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ARTICLE

Neutral Genetic and Phenotypic Variation within and among Isolated Headwater Populations of Brook Trout

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Abstract

Isolated populations are challenging to manage and conserve, as they are particularly vulnerable to genetic drift, allelic fixation, and inbreeding and may express markedly reduced phenotypic variability. We sought to improve our understanding of how spatial isolation, occupancy range, and restricted gene flow influence contemporary phenotypic variation within and among native populations of Brook Trout *Salvelinus fontinalis* by examining the neutral genetic and phenotypic characteristics of 35 isolated headwater populations from Great Smoky Mountains National Park. Across a suite of 13 neutral microsatellite loci, we observed high levels of allelic fixation and considerable genetic differentiation among populations, subwatersheds, and watersheds that were consistent with patterns of isolation. We found significant positive correlations between allelic diversity and estimates of effective population size. In contrast, we observed considerably less phenotypic structure among streams, subwatersheds, and watersheds. Much of the

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observed phenotypic variation occurred among individuals within populations. Pairwise Mann–Whitney tests revealed no significant phenotypic differences among the populations of Brook Trout we examined. Similarly, there was no significant relationship between the amount of phenotypic variation within populations and any of the examined measures of genetic diversity or the amount of occupied habitat sampled, which suggests that unmeasured variables may be influencing morphometric and meristic variation within isolated populations. The observed patterns of isolation, genetic drift, and allelic fixation highlight the importance of enhancing population connectivity but also suggest that considerable phenotypic variability may persist within small, fragmented populations. Our results elucidate some challenges associated with managing and conserving isolated populations of Brook Trout and reinforce the importance of conducting genetic studies on fragmented populations to inform management decisions.

Brook Trout *Salvelinus fontinalis* inhabit thousands of habitat patches scattered across the southern half of the Appalachian Mountains (EBTJV 2016). Anthropogenic stressors, such as land use and development, acid deposition (Robinson et al. 2008; Neff et al. 2009; Fakhraei et al. 2016), and the introduction of exotic salmonids (McCracken et al. 1993; Kanno et al. 2017), have restricted Brook Trout to headwater tributaries in watersheds that are thought to have formerly harbored metapopulations. The overwhelming majority of these extant populations are restricted to small, isolated stream reaches with limited carrying capacity and little to no opportunity for genetic exchange with neighboring populations (see Whiteley et al. 2013). Furthermore, climate change projections suggest that harsher environmental conditions may become more prevalent across much of the native range of Brook Trout, further restricting connectivity among geographically proximate populations (DeWeber and Wagner 2015).

The conservation of many small, fragmented populations across broad landscapes presents significant challenges for fisheries managers. Small, isolated populations are especially sensitive to stochastic events, such as flood, drought, and disease and are therefore more likely to become extirpated. The effects of isolation and fragmentation (e.g., novel competitive interactions, decreased body size, and mate pairing success) on population demographics are mixed among taxa (Wilcox and Murphy 1985; McGarigal and Cushman 2002; Lampila et al. 2005; Letcher et al. 2007) but are not well documented for fishes. Even beyond demographic considerations, such populations are at risk for losses of genetic diversity that place them in further jeopardy (Frankham 2005; Frankham et al. 2017). Understanding how small populations have been affected by isolation is important for conservation planning, as demographically limited populations are expected to experience greater rates of inbreeding, genetic drift, and allelic fixation, which could ultimately impact the effectiveness of natural selection and potentially reduce the genetic resources available for coping with future environmental changes (Ralls et al. 2017).

Genetic diversity is the foundation of heritable phenotypic diversity (Conner and Via 1993). In the absence of migration or selection, theory suggests that genetic variation will be eroded—particularly in small, isolated populations affected by drift, fixation, and inbreeding (Allendorf et al. 2013). Moreover, genetic and demographic declines often precede the loss of phenotypic diversity (Allendorf et al. 2013), which, when observed, may suggest deeper losses of evolutionary potential. Isolated populations may also exhibit locally adapted traits and life history variations (Conner and Via 1993; Hutchings 1996; Ghalambor et al. 2007), but these responses are not well understood. For example, Wood et al. (2015, 2016) provided evidence to suggest that coastal populations of Brook Trout from Newfoundland and Labrador, Canada, are capable of maintaining phenotypic diversity despite variable population sizes, spatial fragmentation, and isolation; those authors concluded that their observations might have been due in part to habitat variation among collection sites. Although gene flow may slow the rate of genetic drift and allelic fixation, many populations are completely isolated and, in the absence of active management (i.e., population/habitat restoration, nonnative species removals, barrier removals, etc.), have limited opportunity for connectivity. As a result, the future prospects for small, isolated headwater populations of Brook Trout across southern Appalachia remain uncertain.

To address these challenges, managers have been encouraged to improve connectivity among existing populations, re-establish populations that have been extirpated, or rescue populations that have been deemed imperiled. As such, managers have invested considerable resources into the removal of migration barriers (e.g., culverts, dams, and nonnative species) between formerly isolated demes, thus providing the necessary pathway for increased dispersion, recolonization, connectivity, and migration success (Petty and Merriam 2012). In some cases, however, isolated populations may have developed unique adaptations, and certain conservation efforts (e.g., translocations and genetic rescue) may result in unforeseen obstacles (i.e., outbreeding depression; Houde et al. 2011, 2015; Orsini et al. 2013). There is a growing body of literature suggesting that imperiled populations more often experience benefits (i.e., increased genetic, phenotypic, and demographic diversity) rather than negative impacts (Ralls et al. 2017; Robinson et al. 2017). Nonetheless, managers

need to balance the risks associated with such conservation efforts against continued losses of genetic diversity, range contractions, and future evolutionary potential to ensure the preservation of imperiled populations.

For this study, we sought to improve our understanding of how spatial isolation, habitat fragmentation, and restricted gene flow might shape contemporary phenotypic (morphologic and meristic) differentiation within and among 35 endemic populations of Brook Trout in Great Smoky Mountains National Park (GRSM). We predicted that isolation has resulted in broad neutral genetic and phenotypic differentiation among such populations and that diverse populations with larger effective population sizes (N_e) would stem from larger occupied habitat reaches. Our specific objectives were to (1) characterize neutral genetic and phenotypic (morphology and meristics) variation of Brook Trout within and among streams, subwatersheds, and watersheds; (2) evaluate whether morphology and meristics—fundamental expressions of phenotype—are conserved among streams, subwatersheds, and watersheds, despite isolation and neutral genetic drift; and (3) assess whether reduced phenotypic diversity within and among populations is associated with patterns of isolation, genetic drift, and/or the length of reach sampled.

METHODS

Study area and sample collection.— Great Smoky Mountains National Park covers an area of over 2,100 km^2 and represents an important stronghold for Brook Trout in the southern Appalachian Mountains. Even in the cool, montane waters of GRSM, historical logging, stream acidification, and the introduction of nonnative Rainbow Trout *Oncorhynchus mykiss* and Brown Trout *Salmo trutta* have combined to extirpate Brook Trout from an estimated 75–80% of historically occupied streams (Larson and Moore 1985). These perturbations have largely restricted modern populations of Brook Trout to disjunct headwater reaches, presumably isolating formerly interconnected populations.

Our study included 35 small (National Hydrography Dataset, 1:24,000 scale: first through third order; Strahler 1957) headwater streams with a mean elevation of 1,044 m (range = 800–1,417 m) and a mean gradient of 13% (range = 2–41%), located within three GRSM watersheds (hydrologic unit code 6): French Broad–Pigeon River (n = 19), Little Tennessee River (n = 14), and Little River (n = 2). Euclidean distance between sampled streams averaged 25.80 km (range = 0.06–69.49 km). Historical logging, settlement, fires, and acid deposition have impacted the study streams to varying degrees. The specific effects of such anthropogenic perturbations upon Brook Trout are well documented (see Marschall and Crowder 1996; Argent and Flebbe 1999; Nislow and Lowe 2006) and generally result in population declines, range contraction, and, in some cases, extirpation (Larson and Moore 1985). Historical records indicated that 37,295 hatchery-reared Brook Trout (of unknown strain; data solicited from D. C. Booth National Fish Hatchery and GRSM Archives, Spearfish, South Dakota) were stocked into six of our sampled streams between 1936 and 1973; precise stocking localities were not reported. However, contemporary molecular data, collected herein, showed little to no signature of genetic hatchery introgression by northern stocks (Kazyak et al. 2018; D. C. Kazyak, personal observation). The only exception occurred within one sampled stream (Walker Camp Prong), where limited hatchery introgression appeared to have occurred, yet microsatellite genotypes were still largely consistent with those expected of Brook Trout native to the southern Appalachians.

We collected mixed-adult/mixed-cohort size-classes (81–169 mm SL) of mature Brook Trout from 35 spatially isolated headwater streams via single-pass electrofishing during summer sampling periods (June–August). Brook Trout generally spawn from late September through mid-November (Kulp et al. 2017) in GRSM; thus, all collections were conducted prior to the onset of spawning. We attempted to collect 30 individuals (n = 10 for phenotype and genotype samples; n = 20 for genotype-only samples) across a majority of available allopatric Brook Trout habitat from each sampled stream. Our sample sizes were similar to those used in other Brook Trout population genetic studies (see Ruzzante et al. 2016; Timm et al. 2016; Nathan et al. 2017) and phenotype investigations (see Rouleau et al. 2010; Stauffer and King 2015). We obtained individual genetic tissue (i.e., adipose fin clips) and whole-body phenotype specimens across each sampled stream's available habitat (mean allopatric stream distance occupied = 1,706 m; range = 663–4,434 m), thus minimizing potential familial overrepresentation bias (Luikart et al. 2010) within each collection. Overall, we sampled a majority (67.8%; 17.22 of 25.40 km) of the available habitat occupied by Brook Trout within our study streams; however, sampled stream reach lengths did vary among collections (mean stream distance sampled = 522 m; range = 200–1,213 m). Sex of fish used as phenotype samples was determined via rapid visual assessment (Kazyak et al. 2013). We observed a near 1:1 sex ratio (females, 49%; males, 51%) among all collections, and our assessments were confirmed via necropsy on a subset of collections (e.g., French Broad–Pigeon River watershed collections). Upon collection, an adipose fin clip was taken from each fish (excluding a single phenotype type specimen from each stream), placed in 95% ethanol, and chilled to allow fixation of DNA. Brook Trout that were only sampled for adipose fin clips were immediately released.

For morphometric and meristic purposes, 10 fish were immediately euthanized using a 250-mg/L concentration of tricaine methanesulfonate (MS-222) for a period of 10 min after cessation of gill movement. Numbered tags were inserted under the right operculum of each phenotype specimen and accompanied corresponding tissue samples. Euthanized Brook Trout were pinned to dissection trays and bathed in a 1% formalin solution for a period of 15 min to assure fin rigidity and enable accurate meristic counts. All fin-rigid specimens were then placed into a 1-L jar filled with 10% formalin and stored at room temperature for a period of 3 weeks to ensure total body preservation. After whole-specimen preservation for phenotype analysis, specimens were washed of formalin, transferred into 70% ethanol, and considered safe for handling and long-term storage.

Genotype methods.— Using a protocol optimized for Brook Trout, we extracted and isolated DNA from small (<25 mg) individual fin clips (n = 1,080). Total DNA yields from isolations were quantified using a NanoDrop 8000 spectrophotometer. Isolated DNA was then diluted to a template standard concentration (25 ng/μL) in preparation for multiplexed PCR amplification and genotyping at 13 microsatellite loci (King et al. 2012). Amplified PCR product fragment analysis was conducted on an Applied Biosystems 3130xl genetic analyzer, and we conducted allele scoring, binning, and multilocus genotyping by using GeneMapper version 5.0 (Applied Biosystems).

COLONY version 2.0 (Jones and Wang 2010) was used to identify family structure within each individual stream collection. Full-sibling individuals comprising familial groups were identified based on the probability ($P > 0.95$) of familial inclusion. In instances where full-sibling families were detected within a collection, we randomly selected and retained only one individual representative of the full-sibling family for later analyses—with the exception being estimates of mixed-cohort N_e (see Luikart et al. 2010)—thus preventing bias from familial overrepresentation in downstream analytics (e.g., population assignment and diversity analyses; Whiteley et al. 2013; Aunins et al. 2015). We obtained estimates of N_e from each mixed-cohort (Luikart et al. 2010; Waples et al. 2014) collection by using the single-sample linkage disequilibrium (LD) method in LDNe version 1.31 (Waples and Do 2008). Specific settings to estimate N_e included a monogamous mating model (Coombs 2010) under the LD method (Waples 2006), with no allele frequency cutoff (observed mean minimum allele frequency = 0.27), and the jackknife method option was selected to obtain 95% confidence intervals.

We used STRUCTURE version 2.3.1 (Pritchard et al. 2000; Falush et al. 2003; Rodríguez-Ramilo and Wang 2012) to conduct a hierarchical examination of population clustering (Evanno et al. 2005; Janes et al. 2017). All STRUCTURE runs (with admixture; 10 replicates per number of clusters K) included an initial burn-in of 500,000 steps, followed by an additional 500,000 steps for data collection. Pritchard et al. (2000) noted that the optimal value of K is often a subjective approximation—one in which careful biological consideration must be applied when interpreting STRUCTURE results (Gilbert et al. 2012). Therefore, we examined log likelihood ($\log_e \Pr[X|K]$; Falush et al. 2003) and ΔK estimates (Evanno et al. 2005) to identify all primary (i.e., uppermost) and subsequent nested population clusters using CLUMPAK (Kopelman et al. 2015). In instances where population clustering was detected among sample units, we re-ran individuals from each inferred cluster separately until a final K equal to 1 was observed, and we summarized individual population cluster membership coefficients (Q) across replicate runs using CLUMPAK. Thus, STRUCTURE analyses were generally conducted via the hierarchical analyses approach outlined by Vähä et al. (2007).

Using GENEPOP version 4.2.1 (Raymond and Rousset 1995; Rousset 2008), we evaluated conformance to Hardy–Weinberg equilibrium (HWE; significant where $P < 0.0038$, $\alpha = 0.05$) and LD across all pairs of loci (significant where $P < 0.0001$, $\alpha = 0.05$) based on a Bonferroni-adjusted critical P-value (Allendorf et al. 2013) to determine whether collections appeared to represent single, interbreeding populations. Next, we calculated the following diversity statistics for each collection: allelic richness (A_R) and the inbreeding coefficient (F_{IS}) were obtained using FSTAT version 2.9.3 (Goudet 2001); and unbiased expected heterozygosity (uH_E) and observed heterozygosity (H_O) were obtained using GenAlEx version 6.5 (Peakall and Smouse 2012). We also calculated population differentiation (F'_{ST}; Bird et al. 2011; Meirmans and Hedrick 2011), allelic fixation (F_{ST}; Wright 1949), and private allele presence and frequency in GenAlEx version 6.5. We assessed genetic variation within and among populations, subwatersheds, and watersheds by using a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) that was implemented within the "pegas" package (Paradis 2010) in R version 3.2.2 (R Core Team 2015).

We conducted a series of pairwise population Mantel tests to measure whether a relationship occurred between genetic fixation and/or differentiation and spatial (i.e., Euclidean) distance separating population clusters. We carried out Mantel tests using the "adegenet" package (Jombart and Collins 2015) in R version 3.2.2, corrected for multiple comparisons, and considered significant ($P < 0.05$) relationships to be a result of processes affiliated with isolation by distance (IBD; Wright 1943). Lastly, we used BOTTLENECK version 1.2.02 (Piry et al. 1999) with the parameters described by Whiteley et al. (2010) to assess whether each of our sampled populations

had experienced recent detectable losses of genetic diversity and expressed heterozygotic excesses.

Phenotype methods.— We collected morphometric data based on a landmark-oriented truss network (Supplementary Figure S.1 available in the online version of this manuscript). We used 21 anatomical landmarks as caliper anchor points to collect 23 body measurements (mm; to the nearest hundredths; Supplementary Table S.1) of Brook Trout. We also collected meristic character counts from 10 external features of Brook Trout (Table S.2). We collected all morphological measurements and meristic counts from the left side of each specimen.

To assess morphological variation among Brook Trout populations, we conducted truss-network-sheared (McCoy et al. 2006) morphometric principal components analysis (PCA) based on a variance–covariance matrix (Stauffer and King 2015) in PAST version 3.12 (Hammer et al. 2001). Use of this approach generated a succession of ranked orthogonal axes (i.e., principal components [PCs]) that explained continuous morphometric variation (Rohlf 1993), in which the first PC of morphology ($morphPC_1$) was expected to reflect variation in body size. In addition, meristic PCA (Stauffer and King 2015) was conducted in PAST version 3.12 based on a correlation matrix (independent of size and shape; Turan et al. 2006). We examined patterns of phenotypic variability independently of body size; consequently, we retained scores from morphometric PCs 2–4 ($morphPC_{2–4}$) and meristic PCs 1–3 ($merPC_{1–3}$) for all subsequent analyses.

To evaluate how variation in morphology (i.e., PC scores) of Brook Trout was partitioned across spatial scales (e.g., streams, subwatersheds, and watersheds), we fitted a hierarchical, random-effects (spatial scale) linear model to each retained PC by using the "lme4" package (Bates et al. 2015) in R version 3.2.2 (R Core Team 2015). We used a global multivariate ANOVA (MANOVA) to examine whether there were significant differences in phenotypic characters ($morphPC_{2–4}$ and $merPC_{1–3}$) among populations. If the global MANOVA generated a statistically significant ($P < 0.05$) result, we conducted ANOVA tests on each retained morphometric and meristic PC independently. Two different post hoc tests were used to conduct multiple comparisons among populations for each retained PC (Duncan's multiple range test [MRT] using least-squares mean PCs; and Mann–Whitney tests using PC medians). We used a Holm's sequential Bonferroni-corrected critical *P*-value to determine significance (Allendorf et al. 2013) for both post hoc analyses. Duncan's MRT results are prone to type I errors when variance is heterogeneous (Duncan 1955); therefore, these tests are expected to provide an anticonservative view of differences among populations. We conducted pairwise population Duncan's MRT analyses using the packages "lsmeans" (Lenth 2016) and "multcompView" (Graves et al. 2015) in R version 3.2.2 (R Core Team 2015). We then generated box plots to illustrate each population's distribution of morphometric ($morphPC_{2–4}$) and meristic ($merPC_{1–3}$) variance. Box plots were arranged according to Duncan's MRT groupings and partitioned according to watershed (i.e., uppermost level of STRUCTURE genetic clustering). To provide an alternative perspective, we ran the same comparisons using nonparametric Mann–Whitney tests. Mann–Whitney tests are robust to differences in variance but have less power to detect significant differences where they occur and are therefore expected to provide conservative results. Mann–Whitney tests were run in PAST version 3.12 (Hammer et al. 2001).

We performed Pearson's product-moment correlation analyses comparing the SDs of morphometric and meristic PC scores within each population against key population genetic diversity indices (e.g., N_e, A_R, H_O, uH_E, and F_{IS}) and sampled stream reach distances in order to assess whether isolated populations with less diversity exhibited reduced phenotypic and neutral genetic variation. Using PAST, we calculated Gower coefficient scores (Gower 1971) based on each population's mean retained morphometric and meristic PCs to quantify the amount of phenotypic differentiation among populations. We then ran a series of Mantel tests to assess whether a significant relationship existed between phenotypic differentiation (i.e., Gower coefficient scores) and the geographic distance among populations, genetic fixation (F_{ST}), or genetic differentiation (F'_{ST}). Correlation analyses and Mantel tests were conducted using the "adegenet" package (Jombart and Collins 2015) in R version 3.2.2. In each of the two analyses above, we considered relationships to be significant when *P*-values were less than 0.05.

RESULTS

Neutral Genetics

We successfully obtained multilocus genotypes of Brook Trout from 94.3% (985 of 1,080) of all tissue samples collected. Two collections (Enloe Branch and the Right Fork of Raven Fork) representing 60 individuals were removed from further analysis, as we were unable to obtain usable genotype data. Independent collection sibship analysis in COLONY indicated the presence of 43 full-sibling families consisting of 113 total individuals among 24 of the sampled streams. After accounting for the removal of 70 full-sibling individuals, the genetic data set consisted of 915 Brook Trout families (Table 1). Overall, the proportion of retained families for genetic analyses (i.e., unique, single-sibling families analyzed per sample reach) was consistently low (mean ± SD = 0.28 single-sibling families/100 m) across collections.

Hierarchical STRUCTURE analyses indicated that the uppermost level of population structure was represented

by two clusters (i.e., $\Delta K = 2$; Figure 1). The first cluster contained all collections from the Little Tennessee River watershed, and the second cluster contained most of the collections from the French Broad–Pigeon River and Little River watersheds (Figure 1). A subsequent STRUCTURE run on the second cluster further partitioned those collections into two distinct subgroups ($\Delta K = 2$), which reflected the French Broad–Pigeon River and Little River watersheds. Subsequent hierarchical STRUCTURE runs differentiated nearly all stream collections as unique population units ($K = 29$; Figure 1). In general, the observed hierarchical population structure corresponded well to stream topology. However, STRUCTURE identified two populations (Buck Fork and Chapman Prong) that occur within the East Prong of the Pigeon River subwatershed (French Broad–Pigeon River watershed) but whose genetics (i.e., STRUCTURE *Q*-scores) closely resembled those of Brook Trout within the Bradley Fork subwatershed (Little Tennessee River watershed; Figure 1). The discrepancy observed between the geographic sampling locations and the clustering results for Buck Fork and Chapman Prong suggests that either ancient stream capture(s) or undocumented fish transfers have occurred.

TABLE 1. Genotyped Brook Trout collections ($n = 33$) obtained across three watersheds (WSs; French Broad–Pigeon River [FB-R], Little Tennessee River [TN], and Little River [LR]) and distance (m) of available habitat sampled (Reach) in Great Smoky Mountains National Park. Sample size after full-sibling removal (Families), mean allelic richness (A_R), observed heterozygosity (H_O), unbiased expected heterozygosity (uH_E), proportion of loci conforming to Hardy–Weinberg equilibrium (HWE), proportion of locus pairs in significant ($P < 0.0038$) linkage disequilibrium (LD), inbreeding coefficient (F_{IS}), and mixed-cohort effective population size (N_e) estimates are provided (CI = confidence interval).

Stream (acronym)	WS	Reach (m)	Families	A_R	H_O	uH_E	HWE	LD	F_{IS}	N_e (95% CI)
Bear Branch (BB)	FB-R	286	26	2.66	0.35	0.36	10/10	0/78	0.02	66 (28–1,918)
Buck Fork (BF)	FB-R	397	30	2.40	0.38	0.38	9/11	0/78	0.02	48 (22–219)
Caldwell Fork (CF)	FB-R	365	27	3.46	0.43	0.44	13/13	0/78	0.02	49 (30–95)
Chapman Prong (CpP)	FB-R	470	28	2.29	0.32	0.32	10/11	0/78	0.02	30 (17–58)
Correll Branch (CB)	FB-R	710	34	1.97	0.20	0.20	7/7	0/78	0.01	46 (15–568)
Horse Creek (HC)	FB-R	330	26	2.47	0.30	0.31	9/9	0/78	0.02	
Hurricane Creek (HrC)	FB-R	818	26	3.02	0.38	0.39	10/12	1/78	0.02	5 (4–8)
Kephart Branch (KB)	FB-R	207	30	3.00	0.38	0.39	13/13	0/78	0.02	70 (40–168)
Little Cataloochee (LCC)	FB-R	752	29	2.12	0.23	0.24	10/10	1/78	0.02	19 (10–32)
McKee Branch (MkB)	FB-R	957	29	3.90	0.49	0.49	11/12	1/78	0.02	76 (46–174)
Messer Fork (MF)	FB-R	1,213	26	3.29	0.47	0.48	12/12	0/78	0.02	62 (37–133)
Onion Bed (OB)	FB-R	699	28	1.92	0.25	0.26	8/9	0/78	0.02	29 (11–178)
Pretty Hollow (Ph)	FB-R	564	25	2.11	0.24	0.25	10/10	0/78	0.02	25 (12–64)
Road Prong (RP)	FB-R	675	41	2.16	0.27	0.27	12/12	0/78	0.01	64 (33–179)
Rock Creek (RC)	FB-R	378	24	1.71	0.18	0.18	6/6	1/78	0.02	4 (2–13)
Sag Branch (SB)	FB-R	438	19	2.93	0.37	0.38	11/11	0/78	0.03	9 (5–13)
Straight Creek (SC)	FB-R	380	30	3.51	0.45	0.45	11/11	0/78	0.02	53 (33–106)
Walker Camp Prong (WCP)	FB-R	585	30	3.29	0.54	0.55	12/12	0/78	0.02	28 (19–42)
Winding Stair (WS)	FB-R	1,002	27	1.95	0.20	0.20	8/8	0/78	0.02	224 (28–∞)
Indian Flats Prong (IFP)	LR	709	9	1.08	0.03	0.04	1/1	0/78	0.06	
Marks Creek (MC)	LR	449	25	1.37	0.10	0.10	5/5	0/78	0.02	147 (1–∞)
Bearwallow Branch (BwB)	TN	403	30	3.10	0.39	0.40	9/11	0/78	0.02	22 (15–32)
Chasm Prong (CP)	TN	451	29	4.07	0.53	0.54	12/12	0/78	0.02	144 (74–790)
Cold Springs Branch (CSB)	TN	981	29	1.60	0.24	0.24	6/7	0/78	0.02	272 (19–∞)
Defeat Branch (DfB)	TN	466	30	3.12	0.47	0.48	9/9	1/78	0.02	23 (15–35)
Desolation Branch (DsB)	TN	251	29	3.38	0.44	0.45	9/9	0/78	0.02	74 (42–191)
Gulf Prong (GP)	TN	200	29	3.90	0.52	0.53	12/12	0/78	0.02	148 (74–1,013)
Haw Gap Branch (HGB)	TN	408	29	2.62	0.37	0.38	9/9	0/78	0.02	102 (39–∞)
Hyatt Creek (HyC)	TN	357	27	2.66	0.34	0.34	13/13	0/78	0.02	37 (21–80)
Louie Camp Branch (LCB)	TN	293	28	3.08	0.46	0.47	11/12	0/78	0.02	39 (25–71)
Noland Creek (NC)	TN	376	28	1.74	0.20	0.21	5/6	0/78	0.02	19 (7–53)
Right Fork Deep Creek (DC)	TN	326	29	3.23	0.39	0.40	13/13	0/78	0.02	138 (180–∞)
Sahlee Creek (ShC)	TN	321	29	3.08	0.40	0.40	11/11	0/78	0.02	66 (39–141)

Within populations, N_e estimates varied (range = 4–148) but were observed to be generally low (median N_e = 43; based on populations with bounded confidence intervals; Table 1). The number of polymorphic loci ranged from 1 (Indian Flats Prong) to 13 (Caldwell Fork, Hyatt Creek, Kephart Branch, and the Right Fork of Deep Creek; Table 1) across all populations. Mean A_R (±SD) across all populations was 2.67 ± 0.75 and ranged from 1.08 (Indian Flats Prong) to 4.07 (Chasm Prong; Table 1). In addition, 19 private alleles were detected

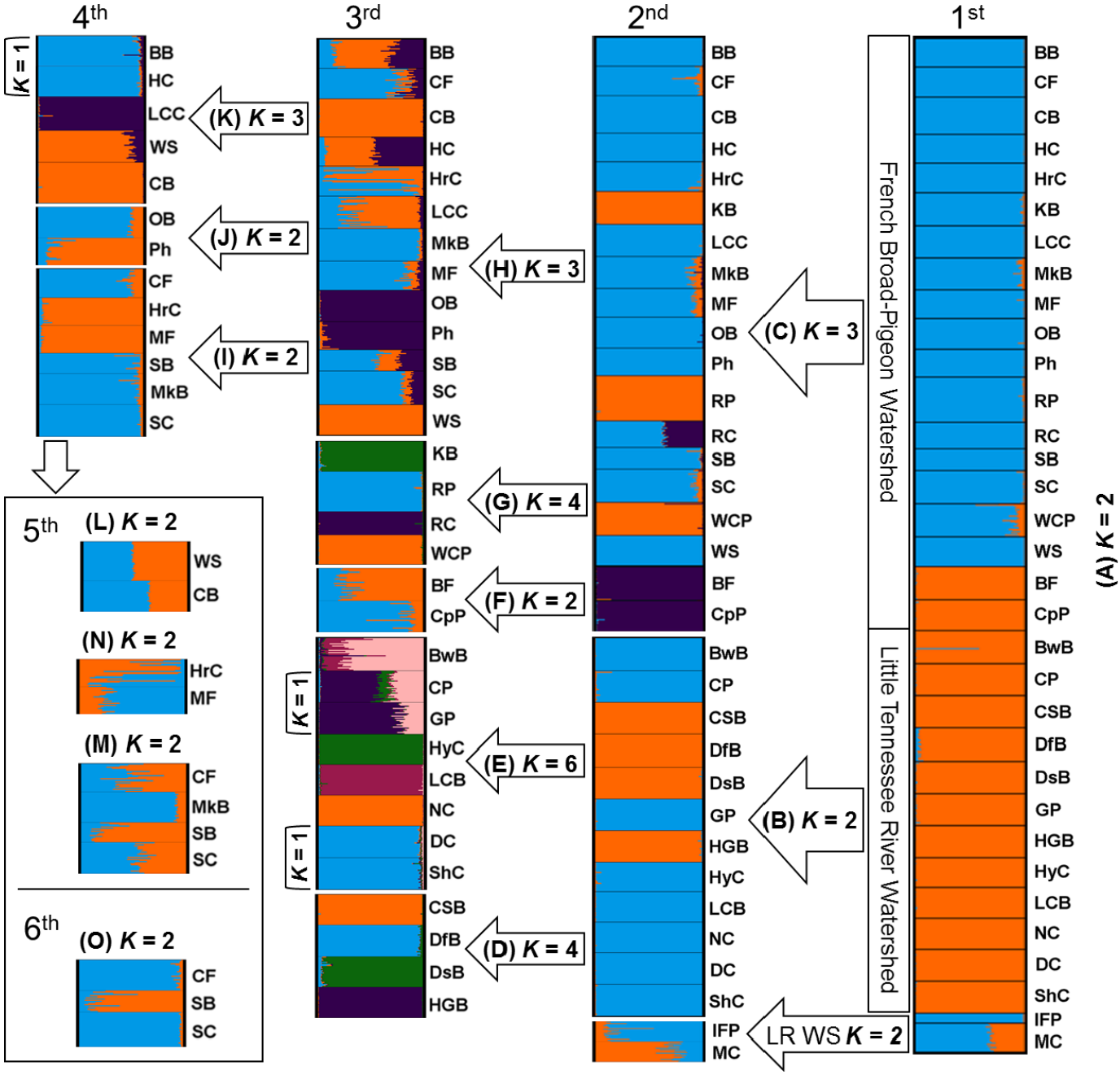

FIGURE 1. Great Smoky Mountains National Park Brook Trout population clusters (K = number of clusters) inferred across six tiers of hierarchical STRUCTURE analyses. Estimated membership coefficient plots (A–O) illustrate patterns of individual Brook Trout (bars) cluster assignments for each level of hierarchical analysis. Collections are sorted based on watershed (French Broad–Pigeon River, Little Tennessee River, and Little River [LR]) and arranged alphabetically according to collection acronym (defined in Table 1). Arrows indicate hierarchical workflow and the number of clusters inferred from each analytical tier; inferred clusters for tiers 5 and 6 are indicated above the corresponding membership coefficient plots. Figure S.2 illustrates the $\log_e \Pr(X|K)$ and ΔK plots used to infer the number of clusters associated with each hierarchical tier (A–O). [Color figure can be viewed at afsjournals.org.]

within polymorphic loci across 10 populations. Each of the populations examined conformed to HWE ($P > 0.0038$), and a majority (28 of 33 populations; 85%) showed little evidence of significant ($P > 0.0001$) LD (Table 1). For collections in which significant LD was detected, only a single pairwise comparison among loci displayed significant LD. There was minimal evidence to suggest that nonrandom mating or inbreeding (F_{IS} range = 0.01–0.06; Table 1) had occurred within each collection. Lastly, there was no evidence that a recent bottleneck or founder event had occurred within any of the populations examined.

Significant neutral genetic differentiation (F'_{ST} range = 0.02–0.94; mean ± SD = 0.64 ± 0.18) and neutral allelic fixation (F_{ST} range = 0.01–0.79; mean ± SD = 0.42 ± 0.35) were detected among nearly all pairs of populations (Figure 2). Three geographically proximate pairs of collections (stream separation distance < 1.0 km; Gulf Prong and Chasm Prong; Sahlee Creek and the Right Fork of Deep Creek; and Straight Creek and Caldwell Fork) exhibited low yet significant population differentiation ($F'_{ST} < 0.05$). The AMOVA results revealed that genetic variation was evident across all spatial scales examined (i.e., within and among populations, among subwatersheds, and among watersheds; Table 2). Genetic differences among watersheds accounted for 30% of the total observed variation. Within watersheds, differences among subwatersheds represented 18% of the overall genetic variation. Substantial variation was also evident among streams (14%), with the remaining diversity occurring within streams (38%). Additionally, significant ($P < 0.05$) positive relationships were observed between the amount of geographic distance separating populations and both genetic fixation (F_{ST}; $r = 0.28$) and genetic differentiation (F'_{ST}; $r = 0.57$). Thus, genetic variation broadly reflected patterns associated with IBD and stream topology, and there was considerable fine-scale variation among populations. Collectively, these results appear to be consistent with the theoretical expectations associated with isolation and drift and indicate that a large proportion of the collections examined are each functioning as a single interbreeding population.

Morphology and Meristics

Although COLONY identified the presence of 20 full siblings (representing 10 full-sibling families within 16 of the 35 populations sampled) among the 351 fish (5.7%) sampled for morphology and meristics, we included all samples in the phenotype analysis. Therefore, 341 unique families of Brook Trout were examined for differences in morphology and meristics. The morphPC$_1$ accounted for 93.5% of the total morphometric variation and was highly correlated ($r = 0.99$) with SL and thus was considered to reflect body size. The remaining variation in morphology (shape) was largely explained by morphPC$_2$, morphPC$_3$, and morphPC$_4$ (30.3, 20.8, and 9.7%, respectively). The three features that loaded most heavily on morphPC$_2$ were posterior dorsal fin to posterior anal fin (0.57); snout to dorsal fin origin (−0.33); and anterior dorsal fin to pelvic

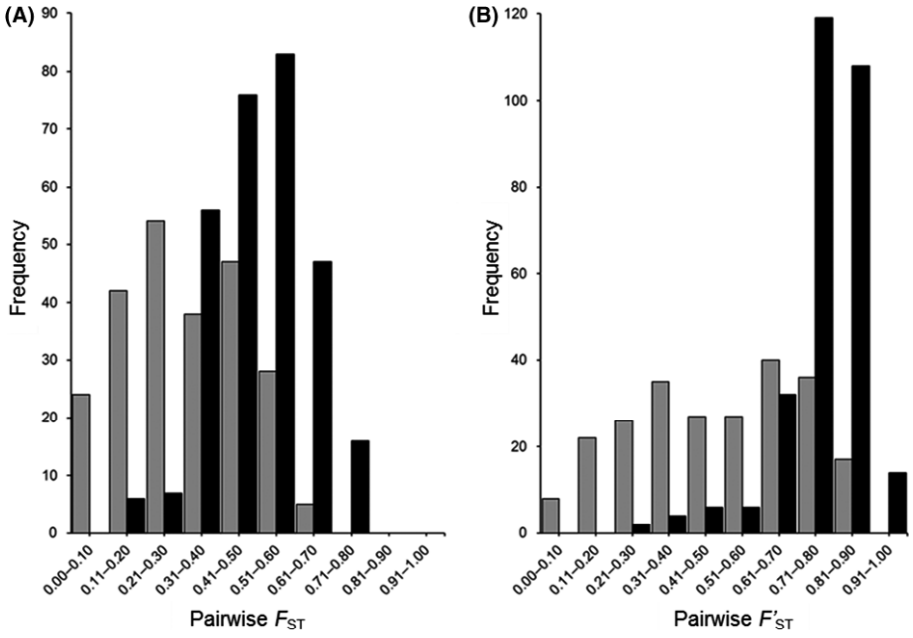

FIGURE 2. Histogram plot illustrating the frequency (*y*-axis) and distribution (*x*-axis) of **(A)** pairwise genetic fixation (F_{ST}) and **(B)** pairwise genetic differentiation (F'_{ST}) among populations of Brook Trout within watersheds (gray bars) and among watersheds (black bars) of the Great Smoky Mountains National Park.

fin origin (−0.33). In addition, $merPC_1$, $merPC_2$, and $merPC_3$ together accounted for 41.3% (15.5, 13.6, and 12.2%, respectively) of the observed variation for this suite of phenotypic characters. The characteristics with the strongest loadings on $merPC_1$ were dorsal rays (0.53), anal rays (0.50), teeth on the lower left jaw (0.44), and ceratobranchial gill rakers (0.34).

Considerable morphological variation was observed among individuals within populations (70.0, 57.5, and 70.3% for $morphPC_2$, $morphPC_3$, and $morphPC_4$, respectively; Table 2). However, as spatial scale increased, morphometric variation decreased among populations (14.2, 42.6, and 12.2%, respectively) and also did so to a lesser extent among subwatersheds (10.8, 0.0, and 2.1%, respectively) and among watersheds (2.1, 0.0, and 15.3%, respectively; Table 2). The global MANOVA test showed significant phenotypic differences among sampled streams. Duncan's MRT groupings showed a continuous gradient of morphological variation among populations and watersheds for both $morphPC_2$ and $morphPC_3$ (Figure 3). Small subsets of populations at the extremes of the observed continuum were found to be statistically different from one another. Additionally, there was no observed statistical difference in Bonferroni-corrected Duncan's MRT groupings at $morphPC_4$. Duncan's MRT results should be viewed with caution, however, as we detected significant heteroscedasticity within each of the retained morphPC scores. Bonferroni-corrected, nonparametric Mann–Whitney pairwise population comparisons, which are expected to be statistically more conservative, showed no significant difference among populations at any morphometric PC score.

Similar levels of meristic variation were observed within and among populations (e.g., 44.1% and 48.2%, respectively, for $merPC_1$; Table 2). However, limited meristic variation was observed among subwatersheds (2.7, 8.2, and 8.9% for $merPC_1$, $merPC_2$, and $merPC_3$, respectively) and watersheds (5.0, 0.4, and 0.0%, respectively; Table 2). Duncan's MRT groupings based on meristics showed a broad continuum of variation among populations for each meristic PC score analyzed, with significant differences among populations occurring at the ends of the continuum (Figure 3). Although we detected some significant differences among populations, these observed differences were not consistent among meristic PCs and should be viewed with caution, as we detected significant heteroscedasticity within each of the retained meristic PCs. Additionally, we observed no significant differences among all pairwise population comparisons based on the Mann–Whitney tests.

No significant ($P < 0.05$) relationships were observed between the amount of phenotypic variation (i.e., SDs of morphometric and meristic PC scores; Figure 4) within populations and any of the examined measures of genetic diversity (e.g., N_e, A_R, H_O, uH_E, and F_{IS}) or sampled stream reach. Additionally, we observed no significant ($P < 0.05$) relationship between pairwise population phenotypic similarity measures (i.e., Gower coefficient scores) and the distance separating populations ($r = -0.32$, $P = 0.99$), genetic fixation (F_{ST}; $r = -0.15$, $P = 0.95$), or genetic differentiation (F'_{ST}; $r = -0.29$, $P = 1.00$).

DISCUSSION

The present results support our prediction that isolation has resulted in widespread neutral genetic differentiation among Brook Trout populations endemic to GRSM. Moreover, our study populations showed clear indications of both genetic isolation and genetic drift, and these findings appear to be typical across populations of Brook Trout that are endemic to southern Appalachia (see McCracken et al. 1993; Kriegler et al. 1995; Hudy et al. 2008). Although the observed patterns of genetic variation broadly reflected stream topology and IBD, substantial molecular variation existed among neighboring streams within subwatersheds. Furthermore, we found high levels of neutral genetic differentiation and allelic fixation among most populations, which suggests that there is little to no contemporary gene flow among Brook Trout inhabiting geographically proximate streams. To our

TABLE 2. Random-effects hierarchical analysis of molecular variance (AMOVA) and morphometric and meristic variation (ANOVA) in Brook Trout collected from Great Smoky Mountains National Park. Percentages of molecular, morphometric (principal component [PC] score), and meristic (PC score) variance partitioned within and among streams, among subwatersheds, and among watersheds are shown. The morphometric PC_1 (size) scores were not retained for shape analyses.

		Morphometric variation				Meristic variation		
Hierarchy	Molecular variation	PC_1	PC_2	PC_3	PC_4	PC_1	PC_2	PC_3
Among watersheds	29.57	0.00	2.06	0.00	15.33	5.04	0.35	0.00
Among subwatersheds	18.28	0.00	10.77	0.00	2.12	2.68	8.21	8.88
Among streams	13.81	31.47	14.16	42.55	12.24	48.16	34.14	44.3
Within streams	38.35	68.53	73.01	57.45	70.31	44.12	57.3	46.82

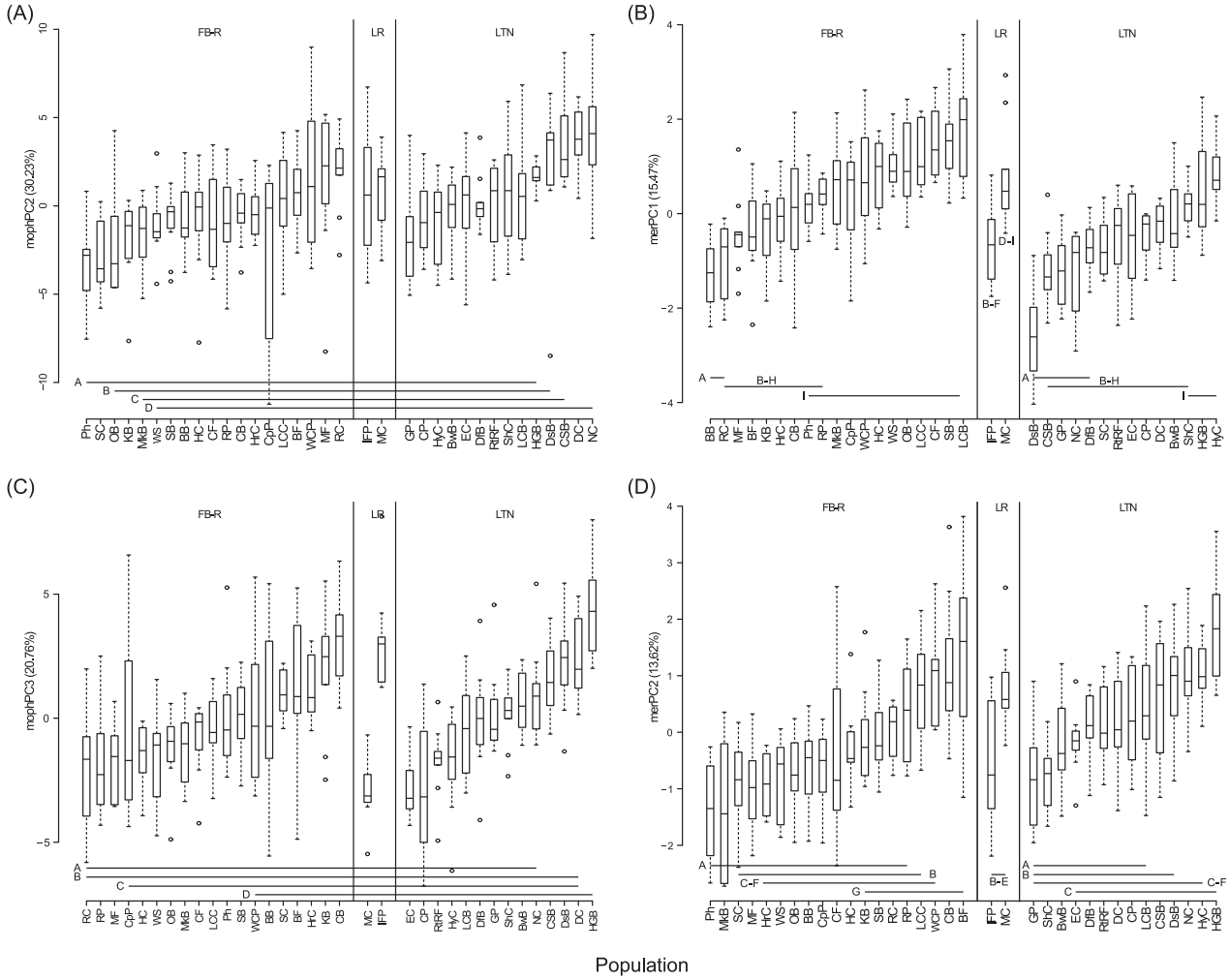

FIGURE 3. Box and whisker plots illustrating the variance of (**A**) the second morphometric principal component ($morphPC_2$), (**B**) the third morphometric PC ($morphPC_3$), (**C**) the first meristic PC ($merPC_1$), and (**D**) the second meristic PC ($merPC_2$) of Brook Trout populations arranged by Duncan's multiple range test groupings (lettered horizontal underbars) and partitioned by watershed in Great Smoky Mountains National Park. Box boundaries indicate 25th and 75th percentiles, whiskers indicate the highest and lowest values, and the horizontal box bar indicates the median PCA value for each population. Watersheds are French Broad–Pigeon River (FB-R), Little River (LR), and Little Tennessee River (LTN). Collection/population acronyms are defined in Table 1, with the exception of Enloe Branch (EB) and the Right Fork of Raven Fork (RF) collected from LTN.

knowledge, these results represent some of the greatest levels of genetic differentiation reported to date for populations of native, wild Brook Trout (see Annett et al. 2012; Pilgrim et al. 2012; Whiteley et al. 2013; Hoxmeier et al. 2015; Kazyak et al. 2015; Kelson et al. 2015).

Most of the populations we examined had small values for N_e. Although our estimates of N_e are likely larger than related measures (i.e., number of breeders [N_b]; see Luikart et al. 2010 for further discussion pertaining to estimation of N_e and N_b for iteroparous species), N_e is generally regarded as more germane to the conservation of isolated populations than N_b (see Frankham et al. 2017). Moreover, theory suggests that N_e drives the rate of drift affecting neutral genetic variation (Willi et al. 2007; Charlesworth 2009) and is often more pronounced in small, isolated populations (Allendorf et al. 2013). Consistent with these expectations, we observed depauperate levels of A_R and heterozygosity (H_O and uH_E) as well as significant genetic fixation (F_{ST}) in nearly all populations. We also found significant positive relationships between N_e estimates, H_O, and A_R despite relatively low global variance. In addition, a significant positive correlation was observed between the distance separating populations and the F_{ST} or F'_{ST} among populations, suggesting a pattern of IBD. Furthermore, we detected no evidence to support our prediction that larger effective populations with more genetic and/or phenotypic diversity would occupy broader stream reaches. Collectively, our observations are consistent with population genetics theory regarding the effect of isolation and the loss of neutral genetic variation in spatially restricted

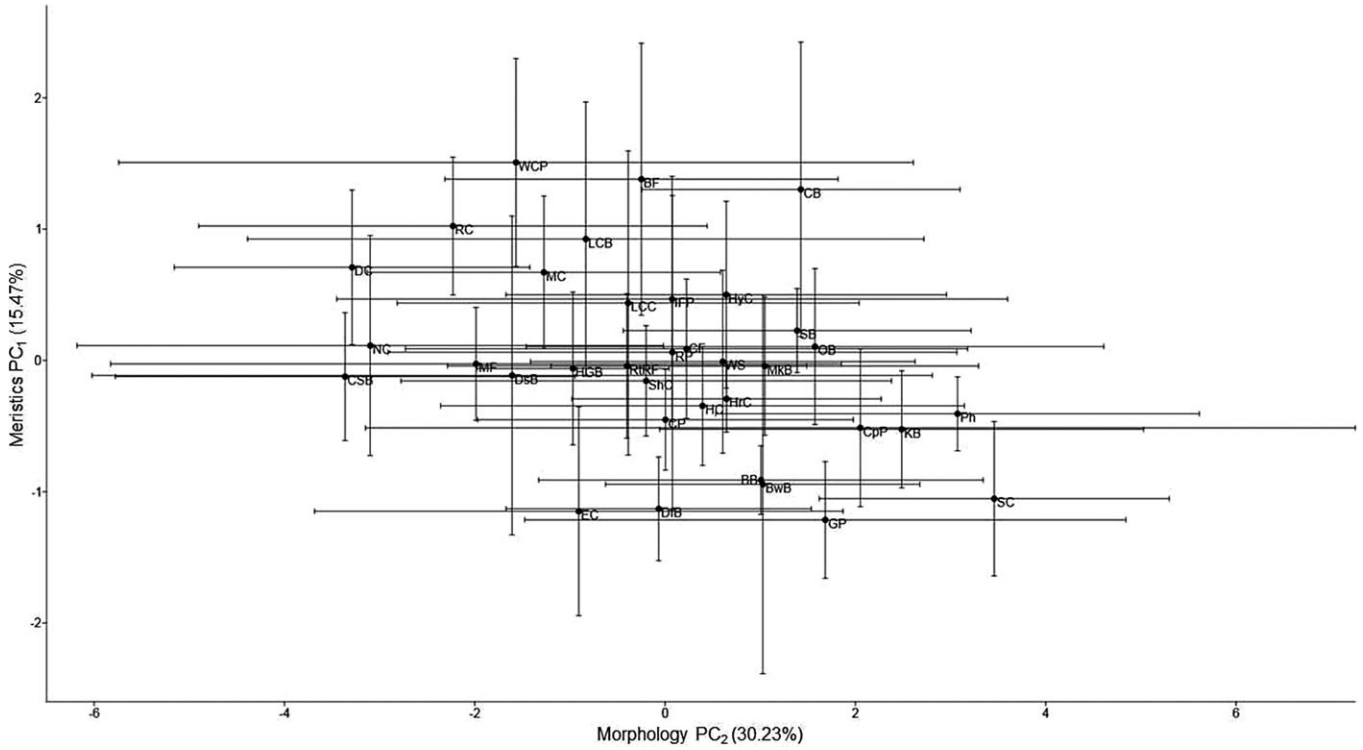

FIGURE 4. Mean meristic PC_1 (15.47%) plotted over mean morphometric PC_2 (30.23%) of Brook Trout population scores with standard deviation bars radiating from the centroid of each population characterizing the amount of phenotypic diversity encapsulated within each geographically isolated Brook Trout population from Great Smoky Mountains National Park.

populations with low N_e (Kalinowski and Waples 2002; Frankham et al. 2017). Therefore, we conclude that genetic drift, isolation, and fragmentation have led to widespread genetic differences among select GRSM populations of Brook Trout, at least at neutral loci.

Despite having observed substantial neutral genetic differentiation among many populations, our results do not suggest that spatial and genetic isolation has resulted in corresponding patterns of broad phenotypic differentiation among populations of Brook Trout sampled within GRSM. Instead, there was comparatively little morphometric structure among isolated populations. Although we observed a greater amount of meristic structure among collections relative to morphological structure, meristic variation was primarily detected within populations rather than among subwatersheds or watersheds (see Table 2). Only a small number of significant differences between pairs of populations was found using the anti-conservative Duncan's MRT, and visual examination of PC scores reflected broadly overlapping morphometric and meristic variation among most populations. Where significant pairwise differences did occur, they were located between populations at the periphery of overall observed variability. These differences, however, were inconsistent among populations across the range of phenotypic characters considered and may be the result of significant heteroscedasticity within PCs among populations.

Our decision to retain phenotype data for all samples deserves explanation. Studies on fish systematics using morphometrics and meristics generally have not considered family structure, often due to the lack of corresponding genetic data. Here, we identified and acknowledged the presence of full siblings within some collections, but we chose to retain those individuals for several reasons. First, there were relatively few full siblings observed ($n = 20$ of 350; 5.7%), and these were fairly evenly spread among collections. Second, since full siblings are expected to be more similar to one another than to other members of a population (Lynch and Milligan 1994), retention of all full siblings was expected to magnify any apparent phenotypic differentiation among populations. Thus, retention of all individuals regardless of familial structure maximized the probability of observing phenotypic differentiation among populations. However, we did not observe consistent patterns of morphologic and meristic differences among populations that paralleled the observed patterns of neutral genetic differentiation. Nevertheless, we did observe some slight geographic structure in morphology and meristics. Collectively, our results suggest that morphology and meristics are largely conserved among nearly

all of the Brook Trout populations we examined, despite strong evidence for isolation and neutral genetic drift.

There was no indication that N_e, genetic diversity, or the extent of available habitat sampled was related to morphological or meristic variability within these Brook Trout populations. This suggests that small, isolated populations may be capable of maintaining phenotypic variation in spite of drift, isolation, and substantial neutral genetic differentiation, as least within GRSM. However, our study was limited to isolated populations in extreme headwater environments near the southern limit of the species' range, and all populations had relatively low N_e and limited genetic diversity. A more pronounced relationship might exist elsewhere among larger populations with greater neutral genetic diversity that occupy heterogeneous riverscapes. However, such populations are very rare in the southern Appalachians, particularly within GRSM. Interestingly, Wood et al. (2015) recently reported that variable abundance and N_e did not correspond to differences in genetic variation or phenotypic differentiation among populations of Brook Trout sampled in coastal Canadian environments. In combination, these results suggest that isolation, fragmentation, neutral genetic drift, and allelic fixation do not necessarily lead to reduced phenotypic variability or to obvious, consistent morphometric and meristic differentiation among populations of Brook Trout.

Brook Trout inhabit dynamic environments (Power 2002), often with a diverse array of available microhabitats. The ability to exploit varied resources likely helps Brook Trout to thrive in these environments, maximizing productivity and resilience to changing conditions. In the case of isolation, it is paramount that populations possess suitable genotypic and phenotypic variability to adapt to changing conditions, as there is little opportunity for gene flow to mitigate the effects of drift and inbreeding. In effectively small, isolated populations, theory predicts that genetic drift may supersede selection and reduce standing additive genetic variation (Willi et al. 2007) and adaptive potential (Blanquart et al. 2012). If this leads to reduced phenotypic variability, the outlook for a population is diminished (see Blanquart et al. 2012; Grenier et al. 2016). Although our study did not detect evidence of reduced phenotypic variability in the context of isolation and drift, we recommend that future studies continue to examine this important aspect of the evolutionary biology of Brook Trout.

Due to permitting restrictions, we were limited to a modest number of samples for phenotype analyses. Future studies should consider nonlethal sampling approaches (e.g., photography) to allow larger numbers of individuals to be sampled. Despite this limitation, our morphometric results are similar to those generated by other studies (see Kazyak et al. 2015; Wood et al. 2015, 2016). For example, Wood et al. (2015, 2016) and Kazyak et al. (2015) similarly observed inconsistent patterns of phenotypic differentiation among fragmented populations of Brook Trout that were genetically differentiated—despite variable population and sample sizes.

Management Implications

From a management perspective, morphology and meristics appear to be an ineffective tool for consistently delineating populations and management units or for assessing the evolutionary potential of Brook Trout, at least within GRSM. Since southern Appalachian Brook Trout generally exist as effectively small, isolated populations, it is important to survey populations across a broad continuum of habitats and geographic areas to fully characterize the structure and range of phenotypic and genotypic variability within and among populations. Smaller studies could by chance observe significant phenotypic (e.g., morphometric and meristic) differentiation when, in fact, Brook Trout phenotypes reflect an overlapping continuum among populations.

Overall, genetic markers (e.g., microsatellites or single-nucleotide polymorphisms) appear to be effective tools for delineating populations and guiding strategies to conserve biodiversity. We encourage managers to actively monitor demographic and genetic trends, as future perturbations are expected to degrade the suitability of Brook Trout habitat across much of the species' native range (Whiteley et al. 2013; DeWeber and Wagner 2015). The widespread pattern of isolation and drift we observed highlights the importance of promoting and maintaining connectivity, where feasible, among populations that have become fragmented due to human and/or natural events in order to sustain sources of genetic variation and habitat heterogeneity, despite some potential risks (e.g., outbreeding depression; Fausch et al. 2009; Hohenlohe et al. 2013; Ofori et al. 2017). Our study also suggests that it is possible to transfer a fair amount of phenotypic variation to a population by translocating a small quantity of individuals, as in the context of genetic rescue. However, since small, isolated populations still maintain substantial morphologic and meristic variability, it appears that Brook Trout populations may have mechanisms to retain phenotypic diversity in spite of genetic drift. Future studies should continue to explore how small, isolated Brook Trout populations cope with genetic drift and how this relates to their future outlook.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.