JNAH
THE JOURNAL OF NORTH AMERICAN HERPETOLOGY
ISSN 2333-0694
Volume 2021, Number 2 2021 journals.ku.edu/jnah

# HIGH GENE FLOW IN EASTERN RED-BACKED SALAMANDERS (*PLETHODON CINEREUS*) FROM FORESTS IN SOUTH-CENTRAL INDIANA

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*ABSTRACT:* Anthropogenic changes are expected to shape the genetic structure of many herpetofaunal populations. Genetic analyses can determine the loss of genetic diversity in isolated populations, identify barriers to dispersal and gene flow, and inform ways in which populations should be managed. Plethodontid salamanders are ideal for understanding fine-scale and landscape-level genetic structure in continuous landscapes given their high abundance and their sensitivity to ecosystem changes. Our study aimed to assess the landscape-level population structure in Eastern Red-Backed Salamanders (*Plethodon cinereus*) within intact habitat. We investigated genetic variation in 479 individuals from three sites across roughly 26 km of intact hardwood forest. We tested for genetic structure among and within three sites using several metrics, including hierarchical Bayesian analysis, $F_{ST}$ analysis, AMOVA, and genetic isolation by distance. The results revealed a single population throughout the study area and genetic structure within sites was not evident. Importantly, the sites in this study are part of a long-term project on the effects of standard silvicultural practices in the American Midwest, making it ideal for future re-evaluation of this Eastern Red-Backed Salamander population. We now have baseline information to determine if any future disturbances or fragmentation may alter the genetic structure of this population, as well as how this population may change over time if the study area remains undisturbed.

*Key Words: Dispersal, Genetic variation, Microsatellite loci, Population genetics*

## INTRODUCTION

Anthropogenic activities can change the distribution and abundance of alleles in natural populations, which can ultimately reduce genetic variation and threaten biodiversity (Avise and Hamrick 1996). Population genetic analyses are commonly applied in disturbed habitats because they can determine the loss of genetic diversity in isolated populations, identify landscape features that act as barriers to dispersal and gene flow, and inform ways in which populations should be managed (Avise and Hamrick 1996, Lampert et al. 2003, Funk et al. 2005). As a result of most population genetic studies being focused on highly altered landscapes, less is known about genetic patterns of organisms in relatively intact landscapes. Importantly, data from intact systems are needed to make comparisons to fragmented systems and thus, inform ecosystem and species management.

Terrestrial salamanders are ideal organisms for understanding fine-scale and landscape-level genetic structure given they are sensitive to changes within their environment (Burton and Likens 1975, Welsh and Droege 2001). The Eastern Red-Backed Salamander (*Plethodon cinereus*; herein referred to as red-backed salamander) is a frequently used forest health indicator species, as it is one of the most common and widely distributed vertebrate species within forests of the Northeast and Midwest United States (Powell et al. 2016). Red-backed salamanders are typically thought to have small home ranges (5–76 $m^2$; Kleeberger and Werner 1982, Mathis 1991, Muñoz et al. 2016), though individuals have also been observed moving upwards of 90–143 m (Kleeberger and Werner 1982, Sterrett et al. 2015). Ultimately, these life-history characteristics suggest that red-backed salamanders may be ideal for evaluating fine-scale and landscape-lev-

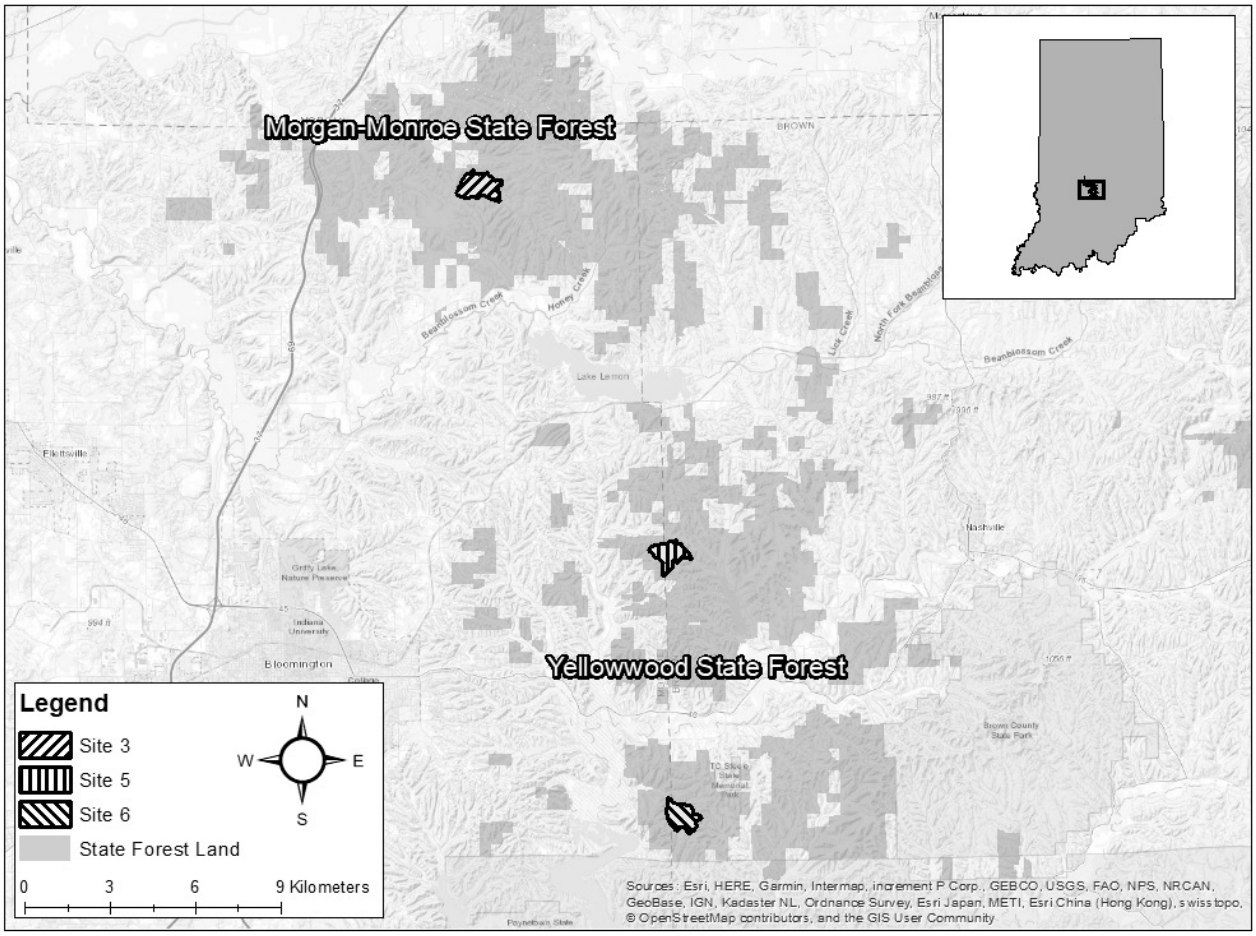


Fig. 1. Map of sampling sites and their position in Morgan-Monroe and Yellowwood State Forests in south-central Indiana.

el genetic structure within intact habitats.

A wealth of literature exists for red-backed salamanders, and a few studies have evaluated neutral population genetic processes. Cabe et al. (2007) sampled red-backed salamanders in continuous hardwood forest habitat across a 2-km transect in Virginia and detected fine-scale spatial structuring between groups of individuals as close as 0.2 km. In contrast, Noël et al. (2007) reported no population differentiation among plots of intact habitat ranging from 0.8–4.1 km apart. Jordan et al. (2009) conducted a study that spanned distances up to 70 km and found weak population structuring across a highly fragmented landscape. Collectively, these studies suggest that gene flow in red-backed salamanders within continuous habitat may (Cabe et al. 2007) or may not (Noël et al. 2007, Jordan et al. 2009) be limited by factors at the landscape-level (e.g., presence of streams and roads; Wiens and Milne 1989). These studies offer seemingly conflicting results, thereby confounding attempts to draw usable management conclusions. Further, they do not document gene flow in this species in continuous habitat at scales greater than 4.1 km, which is likely important for a species that both maintains small home ranges yet is capable of much larger dispersal distances (Waser and Elliott 1991, Newman and Squire 2001, Kimble et al. 2014).

Our study assessed the geographic extent of population genetic structure in red-backed salamanders within intact habitat. We utilized an indirect measure of gene flow by employing a spatially hierarchical genetic sampling regime to provide baseline data on population structure and connectivity from an intact habitat in the midwestern portion of their range. Our study sites are ideal as they occur across a 26-km landscape of relatively intact habitat that has few large roads (Sheets et al. 2013) and streams (Jenkins 2013), have had minimal development for the last 100 years (Swihart et al. 2013), and have a large population of red-backed salamanders (MacNeil and Williams 2014).

## MATERIALS AND METHODS

### *Study Sites*

We sampled red-backed salamanders from three forested sites within Morgan-Monroe and Yellowwood State Forests in south-central Indiana, USA. These sites, each approximately 80 ha, are arranged across 26 km and are part of a larger system of long-term study sites (Swihart et al. 2013; Figure 1). The sites are 12–26 km apart (Table 1) but are within an intact contiguous hardwood forest. The area was not glaciated during the last glacial maximum, was heavily forested before the arrival of European settlers, was cleared for agriculture in the 18th Century, then acquired by state agencies and allowed to succeed back to forest starting in the early 20th Century (Carman 2013). The area is now subject to periodic small-scale harvesting but is protected from large-scale

Table 1. Pairwise $F_{ST}$ values (and associated p-values) below diagonal and approximate Euclidean distance (in km) above diagonal for the three sites of red-backed salamanders (*Plethodon cinereus*) collection in Morgan-Monroe and Yellowwood State Forests, south-central Indiana. No $F_{ST}$ values were significant.

| - | Site 1 | Site 2 | Site 3 |
|---|---|---|---|
| Site 1 | - | 14 | 26 |
| Site 2 | 0.003 (0.075) | - | 12 |
| Site 3 | 0.003 (0.062) | 0.0002 (0.470) | - |

habitat perturbations or development by state governance (Swihart et al. 2013). Its hydrology is characterized by small intermittent streams (Jenkins 2013), most corridors are logging roads (Sheets et al. 2013), and residential and industrial development has been minimal, though increasing (Hoover 2013).

*Salamander Sampling*

Each site was sampled using 24 artificial cover object (ACO; 30 x 30 x 5 cm untreated pine boards) grids, with eight ACO grids per site and 30 boards per grid. Grids within each site were an average of 223 m apart. Additional site details can be found in MacNeil and Williams (2014). Sampling for genetic material occurred during spring (March–May) and fall (September–November) 2011. Grids were sampled every two weeks during each sample period (fall or spring). On each sampling occasion (a single check of a single grid), trained observers lifted all boards in a grid and captured each red-backed salamander encountered. Individual salamanders were batch-marked using a Visible Implant Elastomer (VIE; Anholt and Negovetic 1998) inserted at the venter near a base of a limb on the ventral side so as not to re-sample that individual. If the sample size was < 20 individuals after the grid was searched, natural cover objects within 100 m of the ACO grid were also searched, and subsequent searches every two weeks were also conducted to increase the sample size until it was ≥ 20 (Selkoe and Toonen 2006). Tissue samples were obtained from each salamander by clipping the distal < 5 mm of the tail with scissors, flame-sterilized using 70% ethanol, and were stored in 100% ethanol. Tissue samples remained at ambient temperatures for up to 8 hours, then were stored permanently at -20 or -80 ºC.

*Genetic Data Collection*

Tail clips were digested in a solution containing dithiothreitol (DTT) and proteinase K at 55 ºC for at least two hours. We extracted genomic DNA using a standard phenol chloroform extraction (Sambrook and Russell 2000). Seven microsatellite loci were multiplexed and amplified by PCR using fluorescently labeled primers (Connors and Cabe 2003, Jordan et al. 2009) prior to genotyping individuals using GeneMapper Version 4.1 (Applied Biosystems [ABI], Foster City, CA). We optimized these loci in a 10-µL reaction with ~60 ng of DNA template, 10x buffer, 2.25 mM $MgCl_2$, 0.2 mM of dNTPs, 0.25–0.7 mM of each end-labeled primer, and Taq polymerase and Nanopure water to make the 10-µL reaction. Thermocycler conditions were 94 ºC for 2 min, 94 ºC for 30 s, primer-specific annealing temperature (Table 2) for 30 s, 72 ºC for 30 s for 30 cycles, 72 ºC for 10 min and a final extension at 60 ºC for 45 min. All seven loci produced bands of the expected size when visualized on 2% agarose. DNA was then analyzed by microcapillary electrophoresis on a 3730 DNA analyzer (ABI, Foster City, CA). We assigned genotypes to all samples using GeneMapper and visually confirmed all calls.

*Statistical Analyses*

We tested for the presence of Hardy-Weinberg equilibrium (HWE) within each site using Genepop 4.2 (Raymond and Rousset 1995). Allelic frequencies and expected ($H_E$) and observed ($H_O$) heterozygosity were calculated for each locus. In order to guard against violations of test assumptions associated with any one test, we used multiple approaches to investigate population structure within and among our sites. First, we assessed the genetic differentiation between sites and between grids within sites by calculating pairwise $F_{ST}$ values for each site and grid pair in Genepop 4.2 online. To further quantify population differentiation, we performed an analysis of molecular variance (AMOVA) in Arlequin (version 3.5.1.3; Excoffier and Lischer 2010) dividing samples into four levels: among sites, among grids within sites, among individuals within grids, and within individuals. The significance of the AMOVA results was assessed using permutation tests. Bayesian partitioning using Structure (version 2.3.4; Pritchard et al. 2009) was used to detect population structure based on individual genotypes without spatial priors (Pritchard et al. 2000, Earl 2012). We ran 10 replicate runs of 10,000 iterations for simulation burn-in, followed by 100,000 iterations for each possible value of K between 1 and 24. From these runs, the mean and standard deviation of log likelihoods of K [L(K)] were recorded for each value of K. The optimal value of K was decided by comparing the cumulative mean estimated natural log (Ln Pr(X|K)) of the data (Pritchard et al. 2009), the posterior probability of K (Pritchard et al. 2000), and the ΔK method (Evanno et al. 2005). The cumulative mean estimated natural log was calculated and visualized in STRUCTURE HARVESTER (Earl 2012); the latter two estimators were calculated and visualized in CLUMPAK (Kopelman et al. 2015). Finally, to investigate the role of genetic isolation by geographic distance (IBD) between our sites, we performed a Mantel test on a matrix of pairwise grid-level linearized $F_{ST}$ (Slatkin's D = $F_{ST}$ / (1 - $F_{ST}$)) values and a matrix of pairwise grid-level Euclidean distances in GENALEX (version 6.503; Peakall and Smouse 2012).

## RESULTS

We genotyped 479 red-backed salamanders. All loci within each of the three sites were in HWE (Table 2). Some linkage disequilibrium (2 out of 63 site-specific locus-locus comparisons) was detected but exclusion of these loci did not change any results. Therefore, all loci were included in all analyses.

All of the population genetic structure tests were concordant, showing no evidence of genetic structure within or between our study sites. Specifically, pairwise $F_{ST}$ values among sites were low and not significant ($F_{ST}$ = 0.0002–0.003; Table 1). Median intra-site pairwise $F_{ST}$ values were not significantly different when comparing 95% confidence intervals (data not shown). The hierarchical AMOVA analyses revealed that 95.13% of allelic variation was within individuals, 4.86% was among in-

Table 2. Microsatellite loci amplified in multiplex PCR reactions, annealing temperature (ºC), allelic size range (bp), modification, number of alleles per locus, number of private alleles per locus, expected heterozygosity ($H_E$), and observed heterozygosity ($H_O$) of *Plethodon cinereus* populations from Morgan-Monroe and Yellowwood State Forests in south-central Indiana. [1]Loci from Connors and Cabe 2003. [2]Locus from Jordan et al. 2009. Reactions were conducted in a total volume of 10 µL using the conditions described in text.

| Multiplex | Locus | Annealing temperature (ºC) | Allelic size range (bp) | Modification | Number of alleles | Number of private alleles | $H_E$ | $H_O$ |
|---|---|---|---|---|---|---|---|---|
| I | PcLX16[1] | 63 | 183–239 | HEX | 11 | 4 | 0.655 | 0.497 |
| II | PcII14[1] | 62 | 115–175 | NED | 13 | 4 | 0.324 | 0.298 |
| | PcJX24[1] | 62 | 165–213 | 6-FAM | 16 | 3 | 0.305 | 0.316 |
| | PcJX06[1] | 61 | 98–134 | HEX | 8 | 2 | 0.377 | 0.335 |
| III | PcLX23[1] | 54 | 162–176 | HEX | 6 | 3 | 0.498 | 0.534 |
| | PcXF08[1] | 54 | 173–199 | NED | 12 | 3 | 0.564 | 0.557 |
| | PcA18[2] | 59 | 204–220 | 6-FAM | 8 | 3 | 0.485 | 0.504 |
| Average | | | | | 10.5714 | 3.14286 | 0.458 | 0.435 |

dividuals within grids and only 0.28% was among sites (Table 3). Using Structure, the Ln Pr(X|K) value was largest at K = 1, the posterior probability of K plot was a scatter that gave no suggestion of any value of K other than K = 1, and the ΔK method peaked at K = 2. Though the ΔK method cannot distinguish between K = 1 and K = 2 (Janes et al. 2017) the preponderance of evidence points to K = 1 and this is what we accepted. The Mantel test did not reveal any significant isolation by distance across the three sites (r = 0.061, p = 0.14).

Finally, we provided a summary of population genetic studies on red-backed salamanders showing general measures of genetic variation (Table 4). Average allelic variation ranged from 3.8 (Noël et al. 2010) to 41.5 (Noël et al. 2007). Our study fell within the median range of these values at 10.57 alleles. Additionally, the average expected heterozygosity ($H_E$) and observed heterozygosity ($H_O$) ranged from 0.40–0.75 and 0.35–0.65, respectively. The averages from our study fall within the lower range of these values ($H_E$ = 0.46 and $H_O$ = 0.44).

## DISCUSSION

Our population genetic data on red-backed salamanders suggest there is one continuous population within our study area in south-central Indiana. Several aspects of the natural history of this species may account for the lack of structure at the landscape scale. First, population sizes of red-backed salamanders may be sufficiently high that even with limited individual dispersal ability, realized gene flow may be substantial and genetic drift reduced, discouraging the development of population structure (Wright 1931). Within our study area, 6,274 individuals were encountered over the course of five years (MacNeil and Williams 2014). The second facet that may be relevant is the ability of individuals to travel greater distances than the typically reported home range. Red-backed salamander individuals have been observed moving upwards of 143 m in homing and natural movement behavior experiments (Kleeberger and Werner 1982, Sterrett et al. 2015). Furthermore, salamanders at sites 8,000–9,000 m apart may be expected to trade at least one migrant per generation (Smith and Green 2005), a rate which may be sufficient to genetically harmonize populations (Slatkin 1987). Finally, studies suggest that large roads and streams may alter the dispersal abilities of red-backed salamanders, but smaller roads and streams may not (Marsh et al. 2007, Marsh et al. 2008). Within our study sites, there are 4.15 km of drivable roads, 6.42 km of undrivable or forest roads, and no permanent streams. Therefore, it seems the components of this landscape do not act as dispersal barriers for this population. Ultimately, the high density of red-backed salamanders within our study area and ease of dispersal may contribute to the low level of population structure in our study.

While we suspect the similarities observed between our study and others (Noël et al. 2007, Jordan et al. 2009) are based on current land use, it is important to

Table 3. AMOVA results showing percentage of genetic variation in red-backed salamanders (*Plethodon cinereus*) among sites, among grids within sites, within grids, and within individuals from Morgan-Monroe and Yellowwood State Forests, south-central Indiana. Values in bold are significant at the α = 0.05 level.

| Source of variation | d. f. | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among sites | 2 | 5.775 | 0.004 | **0.28** |
| Among grids within sites | 21 | 31.357 | -0.004 | -0.27 |
| Among individuals within grids | 455 | 757.504 | 0.077 | **4.86** |
| Within individuals | 479 | 723.500 | 1.510 | **95.13** |
| Total | 957 | 1518.136 | 1.588 | |

Table 4. Summary of population genetic studies on red-backed salamanders (*Plethodon cinereus*) showing general measures of genetic variation: number of alleles per locus, average number of alleles across loci, average observed heterozygosity ($H_O$), and average expected heterozygosity ($H_E$).

| Publication | Number of Alleles | | | | | | | Average alleles[1] | Average $H_O$ | Average $H_E$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | PcLX16 | PcII14 | PcJX24 | PcJX06 | PcLX23 | PcXF08 | PcA18 | | | |
| Current study | 11 | 13 | 16 | 8 | 6 | 12 | 8 | 10.57 | 0.44 | 0.46 |
| Connors and Cabe 2003 | 7 | 10 | 7 | 6 | 5 | 9 | N/A | 6.11 | 0.61 | 0.65 |
| Marsh et al. 2007 | 7 | N/A | N/A | 10 | 14 | 14 | N/A | 9.83 | 0.56 | 0.58 |
| Cabe et al. 2007 | 10 | N/A | N/A | 19 | 23 | 18 | N/A | 15.00 | 0.63 | 0.62 |
| Noël et al. 2007 (Pop. 1) | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A | 26.50 | 0.43 | 0.49 |
| Noël et al. 2007 (Pop. 2) | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A[2] | N/A | 41.50 | 0.64 | 0.75 |
| Marsh et al. 2008 | 9.7 | N/A | N/A | 11.7 | 10.7 | 14.8 | N/A | 10.75 | 0.65 | 0.68 |
| Jordan et al. 2009 | 8 | 3 | 5 | N/A | 4 | N/A | 10 | 20.30 | 0.48 | 0.56 |
| Noël et al. 2010 (Pop. 1) | N/A[2] | N/A[2] | N/A | N/A[2] | N/A[2] | N/A[2] | N/A | 4.66 | 0.42 | 0.42 |
| Noël et al. 2010 (Pop. 2) | N/A[2] | N/A[2] | N/A | N/A[2] | N/A[2] | N/A[2] | N/A | 3.8 | 0.35 | 0.40 |
| Noël et al. 2010 (Pop. 3) | N/A[2] | N/A[2] | N/A | N/A[2] | N/A[2] | N/A[2] | N/A | 5.6 | 0.51 | 0.52 |

[1]Average alleles are for the entire study. Except for the current study, not all alleles are shown in this table.

[2] Study used the locus but did not provide individual allele information.

note that past land use and evolutionary events may also play a role in structuring these populations. Red-backed salamanders are part of a species complex that originated in the Appalachian Mountains and then dispersed into Indiana (Radomski 2016). This complex consists of several clades, each with a different history of dispersal and population size changes (Radomski 2016). Evolutionary history suggests that Indiana populations, which are part of the northern clade (Radomski 2016), may be less genetically diverse due to a combination of recent shared ancestry among the individuals sampled, a founder effect, and species range edge effects (Eckert et al. 2008). Given that our study area has been managed for over 100 years, it is likely that enough generations have passed (i.e., 10-20 generations; Gibbs 1998) that historic population genetic levels could be consistent with current levels. Additionally, using empirical data, we show that the levels of genetic variation in our study are similar to other studies that used the same loci on red-backed salamander populations, including those found within the Appalachian Mountains (Table 4). Ultimately, the available evidence suggests that a lack of differentiation on the landscape is less likely to be the result of distant evolutionary events, and more likely to signal strong gene flow given the natural history of the site.

The sites in our study are part of a long-term study on the effects of standard silvicultural practices in the American Midwest (Swihart et al. 2013), making it ideal for future re-evaluation of this red-backed salamander population. We now have baseline information to determine if any future disturbances or fragmentation may cause differences in genetic structure within this population, as well as how this population may change over time if the study area remains undisturbed. Ultimately, these data can be used to inform conservation practices such as maintaining corridors to allow gene flow of red-backed salamander populations in this area, as well as minimizing habitat fragmentation from anthropogenic influences (e.g., roads).

## ACKNOWLEDGMENTS

Data collection on salamanders was carried out in accordance with Purdue Animal Care and Use Committee Protocol #95-019-10 and Indiana Department of Natural Resources Scientific Purpose Licenses and amendments 07-0068 and 10-0076. This paper is a contribution of the Hardwood Ecosystem Experiment (HEE), a partnership of the Indiana Department of Natural Resources, Purdue University, Ball State University, Indiana State University, Drake University, and Indiana University. Funding for the HEE was provided by the Indiana Division of Forestry. We would like to thank M. Hock, J. MacNeil, G. Marin, M. Dillon, M. Winzeler, M. Hamilton, and O. Hernández-Gómez for their efforts in improving this manuscript.

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