power, lower genotyping error rates, and higher amplification success for poor quality samples. These improvements might further increase the precision of the estimates. They might also reduce the cost and labor associated with the generation of multiple replicate genotypes (often >4 in this study). Therefore, we recommend that future population estimation studies adapt the protocols we developed in this study for use with species-specific markers. Genomic sequence data, currently being developed in the laboratory of USGS scientist Timothy King, have yielded scores of microsatellite markers in New England cottontails. These markers will be screened in the summer of 2012 to identify a suite of highly polymorphic markers, which should be suitable for future studies requiring individual identification.

Evaluation of Sampling Scheme

Recapture Rates & Estimate Precision – An appropriate sampling scheme for mark-recapture estimation should yield high capture and recapture rates; these two factors strongly influence the robustness and precision of the estimates. In our population surveys, the number of unique and re-captured individuals varied widely by site (Table 2). This effect appeared to be a function of rabbit density (which was highly variable) rather than patch size. For example, considering only moderate to large-sized sites, on Crescent Beach (12.4 ha) and Stonyfield (4.9 ha), a large number of unique individuals were sampled relative to the total number of samples (21 of 49 and 10 of 18, respectively, or 40-50% unique individuals). Conversely, on Coast Bus (5.4 ha), Kettle Cove (20.4 ha) and CFSP (10.2 ha), unique individuals comprised only 20-33% of the total number of samples collected. On one large site, TSP-301 (26.3 ha), only 7 of 9 genotyped samples were unique. This was likely affected by the poor sample quality on this site, which caused us to discard 8 of the 17 samples collected. On several small sites (<5 ha), a handful of genotypes were sampled multiple times, consistent with the expectation of low abundance. Exemplifying the importance of high recapture rates, confidence intervals were narrower, implying higher precision, for estimates with high recapture rates – lower number of unique individuals relative to the total number of samples. The greatest precision was obtained for estimates from the smallest sites, for which exhaustive sampling resulted in multiple recaptures of each sampled genotype (e.g. WPRE 3 unique individuals captured 33 times, Sliver 4 individuals captured 16 times, and River Road 4 individuals captured 22 times). Overall, our results suggested that patch size was not a strong factor influencing the precision of the estimates, except in the case of the smallest patches. Small sites (<5 ha) typically generated high recapture rates and precise estimates from a single exhaustive sampling. For sites >5 ha, the ratio of recaptures and the precision of the estimates varied.

Table 2 - Population estimates (and confidence intervals) from CAPWIRE derived from the most exhaustive sampling conducted at each site. Also included is patch size, number of pellets processed and successfully genotyped, and number of unique individuals identified, as well as the model selected by CAPWIRE (ECM – even capture rates or TIRM – two innate rates model of capture heterogeneity).

Site Name	State	Size (ha)	# Samples Processed	# Successful Genotypes	# Unique Individuals	Population Estimate N (LCI-HCI)	Model	Sub-sample (m)
Frieze	ME	0.7	4	4	1†	NA	NA	Exhaustive
Sliver	ME	1.0	17	16	4	4 (4-4)	ECM	Exhaustive
WPRE	ME	1.1	34*	33	3	3 (3-3)	TIRM	Exhaustive
Fort Williams	ME	1.5	9	9	4	4 (4-4)	ECM	30
River Road	ME	1.8	22*	22	4	4 (4-6)	TIRM	Exhaustive
Coast	ME	3.3	8	8	5	7 (5-11)	ECM	30
Orchard	ME	3.8	7	7	6	9 (6-19)	ECM	30
Wells Reserve	ME	18.9	5	4	2	2 (2-2)	TIRM	Exhaustive
Kettle Cove	ME	20.4	22	21	7	7 (7-7)	ECM	30
Crescent Beach	ME	12.4	49	49	21	30 (21-41)	TIRM	30
Stonyfield	NH	4.9	19	18	10	18 (10-30)	TIRM	30
Coast Bus	NH	5.4	28*	23	7	15 (7-21)	TIRM	Exhaustive
Bellamy	NH	13.2	4	3	1†	NA	NA	Exhaustive
Weed Mine	NY	10.2	5	3	3†	NA	NA	Exhaustive
CFSP	NY	10.2	26	20	4	4 (4-4)	TIRM	30
TSP-301	NY	26.3	17	9	7	15 (7-33)	ECM	30

Subsampling – The pellet-sampling interval (distance between collected pellets) had a pronounced effect on recapture rates and estimate precision (Table 3). For the 5 sites subsampled at varying intensities, overall we saw a decrease in recapture rates and sample precision with increasing sampling interval, with the most pronounced effect at the 75-m sampling distance. On Crescent Beach, the 50-m sampling interval had similar precision to the 30-m sampling interval. Interestingly, this subsampling decreased the sample size from 49 to 36, but only decreased the number of unique individuals from 21 to 20. Subsampling had greater effects on the other sites, even at the 50-m interval. On one site (Stonyfield), however, the precision increased with increasing sampling distance and the point estimates decreased as well. This is likely due to the fact that a large proportion of unique individuals were sampled on Stonyfield, and subsampling decreased unique individuals and slightly increased recapture rates. Overall, the results of our subsampling indicate that it is important to sample exhaustively, and sampling at >30-m intervals may not be sufficient on most sites, especially where rabbit density is high.

Table 3 – Effects of sampling interval on population estimation. Sample size, number of unique individuals genotyped, the CAPWIRE population estimate (with confidence interval) and selected model is given for each sampling interval tested for each of five sites.

Site Name	State	Size (ha)	Sample Size	# Unique Individuals	Population Estimate N (LCI-HCI)	Model	Sub-sample (m)
Crescent Beach	ME	12.4	49	21	30 (21-41)	TIRM	30
Crescent Beach	ME	12.4	36	20	26 (20-36)	ECM	50
Crescent Beach	ME	12.4	18	13	24 (13-45)	ECM	75
Kettle Cove	ME	20.4	21	7	7 (7-7)	ECM	30
Kettle Cove	ME	20.4	16	7	10 (7-18)	TIRM	50
Kettle Cove	ME	20.4	10	7	12 (7-42)	ECM	75
Sliver	ME	1.0	16	4	4 (4-4)	ECM	Exhaustive
Sliver	ME	1.0	9	4	4 (4-4)	ECM	30
Sliver	ME	1.0	7	4	5 (4-8)	ECM	50
Sliver	ME	1.0	5	4	8 (4-8)	ECM	75
Stonyfield	NH	4.9	18	10	18 (10-30)	TIRM	30
Stonyfield	NH	4.9	12	7	9 (7-18)	ECM	50
Stonyfield	NH	4.9	9	4	4 (4-4)	ECM	75
CFSP	NY	10.3	20	4	4 (4-4)	ECM	30
CFSP	NY	10.3	16	4	4 (4-4)	TIRM	50
CFSP	NY	10.3	9	4	4 (4-4)	ECM	75

Number of Survey Visits – For the three sites that were surveyed in two independent visits, two (River Road and WPRE) yielded consistent results and similar estimates with overlapping individuals identified in each visit (Table 4). For Coast Bus, however, several different individuals were identified and significantly different estimates (with non-overlapping confidence intervals) were generated for the two visits. The estimate from the combined surveys, therefore, was higher than the estimate for either independent survey (but the confidence interval overlapped with that from the first survey). The difference in the two estimates is most likely a result of differences in sampling effort between the two surveys. In the second visit, only half of the patch was surveyed, making the two estimates in fact not comparable. Further, there were a number of pellets from each survey that failed to amplify, which further influenced the sampling effort. These finding, therefore, suggest that a single, thorough survey with exhaustive sampling is sufficient to generate unbiased estimates.

Table 4 – Effects of repeat sampling on population estimation. Sample size, number of unique individuals genotyped, the CAPWIRE population estimate (with confidence interval) and selected model is given for each of two independent surveys and the combined data set for three sites.

Site Name	State	Size (ha)	Visit	Sample Size	# Unique Individuals	Population Estimate N (LCI-HCI)	Model	Sub-sample
Coast Bus	NH	5.4	1	10	5	11 (5-21)	TIRM	Exhaustive
Coast Bus	NH	5.4	2	13	3	5 (3-8)	TIRM	Exhaustive
Coast Bus	NH	5.4	Combined	23	7	15 (7-21)	TIRM	Exhaustive
River Road	ME	1.8	1	9	2	2 (2-2)	ECM	Exhaustive
River Road	ME	1.8	2	13	4	4 (4-6)	TIRM	Exhaustive
River Road	ME	1.8	Combined	22	4	4 (4-6)	TIRM	Exhaustive
WPRE	ME	1.1	1	13	2	2 (2-2)	TIRM	Exhaustive
WPRE	ME	1.1	2	19	2	2 (2-2)	TIRM	Exhaustive
WPRE	ME	1.1	Combined	32	3	3 (3-3)	TIRM	Exhaustive

Capture Heterogeneity – Heterogeneity in individual rabbit movement and pellet deposition rates may affect population estimates derived from pellet sampling approaches. Anecdotally, we noticed that heterogeneity did occur on some sites, where the same one or two genotypes were resampled multiple times and at a much higher rate than other genotypes. To further evaluate heterogeneity in rabbit sample deposition, we mapped the locations of all pellet samples collected from each unique individual. These maps indicated that on some sites, one or two individuals (usually males) deposited multiple pellets over a large portion of the patch, whereas deposition rates of most individuals were restricted to a much smaller area. This heterogeneity appeared to be most evident on small (<5 ha) and low-density patches. Males were sampled more frequently than females, and across all sites the ratio of samples was 57 M: 39 F. The CAPWIRE model selection procedure identified heterogeneity to be a factor, by selecting the TIRM model, on these sites, as well as on the two large sites that had low recapture rates (Crescent Beach and Stonyfield). Given the potential for heterogeneity, the most appropriate sampling consideration is to allow a sufficient number of days after a snowfall for multiple individuals to deposit pellets. Balancing this timing with the risk of DNA degradation upon prolonged environmental exposure is important, and dictates an ideal sampling window of 4-5 days after snowfall.

Evaluation of Algorithms

Our analyses suggested that the intensive transect-sampling approach was appropriate for estimating New England cottontail abundance. This approach is amenable to analysis by either rarefaction or the single session mark-recapture algorithms implemented in CAPWIRE. The approach used by CAPWIRE had greater flexibility and tolerance of low sample sizes and was therefore used to evaluate the effects of subsampling and visitation frequency. For seven sites with sufficient samples sizes, we used the Kohn et al. (1999) rarefaction method for comparison (Table 5). Overall, the estimates generated by rarefaction had lower precision (with the exception of Crescent Beach) and a higher mean (point estimate), in comparison to those from CAPWIRE. For four sites, the standard deviation of the estimates was too large to be informative. Some estimates were similar for the two methods (CFSP, Crescent Beach, and Kettle Cove), while others were markedly higher by the rarefaction method (Coast, Coast Bus,

Orchard and Stonyfield). The rarefaction method appeared to perform the most similarly to the CAPWIRE method in terms of both point estimate and precision for CFSP and Crescent Beach. Both of these sites had relatively large sample sizes – for CFSP, the number of unique individuals was small relative to the number of total samples (4 of 20) and Crescent Beach had the highest sample size of all sites (49 samples with 21 unique individuals). These findings suggest that the performance of the rarefaction approach may be more limited than CAPWIRE by the need for large sample sizes with high recapture rates, and therefore may not be the most applicable to New England cottontail surveys. For both rarefaction and CAPWIRE algorithms, the logistical constraint of a narrow survey window (4-5 pellet deposition days) imposes a significant challenge to obtaining sufficient sample sizes and recapture rates. Nonetheless, our findings suggest that exhaustive (or 30 m) sampling from a thorough survey can yield robust estimates from CAPWIRE and potentially robust estimates from rarefaction on large sites.

Table 5 – Comparison of rarefaction and CAPWIRE algorithms. Estimates generated by the best model selected in CAPWIRE and the Kohn et al. (1999) rarefaction approach are provided for seven sites with sufficient sample sizes.

Site Name	State	Size (ha)	# Samples Processed	# Successful Genotypes	# Unique Individuals	CAPWIRE Estimate N (LCI-HCI)	Rarefaction Estimate N+/- (SD)
Coast	ME	3.3	8	8	5	7 (5-11)	17.7 +/- 20.7
Orchard	ME	3.8	7	7	6	9 (6-19)	31.6 +/- 14.6
Kettle Cove	ME	20.4	22	21	7	7 (7-7)	10.7 +/- 5.2
Crescent Beach	ME	12.4	49	49	21	30 (21-41)	37.2 +/- 8.4
Stonyfield	NH	4.9	19	18	10	18 (10-30)	28.9 +/- 42
Coast Bus	NH	5.4	28*	23	7	15 (7-21)	33.2 +/- 122.9
CFSP	NY	10.2	26	20	4	4 (4-4)	5.1 +/- 1.8

Baseline population estimates

We generated baseline population estimates for 17 sites (Table 2). The estimates varied from a single rabbit on a 1-ha patch (Frieze, ME) to 30 individuals on a 12-ha site in Cape Elizabeth, ME (Crescent Beach). Abundance estimates were not associated consistently with patch size, however, as some large sites had apparently very low rabbit densities (e.g., Wells Reserve with 2 rabbits on a 19-ha site). The 26-ha TSP-301 site in New York was the largest site surveyed, but had a moderate estimate of 15 rabbits (although with an upper confidence interval of 33). The two sites in this study with sympatric eastern cottontails (CFSP and Weed Mine in New York, both 10 ha in size) had relatively low rabbit abundance. In the case of CFSP, most (34 of 42) samples were from New England cottontails and the estimate was quite precise (point estimate of 4, based on 4 unique individuals sampled 20 times). On Weed Mine, only 5 of 46 collected samples were from New England cottontails and an estimate could not be generated from the 3 unique individuals identified. Overall, our results from these 17 sites suggest that rabbit densities may vary widely, given site-specific factors, including rates of sympatry, which may preclude making generalized estimates extrapolated purely from patch size.

Conclusions & Recommendations

In conclusion, we have developed an approach for abundance estimation of New England cottontails from noninvasive genetic sampling of fecal pellets collected during a single, well-timed, systematic (thorough and exhaustive) site survey. The greatest challenge to this approach is timing the survey optimally to balance the need for sufficient pellet deposition days, to obtain sufficient sample size of unique individuals and recaptures, with the constraints imposed by DNA degradation that occurs with prolonged environmental exposure of the pellets. Another significant challenge in the current protocol is the limitation of using genetic markers that were not developed specifically for the target species. We provide below recommendations to address both of these challenges and outline the optimal protocol for monitoring the abundance of New England cottontails. Using these recommendations can yield robust patch-specific abundance estimates range-wide. Application of our protocols to 17 range-wide sites indicated that rabbit densities vary inconsistently with patch size; extrapolating density from generalized estimates of available habitat, therefore, may not provide accurate population assessments.

We provide the following recommendations for monitoring patch-specific abundance:

- Conduct population surveys 4-5 days after a snowfall event.
- In a single site visit, survey the patch thoroughly using systematic, loose transects with approximately 30-m spacing between transects, focusing on suitable habitat throughout the entire patch.
- Collect pellets intensively, either exhaustively or every 30 meters, throughout the patch.
- If pellet density is moderate and logistics allow, analyze all samples in the laboratory.
- If pellet density is high and/or a large number of samples were collected from a large site, analyze a subsample of the total pellets with a 30-m or maximally 50-m sampling interval. If population estimates cannot be obtained with reasonable precision, analyze additional samples to increase capture and recapture rates needed for precise estimates.
- If on a small site a single survey does not yield an estimate with high precision and identifies significant heterogeneity of individuals, conduct a second independent survey.
- Use stringent quality control protocols in the laboratory, including multiple replicate amplifications (minimum of 4) of each sample and evaluation of samples that mismatch by only a few alleles (up to 3 mismatching loci, depending on genotyping error rates and the number of markers used).
- Utilize knowledge of site-specific genetic diversity and probability of identity statistics in evaluating similar genotypes.
- Adapt the laboratory protocols developed in this document for use with species-specific microsatellite markers as soon as they become available.
- When developing a suite of species-specific markers, select a set of no more than 6-8 loci with high polymorphism that yield sufficient power for individual discrimination (*PIsib* <0.05 or preferably <0.001) and which can be co-amplified in 2 multiplexed reactions.

Literature Cited

Fenderson LE (2010) Landscape Genetics of the New England Cottonatial; Effects of Habitat Fragmentation on Population Genetic Structure and Dispersal. University of New Hampshire, Durham.

Fenderson LE, Kovach AI, Litvaitis JA, Litvaitis MK (2011) Population genetic structure and history of fragmented remnant populations of the New England cottontail (Sylvilagus transitionalis). *Conservation Genetics*, **12**, 943-958.

Gervasi V, Ciucci P, Davoli F, Boulanger J, Boitani L, Randi E (2010) Addressing challenges in non invasive capture-recapture based estimates of small populations: a pilot study on the Apennine brown bear. *Conservation Genetics*, **11**, 2299-2310.

Kohn, M.H., E.C. York, D.A. Kamradt, et al. 1999. Estimating population size by genotyping faeces. Proceedings of the Royal Society of London. Series B, Biological Sciences, 226: 657-663.

Kovach, A.I., M.K. Litvaitis, and J.A. Litvaitis. 2003. Evaluation of fecal mtDNA analysis as a method to determine the geographic range of a rare lagomorph. Wildlife Society Bulletin. 31(4): 1061-1065.

Kovach, A.I. and D.R. Brubaker. 2012. Detecting New England Cottontails During Winter Pellet Surveys – A Report for Managers. RCN Grant #2009-04 Deliverable.

Litvaitis MK, Litvaitis JA (1996) Using mitochondrial DNA to inventory the distribution of remnant populations of New England cottontails. *Wildlife Society Bulletin*, **24**, 725-730.

Litvaitis, J.A., J.P. Tash, M.K. Litvaitis, M.N. Marchand, A.I. Kovach, and R. Innes. 2006. A range-wide inventory of the current distribution of New England cottontails. Wildlife Society Bulletin 34(4): 1190-1197.

Marucco F, Boitani L, Pletscher DH, Schwartz MK Bridging the gaps between non-invasive genetic sampling and population parameter estimation. *European Journal of Wildlife Research*, **57**, 1-13.

Miller, C.R. , P. Joyce and L.P. Waits. 2005. A new method for estimating the size of small populations from genetic mark-recapture data.

Otis DL, Burnham KP, White GC, Anderson DR (1978) Statistical inference from capture data on closed animal populations. *Wildlife Monographs*, 7-135.

Pompanon F, Bonin A, Bellemain E, Taberlet P (2005) Genotyping errors: Causes, consequences and solutions. *Nat. Rev. Genet.*, **6**, 847-859.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Research 24: 3189-3194.

Valiere, N. (2002). GIMLET: a computer program for analysing genetic individual identification data. Molecular Ecology Notes 2:377-379.

Woods, J. G., D. Paetkau, D. Lewis, B. N. McLellan, M. Proctor, and C. Strobeck. 1999. Genetic tagging of free-ranging black and brown bears. Wildlife Society Bulletin 27:616-627.