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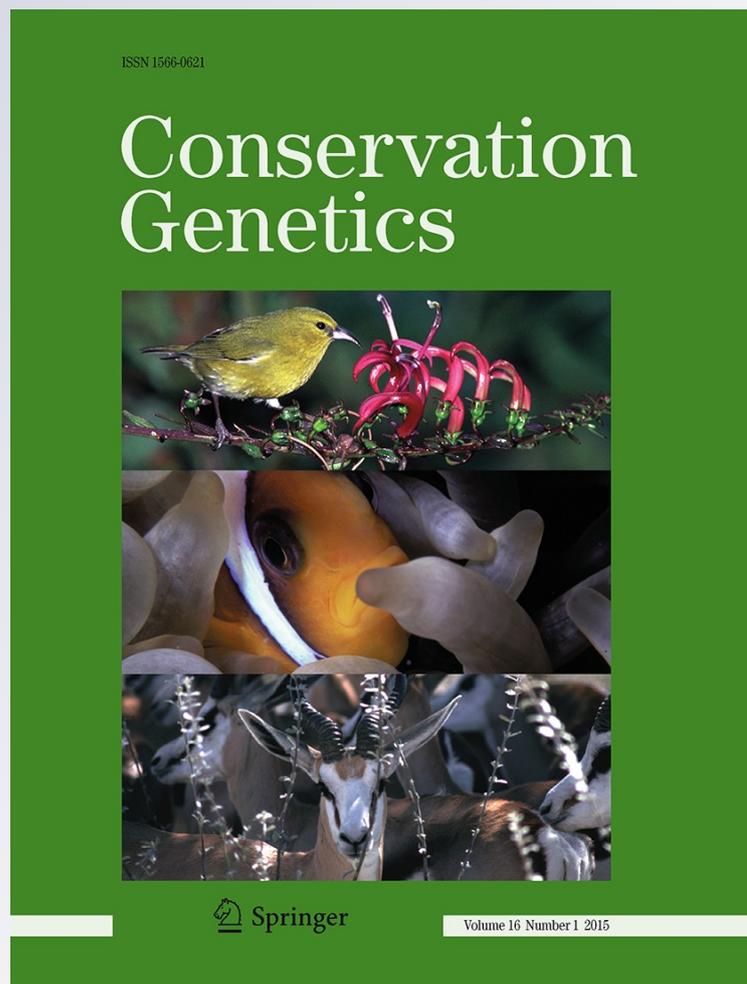
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River mainstem thermal regimes influence population structuring within an appalachian brook trout population

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Abstract Brook trout (*Salvelinus fontinalis*) often exist as highly differentiated populations, even at small spatial scales, due either to natural or anthropogenic sources of isolation and low rates of dispersal. In this study, we used molecular approaches to describe the unique population structure of brook trout inhabiting the Shavers Fork watershed, located in eastern West Virginia, and contrast it to nearby populations in tributaries of the upper Greenbrier River and North Fork South Branch Potomac Rivers. Bayesian and maximum likelihood clustering methods identified minimal population structuring among 14 collections of brook trout from throughout the mainstem and tributaries of Shavers Fork, highlighting the role of the cold-water mainstem for connectivity and high rates of effective migration among tributaries. In contrast, the Potomac and Greenbrier River collections displayed

distinct levels of population differentiation among tributaries, presumably resulting from tributary isolation by warm-water mainstems. Our results highlight the importance of protecting and restoring cold-water mainstem habitats as part of region-wide brook trout conservation efforts. In addition, our results from Shavers Fork provide a contrast to previous genetic studies that characterize Appalachian brook trout as fragmented isolates rather than well-mixed populations. Additional study is needed to determine whether the existence of brook trout as genetically similar populations among tributaries is truly unique and whether connectivity among brook trout populations can potentially be restored within other central Appalachian watersheds.

Keywords *Salvelinus* · Microsatellite DNA · Population structure · Genetic clustering · Brook trout

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Introduction

The Brook trout (*Salvelinus fontinalis*) is an iconic salmonid fish valued throughout its entire native range for its recreational and aesthetic qualities, as well as its ecological importance as a keystone species. Especially within the Appalachian portion of its range in the eastern United States, brook trout have increasingly become a focus of conservation efforts due to population declines that have intensified over the last century (Hudy et al. 2008; Richards et al. 2008). The causes of these continuing declines include overharvest, acid precipitation, habitat degradation, competition with non-native species, and climate change (Petty and Thorne 2005; Flebbe et al. 2006; McClurg et al. 2007; Hudy et al. 2008). An important consequence of habitat loss and/or degradation for stream dwelling fishes

like brook trout is isolation, which may lead to reduced gene flow among populations making them more susceptible to stochastic environmental events and elevated genetic drift, potentially leading to the loss of unique adaptive variability (Fagan 2002; Allendorf and Luikart 2007; Whiteley et al. 2013). Consequently, there is considerable demand for implementation of restoration programs that will maximize population recovery and resilience of brook trout (EBTJV 2013; Petty and Merriam 2012).

Developing a clear understanding of spatial population structure, gene flow, and life history diversity (e.g., resident versus migratory behavior) is critical for salmonid species conservation (Rieman and Dunham 2000; Neville et al. 2006). This information is used to make decisions about whether management should focus on protection and restoration of isolated habitats and populations or whether it should focus on restoring connectivity of habitats and metapopulations at the scale of whole watersheds (Fausch et al. 2002). Genetic studies of contemporary brook trout populations in the central and southern Appalachians show that they persist primarily as fragmented and genetically isolated units (Richards et al. 2008; Hudy et al. 2010; Kanno et al. 2011; Whiteley et al. 2013). Typical explanations for the observed population structuring include natural and artificial barriers to dispersal (e.g., waterfalls, dams, reduced flow), dependence of brook trout on cold water, competition with exotic fishes such as rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*), and habitat degradation and warming in larger river main stems that historically served as dispersal corridors. In Appalachian brook trout, maintaining connectivity among tributaries in stream networks is necessary to allow tributaries to serve as sources of migrants to maintain demographic and genetic variability in sub populations (Letcher et al. 2007).

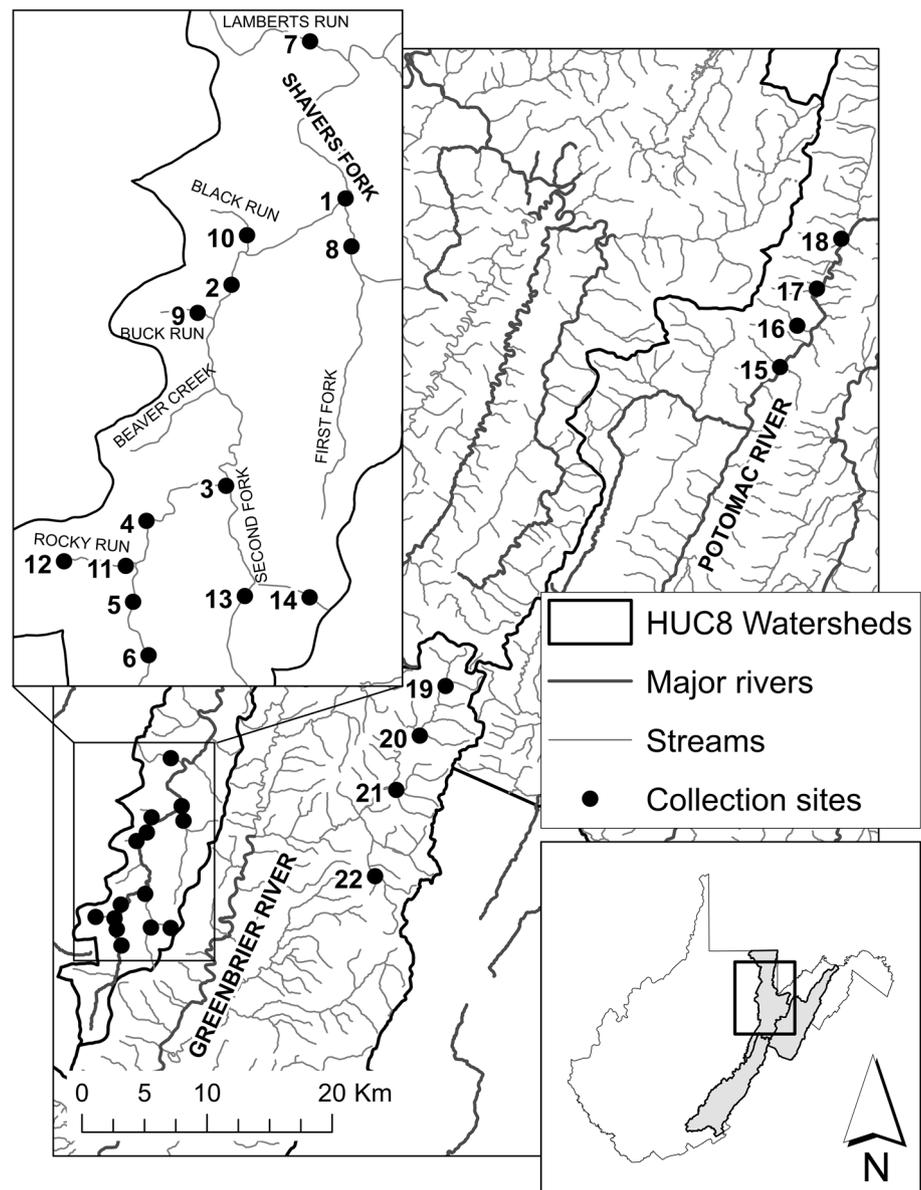
Recent mark-recapture and telemetry data suggest that while large brook trout may undertake extensive movements among tributaries and mainstems in some river systems (Petty et al. 2005, 2012), the propensity to disperse among tributaries may be limited by habitat restrictions such as dams, culverts, or insufficient flow (Poplar-Jeffers et al. 2009; Hudy et al. 2010). For example, Hudy et al. (2010) found that within an 1,800 m reach of typical Appalachian brook trout habitat in the Fridley Gap watershed in north-central Virginia, dispersal of age-0 brook trout and their parents within the first year was highly restricted, supporting the observation that brook trout show strong spatial population structure in stream habitats. However, within 16 months the spatial structure signal was reduced due to dispersal of older fish distances averaging approximately 200 m away from their original inferred redd locations. The spawning habitat in Fridley gap is bounded on the downstream end by an impassable dam and

intermittent flows on the upstream side. Thus, it is unknown whether brook trout would disperse further downstream or upstream if habitat were available, though the dispersal of older fish suggests this may be a possibility. Unfortunately, there are presently no published studies of brook trout population structure in larger, more connected watersheds within the Appalachians for comparison.

Recent ecological studies in the Shavers Fork (Monongahela River, WV), a large, high elevation watershed, suggest that brook trout populations are strongly dependent on post-reproductive movement processes and may be more appropriately defined as a network of closely related populations. Petty et al. (2005, 2012, 2014) observed high levels of adult brook trout physical dispersal and seasonal redistribution in response to temporal shifts in habitat quality at the watershed scale. The patterns observed in the Shavers Fork brook trout population are consistent with studies of other salmonids, including the congeneric Bull trout (*Salvelinus confluentus*) that exhibits relatively high physical and effective migration rates among tributaries in some systems (Rieman and Dunham 2000; Warnock et al. 2010; Nyce et al. 2013). However, observations of brook trout in Shavers Fork are exceptional when compared to most studies of contemporary populations of brook trout in the Appalachians. Shavers Fork tributaries are connected by a mainstem conducive to dispersal for much of the year, and there are no barriers restricting movement among tributaries (Petty et al. 2012). The presence of cold water refugia within the Shavers Fork mainstem serves to expand suitable habitat beyond the tributaries, with the result being increased brook trout growth potential due to the ability to forage within the productive mainstem (Petty et al. 2014). If dispersal among tributaries is common in systems like Shavers Fork, which may be more typical of historical levels of connectivity in Appalachian watersheds prior to habitat degradation and/or alteration, this information could help managers by providing a baseline target for restoration of contemporary brook trout populations.

A clearer understanding of brook trout population structure is needed for developing effective conservation programs in the central Appalachian region. While mark recapture and telemetry data show physical movements among tributaries in Shavers Fork (Petty et al. 2005, 2012), molecular genetic approaches are needed to confirm whether these movements represent effective migration and influence genetic relationships among tributaries. Therefore, the objective of this research was to use genetic approaches to determine if Shavers Fork brook trout more closely resemble a network of genetically similar populations or that of fragmented isolates. Specifically, we describe the genetic population structure of brook trout residing in the upper Shavers Fork mainstem and tributaries. We then compare genetic patterns in brook trout in

Fig. 1 Map of the Shavers Fork, Potomac, and Greenbrier drainages in WV, USA sampled for *Salvelinus fontinalis*. Each numbered dot represents an individual collection, and the collection corresponding to each numbered dot is listed in Table 1. Samples collected in Shavers Fork were from the Cheat River watershed, Potomac drainage samples were from the North fork of the South Branch Potomac River watershed, and Greenbrier samples were from the Greenbrier River watershed. The waterfall between collections 13 and 14 within Second Fork is indicated by a solid line



Shavers Fork to those in tributaries from the nearby Greenbrier (a tributary of the Kanawha River) and North Fork South Branch Potomac River drainages that are each connected by warmer mainstems. We hypothesized that populations inhabiting small tributaries within Shavers Fork would exhibit low levels of differentiation due to effective migration via the mainstem, whereas populations inhabiting the Shavers Fork mainstem would be migratory and represent an admixture of individuals originating from several different tributaries. In addition, we hypothesized brook trout in the Greenbrier and Potomac drainages would show stronger differentiation due to isolation effects of the warm-water mainstems.

Materials and methods

Study area and sample collection

Shavers Fork is a large sub-basin (i.e., basin area of 150 km²) of the Cheat River drainage, flowing north to its confluence with the Monongahela River in east central West Virginia (Fig. 1). Land cover within the drainage is dominated by a mixed deciduous-coniferous forest. Watersheds throughout this region have been negatively impacted by acid precipitation (McClurg et al. 2007), and impacts are prevalent in the Shavers Fork watershed (Petty and Thorne 2005). Shavers Fork is unique in comparison to

Table 1 Summary genetic data for brook trout *Salvelinus fontinalis* collections from the Shavers Fork, Potomac, and Greenbrier drainages in WV, USA

Drainage	Collection	Location	Km ²	Elev.	Abbr.	<i>n</i>	<i>uH_E</i>	<i>H_O</i>	<i>A_E</i>	<i>A_R</i>	HWE	<i>F_{IS}</i>
Shavers Fork	1	Mainstem 1	114.45	3,614	SFMS1	23	0.55	0.50	2.74	5.01	0.1843	0.09
	2	Mainstem 2	79.53	3,643	SFMS2	14	0.61	0.61	3.02	5.69	0.9184	0.00
	3	Mainstem 3	60.41	3,707	SFMS3	36	0.53	0.52	2.71	4.40	0.2716	0.07
	4	Mainstem 4	44.20	3,717	SFMS4	26	0.56	0.54	2.95	4.72	0.7839	0.03
	5	Mainstem 5	32.12	3,781	SFMS5	23	0.57	0.57	3.01	4.92	0.9043	−0.03
	6	Mainstem 6	30.60	3,791	SFMS6	25	0.55	0.52	2.87	4.70	0.2648	0.05
	7	Lambert Run	7.64	3,572	Lambert	15	0.59	0.58	2.99	5.14	0.9041	−0.01
	8	First Fork	24.65	3,616	First	30	0.52	0.52	2.45	4.58	0.8539	−0.01
	9	Buck Run	2.79	3,670	Buck	27	0.49	0.53	2.50	3.80	0.1045	−0.08
	10	Black Run	5.18	3,643	Black	26	0.55	0.53	2.97	4.54	0.6001	0.01
	11	Rocky Run 1	6.79	3,781	Rocky1	33	0.53	0.52	2.83	4.61	0.4535	0.00
	12	Rocky Run 2	1.53	3,929	Rocky2	31	0.55	0.54	2.79	4.55	0.5879	0.00
	13	Second Fork 7	5.66	3,911	Second7	35	0.50	0.47	2.64	3.77	0.4982	0.04
	14	Second Fork 10	1.32	4,091	Second10	17	0.47	0.46	2.31	3.11	0.4575	−0.01
Potomac		All Shavers Fork				361	0.55	0.53	2.77	4.54	0.7,957	0.01
	15	Powdermill Run	8.15	1,422	POSB4	27	0.65	0.54	3.50	5.40	0.0001	0.16
	16	Zeke Run	6.37	1,330	POSB5	20	0.35	0.38	1.79	2.30	0.8780	−0.09
	17	High Ridge Run	6.87	1,264	POSB7	30	0.59	0.58	2.78	4.60	0.0219	0.01
	18	Samuel Run	6.24	1,152	POSB8	30	0.67	0.60	3.74	5.01	0.0000	0.11
	All Potomac				107	0.67	0.54	2.95	4.33	0.0000	0.05	
Greenbrier	19	East Greenbrier	15.67	3,520	OHGK3	27	0.69	0.67	4.31	6.35	0.3901	0.01
	20	Abes Run	7.63	3,302	OHGK5	23	0.63	0.57	3.67	5.58	0.0519	0.11
	21	Long Run	13.11	3,002	OHGK6	19	0.61	0.60	3.39	5.61	0.0002	0.03
	22	Little River	15.02	3,105	OHGK7	22	0.58	0.59	3.37	5.36	0.3661	−0.04
	All Greenbrier				91	0.66	0.612	3.69	5.73	0.0013	0.03	

The Collection number corresponds to the number designations in Fig. 1, Location is the actual location name, and Abbreviation is the sample designation used throughout the text. The 1 and 2 within Rocky Run, and the 7 and 10 within Second Fork refer to subsections of those tributaries. Data include the *n* sample size, *uH_E* unbiased expected heterozygosity, *H_O* observed heterozygosity, *A_E* effective number of alleles, *A_R* allelic richness, the multi-locus HWE *P* value determined using Fisher's method output by Genepop, and the inbreeding coefficient (*F_{IS}*) output by Genepop averaged over loci. Values for the diversity measures are averaged over the 13 loci. Bold and italicized *P* values are those which remained significant after sequential Bonferroni correction

most central Appalachian drainages, because the tributaries within Shavers Fork are connected by a cold-water mainstem that appears to enable movement of brook trout among tributaries (Petty et al. 2012). Tributary temperatures rarely surpass 19 °C, and while temperatures in the mainstem may fluctuate from 14 to 25 °C the presence of cold-water refugia enable brook trout populations to persist year-round (Petty et al. 2012). In addition, the upper Shavers Fork watershed is nearly roadless, and the absence of culverts means that tributary and mainstem habitats are well connected (Poplar-Jeffers et al. 2009). All sampled tributaries are open to bi-directional dispersal with the Shavers Fork mainstem throughout the year even during periods of low flow, with the exception of Second Fork. In Second Fork, a waterfall limits upstream dispersal from the Second Fork 7 (Collection 13) to the Second Fork 10 site (Collection 14; Table 1 and Fig. 1). In addition, the

presence of a wide alluvial fan at the mouth of Buck Run has been observed to become dry in some years, which serves to occasionally isolate brook trout in Buck Run during low flow conditions.

Brook trout fin clip samples (*n* = 371) were collected from 14 different sites distributed among mainstem and tributary habitats of the Shavers Fork watershed (Fig. 1) during May and June 2001 by teams using backpack electrofishing units (DC, 60 Hz, 400–600 V, Smith Root, Vancouver, WA) and preserved in 95 % ethanol. Collections included a mixture of young of the year, juveniles, and adults (Petty et al. 2005). To provide a broader regional comparison to the population structure observed within Shavers Fork, samples were also collected from the nearby North Fork South Branch of the Potomac River watershed (hereafter referred to as Potomac) of the Potomac drainage (*n* = 107) and the Greenbrier River watershed (hereafter

referred to as Greenbrier) of the Kanawha River drainage ($n = 91$) during 2006 and 2007. Like within Shavers Fork, acid precipitation, historical timber harvest, and sedimentation negatively impact brook trout in the Potomac and Greenbrier drainages. Sampling in all three drainages occurred during early June over similar spatial scales (Fig. 1) using the same collection methodology. In contrast to Shavers Fork, mainstem habitats in the Potomac and Greenbrier River study areas are not known to support year-round brook trout populations due to the lack of coldwater refugia and could not be sampled sufficiently enough to characterize population genetic structure. Consequently, only tributary habitats were sampled in the Greenbrier and Potomac study areas (Fig. 1). There are no barriers among the areas we sampled within the Potomac and Greenbrier watersheds, and connectivity was maintained among the sites regardless of time of year or flow conditions.

Samples were brought to the U.S. Geological Survey (USGS) Leetown Science Center, Kearneysville, WV for molecular analyses. Whole genomic DNA was extracted from fin clips using the Puregene Kit (Gentra Systems, Minneapolis, MN). All samples were screened for 13 brook trout microsatellite loci (*SfoB52*, *SfoC24*, *SfoC28*, *SfoC38*, *SfoC79*, *SfoC86*, *SfoC88*, *SfoC113*, *SfoC115*, *SfoC129*, *SfoD75*, *SfoD91*, *SfoD100*; King et al. 2012). Details of the master mix composition, thermal cycling parameters, and multiplexing are provided in (King et al. 2012). Microsatellite allele sizes were characterized on an Applied Biosystems (Foster City, CA) ABI 3100 Genetic Analyzer. Genescan™ 3.7 and Genotyper™ 3.6 Fragment Analysis software (Applied Biosystems) was used to score, bin, and output allelic data.

Genetic data analysis

The allelic data generated for all collections was initially examined using MicroChecker (van Oosterhout et al. 2004) to identify the presence of null alleles, scoring errors, and/or large allele drop-out. Because of past supplementation within the Shavers Fork watershed, it is possible some hatchery fish were among those sampled. To avoid the confounding influence these individuals may have on characterization of natural population structure within the Shavers Fork watershed, GeneClass (Cornuet et al. 1999) was used to determine the probability of each individual's multilocus genotype being found among 16 potential hatchery source populations used for supplementation throughout numerous Atlantic slope drainages (Online Resource Table 1). These hatchery strains represent all of the sources of broodstock that would possibly have been used for stocking. Given the strong genetic differentiation between each hatchery

strain and the brook trout inhabiting the drainages we examined (average $F_{ST} = 0.81$, range = 0.39–0.98, TLK, unpublished data), assignment testing is a reliable tool to detect the presence of hatchery fish, and should also assist in identifying any introgression of hatchery alleles. Because detailed records of supplementation were not available for the Shavers Fork, Potomac, and Greenbrier drainages, we assumed any hatchery source could have been stocked in any drainage and therefore tested for the presence of all hatchery strains in each sample. Population allele frequencies were estimated in GeneClass using the Bayesian option (Rannala and Mountain 1997). The probability an individual belonged to one of the hatchery populations was calculated by simulating 10,000 genotypes and calculating the probability of that individual's genotype being observed in that simulated hatchery population. Individuals assigned to a hatchery population with a probability of >0.01 were determined to be of hatchery origin and culled from additional analyses.

Unidentified family structure can be problematic for detection of hidden population structure using Bayesian clustering programs like STRUCTURE, as samples being dominated by one or a few families can lead to the false interpretation of an entire population as being out of Hardy–Weinberg Equilibrium (HWE) (Ramilo and Wang 2012). Therefore, to determine if our samples consisted of a small number of families, we analyzed each collection for the presence of full sibling families using the program COLONY v2.0 (Wang and Santure 2009). Settings for COLONY analyses included the assumption of male and female polygamy, no per locus genotyping error information, no inbreeding, long run length with the full likelihood analysis method, high likelihood precision, no allele frequency updates, and no sibship prior. Samples were analyzed as offspring without assignment of individuals as candidate males or females, as these data were not available for the samples. While the inference of family relationships is weakened in this situation with no sex, age, relationship information, and the assumption of polygamy for both sexes, COLONY is predicted to be more accurate than pairwise estimates of relationships (Wang and Santure 2009).

Genetic diversity of all collections was quantified using GenAIEx (Peakall and Smouse 2006, 2012) to calculate allelic frequencies, number of alleles per locus (N_A), effective number of alleles (A_E), observed heterozygosity (H_O), and unbiased expected heterozygosity (H_E). Rarefied allelic richness (A_R) was calculated in HP-rare (Kalinowski 2005), which enables comparison of the number of alleles across collections with different sample sizes. The sample size was set to 28 for rarefaction calculations. Exact tests in Genepop (Raymond and Rousset 1995) were used to test

the genotypes at each locus for each collection for conformance to HWE. Multi-locus tests of conformance to HWE for each population were completed using Fisher's method output by Genepop. Linkage disequilibrium (LD) was tested for all pairs of loci using contingency tables in Genepop. All tests of HWE and LD tests in Genepop used the default Markov chain parameters. Significance levels for HWE and LD tests were adjusted using the sequential Bonferroni correction (Rice 1989).

Population structuring between each collection was characterized using the fixation index F_{ST} calculated in GenAlEx in an AMOVA framework. For highly variable molecular markers like microsatellites, F_{ST} is not a measure of genetic differentiation among collections because the maximum value of $F_{ST} = 1$ cannot be obtained even when collections have completely non-overlapping sets of alleles (Hedrick 2005). Therefore, we also calculated F'_{ST} , which is a true measure of allelic differentiation between populations, where $F'_{ST} = 1$ when populations share no alleles. F'_{ST} was calculated in GenAlEx using the scaling approach of Meirmans (2006). Significance of all pairwise F_{ST} and F'_{ST} comparisons were assessed through 9,999 permutations. Principal coordinates analyses (PCoA) of the pairwise F_{ST} and F'_{ST} matrices were used to graphically illustrate the patterns of genetic relationships using the program PAST (Hammer et al. 2002). We estimated the effective number of migrants ($N_e m$) between collections using the private alleles method of Barton and Slatkin (1986) implemented in Genepop. These estimates of $N_e m$ are intended only to approximate the relative magnitude of gene flow among collections, and not as accurate estimates of $N_e m$.

To describe among drainage differentiation, we used a hierarchical AMOVA (Excoffier et al. 1992). AMOVA was used to partition the genetic variance among drainages relative to the total variance (F_{RT}), as well as among collections within drainages (F_{ST}). Within Shavers Fork, the collections were further divided into mainstem and tributary collections to investigate whether there was significant partitioning of genetic variance between these groups. Significance of all hierarchical AMOVA analyses was assessed through 9,999 permutations.

The evolutionary relationships among the brook trout collections from the different drainages were visualized through the construction of a Neighbor-Joining tree (Saitou and Nei 1987). Genetic distances between each pair of collections were summarized with genetic distance matrices calculated using the Cavalli-Sforza and Edwards (1967) chord distance in MEGA5 (Tamura et al. 2011). The strength of support for each node in the phylogenetic tree was tested by bootstrapping over loci using *njbpop* (J.M. Cornuet, INRA, France). TreeView software (Page 2001) was used to visualize the tree.

Both Bayesian and maximum likelihood based clustering methods were utilized for comparison with F_{ST} and F'_{ST} based estimates of population structure. In the first approach, the program STRUCTURE 2.3.1 (Pritchard et al. 2000), a Bayesian clustering algorithm, was used to determine the number of populations (or clusters, K) present among the collections sampled. Because a single STRUCTURE analysis on a set of populations may only reveal the uppermost level of population structure (Evanno et al. 2005), we performed a hierarchical STRUCTURE analysis similar to that employed by Vähä et al. (2007). In the initial phase, $K = 1$ –25 clusters were considered for the Shavers Fork, Potomac, and Greenbrier collections pooled together using 100,000 iterations discarded as burn-in followed by 200,000 iterations, and ten independent runs for each K using the admixture model and un-correlated allele frequencies. Subsequent analysis of each cluster tested $K = 1 - C + 3$ (the number of collections (C) included in the subset plus three) using the admixture and correlated allele frequencies model with the same number of burn-in and iterations. Sample location was incorporated as prior information for analyses of the Greenbrier and Potomac collections, as these drainages do not possess cold-water mainstem habitat and presumably do not exchange migrants among tributaries. The number of clusters for each analysis was determined using the 'ΔK' method of Evanno et al. (2005) performed in the program Structure Harvester (Earl and vonHoldt 2012). The optimal alignment among the ten independent iterations of STRUCTURE was found using the program CLUMPP (Jakobsson and Rosenberg 2007). Barplots visualizing member coefficients in the STRUCTURE clusters using CLUMPP output were made using the program DISTRUCT (Rosenberg 2004).

In the second approach we used the program FLOCK (Duchesne and Turgeon 2012). Like STRUCTURE, FLOCK takes as input genotypes for each collection to be analyzed, and a user defined value of K . The collections are randomly partitioned into K groups initially, and then each multi-locus genotype within each group is re-allocated using the Paetkau et al. (1995) multi-locus maximum likelihood assignment method to the cluster with the highest likelihood. The process is repeated for the number of iterations specified by the user. FLOCK has been shown to provide more reliable estimates of K than STRUCTURE for many datasets, and convergence is typically reached quickly in less than 20 iterations (Duchesne and Turgeon 2012). FLOCK differs from STRUCTURE since it is not based on Bayesian Inference, does not use Markov Chain Monte Carlo resampling, and does not build clusters based on optimization of HWE or minimization of LD (Duchesne and Turgeon 2012). For our FLOCK analyses, we first analyzed all collections together using 200 runs and 20

iterations with an LLOD threshold of 0 for values of $K = 1:14$. K was determined by using the stopping and estimation rules published in Duchesne and Turgeon (2012), where the plateau length for a significant result was adjusted to 24 to adjust for the increased number of runs (Duchesne et al. 2013). If there was evidence for $K > 1$ within drainages, we analyzed the remaining collections in FLOCK that were grouping together for additional evidence of population structuring analogous to the hierarchical STRUCTURE approach.

Results

Thirteen microsatellite loci were surveyed in 569 *S. fontinalis* collected from four locations within the Potomac ($n = 107$), four locations within the Greenbrier ($n = 91$), and 14 locations within Shavers Fork ($n = 371$; Fig. 1). Results of Microchecker did not suggest the presence of null alleles, scoring errors, or large allele dropout. Ten individuals from the Shavers Fork collections (two from SFMS1, three from SFMS2, and five from Lambert) were culled from all subsequent analyses after assignment testing suggested these individuals were all stocked from the Bellfonte Hatchery in Pennsylvania. The location of these ten individuals is consistent with a known stocking location slightly downstream from the mouth of the Lambert collection site in Shavers Fork. All subsequent analyses of Shavers Fork brook trout consisted of the remaining $n = 361$ individuals. No hatchery origin fish were identified in the Potomac or Greenbrier collections.

Analyses of family structure in COLONY did not suggest that any of the collections were dominated by a small number of families (Online Resource Table 2). The largest single full sibling family was observed in the OHGK7 collection from the Greenbrier and consisted of six individuals. Elsewhere, several sets of full-sibling dyads were uncovered, but the most found in once collection was five dyads in the POSB5 collection. Overall, eight collections from Shavers Fork and one collection from the Potomac and one from the Greenbrier did not have any full sibling families identified by COLONY. Because no collections appeared to consist of a small number of families, all individuals from each collection were retained for subsequent analyses.

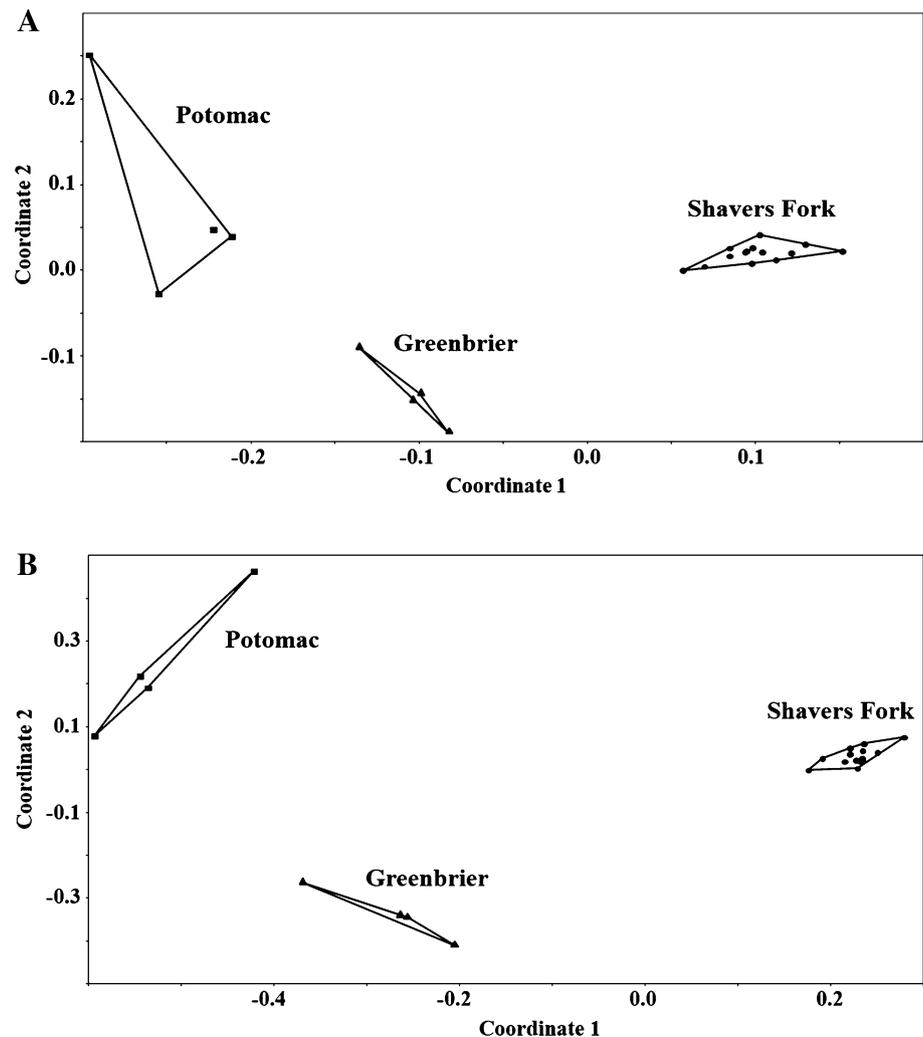
Before correction for multiple testing, 31 individual locus/collection combinations significantly deviated from HWE ($P < 0.05$; Online Resource Table 3). However, after sequential Bonferroni Correction (initial corrected $\alpha = 0.05/271 = 0.0002$) only one locus significantly deviated from HWE (locus D100 in the POSB8 collection). Multi-locus tests for conformance to HWE of each collection using Fisher's method indicated all Shavers Fork

collections were in HWE before and after correction for multiple testing (Table 1). In contrast, the POSB4, POSB7, and POSB8 were out of HWE before Bonferroni correction, with the POSB4 and POSB8 remaining significant after Bonferroni correction. In the Greenbrier, the OHGK6 population was not in HWE before or after correction for multiple testing. When collections within drainages were pooled and analyzed as single populations for conformance to HWE, Shavers Fork was in HWE while the Greenbrier and Potomac were both out of HWE due to heterozygote deficiencies. Each collection possessed at least one locus pair exhibiting significant LD ($P < 0.05$) before sequential Bonferroni correction, totaling 148 loci pairs ranging from 2 loci pairs in SFMS 7 to 17 loci pairs in OHGK7. After sequential Bonferroni correction (initial corrected $\alpha = 0.05/1,576 = 0.00003$), 6 loci pairs remained significant for tests of LD (loci C88 and C115, C88 and D91, C115 and D91 in POSB4; loci B52 and C88, B52 and D91, loci C88 and D91 in OHGK7).

The allelic richness and effective number of alleles among drainages were variable, ranging from 2.77 to 4.54 in Shavers Fork, to 3.69 and 5.73 in the Greenbrier (Table 1; locus specific data are provided in Online Resource Table 3). Observed heterozygosity was the lowest in Shavers Fork (0.53) and Potomac (0.54), but higher within the Greenbrier (0.61; Table 1). Expected heterozygosity was lowest in Shavers Fork ($H_e = 0.55$), and higher within the Potomac and Greenbrier ($H_e = 0.67$ and 0.66 respectively). Within Shavers Fork, the lowest diversity measures were all within the Second 10 collection, though the magnitude of the differences compared to other collections were small. While this may be attributed to sampling error in Second 10 ($n = 17$), it is notable that Second 10 is the only collection separated from downstream samples due to a waterfall, which may contribute to the low diversity observed through reduced gene flow. Genetic diversity among the Potomac collections was comparable with the exception of the POSB5 collection, which had the lowest genetic diversity estimates among all Potomac collections and among all drainages. Within the Greenbrier, genetic diversity was similar among collections although the OHGK3 collection possessed the highest genetic diversity at all measures.

Pairwise F_{ST} estimates among collections from the three drainages ranged from 0.1550 between a collection from the Potomac (POSB8) and Greenbrier (OHGK3) to 0.5262 between a collection from the Potomac (POSB5) and Shavers Fork (Second 10; Online Resource Table 4). In contrast, differentiation among collections from the three drainages measured by F'_{ST} ranged from 0.4784 between POSB8 and OHGK3 to 0.9219 between POSB5 and OHGK7. Patterns of statistical significance were identical between F_{ST} and F'_{ST} . Of the 128 inter-drainage tests of

Fig. 2 Principal coordinates analysis of the pairwise F_{ST} (A) and F'_{ST} (B) matrices of 22 brook trout *Salvelinus fontinalis* collections sampled from the Shavers Fork, Potomac, and Greenbrier drainages in WV, USA. For F_{ST} , the two axes combined accounted for 86 % of the total variation, and for F'_{ST} the two axes combined accounted for 85.2 % of the total variation



significance of pairwise F_{ST} values, all were significantly greater than zero before and after sequential Bonferroni correction for both F_{ST} and F'_{ST} , indicating the presence of strong population structure among the three drainages. All of the collections within the Greenbrier and Potomac exhibited significant pairwise differentiation, with F_{ST} ranging from 0.0399 to 0.3506 in the Potomac, and 0.0282–0.1045 in the Greenbrier. F'_{ST} was higher and ranged from 0.1120 to 0.6735 in the Potomac, and 0.0805–0.2897 in the Greenbrier. The only non-significant F_{ST} values were observed among some collections within Shavers Fork. Of the 91 pairwise comparisons within Shavers Fork, F_{ST} ranged from 0.0000 to 0.1189, and F'_{ST} ranged from 0.0000 to 0.2300. Sixty of the tests were non-significant with F_{ST} values ranging from 0.0000 to 0.0386, and F'_{ST} values ranging from 0.000 to 0.0849, suggesting elevated gene flow among many of the collection sites. The Second 10 site, located above a natural waterfall, was significantly different from all of the other collections within the Shavers Fork watershed. The First and Buck

collections also exhibited significant differentiation against most other pair-wise collections within Shavers Fork.

Principal coordinates analysis of F_{ST} (Fig. 2a) strongly supported the existence of three distinct and separate drainages, with the first two axes explaining 86 % of the variation in the data. Samples within Shavers Fork clustered tightly together indicating minimal population structuring. In contrast the four samples from the Potomac were spaced more widely reflecting the lower F_{ST} values, whereas the Greenbrier samples were more tightly clustered. Principal coordinates analysis of the F'_{ST} values revealed the same general pattern as F_{ST} with the first two axes explaining 85.2 % of the variation in the data, but among drainage differentiation was even more pronounced (Fig. 2b).

The effective number of migrants per generation, N_{em} , estimated between all pairs of collections (Online Resource Table 5) was consistent with the patterns of population structure (F_{ST}) and differentiation (F'_{ST}). The average N_{em} within Shavers Fork was 3.51 (range 0.89–7.41), which

Table 2 Analysis of molecular variance partitioning genetic variation within and among collections of brook trout *Salvelinus fontinalis* from the Shavers Fork, Potomac, and Greenbrier drainages in WV, USA

Source of variation	Sum of squares	% of variance (total)	Estimated F statistic	P value
Shavers Fork, Potomac, Greenbrier drainages				
Among drainages	862.0901	27 %	$F_{RT} = 0.2694$	0.0001
Among populations	294.5365	4 %	$F_{SR} = 0.0604$	0.0001
Within populations	3,991.9360	69 %	$F_{ST} = 0.3136$	0.0001
Potomac				
Among populations	142.2137	18 %	$F_{ST} = 0.1774$	0.0001
Among individuals within populations	419.1798	6 %	$F_{IS} = 0.0752$	0.0001
Among individuals	374.5523	76 %	$F_{IT} = 0.2393$	0.0001
Greenbrier				
Among populations	51.5368	6.5 %	$F_{ST} = 0.0651$	0.0001
Among individuals within populations	368.2217	3 %	$F_{IS} = 0.0310$	0.0361
Among individuals	361.9544	90.5 %	$F_{IT} = 0.0942$	0.0001
Shavers Fork				
Among populations	100.7990	2 %	$F_{ST} = 0.0229$	0.0001
Among individuals within populations	1,233.6753	2 %	$F_{IS} = 0.0194$	0.0208
Among individuals	1,234.3526	96 %	$F_{IT} = 0.0420$	0.0001
Shavers Fork as tributary and mainstem regions				
Among regions	3.9060	0 %	$F_{RT} = -0.0056$	1.0000
Among populations	98.8108	3 %	$F_{SR} = 0.0254$	0.0001
Within populations	2,499.8553	97 %	$F_{ST} = 0.0200$	0.0001

Significance was assessed through 9,999 permutations

The ‘R’ notation (e.g., F_{RT} , F_{SR} etc.) indicates cases where collections were pooled and treated as separate regions/groups

was higher than that observed in the Greenbrier (average $N_e m = 1.48$, range 0.77–2.61) and Potomac (average $N_e m = 0.99$, range 0.31–2.1) indicating higher gene flow within Shavers Fork. When $N_e m$ was averaged for each

collection versus each other collection within Shavers Fork (i.e., SFMS1 vs SFMS2, SFMS1 versus SFMS3, etc.), the lowest average was observed for Second 10 versus other collections (1.78) and the highest was observed for SFMS1 versus all other collections (5.22).

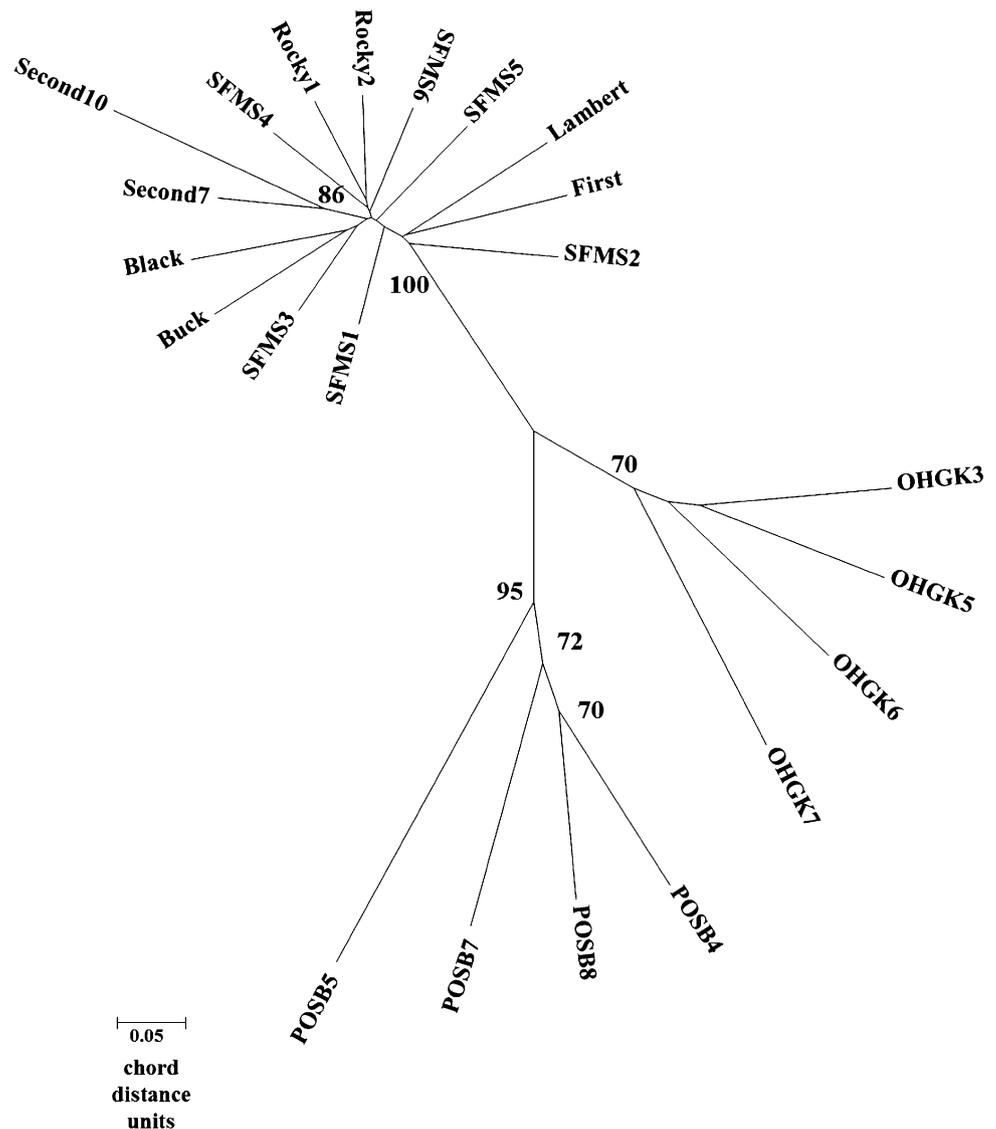
The hierarchical AMOVA reinforced the interpretation of strong among drainage differentiation, indicating 27 % of the genetic variance was due to differences among drainages ($F_{RT} = 0.2694$, $P < 0.0001$; Table 2). Within Shavers Fork, 2 % of the genetic variance was attributed to differences among collections, while the Greenbrier and Potomac had 7 and 18 % of the genetic variance, respectively, partitioned among collections reflecting the stronger population structure within the Potomac and Greenbrier. When Shavers Fork was divided into mainstem and tributary regions, none of the genetic variance was attributed to differences among mainstem and tributary populations ($F_{RT} = -0.0056$, $P = 1.0000$).

The neighbor joining tree (Fig. 3) illustrated the high levels of among drainage differentiation identified by the AMOVA with high bootstrap support, with each collection grouping with other collections from the same drainage. Within Shavers Fork, some of the mainstem collections grouped with tributary collections indicating the low levels of differentiation observed among many mainstem and tributary collections. The Second 10 collection from above the waterfall was the most strongly differentiated from other collections within Shavers Fork, and grouped with the Second 7 collection, presumably due to unidirectional gene flow downstream from Second 10 to Second 7.

Initial STRUCTURE analysis yielded an estimate of $K = 2$, consisting of the Shavers Fork collections comprising one cluster, and the Potomac Greenbrier collections a second cluster (Fig. 3). Subsequent analysis of Shavers Fork alone and applying the ΔK criterion for choosing K resulted in an estimate of $K = 3$ for Shavers Fork (Online Resource Fig. 1), where the Buck and Second 10 collections appeared to constitute distinguishable groups within Shavers Fork. This observation is consistent with the results of pairwise estimates F_{ST} and F'_{ST} within Shavers Fork collections, where Buck and Second 10 exhibited some of the highest pairwise values.

The Potomac and Greenbrier separated into two distinct drainages when analyzed separately from Shavers Fork (Fig. 4). Subsequent analysis of the Potomac drainage suggested $K = 3$, with the POSB5 and POSB7 forming distinct clusters, and the POSB4 and POSB8 collections forming another cluster. Another round of STRUCTURE analysis of the POSB4 and POSB8 collections yielded $K = 3$ using the ΔK criterion. The barplot for $K = 3$ showed two separate lineages comprising most of POSB4 and POSB8, with interspersed largely non-admixed individuals from a third lineage. Omission of one member of

Fig. 3 Evolutionary relationships of brook trout *Salvelinus fontinalis* genotyped at thirteen microsatellite loci inferred using the neighbor-joining algorithm (Saitou and Nei 1987) applied to the Cavalli-Sforza and Edwards (1967) chord distance for 22 collections from three drainages in WV, USA. The phylogenetic tree was generated using *njbpop* (J.M. Cornuet, INRA, France). Numbers along branches represent bootstrap support for nodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The optimal tree with the sum of branch length = 3.51450314 is shown



each identified full-sibling relationship from the COLONY analyses and re-running STRUCTURE for the Potomac samples did not alter the ΔK results (data not shown). Therefore, the Potomac samples appear to constitute five genetically distinct collections.

Within the Greenbrier, an initial STRUCTURE analysis suggested $K = 2$, corresponding to the OHGK7 as one cluster, and the remaining three collections comprising a separate cluster (Fig. 3). When the remaining three collections were analyzed and K determined using the ΔK criterion, K was determined to equal 4, consisting of OHGK3, OHGK5, and OHGK6 as separate clusters with four largely non-admixed individuals mixed into OHGK6. Comparison of these four individuals with the COLONY results showed that two of these individuals were part of a full-sibling family, and all were members of full-sibling dyads. Re-running STRUCTURE with one individual of

each full-sibling relationship removed resulted in an estimate of $K = 3$ among the OHGK3, OHGK5, and OHGK6.

An initial analysis of all collections together in FLOCK suggested the presence of $K = 3$ clusters using plateau analysis (Duchesne and Turgeon 2012), corresponding to the Greenbrier, Potomac, and Shavers Fork drainages. Subsequent analysis of the Shavers Fork collections resulted in an undecided estimate, suggesting there was no detectable genetic structure in Shavers Fork and therefore $K = 1$. Within the Potomac, the initial analysis of all of the Potomac collections resulted in $K = 5$ using plateau analysis. In general, the clusters corresponded closely to each collection with the exception of a cluster of ten individuals primarily from POSB4 and POSB8. FLOCK analyses of the Greenbrier initially identified $K = 2$ clusters, consisting of OHGK7 as one cluster and OHGK3, OHGK5, and OHGK6 in the other. Another round of FLOCK analysis

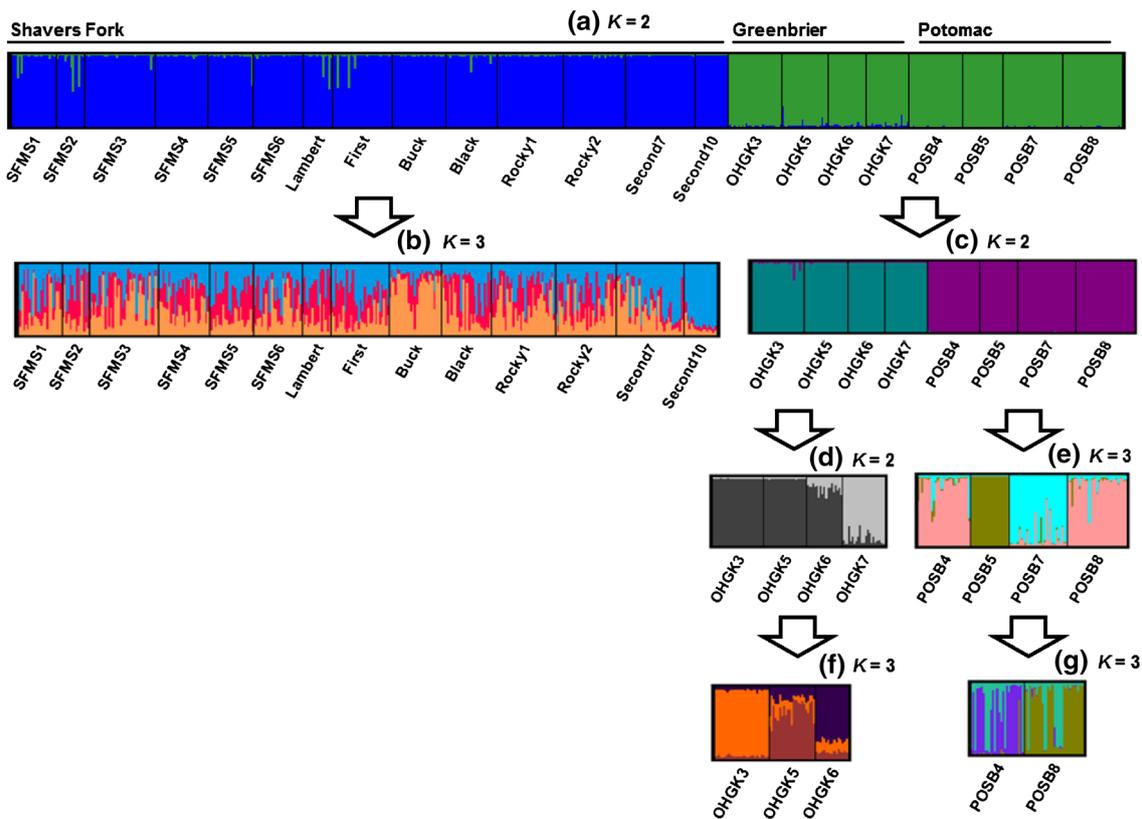


Fig. 4 Summary cluster membership barplot generated by hierarchical cluster analysis in the program STRUCTURE performed on brook trout *Salvelinus fontinalis* collected from three drainages in WV, USA. The letters a–g denote the number of clusters as determined using the Delta K method of Evanno et al. (2005), and plots of the

Delta K statistic for each letter can be found in Online Resource Fig. 1. Each individual is represented by a single vertical line, broken into K colored segments, the length of which is proportional to the membership fraction in each of the K clusters

resulted in $K = 2$ clusters, with the OHGK3 collection forming one cluster, and the OHGK5 and OHGK6 forming another. An additional run of FLOCK on the OHGK5 and OHGK6 cluster resulted in $K = 2$ using plateau analysis yielding an overall estimate of $K = 4$ for the Greenbrier.

Discussion

The results of our genetic analyses are consistent and provide convincing evidence in support of previous telemetry and mark recapture studies (Petty et al. 2005, 2012) hypothesizing brook trout within the Shavers Fork drainage exhibit levels of gene flow sufficient to prevent differentiation among tributaries. All mainstem and tributary samples within Shavers Fork conformed to HWE, as well as all samples pooled and analyzed as a single population. Pairwise F_{ST} and F'_{ST} values among Shavers Fork tributaries were generally low ($F_{ST} < 0.04$, $F'_{ST} < 0.08$) and mostly non-significant indicating appreciable gene flow among tributaries. Estimates of the $N_e m$ suggested elevated gene flow within Shavers Fork versus that

observed in other drainages. Similarly, hierarchical AMOVA showed that only 2 % of the genetic variance in Shavers Fork was due to differences among collections. An analysis of the evolutionary relationships among populations using a neighbor joining tree indicated that mainstem populations grouped with other tributary populations in Shavers Fork, suggesting genetic similarity among those collections. Finally, Bayesian and maximum likelihood clustering methods found little evidence of population substructure within Shavers Fork. In contrast to recent studies documenting strong population structuring in systems with barriers to dispersal (e.g., Whiteley et al. (2013)), our results show that in the absence of barriers, brook trout may exhibit low levels of differentiation among tributaries.

The results of STRUCTURE and FLOCK were largely complementary, and provided similar estimates of population structure. Both methods partitioned the POSB4 and POSB5 collections into three clusters. POSB4 and POSB8 had the lowest F_{ST} value of 0.0399, though these collections were the furthest apart within the Potomac. While the present day mainstem in the North Fork South Branch of

the Potomac is not conducive to inter-tributary movement, there may have been historical gene flow similar to what we observed in Shavers Fork. It is also possible that there may be additional within tributary population structure in the POSB4 and POSB8 collections that was not represented in our samples, resulting in the formation of an extra cluster. The fact that neither the POSB4 or POSB8 populations were in HWE suggest this as a possibility. In Shavers Fork, STRUCTURE identified the Second 10 site located above a waterfall, and the Buck collection as genetically distinguishable from other collections, whereas FLOCK detected no structure within Shavers Fork. The reason for the discrepancy between the methods is unknown, but because STRUCTURE forms clusters based on maximization of HWE and FLOCK forms clusters based on iterative reallocation using the Paetkau et al. (1995) assignment test method, the two approaches may not always yield the same results.

Despite the high levels of connectivity within Shavers Fork, the Second 10 and Buck collections, which are the only two collections within Shavers Fork that experience any isolation due to natural features, were identified as separate clusters using STRUCTURE. The separation of Second 10 due to a waterfall has resulted in significant differentiation of itself from all other collections throughout Shavers Fork. In contrast Second 7, located immediately downstream of Second 10, was not significantly different from many other collections within Shavers Fork. This result highlights the impact that barriers to dispersal can have on levels of population structure in brook trout populations, where barriers in the headwaters of tributaries can cause elevated genetic differentiation (Whiteley et al. 2013). The differentiation of Buck Run is likely due to the fact that it is a small stream and drains into a relatively warm region of Shavers Fork. The mouth of Buck Run is also characterized by a wide alluvial fan that may become dry during extended low flow periods. Either of these factors may reduce gene flow between Buck Run and the rest of Shavers Fork, and may contribute to elevated genetic drift in Buck Run from an unstable population size. An understanding of what causes population isolation or not on different streams is an important direction for future research in this region.

All of the Greenbrier and Potomac collections exhibited significant levels of genetic differentiation among all tributary collections in contrast to Shavers Fork, and more closely conform to observed patterns of genetic structure seen elsewhere in the Appalachians. Among tributary population structure ranged from $F_{ST} = 0.0399$ to 0.2527 in the Potomac, and $F_{ST} = 0.0282$ to 0.1045 in the Greenbrier. These levels are similar to those observed within “patches” (e.g., a group of occupied contiguous catchments) of similar size examined in Whiteley et al.

(2013). F_{ST} among the Shavers Fork, Potomac, and Greenbrier collections were also similar to levels observed among patches in Whiteley et al. (2013). Consequently, it appears that elevated gene flow among tributaries and low levels of differentiation may be a unique characteristic of the Shavers Fork drainage. What remains unclear, however, is whether or not the structure of Shavers Fork populations was more common historically throughout the Appalachian region or whether most brook trout populations have always tended to be structured as fragmented isolates.

While there are no other genetic studies of Appalachian brook trout exhibiting population structure similar to that observed in Shavers Fork, studies of the congeneric bull trout, a species with similar life history characteristics as brook trout, indicate bull trout may exist as metapopulations in relatively undisturbed drainages (Nyce et al. 2013). Bull trout occupying headwater stream habitats within their native range of the northwestern United States and southwestern Canada may consist of solely resident individuals, or a mix of migratory and resident life history strategies, where large adults migrate long distances (among tributaries, to hundreds of kilometers; Swanberg 1997). Nyce et al. (2013) noted that most studies of *S. confluentus* population structure were conducted in disturbed watersheds impacted by fragmentation and/or degradation, where tributary populations exist as highly differentiated subpopulations (e.g., Kanda and Allendorf 2001). However, in the East Fork Bitterroot River drainage in eastern Montana, which has few barriers to dispersal, Nyce et al. (2013) documented metapopulation structure of bull trout where genetic differentiation was relatively low among tributaries (global F_{ST} in the East Fork Bitterroot was 0.063). The findings of metapopulation structure of bull trout in the East Fork Bitterroot parallel the population structure we observed in brook trout occupying Shavers Fork. Our results therefore reinforce the position of Nyce et al. (2013) that a thorough understanding of salmonid population structure requires analyses of population structure across a diversity of systems and scales to help set appropriate goals for population restoration (Petty and Merriam 2012).

The ecology of upper Shavers Fork is unique relative to other Appalachian brook trout populations because of the mobility of large adults in the mainstem, and the use of the mainstem as foraging but not spawning habitat (Petty et al. 2005, 2012). However, despite this mobility Shavers Fork brook trout displayed lower estimates of genetic diversity (effective number of alleles, observed and expected heterozygosity, and allelic richness) than was observed in the Greenbrier and Potomac. It could be that past impacts of harvest, acid precipitation, and general habitat degradation have impacted the brook trout in Shavers Fork to a greater

extent than within the Greenbrier or Potomac, through all of these drainages have experienced similar problems. As noted by Petty et al. (2012, 2014), the maintenance of genetic diversity of Shavers Fork brook trout will require the protection of existing coldwater sources in the mainstem and possibly even the construction of new coldwater refugia (artificial deep pools, lateral groundwater seeps, etc.) to cope with the warming climate, which is predicted to reduce suitable brook trout habitat in the Appalachians (Flebbe et al. 2006).

Because previous studies have shown that tributaries are the source of brook trout that inhabit the mainstem in Shavers Fork (Petty et al. 2005), one of the original objectives of this study was to identify specific tributaries that may be serving as dominant sources of mainstem immigrants in order to provide information about critical areas on which to focus conservation efforts (e.g., Neville et al. 2006; Nyce et al. 2013). High rates of gene flow among tributaries within the Shavers Fork watershed make it difficult to confidently quantify relative rates of dispersal from individual tributaries to the mainstem populations using traditional molecular genetic approaches such as assignment testing or mixed stock analyses (data not shown). Nevertheless, estimates of gene flow among tributaries indicate that the Second 10 site is the most isolated from the mainstem and other tributaries. There is some evidence, also, that Rocky Run may provide a substantial number of migrants to the upper sections of the mainstem based on the relatively low F_{ST} values between Rocky1 and Rocky2 with the mainstem collections. The overall high rates of dispersal suggests that nearly all tributaries have the potential to serve as a source of individuals to the mainstem and relative importance as a source may be most strongly influenced by variation in brook trout densities within tributaries rather than variation in emigration rates from tributaries.

The brook trout we collected from Shavers Fork tributaries consisted of a mixture of young of the year, juveniles, and adults. Sampling a variety of life history stages was necessary to obtain adequate sample sizes for each collection. Unlike later life stages, young of the year brook trout can always be assumed to be residents of their sampled tributary, as young of the year brook trout show limited dispersal on the order of tens of meters or less during the first year (Hudy et al. 2010; Kanno et al. 2011). Therefore, genotyping young of the year exclusively may provide the most accurate characterization of population structure within Shavers Fork. In contrast, sampling large adults only in tributaries may result in a mix of resident and non-resident brook trout, providing a less accurate picture of the true population structure. While we are confident that genetic differentiation is low among tributaries in Shavers Fork, future studies should strive to collect sufficient numbers of both young of the year and adults for

comparison in the same study, and if the levels of differentiation are low in both groups, one can be more confident in the conclusion of extensive effective migration among tributaries.

The isolation of brook trout in small headwater tributaries may explain the high levels of genetic differentiation observed among contemporary brook trout populations throughout the central and southern Appalachian range. What remains unclear, however, is what the primary mechanisms leading to this population fragmentation are. Potential mechanisms of fragmentation include: 1- warming of main stem habitats that historically served as foraging habitats and dispersal corridors, 2- dispersal barriers 3- high rates of harvest, and 4- competition with exotic species such as brown and rainbow trout. In most systems, many of these factors probably interact with each other to fragment suitable habitat. Nevertheless, additional research is needed to determine what the role of each of these factors is in impacted watersheds. Finally, our results raise a sequence of questions critical for brook trout conservation within their native range. Although well mixed networks of populations are currently rare, were they more common historically? Are there other systems in the native range of brook trout that may support well mixed populations or metapopulations? Can we find them, validate them genetically, and manage for them? Also, can we find areas where isolated populations could be restored to mixed metapopulations?

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