


Contrasting Ozark and Great Lakes populations in the endangered Hines emerald dragonfly (*Somatochlora hineana*) using ecological, genetic, and phylogeographic analyses

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Abstract

The federally endangered species Hine's emerald dragonfly (*Somatochlora hineana*) is found in fens surrounding the Great Lakes region and in a small portion of the Missouri Ozarks. Most previous work has focused on the populations in the Great Lakes region. We present mark/recapture studies and genetic surveys to address the status of the Ozark populations. The densities and genetic diversity tend to be higher in the Ozarks than in the Great Lakes region. A phylogeographic analysis indicates that the Ozarks, with its unglaciated fens, is the likely source for the populations currently inhabiting the formerly glaciated Great Lakes region, and genetic diversity decreases with increasing distance from the Ozarks. This work illustrates the inadequacy of using geography alone to identify a population as marginal and of less conservation concern. We also reanalyzed genetic data on the Great Lakes populations, where several populations have been extirpated over the last several decades. We show that the populations in the Great Lakes region have already lost more than 30% of their genetic diversity over just several decades,

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and the phylogeographic analysis indicates that increased fragmentation is a possibility in this region due to local extirpations. Ecologically and genetically, the Ozark populations should have a high priority in management plans, and the high rate of loss of genetic diversity and potential fragmentation indicates that continued monitoring and management is needed in the Great Lakes region for this highly endangered dragonfly.

KEYWORDS

genetic diversity, mark/recapture, mtDNA, peripheral population

1 | INTRODUCTION

Limited funding and resources are often a constraint in designing and implementing a recovery plan for an endangered species (Gerber et al., 2018). One common conundrum is how to allocate resources between populations residing in the bulk of a species' range versus geographically peripheral populations, particularly if those peripheral populations are found in small and distant geographic areas relative to the main range. Often the decision of identifying a peripheral or marginal population is made by geography alone. Evolutionary biologists, going back to Darwin (1859, pp. 69–78), drew attention to the evolutionary and adaptive significance of the contrast between core (central) versus peripheral (marginal) populations (Pironon et al., 2017). Brown (1984) made an influential generalization that populations in the center of the species' geographic distribution inhabit the optimal habitat for a species and would therefore have the highest densities, although he did acknowledge that exceptions could occur. A common addendum to this generalization is that genetic diversity also tends to be higher in the central populations (Soulé, 1973). Many studies revealed a variety of density and genetic diversity patterns in core versus peripheral contrasts that reflect variation in how “peripheral” populations are defined (Pironon et al., 2017). These diverse patterns indicate the need to take an interdisciplinary approach that integrates genetics, ecology, history, and geography to understand the multifaceted nature of species' boundaries (Holt & Keitt, 2005).

For many endangered species we have data on their geographic distribution but little information about the underlying genetic, ecological, and historical factors that may have influenced the evolutionary potential and importance of current geographic peripheral populations. For example, the known geographic distribution of extant and extirpated populations of the federally endangered dragonfly *Somatochlora hineana* Williamson (Odonata: Corduliidae) is depicted in Figure 1. The geographic distribution of this species is limited by restrictive habitat

requirements that include wetlands with calcareous seepage flow (Cashatt & Vogt, 2001; Vogt & Cashatt, 1994), the presence of crayfish burrows that nymphs use as refugia (Pintor & Soluk, 2006; Soluk et al., 2000), and nearby meadows or pasture for adult foraging (Foster & Soluk, 2006). Habitats satisfying these criteria include

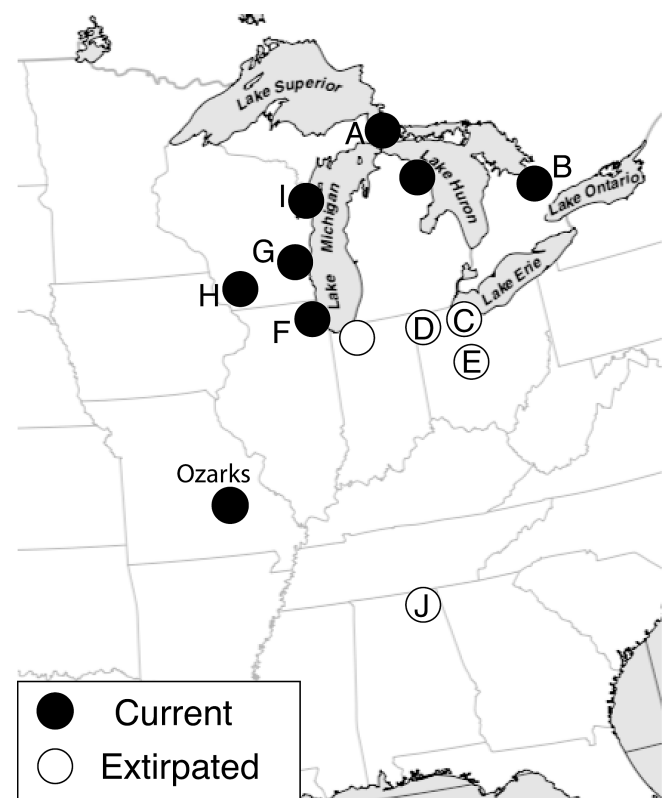
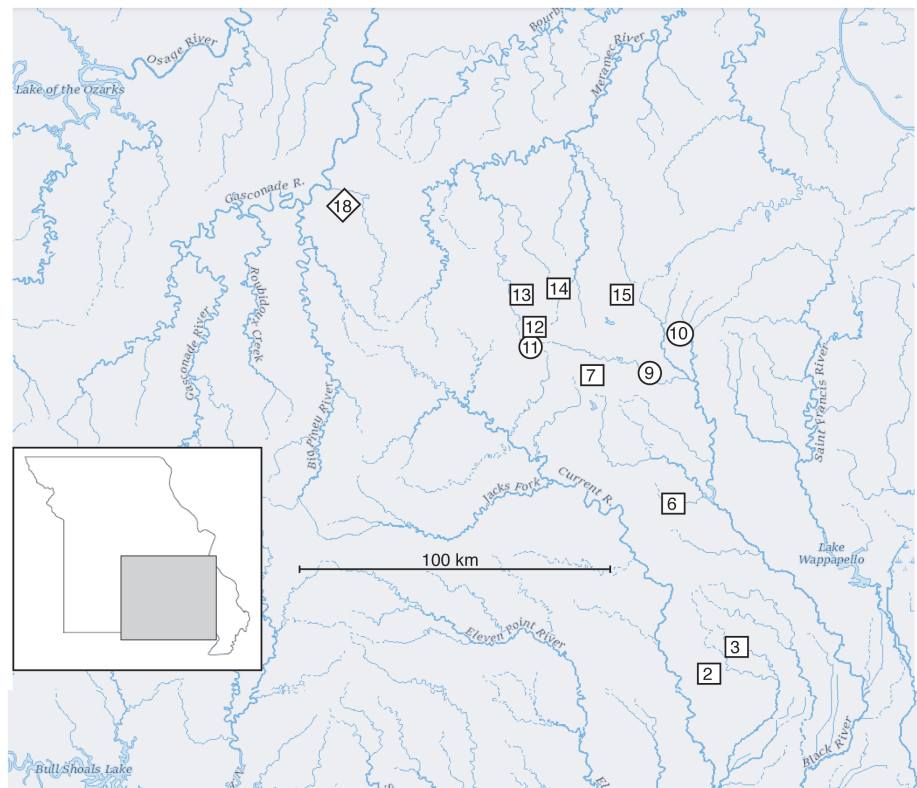


FIGURE 1 The known current and past areas where *Somatochlora hineana* has been collected. Letters indicate sites included in a previous mtDNA genetic survey: (a). Mackinac County, Michigan; (b). Simcoe County, Ontario; (c). Lucas County, Ohio; (d). Williams County, Ohio; (e). Logan County, Ohio; (f). Cook, DuPage, and Will Counties, Illinois; (g). Ozaukee County, Wisconsin; (h). Iowa and Richland Counties, Wisconsin; (i). Door County, Wisconsin; (j). Jackson County, Alabama. Many of these site designations include one or more nearby fens (see Table S4)

FIGURE 2 Known sites with *Somatochlora hineana* in Missouri. The small inset map of the State of Missouri shows the area covered by the larger map, which shows the major rivers and streams. Some sites cover more than one fen. The sites are as follows: 2) Glass Lizard Fen, Overcup Fen, Montgomery Fen; 3) Emerald Fen, Cottonmouth Fen; 6) Deckard Hollow, Ruble Meadow; 7) Bee Fork West, Bee Fork Central, Bee Fork East, Grasshopper Hollow; 9) Centerville Slough; 10) Johnson Shut-Ins (Onoclea Fen); 11) Kay Branch Fens; 12) Wisdom Fen, Lanham Fens, Parker Branch Fen; 13) Fortune Hollow; 14) Bates Hollow; 15) Barton Fen; 18) Kaintuck Hollow. Circled sites were included in the mark/recapture and genetic survey, square sites were in the genetic survey, and diamond sites had no specimens in the genetic survey. Non-consecutive numbers resulted when genetic surveys of specimens from previously reported fens with *S. hineana* were revealed to be the sibling species *Somatochlora tenebrosa*. The base map is from the USGS National Map (www.viewer.nationalmap.gov)



cattail marshes, sedge meadows, and fens (Cashatt & Vogt, 2001; Foster & Soluk, 2006; Pintor & Soluk, 2006; Vogt & Cashatt, 1994), but these Hine's emerald dragonfly (HED) habitats will all be referred to as 'fens' in this paper as fens are the exclusive HED habitat in the Ozarks and the most common HED habitat in the Great Lakes region. Most fen habitat in the contiguous United States is found in the Great Lakes region. The bulk of the HED distribution is in the Great Lakes region, with only one current geographic peripheral area (Figure 1): a small portion of the Missouri Ozarks located far away from the Great Lakes, as shown in more detail in Figure 2. A site in Alabama represented a second peripheral area based upon a single museum specimen from 1978, but this population was extirpated (USFWS, 2001, 2013).

Previous studies on the demography, genetics and conservation of HED have focused on the Great Lakes sites as this region appeared to be the geographical and ecological core for this endangered species. Several studies in this region estimated population size and site presence (Cashatt & Vogt, 1996; Foster & Soluk, 2004, 2006; Kirk & Vogt, 1995; Mierzwa et al., 1995), performed habitat monitoring and modeling (Cobb & Bradbury, 2008; Sampath, Liao, Curtis, Li, & Deloria, 2015; Soluk,

Satyshur, Holmes, & Blas, 2006), and executed genetic surveys of both microsatellites (Monroe & Britten, 2014) and mitochondrial DNA (Purdue, Gaige, Cashatt, and Vogt, 1999). In contrast, before this study, our knowledge of HED in Missouri was based only on presence-absence and habitat surveys by various members of the group of authors of this study and by a monitoring count done by the U.S. Forest Service at one Ozark fen in 2010. However, presence-absence monitoring provides limited information on the status of a species, and the 2010 monitoring count by the U.S. Forest Service yielded low numbers, which reinforced the hypothesis that the small fens in the Ozarks had small and marginal populations with less priority for conservation of this species. However, these counts did not estimate the size of the HED population, and this lack of population size estimates is a major limitation of presence/absence surveys in general.

One goal of this paper is to add ecological (population sizes and densities, dispersal behavior, and other demographic variables) and genetic (mitochondrial DNA variation) knowledge about the HED populations in the Missouri Ozarks and to compare the results to those found previously in the Great Lakes region. Our work includes new demographic and genetic analyses within

both the Ozarks and the Great Lakes region as well as statistical comparisons between these regions. In addition, we execute a phylogeographic analysis on an integrated Ozark/Great Lakes genetic data set. With these additional datasets and analyses we can reassess the status of the Great Lakes and Ozark populations in the context of their genetics, ecology, evolutionary history, as well as geography.

A second goal is to assess the conservation status of the populations in the Great Lakes region by combining genetic surveys from our study with earlier work on extant and extirpated populations in this region. The sites in Ohio and Indiana shown in Figure 1 are based on museum specimens collected between 1929 and 1961. In surveying these sites for a genetic collection in 1992, Mierzwa et al. (1995) determined that the wetland habitats at these sites had been severely altered by human activity and no longer supported HED populations. The inclusion of specimens from these extirpated populations allows us to investigate the loss of genetic diversity over time in an endangered species. Genetic diversity underpins adaptive flexibility, which is more important than ever in this time of global environmental change (Templeton, 2017). Overall, the rate of loss of genetic diversity is 6% since the start of the industrial revolution (Leigh, Hendry, Vázquez-Domínguez, & Friesen, 2019). Here, we have an opportunity to access the loss of genetic diversity in an endangered wetland species over the course of several decades.

2 | METHODS

We performed mark/recapture studies on three fens in the Ozarks (Figure 2) to obtain ecological information comparable to previous studies in the Great Lakes region. We also obtained samples for a genetic survey from 15 fens in the Ozarks (Table S4) at 13 sites (Figure 2) to extend the previous genetic information available on the Great Lakes region to the Ozarks.

2.1 | Three sites in the Ozarks for mark/recapture studies

The first of the three sites used for a mark/recapture study in the Ozarks is the Kay Branch Fen Complex of nine fens, a privately owned site located on a headwater stream of the West Fork of the Black River (Site 11, Figure 2), (Walker & Smentowski, 2006). Fens in the Kay Branch Complex are generally located along the toe slopes of elevated ridges that border both sides of Kay Branch Creek (Figure S1). The surface water varies in

width from 1–10 m. Past ditching and draining has greatly reduced the area with surface water. The areas of the fens in this complex and others were measured by a combination of on-site mapping and observations coupled with the polygon area tool in Google Earth Pro. We collected and marked HED on fen 7 of this complex (Figure S1) that borders the base of a hill toe slope for approximately 400 m. We subdivided the fen into twelve 30-m sections A–M (Figure S1) and used these subdivisions as locators for dragonflies at the time of capture. Marking locations were recorded to the nearest station or midway between stations.

Onoclea Fen (Site 10 in Figure 2) is owned and managed by the Missouri Department of Natural Resources-Division of State Parks. It was added to Johnson Shut-Ins State Park in 2006 as part of a mitigation settlement with Ameren-Missouri Utility Co. over damage suffered to the park when the Taum-Sauk reservoir was breached in 2005 (Walker & Smentowski, 2014). Part of the fen was used as a horse pasture by previous owners but the ecological integrity of a major portion of the fen has remained intact. This fen drains into a small creek which immediately empties into the East Fork of the Black River.

Centerville Slough (Site 9 in Figure 2) is adjacent to the West Fork of the Black River, which runs along its southwestern boundary. The northern boundary is a field adjacent to Highway 21 to the west. The southeastern boundary is an oak/hickory woodland that rises to a ridge.

Previous studies in the Great Lakes region estimated HED population sizes through mark/recapture studies at two sites in Illinois (Lockport Prairie and River South, Cashatt & Vogt, 1995) and three sites in Wisconsin (Ridges Sanctuary, Mud Lake, and Three Springs Creek, Kirk & Vogt, 1995). We use the data from these sites for a comparison to the Ozark fen mark/recapture sites.

2.2 | Field methods for the mark/recapture studies

Our overall goal in the mark/recapture studies was to intensively sample and mark dragonflies during the height of their flight season to obtain accurate population estimates followed by opportunistic samples for recaptures throughout the following month to estimate longer-term survival. Dragonflies were collected with aerial insect nets. We captured and marked HED from 0800–1,200 hours Central Daylight Time (CDT), although occasionally the times were slightly altered due to weather or other logistical constraints. The number of netters varied from day to day depending on the

availability of permitted individuals (training was required to reduce incidental deaths of dragonflies). Captured dragonflies were placed in holding cages (Port-a-bugs InsectLore, Inc.), transported to the marking station, and given a unique identification number. We marked dragonflies with Craftsmart Non-toxic Paint Pens. By using two visible colored dots and four wings, we were able to individually mark 99 dragonflies before changing to a new set of two different colors (Mierzwa et al., 1995). Mierzwa et al. (1995) also performed tests that indicated that this marking system does not impair flight ability and did not result in detectable deleterious effects on survival. Information collected on each capture included: identification number, time of capture, sex of dragonfly, collector, site of capture, behavior at time of capture, presence of predators, and additional remarks. The following behaviors were noted: feeding flight, male territorial patrol, male aggression, females in pre-oviposition mode, and ovipositing females. All of this information is given in Data S4.

We attempted to mark and recapture dragonflies for five consecutive days, weather permitting. Additional collections without marking were performed at one or more intervals of several days after the consecutive marking phase. Specific dates are included in Data S4. To reduce potential incidental take due to net strikes, we limited our netting attempts to perched dragonflies, males on territorial patrol, and ovipositing females. Color patterns could be determined in most cases with the aid of binoculars, reducing the need for re-netting in scoring recaptures. In cases where we observed a marked individual but could not ascertain the exact number, we so noted that on the tally sheet. In most cases, the possible alternative numbers would all result in the same MARK input line (that is, the vector of 1's and 0's indicating days of capture and no capture would be identical), so the ambiguity had no effect on the mark/recapture analysis. When ambiguity was extreme enough to alter the input vectors and that individual was not re-netted, we did not treat that specimen as a recapture. Hence, our estimates are conservative.

Although our netting protocols and use of observational recaptures whenever possible reduced the number of incidental takes to a low level, we did have some (see Section 3). All incidental takes were injected with 95% ethanol, stored in vials of 95% ethanol, and chilled for later genetic analysis at the Illinois State Museum. The details on every capture and recapture are given in Data S4.

2.3 | Statistical methods for the mark/recapture studies

The mark-recapture data were analyzed with MARK (Cooch & White, 2014) (<http://www.phidot.org/software/>

[mark/index.html](http://www.phidot.org/software/mark/index.html)). Data were stratified by sex. Individuals that were removed from the study due to death or injury were treated as uncappable thereafter. We assumed an open population for a maximum likelihood analysis using a general Cormack-Jolly-Seber (CJS) model for the recaptures (Pollock & Alpizar-Jara, 2005). We assessed the goodness of fit of the data to a general time-dependent, stratified CJS model through RELEASE, which is part of the MARK package. The general CJS model fit the data well, but had many unidentifiable parameters. Upon examining the estimated parameters from the complete model, we defined models with fewer parameters to find a reduced model that still fit the data well as determined through log-likelihood ratio tests, Akaike's Information Criterion (AIC), and normalized Akaike weights (AIC weight, an index of relative plausibility on a 0–1 scale) through MARK. This process was used recursively, using the estimated parameters under one well-fitting model to suggest further reductions in the number of parameters followed by statistical testing for goodness of fit. This procedure was repeated until a model of lowest dimension was identified that had no significant reduction in fit relative to the complete model. We obtained maximum likelihood estimates of male and female survivorship and encounter probabilities from the lowest dimension, best-fitting CJS model.

Once we determined the optimal CJS model, we added the assumption of equal catchability for marked and unmarked animals to estimate population size using the Jolly-Seber (JS) method as implemented by the POPAN module that is part of the MARK package. This analysis also generated maximum likelihood estimates of entry probabilities (entry of new individuals into the open population through emergence from the nymphal phase and/or immigration) throughout the study period.

The populations sizes from the Great Lakes Region (Cashatt & Vogt, 1996; Kirk & Vogt, 1995) had been estimated with the Fisher-Ford model and a modified Lincoln Index on a daily basis. These models make more assumptions than are necessary in a MARK analysis, and did not provide an estimate of the population size based upon the total, multi-day sampling. We reanalyzed previous mark-recapture data collected from the Great Lakes region with MARK in the same manner as the Ozark analyses to compare population size estimates across regions.

Because the mark/recapture study at Kay Branch was highly successful in terms of numbers captured and recaptured, additional statistical analyses were pursued. These additional studies yield insight into the biology of HED and the appropriate designs for mark/recapture studies on this endangered species, but are tangential to the main focus of this paper: the conservation importance

and relationship of the Ozark and the Great Lakes populations and genetic diversity loss over time. Therefore, the methods, results, and discussion of these additional studies at the K Branch site are found in Data S1.

2.4 | Obtaining specimens for the survey of mtDNA variation

All specimens were adults (either single legs or whole individuals), except one nymph (Dent Co., MO), and were preserved in 95% ethanol. In the case of the legs, the remainder of the specimen was sent to Enns Entomology Museum, University of Missouri, Columbia, as a site voucher. All other specimens were retained at the Illinois State Museum, Springfield, IL. Samples from Ontario, Canada, were whole adults preserved with acetone and borrowed courtesy of Ontario Ministry of Natural Resources and Forestry.

Our survey was combined with that of previous surveys on mtDNA variation in HED primarily in the Great Lakes region that also included specimens from extirpated populations (Purdue et al., 1999; Purdue, Cashatt, & Vogt, 1996). The sites, locations, and number of specimens per site are given in Tables S4 and S5.

We performed DNA extractions with Qiagen DNeasy spin columns using the muscles and coxa of the single legs or from coxa and thoracic muscle from the whole animals. We amplified a 541 base-pair fragment of mitochondrial DNA consisting of the ND3 protein-coding region plus flanking tRNAs using primers TG-J-5584 (5'-AGTATATTTGACTTCCAATC-3') and TN-N-6160 (5'-TCAATTATATCATTAACAGTGA-3'; Beckenbach, Heed, & Etges, 2008). We used standard PCR conditions with T_m ranging from 45° to 48° for 35–40 cycles. We ran the PCR reactions on 1% agarose gel, stained them with ethidium bromide or GelRed Nucleic Acid stain (Phenix Research Products) and visualized under UV light. We cleaned successful amplification reactions with QiaQuick PCR purification spin columns. Sanger sequencing was performed at UIUC Core Sequencing Facility (University of Illinois Urbana-Champaign). We examined sequencing results with Sequencher software and performed manual alignment in MacClade v. 4.06 (<http://macclade.org/macclade.html>) or Mesquite v. 2.75 (<https://www.mesquiteproject.org>).

2.5 | Statistical analysis of mtDNA variation

We estimated genetic diversity at a site by the number of haplotypes present at that site and by the haplotype

heterogeneity; that is, the probability that two randomly drawn haplotypes (with replacement) from the sample are different. Haplotype heterogeneity is most readily calculated as one minus the probability that two randomly drawn haplotypes are identical:

$$1 - \sum_{i=1}^h p_i^2$$

where h is the number of haplotypes in the sample and p_i is the frequency of haplotype i in the sample. A sample size of one precludes any estimate of genetic diversity at a site. Moreover, the nested clade phylogeographic analysis (to be described shortly) uses an exact permutation procedure to evaluate statistical significance, which works best when site sample sizes are four or more, although even sites with a single observation can be used when pooled into a higher level haplotype clade. For this reason, it is recommended that sites with small sample sizes be pooled (Templeton, 2002). Because the nested clade analysis uses the geographical location of the sample sites, it is important to only pool nearby sites such that a central locality can be assigned to them that gives an accurate location relative to the total geographic scale of the analysis (Templeton, 2004a). Because many of the HED sites had few individuals (often 1 or 2, see Table S4), sites with fewer than four specimens were pooled with other sites within 5 km because previous marking studies in Illinois showed a maximum observed dispersal distance of 5.4 km (Cashatt & Vogt, 1996). This pooling reduced the number of sample localities from 49 in Table S4 to 30 in Table S5. The pooling still left a few sites with fewer than four specimens. The average number of haplotypes and the average haplotype heterogeneity were calculated only within sites with four or more individuals. We pooled specimens from all sites within larger regions and calculated the number of haplotypes and average haplotype heterogeneity for the entire pool. Because there was only one site (now extirpated) and one individual from Alabama, that site was excluded from all analyses.

Statistical comparisons between many of our regions are complicated by unequal sampling. One source of unequal sampling is sample size differences. A computer program was written in Mathematica (<http://www.wolfram.com/mathematica/>) to randomly resample, without replacement from the larger sample, the same number of specimens that are in the smaller sample (this program is included in Data S5). The program also estimates the exact probabilities that the larger sample has more haplotypes and greater haplotype heterogeneity than the smaller sample by repeating the random subsampling 1,000 times.

For a two tailed 5% level of significance, an exact probability ≥ 0.975 indicates that the larger sample has more genetic diversity than the smaller sample, and an exact probability ≤ 0.025 indicates that the larger sample has less genetic diversity than the smaller sample.

A second source of unequal sampling is temporal bias. Some of the samples from the Great Lakes region include specimens from extirpated populations that were collected between 1929 and 1961 (Purdue et al., 1996). Since there has been much destruction of wetland habitats since those earlier times, including the Ozarks, it is likely that some genetic diversity has been lost over time, biasing comparisons between recent collections in the Ozarks versus older collections in the Great Lakes region (the extirpated population plus additional specimens collected in the 1990's). We test the hypothesis of a temporal loss of genetic variation by contrasting the Great Lakes specimens collected in the 1990's versus the specimens collected between 1929 and 1961. We can somewhat correct for the temporal bias by comparing the Ozark specimens from the 2010's just to the Great Lake specimens from the 1990's with the resampling program (that is, excluding all extirpated populations). This comparison does not account for genetic diversity that may have been lost in non-extirpated populations over the last two decades, but it would reduce without completely eliminating the temporal sampling bias in favor of greater genetic diversity in the Great Lakes region.

The third sampling bias arises from differences in geographic range. In almost all genetic surveys, diversity increases with increasing sampling range because almost all species show some degree of population structure in which some genetic diversity is found between geographic locations (Templeton, 2006). The Great Lakes region is so much larger than the Ozarks that it is impossible to fully correct for this geographic range disparity. As can be seen from Figure 2, the genetically sampled Ozark populations lie primarily on rough N-S axis between sites 14 and 2 that is 111-km long. No partitioning of the Great Lakes region could include two or more sampling sites and be roughly 100 km long. The closest we could get was to partition the current Great Lakes population into three subsets: A and B (Michigan and Ontario) at 424 km, F and H (NE Illinois and southern Wisconsin) at 274 km, and I and G (Wisconsin) at 204 km. The most comparable range size comparison would be the Ozarks versus the extirpated Ohio populations (C, D and E at 149 km), but this comparison would also have the most extreme temporal bias. In all cases, these comparisons are made with random resampling, so sample size bias is fully corrected, temporal sampling bias is partially corrected (except for the

Ohio populations), and range sampling bias is partially corrected. All partial biases favor greater apparent genetic diversity in the Great Lakes region.

A nested clade phylogeographic analysis was performed on the pooled 30 sites data. We first construct a haplotype tree, which is appropriate because there is no recombination in mtDNA. The haplotype tree was estimated with statistical parsimony using the program TCS (<http://darwin.uvigo.es/our-software/>), a technique specifically developed for intra-specific data that both estimates the tree and quantifies ambiguity (Templeton, Crandall, & Sing, 1992). The tree was rooted by using outgroup data from the sister species *Somatochlora tenebrosa* (Purdue et al., 1996). The next step converts the haplotype tree into a hierarchical set of nested clades (groups of haplotypes on a common evolutionary branch) within the tree using a few simple rules that account for ambiguity in the tree (Templeton, Boerwinkle, & Sing, 1987; Templeton & Sing, 1993). In this case the only rule needed was to form a clade from the union of all the haplotypes (or previously defined clades of haplotypes at higher nesting levels) that are on the tip of the evolutionary tree that are connected by a single mutational change to a common interior haplotype (or previously defined clade of haplotypes) plus this interior haplotype (or clade). The geographical information is captured by a set of summary statistics that measure the geographical extent of a clade, its distance from the evolutionary center of the clades nested with it at the next hierarchical level, and contrasts of both of these geographical measures of old versus younger clades (as determined by the outgroup data). A description of these statistics is given in Posada, Crandall, and Templeton (2006), and all statistics can be calculated with the program GEODIS (<http://www.softsea.com/download/GEODIS.html>). GEODIS also determines the statistical significance of these statistics under the null hypothesis of random geographic distributions using a random permutational procedure. The nested design results in statistical independence of all nested clades, making correction for multiple testing straightforward. The overall type I error rate corrected for multiple testing was set to 0.05. The statistically significant summary statistics are used to make phylogeographic inferences using a key that comes with the program. This key has been subjected to the most extensive validation for any phylogeographic technique using positive controls from actual data (Templeton, 2004b), and its type I error rate from simulated data (Knowles & Maddison, 2002; Panchal & Beaumont, 2010) was at or below the pre-set level of 0.05 after correction of misrepresentations of the technique in the original simulation papers (Templeton, 2009; Templeton, 2015). Most phylogeographic programs require a detailed simulation

of an *a priori* phylogeographic hypothesis, but GEODIS is unique in that it requires no *a priori* hypothesis but rather reconstructs the phylogeographic history directly from the data (Templeton, 2010).

3 | RESULTS

3.1 | Mark/recapture studies in the Ozarks and Great Lakes region

At the Kay Branch fen, we marked 337 dragonflies and released 332 (231 males and 101 females) with 86 recaptures. Ten dragonflies were kept as incidental take (2% of the total mark and recapture sample). We observed direct attacks by Dragonhunter (*Hagenius brevistylus*) or Gray Petaltail (*Tachopteryx thoreyi*) on HED and on a few occasions the netting of predator and prey immediately after attacks enabled us to secure HED specimens. However, one HED was already severely injured by the predator attempt and was kept as an additional incidental take. We tested several models with program MARK, as shown in Table S1, with the best fitting model having a constant encounter probability, constant male survivorship that was also identical to female survivorship during the initial 5-day period of the study, but with a significantly different female survivorship during the later observation and sampling periods.

At Onoclea Fen, we marked 114 dragonflies and released 112 (80 males and 32 females) with 154 recaptures. Five dragonflies were kept as incidental take (2% of the total mark and recapture sample). We tested several models with program MARK, as shown in Table S2, with the best fitting model having a constant encounter probability, different probabilities of survival for females and males; and two different survival probabilities each for females and males—one for the consecutive days and one for the additional observation days after the marking period.

At Centerville Slough, despite poor weather conditions, 99 dragonflies were marked and released (70 males

and 29 females) with 49 recaptures. Seven dragonflies were kept as incidental take (5% of the total mark and recapture sample). We tested several models with program MARK, as shown in Table S3, with the best fitting model having a constant encounter probability, constant probabilities of survival for males and females captured in the consecutive days of marking, and a second probability of survival for females during the additional observation days after the marking period.

We present the estimated population sizes, confidence intervals, and densities for these three Ozark fens in Table 1. The density at Kay Branch was estimated in two ways. First, only the area at Fen 7 (Figure S1) was used to estimate the density. However, because some marked individuals were observed at the other fens in this complex (Data S1), the total area of all the nearby fens was used to estimate the density of the entire complex. Because of dispersal, the first estimate of density in Table 1 for Kay Branch could be an over-estimate, and if dispersal is restricted over these fens, the second estimate of density could be an under-estimate.

The mark/recapture data from 1995 in the Great Lakes region in general had much lower recapture rates than in our Ozark studies. In particular, the goodness of fit test indicated that the data at The Ridges Sanctuary in Wisconsin should not be used for a CJS model, so we excluded it. The parameter estimators would not converge with the River South data from Illinois, but did converge using a Monte-Carlo, Markov-Chain (MCMC) option in MARK. Hence, we have four Great Lakes region sites with population sizes estimated in a manner comparable to our estimates for the Ozark populations. We present the estimated population sizes, confidence intervals, and densities for the Great Lakes region sites also in Table 1.

3.2 | Survey of mtDNA variation

We present in Table 2 the 30 pooled sites, their locations, sample sizes, number of haplotypes, and

TABLE 1 Estimated population sizes in three Ozark fens and four Great Lakes fens, with 95% confidence intervals (CI), estimated fen areas (in hectares), and densities

Region	Location	Year	Est. N	95% CI	Sampled area	Sampled density	Total area	Total density
Ozarks	Kay branch	2013	1,024	886–1,222	0.57	1,796	1.56	656
	JSI	2014	176	146–206	1.53	115	NA	NA
	Centerville	2015	272	194–412	2.23	122	NA	NA
Great Lakes	Mud Lake, WI	1995	2,390	971–6,571	18.92	126	NA	NA
	3 springs, WI	1995	142	61–305	1.79	79	4.47	32
	Lockport, IL	1995	86	66–118	115.8	0.74	NA	NA
	River south, IL	1995	2,830	2047–3,840	33.8	84	NA	NA

Abbreviation: NA, not applicable because the sampled area was also the total area.

TABLE 2 Results of the mtDNA genetic survey

Site name	Map site	n	No. Hap.	Hap. Het.
Barton Fen, Iron Co., MO	15	9	4	0.333
Bates Hollow, Dent Co., MO	14	1	1	NA
Bee Fork Complex, Reynolds Co., MO	7	11	4	0.355
Centerville Slough, Reynolds Co., MO	9	18	2	0.105
Deckard Hollow & Ruble, Reynolds Co., MO	6	12	4	0.257
Emerald Fen, Ripley Co., MO	3	4	1	0.000
Grasshopper Hollow, Reynolds Co., MO	7	4	4	0.375
Johnsons Shut-ins, Reynolds Co., MO	10	6	2	0.278
Kay Branch Fen, Reynolds Co., MO	11	24	4	0.276
Ripley Co., MO	2	6	2	0.278
Wisdom/Parker, Reynolds Co., MO	12	7	4	0.306
Fortune Hollow Fen, Dent Co., MO	13	1	1	NA
Mackinac Co., MI	A	4	1	0.000
Indian Lake, Logan Co., OH	E	1	1	NA
Oak Openings, Lucas Co., OH	C	9	6	0.395
Mud Lake, Williams Co., OH	D	1	1	NA
Bridgewater Township, Williams Co., OH	D	1	1	NA
Simcoe Co., Ontario, Canada	B	4	1	0.000
Scottsboro, Jackson Co., Alabama	J	1	1	NA
Black Partridge Woods Preserve, Cook Co., IL	F	2	1	NA
Palos Park, Dan McMahon Woods, Cook Co., IL	F	1	1	NA
Waterfall Glen Forest Preserve, DuPage Co., IL	F	1	1	NA
Will Co., IL	F	17	6	0.336
Mud Lake & Pioneer Rd., Door Co., WI	I	6	1	0.000
Piel Creek, Door Co., WI	I	2	1	NA
Ridges & Toft, Door Co., WI	I	2	1	NA
3 Springs, N. Bay, N. Mud Lake, Door Co., WI	I	4	1	0.000
Ozaukee Co., WI	G	5	2	0.320
Rd between Mascola and Avoca, Iowa Co., WI	H	1	1	NA
Knapp Creek Wetlands, Richland Co., WI	H	1	1	NA

Note: The Map Site refers to the locations shown in Figures 1 and 2, *n* is the sample size, No. Hap. is the number of haplotypes, and Hap. Het. is the haplotype heterogeneity. Sites with *n* < 4 are marked with NA (non-applicable) for haplotype heterogeneity, which is not meaningful for such small samples.

haplotype heterogeneity. We give the numbers of each haplotype found at each of these sites in Table S5 in the online supplementary information. In the Ozarks as a whole there are 13 haplotypes and a haplotype heterogeneity of 0.759, and in the Great Lakes Region as a whole there are 11 haplotypes and a haplotype heterogeneity of 0.736 (Data S3 and Figure 3). In the entire data set there are 21 haplotypes, with 10 being unique to the Ozarks and 8 unique to the Great Lakes Region.

The Data S3 gives the output of the random subsampling program that adjusts for biases caused by unequal sample size. First, consider the results obtained within the Great Lakes region. The 50 specimens collected in the 1990's have highly significant reductions in haplotype number ($p \leq 0.001$) and haplotype heterogeneity ($p \leq 0.001$) relative to the 12 specimens from 1929–1961. Within the three Great Lakes subregions for the sites from the 1990's, the sites from northeastern Illinois and southern Wisconsin have significantly more

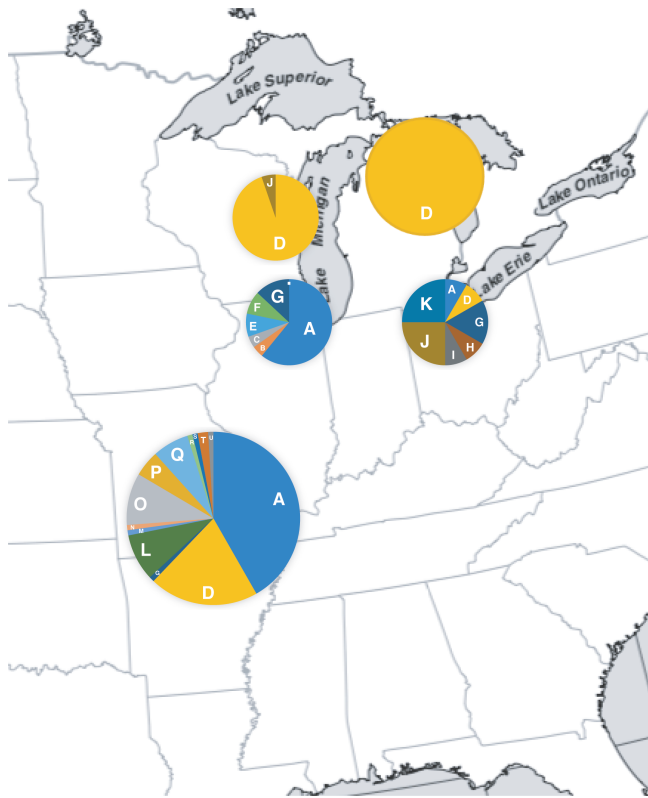


FIGURE 3 A map showing the distribution and frequencies of the haplotypes in the Ozarks and four subregions of the Great Lakes region. The areas of pie charts are roughly proportional to their actual geographical range in the Great Lakes region, but the Ozark pie chart is associated with an area that is smaller than any of the Great Lakes subregions. The Ozark pie chart has been increased in size beyond its proportional area because of its greater haplotype diversity

haplotypes and higher haplotype heterogeneity than the central/northern Wisconsin sites and the Michigan/Ontario sites (see Figure 3). The 23 specimens from the northeastern Illinois and southern Wisconsin sites have highly significant reductions in haplotype number ($p < 0.001$) and haplotype heterogeneity ($p < 0.001$) relative to the 12 specimens from the extirpated Ohio sites (see Figure 3).

The Ozark specimens are not significantly different from all of the Great Lakes region specimens for either haplotype number or haplotype heterogeneity, but the Ozarks have significantly more haplotype heterogeneity ($p = 0.978$; recall from the Section 2 that Data S5 generates a two tailed test, so p values less than 0.025 and p values greater than 0.975 define the 0.05 critical region for the test) when the specimens from 1929–1961 in extirpated sites are excluded to partially correct for temporal bias. When geographical range is partially adjusted and extirpated sites are excluded, the Ozarks has significantly greater haplotype heterogeneity than all three Great

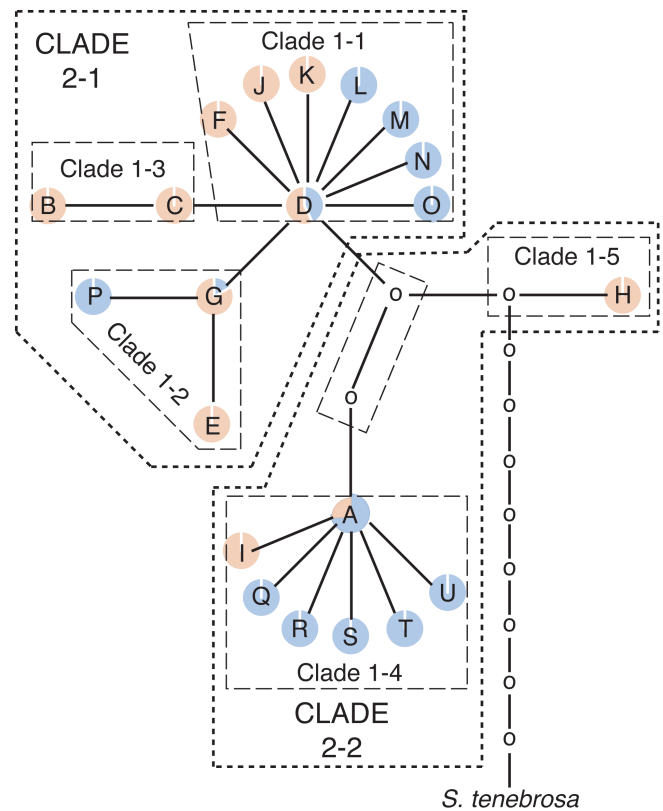


FIGURE 4 The statistical parsimony network for the haplotypes (A through U) found in the mtDNA survey of *Somatochlora hineana* using *Somatochlora tenebrosa* as an outgroup. Each solid line represents one mutational event. When haplotypes are separated by more than one mutational event, an “o” is used to indicate the unobserved intermediates. Thin dashed lines indicate the one-step clades, and thick dotted lines indicate the two-step clades in the nested haplotype tree. The colored pie diagrams are proportional to the number of times the haplotype was found in the Ozarks (light blue) versus the number of times it was found in the Great Lakes region (light orange)

Lakes subregions ($p = 0.978$ for Illinois and Southern Wisconsin, $p = 1$ for central and northern Wisconsin, and $p = 0.999$ for Michigan and Ontario), and has significantly more haplotypes than the central/northern Wisconsin sites ($p = 1$) and the Michigan/Ontario sites ($p = 0.999$). Finally, there are no significant differences in either haplotype number ($p = 0.026$) or haplotype heterogeneity ($p = 0.034$) between the Ozarks and the extirpated Ohio sites from 1929–1961.

Figure 4 depicts the statistical parsimony network, which had no significant ambiguity, along with the nested clade design. The two major clades in Figure 4 are labeled 2–1 and 2–2, and the closest haplotypes between these two clades are separated by three mutations. The rest of the haplotypes are only a single mutational step away from their nearest neighbor. The phylogenetic diversity of the mtDNA haplotypes can be measured by

TABLE 3 Significant geographic associations with haplotypes and clades of haplotypes revealed by nested clade phylogeographic analysis and their biological interpretation

Clade	Nesting clade	D_c	D_n	Inference
D		481 ^L	501 ^L	
J		NS	219 ^S	
L	1-1	14 ^S	NS	1-2-3-4 no \Rightarrow restricted gene flow with
O		4 ^S	NS	Isolation by distance
I-T		465 ^L	NS	
P	1-2	11 ^S	NS	1-19-20-2-3-4-9 no \Rightarrow fragmentation
1-2		NS	358 ^S	
1-3	2-1	NS	72 ^S	1-2-11-12 no \Rightarrow contiguous range expansion
I-T		NS	154 ^L	
1-4	2-2	286 ^S	287 ^S	1-2-3-4 no \Rightarrow restricted gene flow with
I-T		-286 ^L	NS	Isolation by distance
2-1		458 ^L	464 ^L	
2-2	Total cladogram	301 ^S	297 ^S	1-2-11-12 no \Rightarrow contiguous range expansion
I-T		-157 ^S	-167 ^S	

Note: D_c is the clade distance, and D_n is the nested clade distance. A superscript "S" indicates the distance is significantly small after correcting for multiple testing, and a superscript "L" indicates the distance is significantly large after correcting for multiple testing. The biological inference chain is indicated by a series of numbers that indicate the questions in the inference key available at <http://www.softsea.com/download/GEODIS.html>.

the evenness of their distribution across these two clades. From Table S5 or Figure 4, the Ozarks has 48 observations of clade 2-1 and 55 of clade 2-2, the older clade; the Great Lakes Region has 46 observations in clade 2-1 and 17 in clade 2-2. These differences are significant ($p < 0.001$) using the Freeman and Tukey (1950) test of proportions. Equal sample sizes are not required for this test.

Data S2 gives the GEODIS output. Several significant patterns remained after correcting for multiple testing. The significant patterns and their interpretation using the inference key are presented in Table 3.

4 | DISCUSSION

Our studies reveal much about the conservation status and evolution of HED populations in the Great Lakes region and the Ozarks, and the relationship between these two regions. One prevalent tenet in the literature on central versus peripheral populations is that geographically peripheral populations are ecologically marginal (reviewed in Pironon et al., 2017) and inhabit sub-optimal habitats as measured by density (Brown, 1984). We found that the highest densities are in the Ozarks, and three out of the four Great Lakes region populations had densities below all of the Ozark fens (Table 1). With only three Ozark and four Great Lakes fens, we cannot test the hypothesis that the densities differ in the two

regions overall, but there is certainly no indication that the Ozarks is an ecologically marginal area *in sensu* Brown (1984). One potential bias is that 20 years separate these two sets of estimated population sizes and densities. It is likely that further habitat degradation has occurred in this time period, which would tend to bias the densities in favor of the older samples. Despite this bias, the Ozark fens appear to be at least as ecologically suitable for HED as the Great Lakes fens.

A second common prediction is that geographically peripheral populations should display decreased genetic diversity (Soulé, 1973). The Ozark populations have 13 mtDNA haplotypes and an overall haplotype heterogeneity of 0.759, whereas the Great Lakes region has 11 haplotypes and an haplotype heterogeneity of 0.736 (Data S3, Figure 3). After adjusting for sample size differences, neither of these measures of genetic diversity is significantly different between the two regions.

The above result is biased in favor of greater genetic diversity for the Great Lakes region through both temporal and geographical sampling biases. First, consider temporal bias. Since the samples in the Great Lakes region were collected two to eight decades before the Ozark samples, the Ozark samples would be subject to greater loss of genetic variation than the Great Lake samples if there is a temporal trend to lose genetic diversity. We test the hypothesis of temporal loss by comparing the Great Lake samples from the 1990's to those specimens collected between 1929 and 1961. This contrast indicates a

significant reduction in haplotype diversity (a decrease of 41% after adjustment for sample sizes) and haplotype heterogeneity (a decrease of 27%) between these two sampling periods (Data S3). However, genetic diversity is not uniform throughout the Great Lakes region, but rather is much higher in the south (Figure 3). Because the extirpated populations were all from Ohio (Figure 1), a better contrast for temporal loss is to compare the older Ohio samples to the F and H sites from Illinois and southern Wisconsin that were sampled in the 1990's. These two regions are of a comparable geographic range and are geographically close in the southern Great Lakes region (Figure 3). After adjusting for sample size difference (Data S3), there are still significant losses of haplotype diversity (36%) and haplotype heterogeneity (31%) between the extirpated Ohio sites and the nearby Illinois and southern Wisconsin sites (Data S3). Hence, temporal loss of genetic diversity is occurring in HED. The rate of loss is greater than 30% over just several decades, which is truly alarming when compared to the overall rate of loss of 6% over centuries (Leigh et al., 2019). These estimates confirm the endangered status of HED in the Great Lakes region and indicate the desirability of further genetic surveys in this region to monitor the success of the recovery plan (USFWS, 2001, <https://www.fws.gov/midwest/endangered/insects/hed-recplan.html>).

The temporal bias between the Ozarks and Great Lakes can be partially corrected by using only the collection from the 1990's, thereby reducing the time difference to about 2 decades. This comparison results in no significant difference in haplotype number, but a significantly greater haplotype heterogeneity ($p = 0.998$) in the Ozarks (0.751 after adjustment for sample size) than in the Great Lakes populations from the 1990's (0.643) (Data S3).

The Great Lakes samples are still biased due to a greater range of geographic sampling than the Ozarks. When the Great Lakes samples are split into smaller subregions, each still larger than the Ozark sampling range, the Ozarks has significantly greater haplotype heterogeneity than all three Great Lakes subregions present in the 1990's, and the Ozarks has a significantly larger haplotype number than the two more northern subregions of the Great Lakes (Data S3, Figure 3). These analyses indicate that the Ozarks has greater genetic diversity than the Great Lakes region when controlling for sample size, temporal bias, and geographic scale, so the Ozarks is not a marginal region as a source of genetic diversity for this species. Even without these bias controls, the small Ozark region has comparable genetic diversity to the entire Great Lakes region, once again emphasizing its importance as a source of genetic diversity and its significance to range-wide recovery for the species (Figure 3).

Another measure of genetic diversity is phylogenetic diversity (Crozier, 1997). The Freeman and Tukey test shows a highly significant deficiency ($p < 0.001$) of haplotypes from the older clade in the Great Lakes regions compared to the Ozarks. Hence, the Ozarks has greater phylogenetic depth in its haplotype diversity than the Great Lakes region, having a more even distribution of both older and younger haplotypes (Figure 4). The Ozark populations are of high conservation priority to preserve ancestral haplotype diversity that may be beneficial to future adaptability (Templeton, 2017).

Dispersal and gene flow greatly influence the amount of genetic diversity within a population or region (Templeton, 2006). Our mark/recapture studies were not designed to measure dispersal, but they did yield some information about dispersal at the Kay Branch site (Data S1). Most of our observed dispersal events involved distances less than 150 m, with only two males showing dispersal distances close to the maximum possible with our sampling design. This indicates that our sampling area was broad enough to include most dispersal events. However, many species display more than one mode of dispersal (Dingle & Drake, 2007). One common mode is relatively short daily movements associated with individual foraging or territorial behavior. The fact that there was no significant association between the number of days available for an observed movement and the distance moved (Figure K3 in Data S1) indicates that the movements that we detected were primarily daily movements. Note, Figure K3 includes same-day recaptures (which were not scored as recaptures in the MARK analyses as these analyses required an interval of 1 day or more to be regarded as a recapture). Figure K3 therefore indicates that mixing of dragonflies within the population occurs within a time period of less than a day, emphasizing again that these are daily movements. Many species also have long-distance movements, but our design misses dispersal between fen complexes. This gap in our knowledge can be addressed indirectly with genetic studies.

The nested clade analysis revealed significant patterns of isolation by distance within non-overlapping clades 1–1 and 2–2 (Table 3). These clades involve one group of haplotypes restricted primarily to Ohio and a second group limited to the Ozarks, indicating isolation by distance in both Ohio and the Ozarks. In clade 2–2, the Ozark sites are all in the upper northwestern headwaters of the Black River drainage (sites 7, 11 and 15 in Figure 2). Isolation by distance here could be explained by movements along rivers and creeks. In addition to isolation by distance, Clade 1–2 indicates a significant break in gene flow (fragmentation) along the Black River (Table 3) that is associated with a long stretch with no

known intermediate populations despite extensive searches in this area. This significant fragmentation event (Table 3) indicates that a sufficiently long distance with no available habitat could be an effective dispersal barrier. This possibility makes the extirpation of the Ohio and Indiana populations (Figure 1) all the more alarming for the long-term maintenance of genetic diversity in the Great Lakes region as it may represent a severing or at least reduction in gene flow between the eastern and western populations of HED surrounding the southern Great Lakes.

The nested clade analysis detected two range expansions (Table 3), nested within one another (clade 2–1 and the total cladogram) so they are likely the same event. The inferred expansion within clade 2–1 was ambiguous as to direction, as the older clade within it (clade 1–1) had a geographical center that was intermediate between the geographical centers of the more southern clade (1–2) and the more northern clade (1–3). However, at the total cladogram level, the older clade 2–2 has a geographical center south of that of the younger clade 2–1, indicating a range expansion from the Ozarks to the Great Lakes region. As previously pointed out, the Great Lakes region has significantly fewer haplotypes from the older clade, which further supports the conclusion that the Ozarks is the ancestral region. This conclusion is consistent with paleoclimatic data, which indicates there were no fens in the current Great Lakes region until the Last Glacial–Interglacial transition (13,000–8,500 C-14 BP) (Yu, 2000). We speculate that there could have been many geographically intermediate habitats available for HED as the glaciers retreated, so this expansion into the Great Lakes region could have involved large numbers of individuals. We use Tajima's D statistic (Tajima, 1989) to test the null hypothesis of no population size bottleneck in the past for the Great Lakes region. Using the data in Figure 4 and Table S5, the D statistic for the entire Great Lakes region sample is -0.902 . This statistic converges to a normal with mean 0 and variance of 1 under the null hypothesis, and we fail to reject the null hypothesis. The expansion out of the Ozarks into the Great Lakes region was therefore not associated with a severe founder or bottleneck effect during the colonization of the Great Lakes region.

Further insight into the colonization of the Great Lakes region is provided by the pattern of genetic diversity within this region. As mentioned above, the most diverse populations in the Great Lakes region are in the southern part of that region (Figure 3). These southern populations would be the first populations to be colonized from the Ozarks after the Last Glacial–Interglacial transition. As one goes north from the southern Great Lakes region, the genetic diversity decreases significantly

for both haplotype diversity and haplotype heterogeneity (Data S3, Figure 3). Hence, the pattern of genetic diversity fits a south to north gradient with Ozarks > southern Great Lakes > northern Great Lakes. This pattern of genetic diversity dropping off with increasing distance from the source populations is found in other species in which there was a population range expansion, such as modern humans expanding out of Africa (Ramachandran et al., 2005; Templeton, 2002), and reinforces the conclusions from the nested clade analysis of an out-of-Ozarks range expansion event to the north.

The above discussion illustrates the limitations of making conservation decisions based solely on current geographic distributions. Population density, genetic diversity, phylogenetic diversity, and phylogeography all indicate that the Ozark region—despite its small geographic size and being separated from the bulk of the species' range—has high conservation value for this endangered species. Given that populations on the low latitude boundaries of a species' range are the ones disproportionately at risk for local extinction from global climate change (Cahill et al., 2013; Chen, Hill, Ohlemüller, Roy, & Thomas, 2011), it is particularly important to maintain the reservoir of genetic diversity found in the Ozark HED as genetic diversity is an indicator of adaptive potential (Templeton, 2017). The HED in the Ozarks therefore represent a conservation resource that should be preserved and not marginalized.

This study also indicates the need for continued monitoring of the HED populations in the Great Lakes region. These populations appear to be losing their genetic diversity at an exceedingly high rate just within the 20th century, and the geographic pattern of extirpation indicates the potential for fragmenting the populations in this region. The recovery of this highly endangered species will depend upon conservation action in both the Ozarks and the Great Lakes region.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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