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Population genetics of Brook Trout (*Salvelinus fontinalis*) in the southern Appalachian Mountains

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<A> **ABSTRACT**

Broad-scale patterns of genetic diversity for Brook Trout remain poorly understood across their endemic range in the eastern United States. We characterized variation at 12 microsatellite loci in 22,020 Brook Trout among 836 populations from Georgia, USA, to Quebec, Canada, to the western Great Lakes region. Within-population diversity was typically lower in the southern Appalachians relative to the mid-Atlantic and northeastern regions. Effective population sizes in the southern Appalachians were often very small, with many estimates less than 30 individuals. The population genetics of Brook Trout in the southern Appalachians are far more complex than a conventionally held simple "northern" versus "southern" dichotomy would suggest. Contemporary population genetic variation was consistent with geographic expansion of Brook Trout from Mississippian, mid-Atlantic, and Acadian glacial refugia, as well as differentiation among drainages within these broader clades. Genetic variation was pronounced among drainages (57.4% of overall variation occurred among Hydrologic Unit Code (HUC)10 or larger units) but was considerable even at fine spatial scales (13% of variation occurred among collections within HUC12 drainage units). Remarkably, 87.2% of individuals were correctly assigned to their collection of origin. While comparisons with fish from existing major hatcheries showed impacts of stocking in some populations, genetic introgression did not overwhelm the signal of broad-scale patterns of population genetic structure. Although our results reveal deep genetic structure in Brook Trout over broad spatial extents, fine-scale population structuring is prevalent across the southern Appalachians. Our findings highlight the distinctiveness and vulnerability of many Brook Trout populations in the southern Appalachian Mountains and have important implications for wild Brook Trout management. To facilitate application of our findings by conservation practitioners, we provide an interactive online visualization tool to allow our results to be explored at management-relevant scales.

<A> INTRODUCTION

Over the course of millennia, the distribution and genetic structure of Brook Trout (*Salvelinus fontinalis*) have been shaped by a long history of repeated glaciation and recolonization of eastern

North America (Andersen and Borns 1994; Power 2002; Pilgrim et al. 2012). Following deglaciation, Brook Trout recolonized much of northeastern North America from unglaciated refugia (Danzmann et al. 1998). As a charr, Brook Trout are able to exploit a broad variety of coldwater habitats through considerable life history diversity and adaptation (Power 2002). The current native range of Brook Trout extends from the southern Appalachian Mountains, north to the Canadian Maritimes, and west to the Hudson Bay drainage (MacCrimmon and Campbell 1969). Across this vast area, Brook Trout were found historically in nearly all coldwater habitat types, including streams, rivers, lakes, and nearshore marine environments, providing opportunities for recreational angling and serving as an iconic indicator of high-quality coldwater habitats (Power 1980). However, widespread declines have been documented across their native range, with the most precipitous decline in the southeastern United States (Smith 1833; Larson and Moore 1985; Stranko et al. 2008; Hudy et al. 2008).

In the southern Appalachian Mountains (considered here as the area from Maryland to Georgia), nearly all remaining populations of Brook Trout are found in small, higher-elevation, headwater streams. Here, their occurrence in small, isolated populations make Brook Trout vulnerable to local extirpation (King 1937; Lennon 1967; Guffey et al. 1999). Small populations suffer heightened risk of the deleterious effects of genetic drift and inbreeding depression (Whitlock 2000; Hedrick and Kalinowski 2000). They are also at greater risk of extirpation by stochastic events (Lande 1993), which are known to cause erratic population dynamics of even robust populations of stream-dwelling Brook Trout (Roghair et al. 2002; Kazyak 2015; Kanno et al. 2016, 2017). Typically, these habitats are isolated from one another by impediments to connectivity, such as waterfalls, reaches with exotic competitors, and thermally unsuitable areas (Timm et al. 2016; Moore et al.1986; Aunins et al. 2014; Weathers et al. 2019). The Eastern Continental Drainage Divide has isolated some populations for millions of years, with marked genetic differentiation observed between nearby sites (Danzmann et al. 1998; Hall et al. 2002; King et al. 2012; Kazyak et al. 2015).

There is little opportunity for natural recolonization of Brook Trout in most streams across the southern Appalachian Mountains. In addition, more than a century of supplementing and restoring trout fisheries with hatchery-raised Brook Trout is thought to have resulted in introgression of

hatchery genotypes of northern origin into endemic southern populations (Hayes et al. 1996; Kazyak et al. 2018; Printz et al. 2018), possibly resulting in a loss of regional diversity and local adaptations (Laikre et al. 2010). Given recent declines and the continued vulnerability of these populations, it is important to understand the current population structure and biogeographic context of Brook Trout in the southern Appalachian Mountains to guide management and conservation efforts.

Previous studies have identified unique characteristics of Brook Trout in the southern Appalachians. Because food availability is a limiting factor in this region (Whitworth and Strange 1983; Cada et al. 1987; Ensign et al. 1990; Romaniszyn et al. 2007), adult fish are typically small (Harris et al. 2021) and life span seldom exceeds three years (Konopacky and Estes 1986; Habera et al. 2001). Wesner et al. (2011) reported that Brook Trout native to the southern Appalachian Mountains and introduced northern-origin Brook Trout differed in terms of survival in the laboratory and diet in a natural stream. Early molecular studies observed putatively fixed differences in the allozymes of creatine phosphokinase between northern and southern populations of Brook Trout, and this was widely adopted as a diagnostic marker (Stoneking et al. 1981; McCracken et al. 1993; Hayes et al. 1996). These studies fostered a widespread perspective that southern Appalachian Brook Trout represent a distinct entity (i.e., "northern" versus "southern" strains) with a sharp transition area near the New River drainage (Figure 1; Guffey 1998; Palmer and Hallerman 2000; Davis 2008; Printz et al. 2018), and potentially even warranting a taxonomic revision (Stoneking et al. 1981). In their study of mitochondrial haplotypes across the native range of Brook Trout, Danzmann et al. (1998) found that the single population they analyzed from south of the New River had a distinct haplotype not observed in 154 other populations in the north. Moreover, it is thought that Brook Trout from the southern Appalachian Mountains may have diverged from their northern form over 1.6 million years ago (Fausch 2008). Based on these studies, management guidelines for southern Appalachian Brook Trout have been developed and implemented (Habera and Moore 2005), but the underlying science has not been reevaluated with more contemporary molecular genetic techniques using a larger number of markers.

The advent of more powerful molecular tools provides an opportunity to review and enhance our understanding of Brook Trout in the southern Appalachian Mountains. The purposes of this manuscript are to: (1) characterize the population genetic patterns of Brook Trout across their native range, with an emphasis on those populations in the southern Appalachian Mountains; and (2) in doing so revisit the biogeography of this species. Our geographic scope is much broader than previous genetic assessments of Brook Trout (e.g., Stoneking et al. 1981; McCracken et al. 1993; Printz et al. 2018), allowing us to assess the putative genetic break between "northern" and "southern" Brook Trout at the New River drainage and to identify other zones of discontinuity where they occur. This information may help provide the foundation for ongoing conservation and management activities across the region.

<A> METHODS

We obtained samples (n = 22,020) collected across the native range of Brook Trout by many agency and academic partners. Among 836 total collections (Figure 1, Supplemental Material 1), 818 collections were taken from wild Brook Trout. We focused primarily on Brook Trout collected in the southern Appalachian Mountains (i.e., Georgia to Maryland; these 718 collections consisted of 17,938 individuals). The northern edge of this focal area corresponds roughly to a key transition area for Brook Trout, near the maximum extent of past glaciation and at a latitude north of which Brook Trout can be found in lower-elevation systems and in a broader diversity of habitats (e.g., lakes, larger rivers, and coastal environments; Batchelor et al. 2019). We included 100 additional genetic collections (comprising 3,294 individuals) from elsewhere in the native range of the species to provide context to the patterns observed in the southern Appalachian Mountains. The remaining 18 collections (comprising 788 individuals) were sampled from captive fish used for production activities. Seventeen hatchery collections represented northern-origin hatchery strains used for conventional stocking (Kazyak et al. 2018). The Tellico collection is unique, in that this facility does not rear domestic stocks but instead propagates progeny of wild Brook Trout from selected streams in the southern Appalachians to be used in restoration (this collection was omitted from all hatchery analyses but is presented for contrast). Collection protocols varied, but the majority of samples were

fin clips taken from trout collected in wadeable streams using backpack electrofishing and preserved in 95% ethanol. Sample sizes varied among collections (range: 2–152) but averaged 26 individuals. Most collections represent mixed-age samples drawn from several hundred meters of contiguous stream habitat. A subset of samples (12 collections) represents single-cohort samples that focused on age-0 (young-of-year, YOY) individuals. YOY were sampled from approximately three spatially distinct sites, each approximately 100 meters in length, within contiguous stream habitat (Pregler et al. 2018).

[C] *DNA Extraction and Microsatellite Genotyping*.— Molecular analyses were performed at the United States Geological Survey (USGS) Eastern Ecological Science Center, Kearneysville, WV. Genomic DNA was isolated from fish tissue using the Puregene Tissue Kit (Gentra Systems, Minneapolis, MN) or the E-Z 96 Tissue DNA Kit (Omega Bio-Tek, Norcross, GA). DNA concentrations were evaluated using a Tecan Spectrafluor Plus (Tecan Group Ltd., Männedorf, Switzerland), Nanodrop ND-1000 or 8000 Spectrophotometer (Thermo Fisher Scientific), or a Qubit Fluorometer (Thermo Fisher Scientific). Stock DNA was diluted and normalized prior to polymerase chain reactions (PCR).

All samples were screened for 12 microsatellite loci (*Sfo*B52, *Sfo*C24, *Sfo*C28, *Sfo*C38, *Sfo*C79, *Sfo*C86, *Sfo*C88, *Sfo*C113, *Sfo*C115, *Sfo*C129, *Sfo*D75, *Sfo*D91) designed for Brook Trout (King et al. 2012). PCR amplification of microsatellite loci was carried out on either a PTC-225 Tetrad thermal cycler (MJ Research), PTC-200 thermal cycler (MJ Research), or T100 thermal cycler (BioRad) using the following procedure: initial denaturing at 94°C for 2 min; 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 2 min; and a final extension at 72°C for 10 min. Four multiplexed PCR reactions were generated to genotype the 12 microsatellite DNA markers. PCR master-mix composition, thermal cycling parameters, and multiplexing were generally as provided in King et al. (2012); more recent laboratory work had slight changes to PCR composition and fragment analysis multiplexes (Kazyak et al. 2018). PCR products were combined, diluted, and ran in two separate reactions on an Applied Biosystems (Foster City, CA, USA) ABI 3100 or 3130XL Genetic Analyzer using an internal size standard (LIZ-500, Applied Biosystems). A positive control sample (of known

multi-locus genotype) was included on each PCR plate for checking success of PCR amplifications and for correct binning success in the analysis software. A negative control sample (containing all the ingredients for PCR amplification except DNA) was included on each PCR plate to check for contamination in the PCR products. Genemapper or Genotyper Fragment Analysis software (Applied Biosystems) was used to score, bin, and output allelic data. All microsatellite scoring was automated and then checked by experienced laboratory personnel. PCR was performed again on all samples with missing data due to weak or unamplified alleles. PCR amplifications that had to be repeated were done with single loci and not in a multiplexed PCR. All Genemapper files were double-checked for scoring errors.

[C] *Sibship*.— Because family structure can obscure comparisons among populations, we used COLONY 2.0.5.0 (Jones and Wang 2010) to identify full-sibling families within each collection. Due to the large number of collections, a custom R-script (R Core Team 2015) was used to run COLONY from the Windows command line and to store results. Model parameters included an assumption of male and female polygamy and the absence of inbreeding. Single-cohort samples with numerous siblings from the same family can cause deviations from Hardy-Weinberg (HW) expectations, elevated linkage disequilibrium (LD), and bias in genetic structure analyses (Whiteley et al. 2013; Waples and Anderson 2017). Since 12 of the collections included in our analysis were single-cohort samples, we performed sibship removal following the 'yank-2' procedure of Waples and Anderson (2017). When families were identified (pairwise sibship probability >0.95), full siblings were retained for all estimated family sizes of either one or two. For larger family sizes, we randomly removed siblings until two representatives remained. This sibling-purged dataset was used for all analyses of among-population differentiation and diversity (e.g., F'_{ST} and hierarchical analysis of molecular variance [AMOVA]).

[C] *Within- and among-population diversity*.— We tested each collection for conformance to Hardy-Weinberg proportions and for linkage disequilibrium using Genepop v. 4.3 (Raymond and Rousset 1995). Descriptive statistics for each collection were generated using GenAlEx 6.502 (Peakall and Smouse 2006, 2012). Allelic richness (N_A), unbiased expected heterozygosity (uH_E), observed

heterozygosity (H_o), and a measure of departure from Hardy-Weinberg proportions (F_{IS}) were calculated for each collection. Rarified allelic richness (A_R) was calculated using HP Rare 1.1 (Kalinowski 2005), based on a sample size of 40 genes (20 diploid individuals). This metric was not calculated for collections with fewer than 20 individuals. Single-sample estimates of effective population size (N_e) based on linkage disequilibrium were produced using NeEstimator v2 (Do et al. 2014), using a rare allele cutoff frequency of 0.02 and jackknifed confidence intervals. We refer to this as an estimate of N_e rather than the effective number of breeders (N_b) because the majority (98.6%) of our collections included samples with mixed cohorts. No estimate of N_e was reported for the single-cohort samples. Measures of allelic fixation (F_{ST}) and differentiation (F'_{ST}, Hedrick 2005) among collections were calculated using the diveRsity package (Keenan et al. 2013) in R.

To assess evidence of genetic drift, we investigated whether there was a negative relationship between genetic differentiation and genetic diversity metrics using linear regression models. Rarefied allelic richness, expected heterozygosity, and effective population size were regressed against mean population-specific F'_{ST} estimates for each population (Coleman et al. 2013). For this analysis, we only used those collections with sample sizes $\geq$ 20 individuals.

To examine the geographic structure of genetic variation, we used a hierarchical AMOVA, implemented with the pegas package (Paradis 2010) in R. Five hierarchical levels were considered: collection, HUC12, HUC10, HUC8, and HUC6 units. Hydrologic Unit Code (HUC) units were established by the U.S. Geological Survey and represent a series of nested units defined by basin topography (Seaber et al. 1987). A small proportion of the sample collections were missing latitude and longitude information. For the purposes of this analysis those collections were not considered in the AMOVA or assignment tests.

To further assess the uniqueness of each collection, we assessed our ability to assign each individual to its source collection based on genotype data. Assignment testing was conducted using GeneClass2 (Piry et al. 2004) based on the Bayesian approach of Rannala and Mountain (1997). We summarized classification efficiencies (i.e., the percentage of individuals correctly assigned) at

different spatial scales (collection, patch, and HUC units). We used patches that were developed by the Eastern Brook Trout Joint Venture (https://easternbrooktrout.org; EBTJV), which are intended to represent contiguous stream habitats that support Brook Trout. Collections that were not located within an existing EBTJV patch or were missing sampling coordinates were omitted from assignment testing.

[C] *Cluster analyses*.— We examined population structure with discriminant analysis of principal components (DAPC) using the *adegenet* package (Jombart 2008) in R. Analyses were performed on the filtered dataset (≥ 20 individuals per collection) that contained 20,220 individuals from 665 collections. We used the *find.clusters* function to detect genetically distinct populations. This function uses *k*-means clustering to decompose the total genetic variance into between- and within-group components. Bayesian information criterion (BIC) scores were evaluated to assess optimal clustering. Patterns of population clustering were examined using the *dapc* function, which transforms the data using principal components analysis and then performs discriminant analysis on the retained principal components (PCs; Jombart et al. 2010). The number of PCs corresponding to the asymptote in cumulative variance explained (N = 100 PCs) was determined visually. We retained all discriminant functions for analysis for each number of clusters examined. The DAPC results were visualized using the *scatter* function and posterior membership probabilities were used to examine individual genetic similarities to each population cluster. Preliminary analyses indicated that clustering using STRUCTURE provided results that were largely congruent with DAPC; STRUCTURE analyses are described in Supplemental Material 3.

To compare overall genetic diversity among the major clusters identified (based on DAPC, K = 3) while standardizing for sampling intensity, we subsampled the overall dataset and retained 20 randomly selected individuals from 47 randomly selected collections in each of the three clusters. Using this subsampled dataset, we compared the total number of alleles as well as the number of private alleles in each of the three genetic clusters. In addition, we used a hierarchical Shannon diversity analysis (Smouse et al. 2015; Sherwin 2015) to compare levels of genetic diversity among regions. Due to limitations of the Genalex implementation of the Shannon diversity analysis, we

compared diversity within each of the regions using a smaller number of random samples (20 random individuals from 20 randomly selected populations within each of the three clusters; populations that were assumed to be introgressed in the southern Appalachians were excluded). The hierarchical Shannon diversity analysis was repeated 10 times with independently selected random samples.

<A> **RESULTS**

[C] *Sibship*.— COLONY identified 17,562 full-sibling families across the 836 collections included in the sibship analysis. Mean family size across all collections was 1.40 with a range of 1 to 84. Eighty-four percent of the identified families contained a single individual. Among the 836 collections, siblings were purged from 12 young-of-year-only samples containing full-sibling families of three or more individuals. Ultimately, sib-purging reduced our sample size from 22,020 total individuals to 21,998.

[C] *Within-population diversity*.— Genotype frequencies generally conformed with Hardy-Weinberg (HW) proportions and showed linkage equilibrium among loci. At a Bonferroni-corrected *p*-criterion of 0.00417 (0.05/12 loci), collections showed a mean of 0.21 loci that deviated from HW proportions. Most collections showed no significant departures; however, four loci in the Greens Creek, NC, collection (sample size = 33) and seven in the Flat Creek, NC, collection (sample size = 19) showed significant departures from HW proportions. At a critical Bonferroni-corrected *p*-criterion of 0.00076 (0.05/66 tests per collection) for tests of linkage disequilibrium, collections showed a mean of 0.89 significant tests results between pairs of loci, with most collections showing no significant results. Thirteen collections (eight from the southern Appalachians, two from the Shenandoah drainage, and three northern collections, all small or known to have been stocked) showed ten or more significant test results (range of sample sizes = 15–152; Supplemental Material 1). Since the majority of tests for departures from HW proportions and linkage disequilibrium showed non-significance, we concluded that collections behaved as populations and that the respective microsatellite loci segregated independently.

Within-population diversity for Brook Trout populations in the southern Appalachians was lower than for most populations from the northern portion of the range (Figures 2–3; Supplemental Material 1). The mean number of alleles per locus (N_A) ranged from 1.00–9.33 (mean = 3.56) and tended to be lower in the southern than in the mid-Atlantic and northeastern parts of the range. Allelic richness (A_R) ranged from 1.00–7.55 (mean = 3.43) and showed a similar geographic trend (Figure 2A and Figure 3). Observed heterozygosities (H_O, range = 0.00–0.76, mean = 0.44) were comparable to unbiased expected heterozygosities (H_E, range =0.00–0.73, mean = 0.43) and tended to be lower in the southern part of the range (Figure 2B). Although some F_{IS} values departed from zero (range = -0.55–0.73), the mean F_{IS} = -0.03 gave no indication of widespread departures from random mating across the populations surveyed. Estimated effective population sizes ranged from one to over 2000 (median = 55.1). Effective population sizes of Brook Trout populations in the south were often less than 30 (Figure 2C and Figure 3; 60.3% of populations in this region), which is consistent with observations across much of the species range and a history of bottlenecks in isolated populations. Notably, one population (Boone Fork Watauga River, NC) exhibited no variation within any of the 12 microsatellite loci, despite an apparently robust census population size (Jacob Rash, North Carolina Wildlife Resources Commission, unpublished data).

Genetic variation tended to be higher within domestic hatchery Brook Trout populations than wild populations, particularly compared to populations in the southern Appalachians. Within the 17 domestic hatchery Brook Trout populations, N_A ranged from 3.00–6.08 (mean = 4.40), and A_R ranged from 2.81–6.08 (mean = 4.10; Supplemental Material 1). Observed heterozygosity (range =0.41–0.70, mean = 0.54) approximated expected heterozygosity H_E (range 0.43–0.68, mean = 0.53). F_{IS} values were near zero (range -0.09–0.08, mean = 0.00). Effective population sizes N_e ranged from 14.2–212.7 (median = 57.3).

Results from our genetic analyses of these Brook Trout populations can be seen in an interactive, web-based viewer located at http://bte.ecosheds.org/. The user can select geographic layers (e.g., state outlines), overlay layers (e.g., continental divide, HUC watersheds), data layers

(e.g., genetic differentiation metrics, STRUCTURE and DAPC results), and histogram and scatter plots of key metrics. Further, the viewer can zoom in to view features of regional interest.

[C] *Among-population diversity*.— Brook Trout showed marked differentiation among wild populations in the study range (mean F'_{ST} = 0.746; range = 0.000–0.998). Clear spatial trends were evident in pairwise comparisons of populations within and among the three genetic clusters identified by DAPC (K = 3, see "Cluster analyses" section; Table 1). Populations within the northern regional genetic cluster were least differentiated (mean F'_{ST} = 0.478; range = 0.040–0.812). In contrast, populations within the southern regional genetic cluster were differentiated to a much greater extent (mean F'_{ST}= 0.722; range = 0.000–0.998). Comparisons within the mid-Atlantic regional genetic cluster showed intermediate levels of differentiation among populations (mean F'_{ST} = 0.666; range = 0.000–0.996). Notably, the average level of differentiation among pairs of populations in the southern genetic cluster was only slightly lower than in comparisons between populations in the southern region and those in the mid-Atlantic or northern regions (mean F'_{ST} = 0.796 and 0.793, respectively). The domestic hatchery collections were highly differentiated from nearly all wild collections, but comparatively similar to one another. Additional comparisons may be viewed in Table 1.

Based on our AMOVA, genetic variation was pronounced among drainages (57.4% of overall variation could be explained by differences among HUC10 or larger units; Table 2), but considerable variation occurred even at fine spatial scales (13.0% of variation reflected differences among populations within HUC12 units). Remarkably, 87.2% of individuals were correctly assigned to their collection of origin (Table 3), even though many collections were taken from geographically proximate locations within the same watersheds. An even greater percentage (94.6%) of Brook Trout were assigned to the correct EBTJV patch. Across broader hydrologic scales, nearly all individuals could be correctly assigned (e.g., 98.2% to the HUC8 level; Table 3).

A comparison of mean population-specific F'_{ST} values with rarefied allelic richness, expected heterozygosity, and effective population size (Figure 4) provided strong evidence that the pronounced among-population differences are due, in part, to genetic drift. Many estimates of effective population

size were very low—conditions which may lead to rapid, random changes in allele frequencies and loss of intrapopulation genetic diversity. Linear regression models revealed a significant negative relationship between F'_{ST} and rarefied allelic richness ($p = 0.03$; effect size = -0.21). Populations that were most distinct (i.e., had the greatest mean F'_{ST}) consistently had very low levels of allelic richness. Conversely, the populations that were least distinct were also among those with the greatest levels of allelic richness observed in this study. There was also a tight, negative linear relationship between mean-population specific F'_{ST} and unbiased expected heterozygosity ($R^2 = 0.76$; $p = 0.02$; effect size = -0.57). Although most estimated effective population sizes were small, there was not a significant relationship ($p = 0.09$) between effective population size and mean population-specific F'_{ST}. Overall, these results suggest that populations have lost diversity through genetic drift, and that the observed distinctness among populations is likely to have been substantively driven by this process.

[C] *Cluster analyses*.— In the discriminant analysis of principal components (DAPC) analyses, BIC values progressively declined for up to 200 evaluated clusters, providing no clear indication of an optimal *K* for this dataset (Supplemental Material 2). We therefore evaluated a set of clusters with the *dapc* function that was reasonable based on STRUCTURE results (*K* = 2 through 7, 10, 15, 20, and 25; see Supplemental Material 3 for a full presentation of STRUCTURE results). At *K* = 2 (Figure 5A; see Supplemental Material 4 for collection-specific DAPC scores), one of the two clusters, shown in blue, was distributed throughout much of the southern portion of the species' range, and presumably represents what has been traditionally referred to as southern Appalachian Brook Trout. Contributions from this cluster were distributed not only to the southwest of the New River drainage, but also farther north on the west side of the Eastern Continental Drainage Divide in West Virginia, with smaller contributions in Pennsylvania, southwestern New York, and Ohio.

We observed additional, likely biologically meaningful, substructure at higher values of *K*. At *K* = 3 (Figure 5B), a northern cluster of populations (shown in green) was distinguished from a central Appalachian cluster (blue) and a southern Appalachian cluster (pink). Several West Virginia and Blue Ridge Mountain, Virginia, populations clustered with the southern Appalachian cluster. At *K* = 4

(Figure 5C), populations in the Pigeon River watershed of North Carolina were clustered separately from other Brook Trout populations. At $K = 5$ (Figure 5D), a new cluster of 21 populations in central Virginia was identified, primarily on the east side of the Blue Ridge Mountains in the Rapidan and Rappahannock river basins. At higher values of K, subdivision became more apparent in the southern-most populations. Additional clusters were added within the southern Appalachian set of populations at $K = 6$ and 7. At $K = 10$, the former central Appalachian cluster was divided into two (while maintaining the Virginia Blue Ridge cluster) and southern populations comprised six clusters that tend to fall within HUC8 watersheds (Supplemental Material 4 and interactive, web-based viewer available at https://bte.ecosheds.org/). Further subdivision within the southern Appalachian region occurred at $K = 15$. At $K = 20$, some geographic structure among the northern populations became apparent. One cluster was located in Maine, New Hampshire, Vermont, and western Massachusetts. Another cluster occurred in coastal drainages in Maine, New Hampshire, Massachusetts, and coastal New York. Northern New York and Great Lakes populations formed a third cluster in this region (Supplemental Material 4 and interactive, web-based viewer available at https://bte.ecosheds.org/). At $K = 25$, clusters were generally similar to those observed at $K = 20$ but with subdivision at increasingly finer spatial scales. For example, collections within the Susquehanna River (Pennsylvania) formed a separate cluster at $K = 25$ with cohesion at the HUC6 level, and farther to the south conformity with HUC8 watersheds further increased.

Results of DAPC analysis of hatchery stocks revealed that at $K = 2$, the captive lineages belonged entirely to the cluster associated with populations in northern areas, with a small amount of southern ancestry in the Paint Bank stock (Figure 5A). Only the Tellico propagation facility, which cultured Brook Trout from the southern Appalachians, was entirely of southern origin (Figure 5A). At $K = 3$, 13 of 17 hatchery stocks were predominantly of northeastern origin while four were predominantly of mid-Atlantic origin (Figure 5B). At higher levels of K, all 17 hatchery stocks showed varying compositions of northeastern and mid-Atlantic ancestry. The Tellico collection showed indications of multiple southern lineages (Figure 5C, D). Within the southern Appalachian Mountains, there was a signature of apparent introgression of the northern Brook Trout lineage into some populations across values of K (Figure 5A-D).

A comparison of allelic diversity among the three broad genetic clusters identified with DAPC (K = 3; using the subsampled dataset to account for sampling intensity) contrasted somewhat with patterns of within-population diversity. The mid-latitude cluster contained the greatest number of alleles (n = 174). However, despite generally low levels of allelic diversity within populations, the southern cluster as a whole showed more allelic diversity (n = 165) than the northern cluster (n = 147). Hierarchical Shannon diversity analysis further indicated that the mid-latitude cluster contained the highest amount of within-region genetic diversity (mean ${}_{S}H(WR_r)$ = 0.625; SD = 0.014), followed by endemic populations in the southern Appalachian Mountains (mean ${}_{S}H(WR_r)$ = 0.560; SD = 0.015), and then by populations in the northern cluster identified by DAPC (k = 3; mean ${}_{S}H(WR_r)$ = 0.514; SD = 0.013). Although genetic diversity was low within most individual populations in the southern Appalachians when compared to other regions, the region harbors considerable total genetic diversity because of high degrees of differentiation among populations.

<A> **DISCUSSION**

This study presents results from the largest population genetic survey of wild and cultured Brook Trout populations in eastern North America yet conducted. Although many studies have examined population genetic structure of this species (e.g., McCracken et al. 1993; Hayes et al. 1996; Danzmann et al. 1998; Kazyak et al. 2016; Printz et al. 2018; Nathan et al. 2019, 2020; Morgan et al. 2021), no previous effort has characterized relationships among populations at such a broad spatial scale with nuclear DNA markers, particularly in the southern Appalachian Mountains. The large number of populations represented in our study allows insights that would not be available with analysis of smaller, more spatially restricted datasets. This underscores the value of collaborative, broad-scale approaches to studying widely distributed taxa. Notably, we made the following observations and inferences: (1) populations in the south tend to have small effective population sizes, and genetic drift has been a strong driver of contemporary population structure; (2) relationships among populations across the landscape are complex, and more complicated than the simple north-south division suggested in earlier studies; and (3) major genetic clusters reflect large-scale dispersal

from Pleistocene refugia. Our findings highlight the distinctiveness and vulnerability of many Brook Trout populations in the southern Appalachian Mountains and have important implications for wild Brook Trout management.

[C] *Within- and among-population genetic variation.*— Genetic variation within native southern Appalachian Brook Trout populations tended to be substantially lower than within populations at higher latitudes. While low estimates of genetic variation have been reported in isolated high-latitude populations within the native range (Kelson et al. 2015; Bernos and Fraser 2016), the proportion of small and isolated populations with low genetic variation is greater at southern latitudes. This pattern appears to be due to strong genetic drift, an inference supported by our observation that populations with the lowest estimates of genetic variation (in terms of expected heterozygosity and allelic richness) were also the most genetically differentiated. This pattern of genetic distinctiveness owing to genetic drift also has been observed in isolated populations on finer spatial scales than the present study in isolated populations of salmonids (Whiteley et al. 2010; Whiteley et al. 2014), an Australian galaxiid (Coleman et al. 2013), and small mammals (Weeks et al. 2016). Small estimates of N_e, often less than 30 in many southern populations that we examined, were consistent with the expectation for strong genetic drift. We are confident that N_e is small in many of these populations, although some of the variation in N_e estimates was likely due to small sample size and, due to violation of the assumption of non-overlapping generations, whether estimates from mixed-age samples were more similar to N_b or N_e (Waples and Do 2010; Luikart et al. 2010).

Given small effective and census sizes, the risk of population extinction is likely to be raised in this large set of isolated populations due to strong genetic drift causing deleterious alleles to shift to high frequency or become fixed. Low genetic variation is also likely to cause limited adaptive potential. Under similar circumstances, others have argued that continued management of fragmented populations in isolation could increase extinction risk (Weeks et al. 2016). Notably, populations at the edge of a species' range are expected to encounter more frequent demographic bottlenecks, which would further increase the rate of genetic drift (Allendorf 1986; Hampe and Petit 2005) and frequency of deleterious alleles in the population. Continued erosion of genetic variation is likely to limit future

adaptive potential and population resiliency under future environmental conditions. Although we found significant positive correlations between allelic diversity and estimates of effective population size, it is worth noting that Weathers et al. (2019) observed no significant correlation between the amount of phenotypic variation within populations and any of the examined measures of genetic diversity or the amount of occupied habitat sampled. However, additional work may be needed to understand the most appropriate scale of Brook Trout management as there is some evidence to suggest Brook Trout populations differ in their upper thermal tolerance and capacity for acclimation (Stitt et al. 2014), at least in part due to differences in routine metabolic rates (Hartman 2019). Among-population differences may, at least in part, be due to regional differences in bioenergetics, as southern populations have had much longer to develop local adaptations to warmer stream temperatures and restricted energy availability (Whitworth and Strange 1983; Cada et al. 1987, Ensign et al. 1990; Romaniszyn et al. 2007) than northern populations. Taken together, this suggests that more work is needed to understand the relationship between genetic drift and differentiation, as well as adaptive traits in isolated populations within and among geographic regions.

Nearly all Brook Trout populations were significantly genetically differentiated, and typically to a great extent. High divergence among populations has been widely reported across the northern portion of the native range of Brook Trout (Angers and Bernatchez 1998; Castric and Bernatchez 2003; Richards et al. 2008; Bruce et al. 2018), but genetic differentiation was even greater across much of the southern Appalachians than has been previously reported. Patterns of strong differentiation may, in part, be due to habitat alteration and competition with introduced Rainbow Trout (*Oncorhynchus mykiss*) and Brown Trout (*Salmo trutta*) which have restricted native Brook Trout to more isolated, higher-elevation habitat patches in the south (Larson and Moore 1985; Hudy et al. 2008).

Despite the limited genetic variation observed within many populations (alpha diversity), most populations in the southern Appalachian Mountains were highly differentiated (beta diversity; Table 1). However, when viewed in aggregate this region contains more genetic diversity than the northern

cluster (gamma diversity; see results of hierarchical Shannon diversity analysis). This finding highlights the importance of conserving endemic genetic diversity within the southern region, as populations are often unique and irreplaceable. Moreover, it challenges the notion that Brook Trout in the south are genetically depauperate (Pregler et al. 2018; Weathers et al. 2019). There is in fact high genetic diversity here, but it is spread among many populations which have had a long time to diversify and adapt to local conditions.

[C] *Population clustering results and natural history*.— The physiographic setting of much of unglaciated eastern North America has been defined by the geologically and ecologically complex Appalachian Mountains (Soltis et al. 2006). Some features of genetic structure observed in our analyses can be related to the Eastern Continental Drainage Divide, to current or past drainage patterns, and to dispersal from glacial refugia. The geographic patterning of genetic clusters was strikingly consistent between the two methods used in this study, although DAPC clusters populations based on allele frequencies and STRUCTURE uses a Hardy-Weinberg model-based clustering algorithm. That the most fundamental differentiation among Brook Trout populations (at $K = 2$ for both DAPC and STRUCTURE analyses) occurred among southern and other Brook Trout assemblages was not surprising, as this distinction has long been suggested on the basis of coloration, morphology and life history (Lennon 1967; Behnke 1980; Power 1980; Bivens et al. 1985), and allozyme frequencies (Stoneking et al. 1981; McCracken et al. 1993; Printz et al. 2018). Our findings based on microsatellite allele frequencies support the distinctiveness of Brook Trout in the southern Appalachian Mountains, which may be in part explained by a zoogeographic boundary along the Eastern Continental Drainage Divide. This assemblage of populations likely expanded from one or more Pleistocene glacial refugia in the Mississippi drainage (Danzmann et al. 1998). Other species showing evidence of genetic discontinuity at the Appalachian Mountains include salamanders (Donovan et al. 2000; Church et al. 2003), turtles (Walker and Avise 1998), and plants (Parks et al. 1994; Sewell et al. 1996; Joly and Bruneau 2004; Mylecraine et al. 2004), suggesting that many elements of the regional fauna and flora expanded from distinct glacial refugia east and west of the Appalachians (Soltis et al. 2006).

At higher latitudes, mid-Appalachian Brook Trout populations on the east side of the continental divide were distinguished from other northerly populations on both sides of the divide (K = 3 for DAPC). A growing body of evidence suggests that some temperate species survived glacial periods in refugia located well north of the Gulf Coast (Soltis et al. 2006). We suggest that the mid-Appalachian Brook Trout populations recolonized the landscape from glacial refugia on the Potomac, Susquehanna, and other east-flowing drainages of the mid-Atlantic region. More northerly populations likely found refuge in the Delaware, Hudson, Connecticut, and more northerly coastal rivers, sometimes collectively referred to as an Acadian refugium. Such populations may have entered the Great Lakes watershed through the St. Lawrence River, and the upper Mississippi system through the Brule glacial spillway in Wisconsin into the St. Croix River. As discussed below, the geographic distribution of mitochondrial DNA variation (Danzmann et al. 1998) also supports the hypothesis that contemporary Brook Trout populations expanded from three glacial refugia. We note that the group of populations in the vicinity of the Greenbrier River, West Virginia, clustered with others on the opposite side of the continental divide. These populations are located in an area with multiple documented stream captures (Hocutt et al. 1978) which may have facilitated localized expansion of this lineage into the Mississippi Basin.

At finer spatial scales (e.g., $K \geq 4$ for DAPC), the clustering results appear to reflect a combination of geophysical processes and supplemental stocking. Within the southern Appalachian Mountains, populations within the upper Pigeon River watershed were among the first to split out in the clustering analyses. Among the possible explanations, this may in part reflect the presence of numerous waterfalls posing barriers to upstream migration and northern-derived hatchery stocks might be poorly adapted to such ecosystems (Galbreath et al. 2001; Kazyak et al. 2018). We present a case study of stocking and limited introgression of hatchery stocks into native populations in Great Smoky Mountains National Park in Supplemental Material 5 accompanying this article.

Another distinct cluster was resolved in the vicinity of Shenandoah National. This group of 21 populations (shown in dark blue in Figure 5D, K =5 for DAPC) occurred mostly but not entirely on the eastern side of the Blue Ridge Mountains of central Virginia. A review of stocking records (David

573 Demarest, Shenandoah National Park, written communication) suggests that this cluster may reflect in
574 part the result of multiple stocking events both inside and outside Shenandoah National Park starting
575 in the early 1900s and continuing through at least the 1950s. Therefore, we infer that the genetic
576 composition of populations within this cluster, which straddles the watershed divide, is likely a
577 mixture of natural and anthropogenic origins.
578
579 In DAPC models with greater complexity (e.g., $K \geq 7$), clusters of populations especially in
580 the south tend to become split more finely among watersheds. The finer-scale variation in the south
581 likely reflects that this region was never glaciated (Hewitt 2000). Greater genetic diversity in
582 unglaciated than in deglaciated regions has been observed in Brook Trout (Bernatchez and Danzmann
583 1993), Walleye (Billington and Hebert 1988; Ward et al. 1989; Billington et al. 1992), Red Shiner
584 *Cyprinella lutrensis* (Richardson and Gold 1995), and European Brown Trout (reviewed by
585 Bernatchez and Wilson 1998).
586
587 [C] *Correspondence with mitochondrial DNA variation*.— Some authors (Radforth 1944; Mandrak
588 and Crossman 1992) have argued that Brook Trout expanded from one Atlantic upland refugium,
589 while others (Bailey and Smith 1981) have argued that northern Brook Trout also arose from a
590 Mississippian refugium. Our interpretations of microsatellite DNA data led to inferences of past
591 expansion of Brook Trout populations from Mississippian, mid-Atlantic, and Acadian glacial refugia
592 to recolonize the deglaciated North American landscape, with subsequent secondary contact among
593 lineages. Our results supporting the view that Brook Trout populations in the Great Lakes region are
594 the product of mixing of ancestral populations from Mississippian and Acadian refugia (results for
595 these collections can be viewed using the web browser) parallel those reached using mitochondrial
596 DNA (Danzmann et al. 1998). The geographic distribution of the Danzmann et al. (1998) sampling
597 sites was mostly in the northern part of the range, which limits direct comparison of their results with
598 ours. Building upon this work, Hall et al. (2002), examining mitochondrial RFLP variation in Brook
599 Trout from ten stream units in five drainages in Maryland, showed three major assemblages, two on
600 the east and one on the west of the Eastern Continental Drainage Divide. Drainage basins nested
601 within the two major drainage basins were the major units of population division, a finding

convergent with our microsatellite nuclear DNA-based results. Further, the inferences that we reached for Brook Trout using microsatellite markers parallel those for other salmonids assessed using mitochondrial markers (reviewed by Bernatchez and Wilson 1998). A range-wide study of Brook Trout mitochondrial genomes would help inform a phylogeographic assessment of the species' natural history, including more direct assessment of expansion from glacial refugia and subsequent secondary contact. Application of a molecular clock to DNA sequence variants would support estimation of times of divergence among lineages, in turn supporting interpretation of natural history events.

[C] *Southern lineage*.— Previous studies have considered southern Appalachian Brook Trout a distinct strain (e.g., Hayes et al. 1996; Galbreath et al. 2001) warranting taxonomic review (e.g., Habera and Moore 2005). We found patterns of population genetic structure of Brook Trout in the southern Appalachians are far more complex than a simple "northern" versus "southern" dichotomy. We did not find evidence for a crisp genetic break between putative northern and southern lineages at the New River watershed (Printz et al. 2018). Rather, we interpret the southern cluster as the descendants of fish radiating from a Pleistocene refugium in the Mississippi drainage that colonized much of North America west of the Eastern Continental Drainage Divide, with evidence of dispersal as far north as Pennsylvania and New York. Further, within the geographic distribution of this lineage, we noted a tremendous amount of fine-scale variation. Nearly all populations were genetically distinct, and populations within the same watershed commonly were very divergent. The Atlantic slope populations that cluster with interior basin populations in the southern region likely reflect expansion via past stream capture events. This explanation is supported by geological evidence indicating repeated shifts in the Eastern Continental Drainage Divide in this region (Gallen 2018, Johnson 2020).

Despite an extensive history of stocking domesticated conspecifics, many Brook Trout populations in the southern Appalachians show little evidence of hatchery introgression (this study; Printz et al. 2018; Pregler et al. 2018). Rather, the vast majority of populations retain genetic characteristics distinct from hatchery strains. However, a small number of populations were genetically similar to stocked hatchery strains, reflecting high levels of admixture or establishment of

631 the population by hatchery-origin individuals. This finding is consistent with those of Kazyak et al.
632 (2018), who used the same techniques to assess hatchery introgression across Brook Trout
633 populations in North Carolina (those populations are included in the present study), and with previous
634 studies across other portions of the southern native range (e.g., Virginia: Humston et al. 2012, Printz
635 et al. 2018; South Carolina: Pregler et al. 2018).
636
637 **<A> IMPLICATIONS FOR MANAGEMENT**
638
639 Our findings pose important implications for management. The American Fisheries Society
640 Southern Division Trout Committee developed a position statement (Habera and Moore 2005) to
641 advocate management approaches suitable for conserving southern Appalachian Brook Trout. After
642 expressing the importance of these fish and promoting comprehensive, region-wide management, its
643 recommendations addressed habitat protection and improvement, population restoration, stocking of
644 hatchery Brook Trout, and angling regulations. Our work constitutes the genetic inventory that was
645 called for in the position statement, and our results can inform management planning and
646 implementation, such as prioritizing protection of habitats supporting native gene pools or selecting
647 source and recipient populations for restoration or enhancement actions. The highest-level goal for
648 genetically based Brook Trout management would be to conserve native genetic variation and to
649 practice population restoration as needed to maintain each population's potential to adapt to
650 environmental change. Ultimately, genetically diverse populations representing endemic lineages are
651 critical to conserving our natural heritage in a changing world (Des Roches et al. 2021; Stange et al.
652 2021).
653
654 In light of our findings, managers may wish to review and update the management actions and
655 guidelines proposed by Habera and Moore (2005). Instead of simply viewing Brook Trout in a
656 "northern" versus "southern" context, our data indicate that substantial genetic differences are
657 widespread among Brook Trout collected from many different regions. Management strategies may
658 be most effective when they consider the substantial amount of fine-scale genetic variation that is
659 characteristic of the species and its evolutionary history.

One such approach would be to classify Brook Trout within the southern Appalachian Mountains as an evolutionarily significant unit, or ESU (Ryder 1986; Waples 1991; Nielsen and Powers 1995), while recognizing the substantial heterogeneity therein as management units (MUs). A population or assemblage of populations meets the criteria for an ESU if: (1) it has been reproductively isolated for long enough that it contains unique evolutionary combinations that are unlikely to re-evolve on an ecological timeframe, and (2) it is ecologically or adaptively distinct, that is, it contains genetic or phenotypic variation that is important for adaptive capacity to changing environmental conditions (Waples 1991). Our work and others' with selectively neutral microsatellite markers and that of other groups using allozyme and mitochondrial DNA markers (Stoneking et al. 1981; McCracken et al. 1993; Danzmann et al. 1998; Guffey et al. 1999; Printz et al. 2018) show that southern Appalachian Brook Trout are reproductively isolated from other conspecific units, even at very small spatial scales. Putatively, adaptive characters exhibited by southern Appalachian Brook Trout would include tolerance of relatively high temperatures, an adaptation that has yet to be assessed for populations across the distribution of the species, and small size and early age of maturity compared to Brook Trout of more northerly origin (Konopacky and Estes 1986; Habera et al. 2001; but note that some populations of Brook Trout in northern areas also are adapted for early maturity - Hutchings 1993). Further studies of local adaptation of Brook Trout populations would be critical to strengthen this line of inference.

Management units (MUs) ideally correspond with populations that are demographically independent from one another (Allendorf and Luikart 2007). Identification of MUs is critical for short-term management, such as managing habitat, setting harvest rates, and monitoring population status. Moritz (1994) suggested that MUs are populations that have substantially divergent allele frequencies at many loci; however, allele frequency differentiation cannot be interpreted directly as evidence for demographic independence (Allendorf and Luikart 2007). Palsboll et al. (2007) proposed that identification of MUs from population genetic data be based upon the amount of genetic divergence at which populations become demographically independent; that is, MU status would be assigned when the observed estimate of genetic divergence is significantly greater than a predefined

threshold value (Ramstad et al. 2004). Until the results of such studies are available, we offer that managers could use watersheds to delineate provisional management units, as our results suggest that a considerable amount of genetic variation is associated with watershed structure (Table 2) and these units are likely to be demographically independent. Our suggestion is convergent with those of Habera and Moore (2005) and other authors regarding use of river sub-basins and watersheds as management units for conserving genetic variation in Brook Trout.

Future Brook Trout translocations will have the goal of either re-establishing extirpated populations (hereafter, reintroduction) or elevating the probability of persistence of extant populations (hereafter, genetic rescue). Population extirpations have occurred in southeastern North America (Hudy et al. 2008), and managers often reintroduce Brook Trout (Pregler et al. 2018). In addition, our study revealed many extant populations with low genetic variation which may be potential candidates for genetic rescue. Genetic rescue focuses on small, isolated populations that may be suffering from the effects of inbreeding, and may increase genetic variation and adaptive potential (Hedrick et al. 2011; Whiteley et al. 2015). While some high-profile studies have shown positive fitness effects after translocations into target populations (e.g., Florida panther – Johnson et al. 2010; bighorn sheep – Hogg et al. 2006, Miller et al. 2012), others have not (e.g., gray wolf – Adams et al. 2011; but note this example was based on a single immigrant in a limited habitat). Examples of genetic rescue in fishes include guppy *Poecilia reticulata* (Zajitschek et al. 2009, Fitzpatrick et al. 2016) and Brook Trout populations in Virginia, where Robinson et al. (2017) found evidence of positive fitness effects through the F_1 generation. Wells et al. (2019) found little evidence of outbreeding depression in Brook Trout populations in Newfoundland; instead, hybridization effects were mostly neutral (60/66 non-hybrid vs. hybrid comparisons) with some support for heterosis (6/66). A growing body of evidence suggests genetic rescue may be beneficial, at least under certain circumstances (Frankham 2015).

Concerns about outbreeding depression have generally limited more widespread implementation of genetic rescue across all taxa (Ralls et al. 2018; Bell et al. 2019). Outbreeding depression is an important genetic concern for both reintroduction and genetic rescue (Whiteley et al.

2015; Ralls et al. 2018), as it can result in the disruption of locally adapted gene complexes such as those that are likely found in wild populations of Brook Trout throughout the southern Appalachians. Even single-source reintroductions carry this risk if gene flow out of reintroduced populations to other nearby natural populations occurs post-translocation. Our results suggest that donor populations should be chosen from within the same watershed to minimize the probability of outbreeding depression. Therefore, our results extend the recommendations of Habera and Moore (2005), who asserted that donor Brook Trout populations should have known genetic origins and that non-native Brook Trout donor populations should be avoided. Further, if single sources are preferred for reintroductions, it may be best to choose source populations with high genetic variation from similar environmental conditions to maximize matches in local adaptations (Kazyak et al. 2021). The number of translocated individuals should be sufficient to maintain genetic variation in both source and recipient populations. Malone et al. (2018) provide guidance for the number of individuals to target to match N_e in source and re-established populations along with a quantitative method to combine information based on habitat matching, genetic variation, genetic differentiation, and fish density to find suitable source populations. The 50:500 rule provides additional guidance for a minimal N_e to avoid concerns about inbreeding depression in either the source or recipient population (Jamieson and Allendorf 2012). An N_e below 50 corresponds to an increase in genome-wide homozygosity greater than 1% per generation and can be a warning of negative fitness effects of inbreeding. If there are demographic or genetic concerns about removal of adults from single source populations, multiple sources can be used. Interbreeding among individuals from multiple source populations, assuming a lack of assortative mating within the reintroduced population, will elevate genetic variation, but could induce outbreeding depression if interbreeding individuals are too genetically divergent (Huff et al. 2011). Finally, we note that there are additional concerns beyond genetics when moving individuals between populations, such as potential introduction of harmful parasites or microbes (Ruiz et al. 2017). Given the risks and uncertainty, we suggest that future Brook Trout translocations (reintroductions or genetic rescue) occur within an adaptive management framework (Robinson et al. 2017) with the goal of achieving a general understanding of the efficacy of these approaches for Brook Trout.

747 Captively reared individuals could serve as the source for either reintroduction or genetic
748 rescue efforts. However, caution is warranted when using captive fish for this purpose because recent
749 studies indicate that hatchery stocks propagated from wild broodfish have lower fitness than wild fish
750 (Araki et al. 2008; Christie et al. 2012a; Evans et al. 2015), lower reproductive success (Theriault et
751 al. 2011; Christie et al. 2012a), decreased allelic richness, higher linkage disequilibrium and levels of
752 genetic drift (Christie et al. 2012b), and often very unequal contributions among individual
753 broodstock (Beirão et al. 2019). Additionally, Le Luyer et al. (2017) identified epigenetic
754 modifications induced by captive rearing as a potential explanation for reduced fitness in hatchery-
755 reared salmon, suggesting a mechanism for trans-generational inheritance of these deleterious effects
756 on gene expression. Due to these concerns, we view the use of hatchery-reared individuals as less
757 preferable than wild individuals for translocation purposes. However, if hatchery individuals are to be
758 used, the use of local genetic source stocks (Olson et al. 2004; Cooper et al. 2010; Fisch et al. 2015;
759 Trushenski et al. 2015) should minimize outbreeding depression risks for reintroductions or genetic
760 rescue attempts. Ongoing work at the Tennessee Aquarium and Conservation Institute and Tellico
761 trout hatchery support the case that propagation of southern Appalachian Brook Trout is a viable
762 technique (Johnson 2016). To support reintroductions, a model of habitat variables determining the
763 suitability of streams for Brook Trout restoration has been developed (Romines 2017). Habera et al.
764 (2001) reported restoration of Brook Trout in 17 Tennessee streams, including extension of their
765 distribution in Sevier County by outplanting the progeny of wild Brook Trout propagated in the
766 Tennessee Wildlife Resources Agency's Tellico hatchery.
767
768 [C] *Caveats and Limitations*.— Although the present study is based on an unusually large genetic
769 dataset, we faced several limitations that could be addressed in future work. First, many of our
770 collections comprised fewer samples than are generally recommended. This reflects sampling of
771 many marginal populations with limited numbers of individuals as well as the reuse of tissue samples
772 that were collected for other purposes. We addressed this issue by restricting much of our analysis to
773 collections with at least 20 individuals. Although sample sizes of at least 25–30 (Hale et al. 2012)
774 have been recommended to provide a reasonable likelihood of observing rare alleles or haplotypes, it
775 can still be worthwhile to report genetic metrics for marginal populations with smaller sample sizes

(Pruett and Winker 2008). Our sampling intensity also varied among collections and among-regions. Uneven sampling is associated with a greater propensity to identify subdivision in more heavily sampled units using STRUCTURE (Peuchmaille 2016; but note that their simulations used far lower levels of differentiation among populations than generally observed within our study). However, the impacts of uneven sampling on DAPC have not been explored (Miller et al. 2020). Given that our sampling effort was more intense within the southern Appalachian Mountains, we may have had greater power to resolve structure within this region. Further sampling in northern areas may shed more light on the lineages present in that part of the range of Brook Trout. However, we note that our general findings were consistent among different analytical approaches and with hypotheses associated with glacial history. The high levels of differentiation observed in many areas likely moderated any impacts of uneven sampling. There were also differences in the length of stream from which the samples were collected. While most collections included multiple cohorts, some collections were restricted to only young-of-the-year. Future population genetics studies of Brook Trout would benefit from the adoption of consistent sampling guidelines that effectively support their goals, with target sample sizes based on guidelines for the class of marker that will be used. To obtain the best possible genetic characterization of a population, it should ideally be sampled along the entire length of its habitat patch and include members of all cohorts present.

[C] *Future directions for studies of genomics and local adaptation*.— We screened variations in microsatellite DNA, which are regarded as indicative of selectively neutral population genetic processes. Such markers are well suited for detecting the signatures of demographic events such as population expansions and contractions, gene flow, and introgression from hatchery-derived Brook Trout. Patterns of microsatellite variation are not, however, indicative of adaptive genetic variation within and between populations of Brook Trout. Fraser et al.'s (2014) examination of coding-gene polymorphisms associated with various biological functions in fragmented Newfoundland Brook Trout populations of varying sizes found that fragmentation affects natural selection and that population size affects adaptive changes and population differentiation. Ferchaud et al. (2020) identified genomic regions associated with anadromy in Canadian Brook Trout, as well as an overrepresentation of transposable elements associated with environmental variables, suggesting the

importance of transposable elements in adaptation. They also observed considerable accumulation of maladaptive mutations, which they associated with genetic drift. Wood et al. (2015) observed that population size was only weakly related to quantitative genetic variation and expression of 15 traits across nine Brook Trout populations, although large studies would be needed to reach strong conclusions. Brook Trout body size, shape, and coloration differences were most frequently and directly linked to habitat variation and operational sex ratio, rather than to population size (Zastavniouk et al. 2017), suggesting that selection may overcome drift at small population sizes and that selection may be acting more strongly on females than on males. Taken together these studies provide fresh insight into the role of genetic variation in adaptation and population resilience; however, there is still much to learn to enhance management outcomes.

Investigation of adaptive genetic variation has not yet been extended to Brook Trout populations across the range of the species. While the genetic basis of adaptation in Brook Trout remains largely unknown, further understanding of adaptive genetic variation would inform management of populations to conserve their long-term adaptive potential. Future research may utilize next-generation genomics technologies to further investigate how the adaptive potential of Brook Trout varies among populations, and to identify putatively resilient populations and management practices that optimize the evolutionary potential for the species. The development of a standardized single nucleotide polymorphism (SNP) panel suitable for reduced representation sequencing would allow range-wide marker comparisons in a similar manner as presented here for microsatellites.

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1486 <A> **TABLES**

Table 1. Pairwise differentiation (F'_{ST}) between populations, summarized within and among the three genetic clusters identified by DAPC (K = 3) and the domestic hatchery collections.

Category	Groups	Pairwise comparisons	Mean F'_{ST}	Minimum F'_{ST}	Maximum F'_{ST}
Wild-type	Northern & Northern	1081	0.478	0.040	0.812
	Mid-Latitude & Northern	7379	0.728	0.201	0.977
	Northern & Southern	17343	0.793	0.289	0.984
	Mid-Latitude & Mid-Latitude	12246	0.666	-0.004	0.996
	Mid-Latitude & Southern	57933	0.796	0.293	0.992
	Southern & Southern	67896	0.722	-0.010	0.998
Comparisons with introgressed populations	Northern & Northern (Introgression)	1974	0.537	0.108	0.905
	Mid-Latitude & Northern (Introgression)	6594	0.703	0.179	0.983
	Northern (Introgression) & Southern	15498	0.764	0.022	0.994
	Northern (Introgression) & Northern (Introgression)	861	0.530	0.007	0.952
Comparisons with domestic lineages	Northern & Hatchery	799	0.924	0.843	0.963
	Mid-Latitude & Hatchery	2669	0.942	0.843	0.998
	Southern & Hatchery	6273	0.935	0.828	0.981
	Northern (Introgression) & Hatchery	714	0.922	0.840	0.975
	Hatchery & Hatchery	136	0.224	-0.015	0.424

Table 2. Hierarchical analysis of molecular variance (AMOVA) for 612 populations of wild Brook Trout. Variance at five strata was assessed, including six, eight, ten, and twelve-digit USGS hydrologic units (HUCs) and collections of Brook Trout.

Hierarchical level	Sum of squared differences	Variance explained
Among HUC6s	32939443	30.1%
Among HUC8s within HUC6s	13642363	12.5%
Among HUC10s within HUC8s	16172066	14.8%
Among HUC12s within HUC10s	10459602	9.6%
Among populations within HUC12s	14244488	13.0%
Among individuals within populations	22029914	20.1%
Total	109487876	100.0%

Table 3. Proportion of individuals correctly assigned to various geographic units with GENECLASS2 using the criterion of Rannala and Mountain (1997). Only collections that fell within an existing Eastern Brook Trout Joint Venture patch (coverage restricted to eastern United States) were considered for this analysis.

Assignment unit	Correct	Total	Percentage
Collection	14282	16371	87.2%
EBTJV Patch	15494	16371	94.6%
HUC12	15729	16371	96.1%
HUC10	15955	16371	97.5%
HUC8	16070	16371	98.2%
HUC6	16122	16371	98.5%

<A> **FIGURE CAPTIONS**

Figure 1. Sampling locations (red dots) for 836 collections representing 22,020 wild Brook Trout from across their native range. Geographic coverage extended from Georgia northwards to Quebec and from Newfoundland westward to Iowa, representing much of the native range of the species. The Eastern Continental Drainage Divide is shown with a heavy gray line. The New River watershed, which has previously been suggested as a key transition area, is shaded in yellow.

Figure 2. Three measures of within-population diversity estimated for wild Brook Trout populations in the eastern United States: (A) mean rarefied allelic richness per locus, (B) unbiased expected heterozygosity, and (C) effective population size. Samples outside of the eastern United States were truncated for visual purposes but were included in the analysis and can be viewed with the online viewer (http://bte.ecosheds.org/). The inset panel shows metrics for each of the hatchery collections.

Figure 3. Observed variation in allelic richness and effective population size across a latitudinal gradient. Points are color-coded by clusters identified with discriminant analysis of principal components ($K = 3$) and represent collections with ≥ 20 samples. For the purposes of this visualization, collections in Cluster 2 which were found in south of the Maryland-Pennsylvania border were considered to reflect hatchery introgression.

Figure 4. Relationships between rarefied allelic richness, expected heterozygosity, effective population size, and mean F'_{ST}. Points are color-coded using clustering results ($K = 3$, distribution of each cluster shown) from discriminant analysis of principal components. Samples outside of the eastern United States were truncated in panel A for visual purposes but were included in the analysis and can be viewed with the online viewer. Only collections with ≥ 20 samples are shown. For the purposes of this visualization in the scatterplots, collections in Cluster 2 which were found in south of the Maryland-Pennsylvania border were considered to reflect hatchery introgression.

Figure 5. Geographic distribution of DAPC-based population-level assignment to $K = 2$, 3, 4, or 5 clusters of multilocus genotypes. The continental divide is shown with a red line. To observe DAPC-based population assignments at finer scale or for populations farther north or west, visit https://bte.ecosheds.orgt/ and using the pull-down menu, select the DAPC data layers. Samples outside of the eastern United States were truncated for visual purposes but were included in the analysis and can be viewed with the online viewer.

<A> **FIGURES**

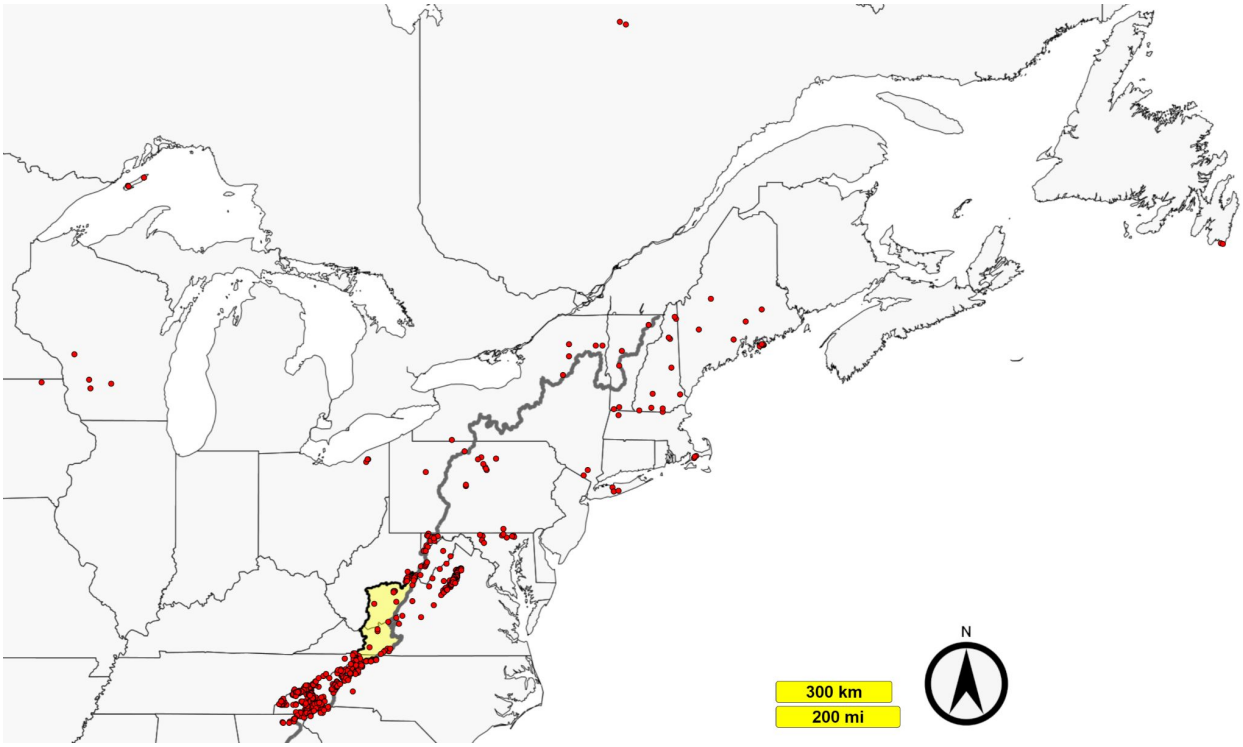


Figure 1

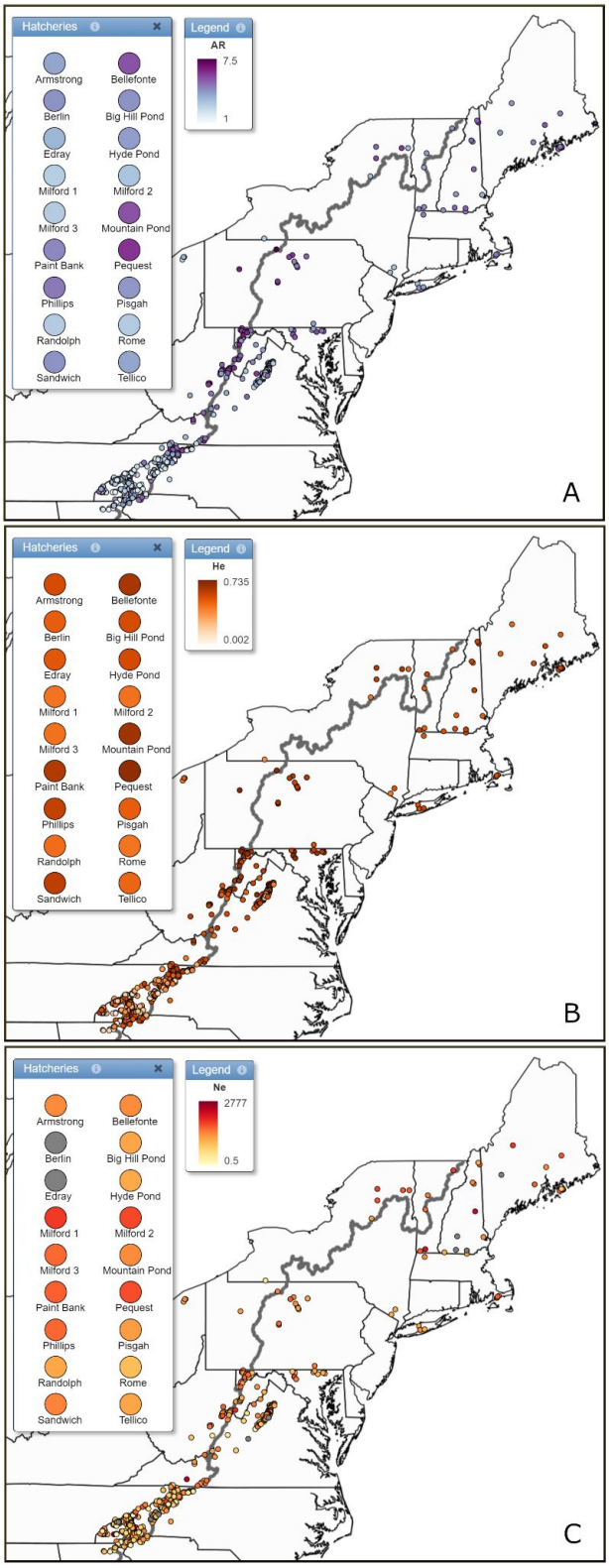


Figure 2

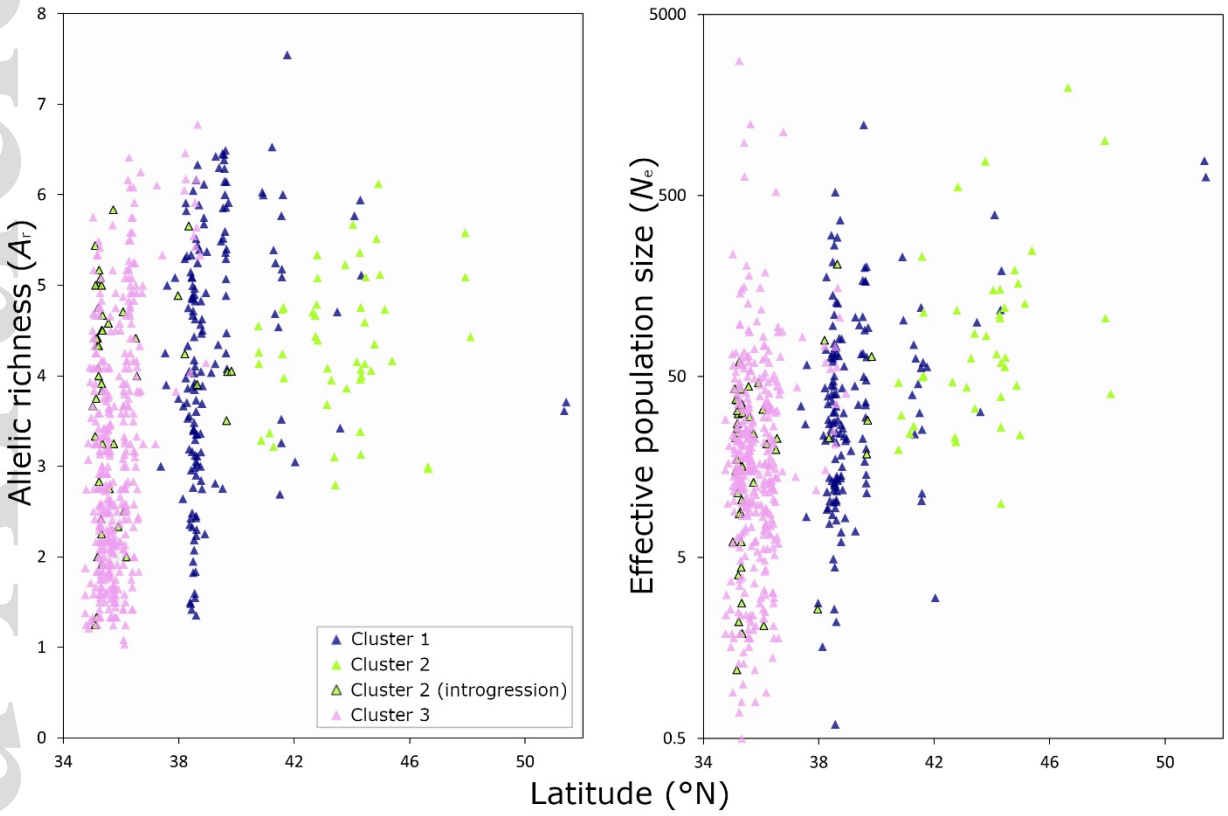
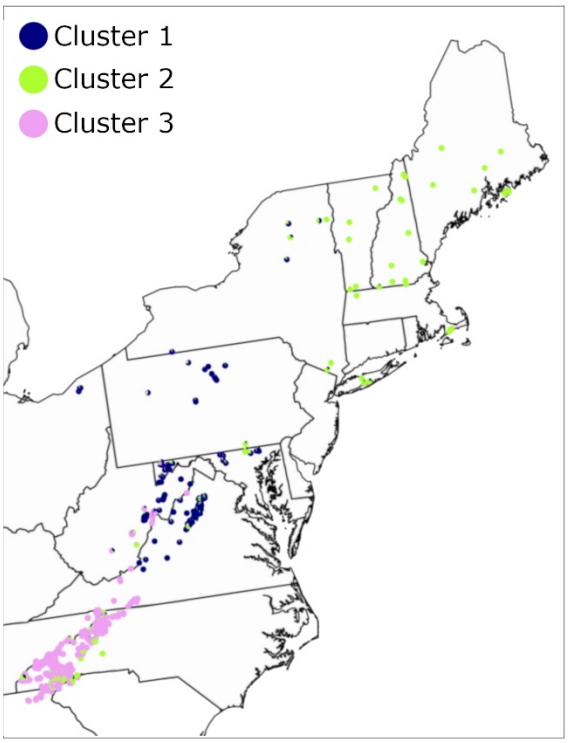
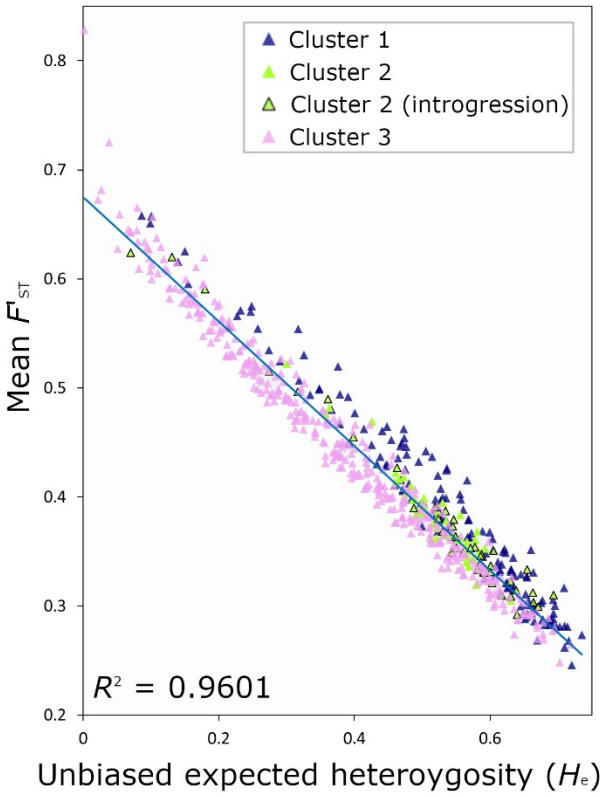
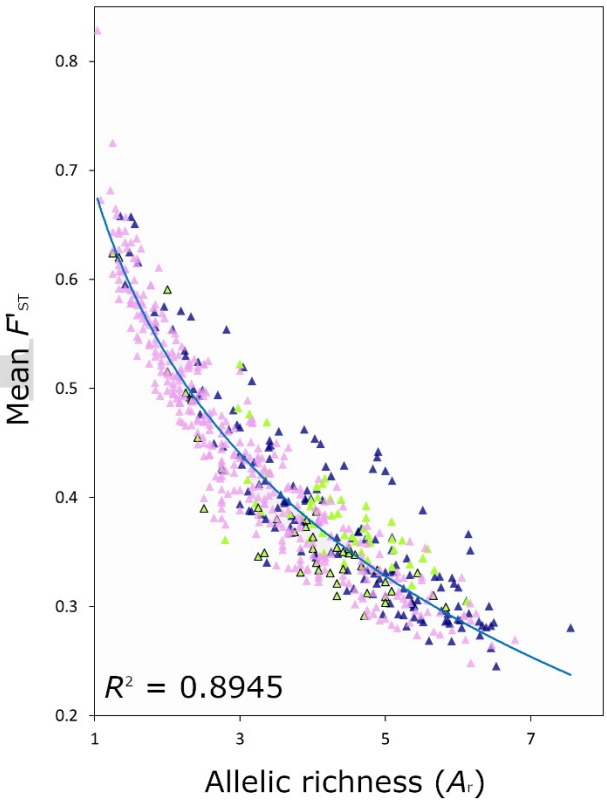


Figure 3







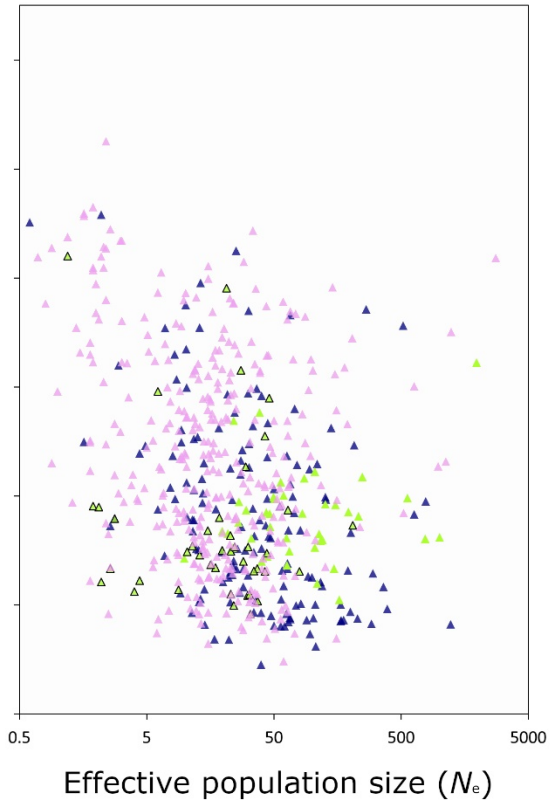


Figure 4

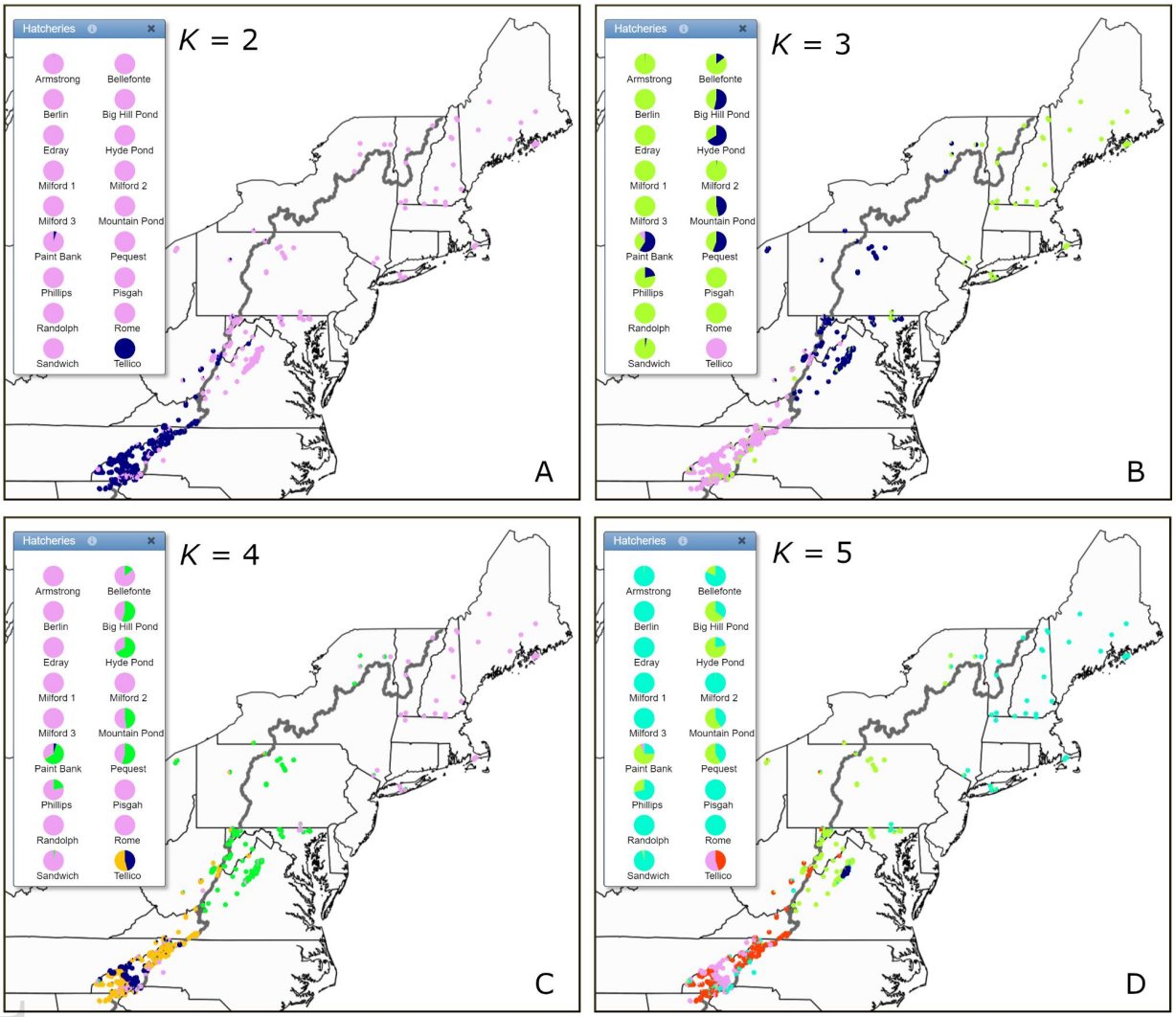


Figure 5