Geographic distribution of southern- and northern-form brook trout populations in southwestern Virginia

Joanne Elizabeth Davis

A thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the
degree of
Master of Science
in
Fisheries and Wildlife Sciences

Eric M. Hallerman, Chair
Brian R. Murphy
C. Andrew Dolloff

June 12, 2008
Blacksburg, Virginia

Keywords:
brook trout, *Salvelinus fontinalis*, population genetics, allozymes, New River

© 2008, Joanne Elizabeth Davis

Geographic distribution of southern- and northern-form brook trout populations in southwestern Virginia

Joanne Elizabeth Davis

ABSTRACT

The brook trout *Salvelinus fontinalis* is the only salmonid native to the southern Appalachian Mountains, and is distributed across eastern North America from Canada to Georgia. This species was once abundant in coldwater lakes and streams throughout its range, but environmental disturbances and the introduction of non-native species have drastically reduced the number and sizes of wild populations. Genetic evidence suggests a division at the subspecies level between southern- and northern-derived brook trout populations, with the break between the two forms occurring roughly at the New River watershed. Before the subspecies structure was recognized, brook trout of northern origin were widely stocked throughout the Southeast. The primary objective of this study was to determine the phylogenetic origin of all wild brook trout populations in southwestern Virginia using allozyme markers. Seventy-eight streams believed to contain brook trout in the New, James, Holston, and Yadkin river drainages were sampled by backpack electrofishing. Muscle tissue samples were collected from 916 individuals from 56 populations using a non-lethal biopsy technique. The samples were analyzed by cellulose acetate gel electrophoresis and histochemical staining techniques. Variation at four polymorphic loci, including the diagnostic creatine kinase (*CK-A2**) locus, was quantified in terms of genetic diversity and population genetic differentiation. Allele frequencies indicated that 19 populations were of putative southern origin, 5 of northern origin, and 32 of mixed genetic origin. The secondary objective was to determine the geographic distribution of southern- and northern-form brook trout populations throughout the native range using data compiled from all known genetic studies. A map of these data showed that the break between the southern and northern form is sharp, occurring at the New/Roanoke-James watershed divide. Populations from the New River drainage expressed the southern allele at a frequency of 85%, suggesting that their historic native character is southern, and that the presence of northern alleles is due to stocking or stream-capture events. The persistence of the southern form, despite the heavy stocking of northern-derived individuals, may be evidence of an adaptive advantage for the southern form of

the species. Existence of adaptive genetic differentiation supports the case for conservation of the southern form of the species in future management of brook trout.

ACKNOWLEDGMENTS

This work was funded through Federal Aid in Sport Fish Restoration Project F-128-R, administered by the Virginia Department of Game and Inland Fisheries (VDGIF). I thank George Palmer, Cliff Kirk, and especially Joe Williams of VDGIF for their assistance with fieldwork and collection of genetic samples. Without complaint, Joe spent many hot summer days negotiating slippery stream beds while bushwhacking through dense rhododendrons and plumes of mosquitoes, all while carrying a net and a heavy backpack electroshocker. His vast knowledge of the local brook trout populations could only be acquired from 25+ years of monitoring these streams and a love for his work. I sincerely thank him for sharing his knowledge and experience with me and making field work a truly enjoyable experience.

I thank my committee members; Eric Hallerman, Brian Murphy, and Andy Dolloff for helping me complete this project by donating their time, support and helpful criticism. Ray Morgan of the University of Maryland Center for Environmental Science kindly provided unpublished data from Maryland populations, and Doug Besler of the North Carolina Wildlife Resource Commission generously provided tissue samples. I would also like to recognize all of the other researchers and brook trout enthusiasts who provided the background information and data necessary for me to complete this project. I thank Chris Printz of ATS International for generously providing his time and expertise in the production of the maps.

I am indebted to my friend and mentor, Michael Milgroom of Cornell University, for his role in getting me interested and involved in conservation genetics. Also, my dear friend Brandon Smith gave me the confidence and encouragement to pursue a graduate education. I thank my entire family and all of my friends for providing me with a lifetime supply of love and laughter. Chris provided stability and balance by offering plenty of welcome excuses to forget about work, and Zoe and Maya welcomed me home every single day with wagging tails, exposed bellies and hopeful expressions. Finally, I dedicate this thesis to my mom who, despite difficult circumstances, raised me and my brothers with unwavering love and boundless encouragement. I thank her, with all my heart, for making me the person I am today.

TABLE OF CONTENTS

ABSTRACT……………………………………………………………………………...........ii
ACKNOWLEDGMENTS……………………………………………………………..…..............iv
TABLE OF CONTENTS………………………………………………………...….............v
LIST OF TABLES………………………………………………………………..…...........vi
LIST OF FIGURES……………………………………………………………..…............vii

INTRODUCTION……………………………………………….........……………………...1

METHODS
Genetic assessment of southwestern Virginia brook trout populations..2

Range-wide genetic assessment of brook trout populations..5

RESULTS
Genetic assessment of southwestern Virginia brook trout populations...5

Range-wide genetic assessment of brook trout populations...7

DISCUSSION
Properties of allozyme markers...8

Genetic assessment of southwestern Virginia brook trout populations...9

Range-wide genetic assessment of brook trout populations...12

Management Recommendations.................………………………………………..........……..13

REFERENCES………………………………………………………………........……….…..16

APPENDICES
Appendix A: Protocol for the non-lethal collection and storage of brook trout muscle tissue...35
Appendix B: Protocol for cellulose-acetate gel electrophoresis of enzymatic proteins................36
Appendix C: Protocol for histochemical staining of cellulose acetate gels for the visualization of enzymatic proteins...37
Appendix D: Buffer and stain recipes for each enzyme system...38
Appendix E: Representative gel images for each enzyme system...40

LIST OF TABLES

Table 1. - Wild brook trout streams in southwest Virginia sampled by electroshocking in 2005-2007. Non-lethal tissue samples collected from brook trout in 78 streams; 22 streams contained no brook trout; 19 streams were dropped from the study (N/S = not sampled)………….......…20

Table 2. - Allele frequencies at four polymorphic loci in wild brook trout populations in 56 southwest Virginia streams, grouped by drainage. Charles Creek, a known southern-form population, was included as a southern-form reference group. Individuals from Paint Bank Hatchery, which cultures the northern form, were included as a northern-form reference group…………………………………………..........................……………………..22

Table 3. - Allele frequencies at two pairs of isoloci in wild brook trout populations in 56 southwest Virginia streams, grouped by drainage. Charles Creek, a known southern-form population, was included as a southern-form reference group. Individuals from Paint Bank Hatchery, which cultures the northern form, were included as a northern-form reference group…………………………………………………….........................……………..24

Table 4. - Genetic diversity of populations of brook trout from 56 streams, based on analysis at four polymorphic allozyme loci (*CK-A2*, G3PDH*, GPI-A*, sMDH-B1,2**). Abbreviations include: number of individuals analyzed (*N*), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (*Ho*), and observed heterozygosity (*He*)………………………...………………………………26

Table 5. - Genetic diversity of brook trout populations, grouped by drainage, genetic origin, and geographic location relative to the eastern continental divide. Based on analysis at four polymorphic allozyme loci (*CK-A2*, G3PDH*, GPI-A*, sMDH-B1,2**). Abbreviations include: number of populations per group (*N*), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (*Ho*), and observed heterozygosity (*He*)………………………………………………...……28

Table 6. - Genetic characterization at the *CK-A2** locus for brook trout populations in southwest Virginia that were not sampled in this study. Data were compiled from all known published and unpublished data sources………...………………………………..29

Table 7. - Genetic characterization of brook trout populations in major river drainages, based on the frequency of the *CK-A2*100* allele using data gathered from all available published and unpublished studies...30

LIST OF FIGURES

Figure 1.- Genetic characterization at the *CK-A2** locus for 56 wild brook trout populations in southwest Virginia. See Table 1.1 for details……………………...31

Figure 2. - UPGMA cluster analysis of genetic distance (Nei 1978) among brook trout populations in southwest Virginia. The major river drainages are shown in parentheses…………………………………………………………………….....…............32

Figure 3. - Genetic characterization at the *CK-A2** locus for 83 wild brook trout populations in southwest Virginia, including populations characterized in this study and populations characterized previously. Spy Run (James River drainage, Augusta County) was out of the range of this map. See Tables 1.1 and 1.6 for details…………...……...….33

Figure 4. - Genetic characterization of brook trout populations in major river drainages, based on the *CK-A2** locus, using data compiled from all known genetic studies of brook trout populations throughout the native range. See Table 2.1 for details.....……..............................….................34

INTRODUCTION

The brook trout *Salvelinus fontinalis* is the only salmonid native to the southern Appalachian Mountains, and is distributed across eastern North America from Canada to Georgia (MacCrimmon and Campbell 1969). This species was once abundant in coldwater lakes and streams throughout its range, but environmental disturbances, such as deforestation, development, pollution, and the introduction of non-native rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* have drastically reduced the number and sizes of wild populations. Hudy et al. (2005) completed an assessment of the distribution, status, and threats to brook trout populations across their native range within the eastern United States, and found that brook trout are extirpated from 21% of the subwatersheds in their potential or historical range.

The decline of native populations became apparent in the mid-1800s (King 1937, Lennon 1967). In an effort to reestablish or supplement depleted streams, fishery managers began transferring and stocking hatchery-reared brook trout extensively throughout its native range. However, hatchery-reared brook trout often exhibit lower growth, yield, survival, and natural reproduction than locally adapted wild populations (Webster and Flick 1981, Lachance and Magnan 1990). The hybridization of hatchery-derived fish with wild populations can compromise the genetic integrity and fitness of receiving populations by introducing foreign genes and breaking up locally adapted gene complexes (Utter 2003, Allendorf et al. 2004).

Owing to the challenges of culturing southern-derived brook trout in captivity, all hatchery-reared brook trout are derived from the northeastern United States (Kincaid 1981, McCracken 1993). Some fishery managers have asserted that brook trout populations from the southern Appalachians are smaller as adults and mature earlier than those from the North (Lennon 1967), and have suggested that these morphological differences may be adaptations to life in the high-elevation headwater streams to which they are restricted. Although the presence of significant morphological differences between brook trout populations has not been rigorously demonstrated (Guffey 1998, Cornelison 2005), various genetic studies have identified significant genetic differentiation between southern- and northern-derived brook trout populations (Stoneking et al. 1981; McCracken et al. 1993; Dunham et al. 1994; Kriegler et al. 1995;

Hayes et al. 1996), with the break between the two forms occurring roughly at the New River watershed (Guffey 1998, Palmer and Hallerman 2000, Hall et al. 2002). Genetic differences between the two forms may be large enough to justify subspecies-level distinction (Stoneking et al. 1981, McCracken et al. 1993). Unfortunately, before the subspecies structure was recognized, brook trout of northern origin already had been stocked extensively in southern waters.

In addition to subspecific-level differentiation, DNA markers have revealed finer-scale population structure throughout the range of brook trout (Stoneking et al. 1981, Morgan and Baker 1991, McCracken et al. 1993, Dunham et al. 1994, Angers et al. 1995, Kriegler et al. 1995, Shull and Walker 1995, Burnham-Curtis 1996, Hayes et al. 1996, Jones et al. 1997, Danzmann et al. 1998, Guffey 1998, Palmer and Hallerman 2000, Castric et al. 2001, Galbreath et al. 2001). Differentiation at smaller geographic scales may reflect different colonization histories, as well as differential effects of selective and non-selective population genetic processes. Some of this differentiation may warrant management of populations or groups of populations as distinct management units.

Against the backdrop of decline of the southern form and history of stocking with non-native fish, genetic characterization of southwestern Virginia brook trout populations is necessary to support informed management decisions. The primary objective of this study was to use allozyme markers to classify all known, extant wild brook trout populations in southwestern Virginia as southern form, northern form, or introgressed. The secondary objective of this study was to determine the geographic distribution of southern- and northern-form brook trout populations throughout the native range by compiling and analyzing data from all known allozyme studies. To my knowledge, these data have never been compiled.

Methods

Genetic assessment of southwestern Virginia brook trout populations

Study Site. - The study area included all known, extant wild brook trout populations in southwestern Virginia. Seventy-eight wild brook trout streams from the New, James, Holston, and Yadkin river drainages were sampled by backpack electrofishing (Table 1). Collections were conducted in collaboration with Virginia Department of Game and Inland Fisheries (VDGIF) personnel through the summer and

fall of 2005 through 2007. Nineteen additional streams in the study area were not sampled because few brook trout had been found over the course of several years of sampling by VDGIF (Table 1).

Sampling. - Muscle tissue samples were collected from 916 individuals from 56 streams (Table 1). Sample sizes ranged from 8 to 26 individuals per stream. Fish were anaesthetized, and two samples of dorsal muscle tissue (from fish greater than 120mm TL) were collected non-lethally using an 18-gauge Monopty Biopsy Instrument (C.R. Bard, Inc., Covington, GA) and immediately placed on dry ice. Anaesthetized fish were fully revived in fresh water prior to release. A limited number of fish of <120 mm total length were sacrificed to increase the sample size for streams from which few adults were collected. Samples were transported to the Department of Fisheries and Wildlife Sciences at Virginia Polytechnic Institute and State University for long-term storage at -80°C.

Protein analyses. - Genetic analysis was performed using cellulose acetate gel electrophoresis to observe variability at nine loci encoding five polymorphic enzymes; creatine kinase (*CK-A2**), aspartate aminotransferase (*sAAT-1,2**), glycerol-3-phosphate dehydrogenase (*G3PDH**), glucose-6-phosphate isomerase (*GPI-A*, GPI-B1,2**), and malate dehydrogenase (*sMDH-B1,2**). Muscle tissue was homogenized in 200 µl of 0.09M tris-HCl (pH 8.0), and subjected to electrophoresis in tris-glycine buffer (pH 7.5 or 8.0) for 45 minutes, followed by staining for enzyme activity. Electrophoretic conditions and histochemical staining procedures (Appendices B, C and D) were modified from those described by Hebert and Beaton (1993) and Galbreath et al. (2001). Individuals from the Paint Bank National Hatchery (U.S. Fish and Wildlife Service), Paint Bank, VA were included in the analysis as a northern reference population because the hatchery is known to culture the northern form (i.e., the broodstock was derived from the northeastern United States). The North Carolina Wildlife Resource Commission (NCWRC) provided tissue samples from individuals from Charles Creek of the North Toe River drainage for use as a southern reference population. Charles Creek previously was characterized as a pure southern population in a study conducted by the NCWRC using allozyme markers; these particular tissue samples came from individuals that were characterized as southern in that study.

Data Analyses. - Allele frequencies for *CK-A2*, G3PDH*, GPI-A**, and *MDH-B1,2** were calculated for all populations using the Excel Microsatellite Toolkit (Park

2001). Allele frequencies could not be calculated for *sAAT-1,2** and *GPI-B1,2** using that program because both enzymes are encoded by isoloci (i.e., duplicated loci with alleles of overlapping mobility), and the software is not designed to handle duplicated loci (see discussion below for detailed explanation). Since genotypes among heterozygous individuals could not be determined with certainty for *sAAT-1,2**, phenotype frequencies were calculated using the program FDASH (Obbard et al. 2006). The *GPI-B1,2** isoloci contain multiple alleles that could not be assigned to either locus with confidence; hence, they were treated as a single tetraploid locus and allele frequencies were estimated using the program AUTOTET (Thrall and Young 2000). Initially, allele frequency data from all nine marker loci were used to calculate genetic distance, population differentiation, contingency-table analysis of heterogeneity among populations, and hierarchical cluster analysis using the program BIOSYS-1 (Swofford and Selander 1981). The same statistics then were calculated using only the five marker loci with unambiguous interpretation of allelic expression (i.e., omitting data from *sAAT-1,2** and *GPI-B1,2**), to determine any effect of omitting these data from analysis. Because similar conclusions were drawn from analysis of both data sets, I report results from analysis of the reduced dataset only.

Initial characterization of the genetic origin of each population was based on allele frequencies at the diagnostic *CK-A2** locus. Allele frequencies at the other markers were compared to those observed in northern and southern populations characterized in previous studies (Stoneking et al. 1981, Morgan and Baker 1991, McCracken et al. 1993, Dunham et al. 1994, Kriegler et al. 1995, Shull and Walker 1995, Hayes et al. 1996, Guffey 1998, Palmer and Hallerman 2000, Galbreath et al. 2001). Individual heterozygosity and polymorphism were calculated across five loci to assess levels of genetic diversity within each population (Park 2001). Observed genotype frequencies were compared with expected genotype frequencies to assess any departure from Hardy-Weinberg equilibrium using Arlequin (Excoffier et al. 2006) to determine whether populations were exhibiting the genetic signatures of recent inbreeding or bottlenecks. Arlequin also was used to perform an analysis of molecular variance (AMOVA) to characterize the distribution of the genetic diversity within and among populations and groups of populations within and among river basins. Cluster analysis using the unweighted pair-group with arithmetic averaging algorithm (UPGMA, Sneath and Sokal

1973) was performed using BIOSYS-1 (Swofford and Selander 1989), and a dendrogram was built based on Nei's unbiased genetic distance (Nei 1978).

Range-wide genetic assessment of brook trout populations

Data collection. - Allele frequency data were compiled from all known and accessible allozyme studies of brook trout populations throughout the native range (Stoneking et al. 1981, Morgan and Baker 1991, McCracken et al. 1993, Dunham et al. 1994, Kriegler et al. 1995, Shull and Walker 1995, Hayes et al. 1996, Guffey 1998, Palmer and Hallerman 2000, Galbreath 2002, NCWRC 2006, current study). To reduce the inconsistencies inherent to comparing data from multiple sources, only data from the diagnostic *CK-A2** locus were used to characterize populations as either southern form or northern form. To account for sample-size bias, frequencies of the *CK-A2**100 (i.e., southern) allele were weighted based on the number of individuals analyzed per population and frequencies were averaged across all populations in each major river drainage.

Results

Genetic assessment of southwestern Virginia brook trout populations

No brook trout were found in 22 of the 78 streams sampled; hence, muscle tissue samples were collected from only 56 of the streams sampled. Of the 56 populations analyzed for allozyme variation in this study, 19 were characterized as putatively pure southern form, 5 populations as northern form, and 32 populations as introgressed (Table 2, Figure 1). In the Holston River drainage, one population was characterized as putatively pure southern, one northern, and four introgressed. The three populations from the James River drainage exhibited alleles characteristic of northern-form brook trout. Of the 45 populations analyzed from the New River drainage, 16 were characterized as putatively pure southern form, one northern form, and 28 introgressed. The two populations from the Yadkin River drainage exhibited alleles characteristic of southern-form brook trout (Tables 2 and 3, Figure 1).

The Cabin Creek population (New River drainage, Grayson County) deviated significantly ($p < 0.05$) from Hardy-Weinberg equilibrium at the *CK-A2** locus; the sample ($n = 20$) was fixed for *CK-A2*100*, with the exception of one individual that was

homozygous for the northern allele. No other deviations from Hardy-Weinberg equilibrium were detected, indicating that all such populations were in reasonable conformance with assumptions underlying the model.

The proportions of polymorphic loci (*P*), the mean number of alleles per locus (*A*), and mean heterozygosities (*H*) for each population are listed in Table 4. Observed mean *P* and H_0 values were lowest in the putative southern populations ($P = 0.05$, $H_0 = 0.004$; Table 5). The introgressed populations exhibited the highest means for metrics of genetic variability ($P = 0.48$, $H_0 = 0.099$), and the northern populations exhibited intermediate means ($P = 0.20$, $H_0 = 0.053$). Grouped by drainage, Yadkin River populations had the lowest means ($P = 0$, $H_0 = 0$), followed by James River ($P = 0.08$, $H_0 = 0.007$), New River ($P = 0.34$, $H_0 = 0.064$), and Holston River ($P = 0.29$, $H_0 = 0.100$) populations. Atlantic-slope populations exhibited lower mean percent polymorphic loci and heterozygosity values ($P = 0.05$, $H_0 = 0.004$) than Gulf of Mexico drainage populations ($P = 0.33$, $H_0 = 0.068$).

Analysis of molecular variance showed that approximately 34% of the total genetic diversity resulted from variation within populations, 18% among populations within drainages, and 48% among drainages. Most of the total limiting variance was attributed to the *CK-A2** locus, meaning that most of the variance that I measured with allozyme markers was due to differentiation among northern and southern forms of the species.

There was no apparent pattern regarding where populations characterized as southern, northern, or introgressed were located geographically within the New, Holston, Yadkin, and James drainages (Figure 2). Cluster analysis of Nei's (1978) unbiased genetic distances among all populations showed that all populations of northern origin or with a high frequency of the *CK-A2*78* allele clustered together; these included populations from the James River drainage (Barbours Creek, Ewin Run, Pickles Branch), the Holston drainage (Henshew Creek), the New River drainage (Pearis Thompson and Little Indian Creek), and Paint Bank Hatchery. The Roaring Fork population in the Holston drainage had a high frequency of the northern allele, but did not cluster closely with the other northern populations due to a high frequency of a rare allele at the *GPI-A** locus. Cluster analysis of Nei's (1978) unbiased genetic distances among populations

showed no geographic patterns of genetic variation among the populations of putative southern Appalachian origin.

In addition to the 56 populations analyzed in this study, allozyme frequency data from 27 other populations in southwestern Virginia were reported previously (Shull and Walker 1995, Guffey 1998, Palmer and Hallerman 2000; Table 6). Combining all of these data (n = 83, Figure 3), 26 populations in southwestern Virginia were characterized as putatively pure southern, 11 as northern and 46 were introgressed. Of the Holston River drainage populations, four were putatively pure southern, one was northern, and six were introgressed. All seven populations analyzed from the James River drainage contained alleles characteristic of northern-form brook trout. In the New River drainage, 20 populations were putatively pure southern, one was northern and 38 were introgressed. Three populations from the Roanoke River drainage were analyzed; two were northern form and one was introgressed. In the Yadkin River drainage, two populations were characterized as putatively pure southern, and one was introgressed.

Range-wide genetic assessment of brook trout populations

In addition to the current study, eleven published and unpublished studies (Stoneking et al. 1981, Morgan and Baker 1991, McCracken et al. 1993, Dunham et al. 1994, Kriegler et al. 1995, Shull and Walker 1995, Hayes et al. 1996, Guffey 1998, Palmer and Hallerman 2000, Galbreath 2002, NCWRC 2006) reported allele frequency data at the *CK-A2** locus for brook trout populations in the Appalachian portion of the native range (i.e., Pennsylvania to Georgia). Although many genetic studies have been conducted on populations north of this region, especially in Canada, allele frequency data for the *CK-A2** locus have not been reported. The allele frequency data compiled from the 12 studies included 8921 individuals from 480 populations in 24 major river drainages in Pennsylvania, Maryland, Virginia, North Carolina, Tennessee, and Georgia (Table 7 and Figure 4).

All river drainages north of the New River, Virginia were characterized as pure northern form, with the exception of the Roanoke River drainage that contained a single population with a low frequency of the southern allele. This latter observation may be due to the transfer of individuals from another location or stream capture. The frequency of the southern allele in river drainages south of the New River ranges from 29% in the Broad River of North Carolina to 100% in the Coosa River of Georgia. However, it is

important to note that the number of populations (i.e., streams) analyzed varied widely between river drainages. For example, several studies focused on the Great Smoky Mountains National Park, so a large number of populations have been analyzed in this region of North Carolina and Tennessee. In contrast, only a single population has been analyzed in each of the Coosa (GA), Chattahoochee (GA), Gunpowder (MD), Patapsco (MD) and Rappahannock (VA) river drainages.

Discussion

Properties of allozyme markers

Certain allozyme markers screened in this study posed complications to interpretation of underlying genotype. Brook trout show a high incidence of duplicated enzyme loci due to the tetraploid ancestry of salmonids (Allendorf and Thorgaard 1984). Polyploidy has been recognized as a potentially important process in the evolution of vertebrates, and recent research suggests that it is more common than once suspected (see Allendorf and Thorgaard 1984). Salmonids are still undergoing the process of “diploidization”, i.e., of restoring disomic inheritance, as some protein-encoding loci are functionally duplicated. Each pair of duplicated loci (“isoloci”) are genetically independent, but exhibit alleles of similar electrophoretic mobility that cannot be unambiguously assigned to either locus. Three of the five enzymes that I screened were encoded by isoloci (i.e., *MDH-B1,2**, *sAAT-1,2**, *GPI-B1,2**). Ambiguous interpretation of the banding patterns of two of these isoloci, *sAAT-1,2** and *GPI-B1,2**, forced me to eliminate data for them from statistical analyses.

Aspartate amino transferase (*sAAT-1,2**) is a dimeric isolocus with three known alleles. Some researchers have attempted to infer genotypes from this isolocus (Morgan and Baker 1991, Dunham et al. 1994, Kriegler et al. 1995, Guffey 1998), but close examination of my banding patterns revealed that they do not fit the expected model for a dimeric enzyme with two loci exhibiting alleles of equal mobility.

Three loci encode glucose-6-phosphate isomerase, *GPI-A** and *GPI-B1,2**. As indicated by the nomenclature, *GPI-B1,2** are isoloci, with three previously known alleles that migrate cathodally. Interaction bands of gene products from all loci can be seen between those for the *GPI-A** and *GPI-B1,2** loci. Previous researchers scored variation at *GPI-B1** and *GPI-B2** by attributing all of the variation to one of the loci

(Morgan and Baker 1991, Dunham et al. 1994, Shull and Walker 1995, Galbreath et al. 2001). However, close examination of the banding patterns in this study revealed that both loci were polymorphic, with at least six different alleles. Since it was not possible to determine which alleles belonged to either locus, *GPI-B1,2** data were eliminated from statistical analyses.

Precise estimation of genetic diversity and differentiation metrics require data from many loci (Kidd and Cavalli-Sforva 1971, Nei and Roychoudhury 1974). Information from only four markers clearly limited the power of statistical analysis of genetic differentiation, especially with small sample sizes for some of the populations (Archie et al. 1989). Genotypic data from more markers likely would reveal genetic differentiation not detected with only four loci. Screening of additional, more highly polymorphic markers, such as mitochondrial and microsatellite DNA markers, would increase the ability to detect population genetic differentiation.

Genetic assessment of southwestern Virginia brook trout populations

Based on fixation for the diagnostic allele at the *CK-A2** locus and allele frequency differences at three other marker loci, 34% (n = 19) of the brook trout populations analyzed in this study were of putative southern Appalachian origin, 9% (n = 5) were of northern origin, and 57% (n = 32) were of mixed genetic origin (Table 2, Figure 1). The level of certainty for precise characterization of a population is directly related to sample size. That is, any population observed to be fixed for the common allele actually may harbor the alternate allele at a low, undetected frequency. For example, with a sample size (s) of 20, the likelihood (p) of detecting an allele with a frequency (p_a) of 5% is 36% (i.e., $p = (1-p_a)^s = 0.95^{20}$, Walsh 2000). Therefore, there is some likelihood that some populations characterized as "pure" southern Appalachian are of mixed genetic origin. Similarly, sample size also affects estimation of within-population diversity statistics such as P and H_0. Sampling of a limited number of populations in a watershed also would affect estimates of between-population genetic variability.

Of the six populations from the Holston drainage analyzed in this study, four were of mixed genetic origin, with the southern allele at frequencies ranging from 0.44 to 0.95. The Grassy Branch population was characterized as putatively pure southern Appalachian, and the Henshew Branch population was characterized as pure northern. Results from

earlier genetic studies (McCracken et al. 1993, Kriegler et al. 1995, Guffey 1998) and its geographic location suggest that the Holston River historically contained the southern Appalachian form, so the presence of the northern allele is likely due to stocking.

The Yadkin (upper Pee Dee) River is an Atlantic slope watershed. Despite the common presumption that Atlantic slope drainages would contain native northern-form brook trout (McCracken et al. 1993, Shull and Walker 1995, Palmer and Hallerman 2000), two putatively pure southern populations (Pauls Creek, South Fork Stewarts Creek) were found in the Yadkin drainage. Although no early sampling efforts are known from the upper Pee Dee in Virginia (Jenkins and Burkhead 1993), the section of the river that flows through North Carolina was excluded from the range of brook trout originally described by Smith (1907). However, several stream capture events have been inferred in this region, suggesting that these populations may be descendants of brook trout captured from the New River drainage (Jenkins and Burkhead 1993). Inspection of stocking records showed that both Pauls Creek and South Fork Stewarts Creek were stocked in the recent past (B. Kittrell, VDGIF, personal communication), implying that the "native" southern form persisted despite stocking.

Earlier genetic study (Guffey 1998) and geographic location suggest that the James River drainage historically contained northern-form brook trout. Three populations from the James River drainage screened in this study were characterized as northern form. This finding leaves little doubt that the New River is the boundary between northern- and southern-form brook trout populations.

In this study, 16 populations from the New River drainage (36%) were characterized as putatively southern Appalachian. No geographic patterns of genetic variation were observed among the populations of putative pure southern origin. There was apparent allele frequency variation at the *sAAT-1,2** and *GPI-B1,2** loci (Table 1), but inability to unambiguously score genotypes at these loci prevented use of these data in statistical analyses. Interestingly, two of these "pure southern" populations (Crooked Creek and West Fork Dry Run) were stocked in the recent past with northern-derived hatchery fish. Crooked Creek is a "put-and-take" fishing area, and 5000 brook trout are stocked annually (J. Williams, VDGIF, personal communication), yet it maintained an apparently pure southern population. Sixty-three percent (n = 28) of the populations from the New River drainage were of mixed origin, with the southern allele at frequencies

ranging from 0.21 to 0.98. Although stocking records are limited, only two of these (Howell Creek and Little Indian Creek) are known to have been stocked with northern-derived hatchery fish (B. Kittrell, VDGIF, personal communication).

Only one population (Pearis Thompson Branch) in the New River drainage was characterized as pure northern, and explaining its genetic origin leads to more questions and generalizations about the evolutionary significance of southern-form brook trout. Both Pearis Thompson Branch and Standrock Branch are first-order streams in Giles County, Virginia that drain into Dismal Creek about 1300 meters from each other. Dismal Creek does not contain wild brook trout and likely becomes dry every few years (J. Williams, VDGIF, personal communication). Pearis Thompson Branch does not have a known history of stocking and has a waterfall at its base, preventing any straying northern-derived stocked fish from migrating upstream, yet it contains a pure northern population. Conversely, Standrock Branch does not have any obvious barrier to migration, yet it maintains a pure southern population. To explain two divergent populations in such close proximity, it seems reasonable to assume that Pearis Thompson Branch was privately stocked using northern-derived individuals. The tendency for Dismal Creek to become dry every few years may limit, but probably does not exclude the migration of individuals between Pearis Thompson and Standrock Branch. The persistence of the southern form in Standrock Branch, despite the close proximity of northern-derived individuals and the lack of a barrier to migration, may be evidence of local adaptation. Southern-derived brook trout populations, subject to selection, may have adapted to the challenges of their local ecosystem (i.e., isolated, high-elevation headwater streams). The process of local adaptation gives rise to particular combinations of alleles at multiple loci that may confer fitness upon the carrier (Hallerman 2003). When favorable, these coadapted gene complexes spread throughout the population and are maintained by selection, which could make the Standrock Branch population resistant to invasion by the northern-derived individuals. The absence of native individuals in Pearis Thompson Branch at the time of stocking would have allowed the non-native fish to become established. Given the population subdivision, limited dispersal, and small population sizes common to brook trout in the southern portion of the range, it is likely that the Standrock Branch population is only one of many instances of local adaptation of

southern-form brook trout. Existence of adaptive genetic differentiation supports the case for conservation of the southern form of the species in future management of brook trout.

Range-wide genetic assessment of brook trout populations

Creatine kinase and other isozyme markers have proven useful in characterizing brook trout populations in geographic extremes of their native range. However, inference based on these markers is less clear-cut in the middle portion of the range where we do not know the native character of the populations before humans altered the landscape and introduced non-native genotypes. The zone of contact between the northern and southern forms lies roughly at the New River watershed (Guffey 1998, Palmer and Hallerman 2000, Hall et al. 2002), but it is unknown whether the New River drainage historically contained pure southern populations, or whether it was a natural mixing zone between the southern and northern forms. If northern alleles occurred naturally in the New River drainage, it would be more difficult to justify protection of the "pure" southern form. Interpreting genetic data from both the local (i.e., southwestern Virginia) and regional (i.e., New River and all surrounding drainages) perspective provides insights into the native genetic structure of New River brook trout populations.

Interpreting population-level data from southwestern Virginia only (Shull and Walker 1995, Guffey 1998, Palmer and Hallerman 2000, current study; Figure 3), it emerges that the New River drainage contains 20 (34%) putatively pure southern-form populations. The large number of pure southern-form populations suggests that the presence of northern alleles is due to either stocking or stream capture events. However, a large proportion (64%) of the populations from this data set is of mixed genetic origin, suggesting that the New River is a zone of natural intergradations.

Interpreting watershed-level data from the larger data set, the New River drainage showed an 85% frequency of the southern-form allele (Table 7). Figure 4 shows that the break between the southern and northern forms is sharp, occurring at the New/Roanoke-James watershed divide. This weakens the hypothesis that the New River is a zone of natural intergradation between the southern and northern forms of brook trout, and supports the hypothesis that the presence of northern alleles is due to either stocking or stream capture. The persistence of the southern form in these populations, despite the heavy stocking of northern-derived individuals, may be evidence of an adaptive

advantage for the southern form of the species. As mentioned above, existence of adaptive genetic differentiation supports the case for conservation of the southern form of the species in future management of brook trout. It is important to qualify this inference by noting that genetic characterization of these populations was based on variation at a single locus. Screening of populations from the New River drainage and surrounding drainages using additional genetic markers, including mitochondrial and microsatellite DNA markers, will be needed to gain insights into patterns of population genetic differentiation, shedding light on the native character of brook trout populations in southwestern Virginia.

Management recommendations

Results from this and other studies demonstrate that planting of non-native hatchery stocks poses long-term genetic impacts and interferes with efforts to conserve native brook trout. Although the negative effects of stocking have become well known, and keeping of detailed records would help determine future management options, hatchery personnel continue to keep incomplete or imprecise stocking records. For example, hatchery personnel often substitute one stock for another depending upon availability, making it difficult to infer what genetic backgrounds to expect in particular streams. Hence, I recommend that all stocking and transfers of fish be well planned and thoroughly and accurately documented.

It is apparent that many of the remaining wild, native brook trout populations in southwestern Virginia are small and isolated; however, I had only limited ability to estimate levels of genetic diversity and differentiation using allozyme markers. I recommend assessment of levels of genetic diversity and differentiation using additional genetic markers, such as mitochondrial and microsatellite DNA markers. Once differentiation among native populations is well quantified, it will be possible to define management units. Until then, precaution suggests that brook trout populations should be managed on a stream-by-stream basis.

Genetic divergence between northern and southern brook trout populations may reflect different colonization patterns and restricted gene flow or local adaptation. Southern-form brook trout may have an adaptive advantage over the northern form that allows them to persist in the high-elevation headwater streams to which they are restricted. The identification of "pure" southern populations, despite heavy stocking of

northern-derived individuals, lends credence to the existence of such an advantage. Conservation of the genetic integrity and evolutionary future of the native southern form may prove critical for the survival of the species. For this reason, I recommend stricter management of brook trout populations in drainages that, to the best of our knowledge, historically supported the native southern form (i.e., the New, Holston and Yadkin River drainages).

New, Yadkin and Holston River drainages. - Those populations characterized as pure southern form should be given conservation priority. All stocking and transfer of fish into these populations should be prohibited. I recommend that introgressed populations that contain less than 5% admixture from northern-form brook trout be treated as 'pure' southern form. If the stocking or transfer of fish is necessary to satisfy angling interests, it should occur only in those streams characterized as northern or those with >95% admixture. Once genetic diversity among brook trout populations is quantified using microsatellite markers, the establishment of one or more southern-form hatchery stocks should be considered. Founders for the hatchery stocks would have to be certified as pure southern form by screening them and their source populations with multiple allozyme and microsatellite markers. Southern-form hatchery stocks then can be used to reestablish extirpated populations or supplement depleted populations in drainages that historically supported the southern form (i.e., New, Yadkin and Holston River drainages). Eradication of northern-form or introgressed populations from these drainages is not necessary to conserve the native form of the species. Using the New River walleye fishery as a model (Palmer et al. 2008), stocking or transferring southern-form brook trout onto the compromised populations could help conserve the southern form by shifting allele frequencies back toward the native state. Until a southern-form hatchery stock is established and more is known about the genetic composition of these populations, it may be wise to stock only infertile triploid brook trout (Allen and Stanley 1978).

James and Roanoke River drainages. - Northern-form hatchery stocks can be used to reestablish extirpated populations or supplement depleted populations in drainages that historically supported the northern form (i.e., James and Roanoke River drainages). However, I caution that any negative consequences of stocking also would apply to native northern-form populations. Allozyme markers do not provide enough resolution to

differentiate between native northern and hatchery populations, so I recommend that all populations, not just those characterized as pure southern form, be screened using microsatellite markers. Until more is known about the genetic composition of these populations, it may be wise to stock only infertile triploid brook trout.

REFERENCES

Allen Jr., S.K., and J.G. Stanley. 1978. Reproductive sterility in polyploid brook trout, *Salvelinus fontinalis*. Transactions of the American Fisheries Society 107:473-478.

Allendorf, F.W., R.F. Leary, N.P. Hitt, K.L. Knudsen, L.L.Lundquist, and P. Spruell. 2004. Intercrosses and the U.S. Endangered Species Act: should hybridized populations be included as Westslope Cutthroat Trout? Conservation Biology 18: 1203-1213.

Allendorf, F.W., and G.H. Thorgaard. 1984. Tetraploidy and the evolution of salmonid fishes. Pages 1-53 *in* B.J. Turner, ed. Evolutionary Genetics of Fishes. Plenum Press, New York, NY.

Angers, B., L. Bernatchez, A. Angers, L. Desgroseillers. 1995. Specific microsatellite loci for brook charr reveal strong population subdivision on a microgeographic scale. Journal of Fish Biology 47(A):177-185

Archie. J.W., C. Simon, and A. Martin. 1989. Small sample size does decrease the stability of dendrograms calculated from allozyme-frequency data. Evolution 43:678-683.

Burnham-Curtis, M.K. 1996. Mitochondrial DNA variation among Lake Superior brook trout populations: Summary of genetic analyses. Project completion report to U.S. Fish and Wildlife Service, Washington, D.C.

Castric, V., B. Forrest, and L. Bernatchez. 2001. Landscape structure and hierarchical genetic diversity in the brook charr, *Salvelinus fontinalis*. Evolution 55:1016-1028.

Cornelison, J.W. 2005. An ecological comparison between two starins of wild brook trout, *Salvelinus fontinalis*. Master's thesis. Western Carolina University, Cullowhee. NC.

Danzmann, R.G., R.P. Morgan II, M.W. Jones, L. Bernatchez, and P.E. Ihssen. 1998. A major sextet of mitochondrial DNA phylogenetic assemblages extant in eastern North American brook trout (*Salvelinus fontinalis*): Distribution and postglacial dispersal patterns. Canadian Journal of Zoology 76:1300-1318.

Dunham, R.A., J. Dibona, L. Robison, K. Norgren, J. Drniak, and M. Spencer. 1994. Biochemical genetics of brook trout, *Salvelinus fontinalis*, in Georgia. Final report, Dingell-Johnson Project F-42 to the Georgia Department of Natural Resources, Atlanta, GA.

Excoffier, L., G. Laval, and S. Schneider. 2006. Arlequin, version 3.01: an integrated software package for population genetics data analysis. Available online at http://cmpg.unibe.ch/software/arlequin3

Galbreath, P.F., N.D. Adams, and S.F. Guffey. 2001. Persistence of native southern Appalachian brook trout populations in the Pigeon River system of western North Carolina. North American Journal of Fisheries Management 21:927-934.

Guffey, S. 1998. Population genetics of brook trout in Virginia. Unpublished report to the Virginia Department of Game and Inland Fisheries, Richmond, VA.

Hall, M. R., R. P. Morgan, and R. G. Danzmann. 2002. Mitochondrial DNA analysis of mid-Atlantic populations of brook trout: the zone of contact for major historical lineages. Transactions of the American Fisheries Society 131:1140-1151.

Hallerman, E.M. 2003. Coadaptation and outbreeding depression. Page 239 *in* E. Hallerman, ed. Population Genetics: Principles and Applications for Fisheries Scientists. American Fisheries Society, Bethesda, MD.

Hayes, J.P., S.Z. Guffey; F.J. Kriegler, G.F. McCracken, and C.R. Parker. 1996. The genetic diversity of native, stocked, and hybrid populations of brook trout in the southern Appalachians. Conservation Biology 10:1403-1412.

Hebert, P.D.N., and M.J. Beaton. 1993. Methodologies for allozyme analysis using cellulose acetate electrophoresis: A practical handbook. Helena Laboratories, Beaumont, TX.

Hudy, M., T. M. Thieling, N. Gillespie, and E. P. Smith. 2005. Distribution, status, and threats to brook trout within the eastern United States. Report submitted to the Eastern Brook Trout Joint Venture, International Association of Fish and Wildlife Agencies, Washington, D. C.

Jenkins, R.E., and N.M. Burkhead. 1993. Freshwater Fishes of Virginia. American Fisheries Society, Bethesda, MD.

Jones, M.W., R.G. Danzmann, and D. Clay. 1997. Genetic relationships among populations of wild resident, and wild and hatchery anadromous brook charr. Journal of Fish Biology 51:29-40.

Kidd, K.K., and L.L. Cavalli-Sforza. 1971. Number of characters examined and error in reconstruction of evolutionary trees. Pages 335-346 *in* F.R. Hodson, D.G. Kendall, and P. Tautu, eds. Mathematics in the Archaeological and Historical Sciences. Proceedings of the Anglo-Romanian Conference. University of Edinburgh Press, Edinburgh.

Kincaid, H.L. 1981. Trout strain registry. National Fisheries Center-Leetown, U.S. Fish and Wildlife Service, Kearneysville, WV.

King, W. 1937. Notes on the distribution of native speckled and rainbow trout in the streams of Great Smoky Mountains National Park. Journal of the Tennessee Academy of Science 12:351-361.

Kriegler, F.J., G.F. McCracken, J.W. Habera, and R.J. Strange. 1995. Genetic characterization of Tennessee brook trout populations and associated management implications. North American Journal of Fisheries Management 15:804-813.

Lachance, S., and P. Magnan. 1990. Performance of domestic, hybrid, and wild forms of brook trout, *Salvelinus fontinalis*, after stocking: the impact of intra- and interspecific competition. Canadian Journal of Fisheries and Aquatic Sciences 47:2278-2284.

Lennon, R.E. 1967. Brook trout of Great Smoky Mountains National Park. U.S. Fish and Wildlife Service Technical Paper 15, Washington, D.C.

MacCrimmon, H.R., and J.S. Campbell. 1969. World distribution of brook trout, *Salvelinus fontinalis*. Journal of the Fisheries Research Board of Canada 26:1699-1723.

McCracken, G.F., C.R. Parker, and S.Z. Guffey. 1993. Genetic differentiation and hybridization between stocked hatchery and native brook trout in Great Smoky Mountains National Park. Transactions of the American Fisheries Society 122:533-542.

Morgan, R.P., B.M. Baker. 1991. Development of genetic inventories for Maryland game fish : brook trout. Final report submitted to the Department of Natural Resources, Annapolis, MD.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583-590

Nei, M. and A.K. Roychoudhury. 1974. Sampling variances of heterozygosity and genetic distance. Genetics 16:379-390.

North Carolina Wildlife Resources Commission (NCWRC). 2006. Brook trout distribution and genetics information. Unpublished data, NCWRC, Raleigh, NC.

Obbard, D.J., S.A. Harris, and J.R. Pannell. 2006. Simple allelic-phenotype diversity and differentiation statistics for allopolyploids. Heredity 97:296-303

Palmer, G.C., and E.M. Hallerman. 2000. Genetic characterization of southwest Virginia brook trout populations. Project completion report to the Virginia Department of Game and Inland Fisheries, Richmond, VA.

Palmer G.C., J. Williams, M. Scott, K. Finne, N. Johnson, D. Dutton, B.R. Murphy, E.M. Hallerman. 2008. Genetic marker-assisted restoration of the presumptive native walleye fishery in the New River, Virginia and West Virginia. In press.

Park, S.D.E. 2001. Microsatellite Toolkit for Microsoft Excel, Version 3.1. Available online at http://www.animalgenomics.ucd.ie/sdepark/ms-toolkit/.

Richardson, B.J., P.R. Baverstock, and M. Adams. 1986. Allozyme electrophoresis: A handbook for animal systematic and population studies. Academic Press, Sydney.

Shull, L., and G. Walker. 1995. An electrophoretic study of brook trout (*Salvelinus fontinalis*) from headwater streams of the Blue Ridge Parkway. Unpublished report to the Natural Resources Division of the Blue Ridge Parkway, Asheville, N.C.

Smith, H.M. 1907. The Fishes of North Carolina. North Carolina Geologic and Economic Survey 2:1-453.

Sneath, P.H.A., and R.R. Sokal. 1973. Numerical Taxonomy. Freeman, San Francisco, CA.

Stoneking, M., D.J. Wagner, and A.C. Hildebrand. 1981. Genetic evidence suggesting subspecific differences between northern and southern populations of brook trout (*Salvelinus fontinalis*). Copeia 4:810-819.

Swofford, D.L., and R.B. Selander. 1981. BIOSYS-1: A FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. Journal of Heredity 72:281-283.

Thrall, P.H., and A. Young. 2000. AUTOTET: A program for analysis of autotetraploid genotypic data. Journal of Heredity 91:348-349.

Utter, F. M. 2003. Genetic impacts of fish introductions. Page 475 *in* E. Hallerman, ed. Population Genetics: Principles and Applications for Fisheries Scientists. American Fisheries Society, Bethesda, MD.

Walsh, P.D. 2000. Sample size diagnosis of conservation units. Conservation Biology 14:1533-1537.

Webster, D. A., and W. A. Flick. 1981. Performance of indigenous, exotic, and hybrid forms of brook trout (*Salvelinus fontinalis*) in waters of the Adirondack, New York. Canadian Journal of Fisheries and Aquatic Sciences 38:1701-1707.

Table 1. - Wild brook trout streams in southwest Virginia sampled by electroshocking in 2005-2007. Non-lethal tissue samples collected from brook trout (BT) in 56 streams; 22 streams contained no brook trout; 19 streams were dropped from the study (N/S = not sampled).

Stream	County	Drainage	No. collected	Comment
Grassy Branch	Smyth	Holston	12	
Henshew Branch	Bland	Holston	20	
Nicks Creeks	Smyth	Holston	0	
Parks Creek	Smyth	Holston	10	
Pennington Branch	Smyth	Holston	12	
Roaring Fork	Tazewell	Holston	8	
Rush Creek	Washington	Holston	0	
Star Hill Branch	Washington	Holston	0	
Sturgill Branch	Washington	Holston	16	
Barbours Creek	Craig	James	20	
Ewins Run	Craig	James	20	
Pickles Branch	Craig	James	20	
Baldwin Branch	Grayson	New	0	
Bear Creek	Carroll	New	23	
Beaverdam Creek	Carroll	New	N/S	5 BT collected in last 3 samples
Big Branch	Carroll	New	N/S	15 BT collected in last 5 samples
Big Fox Creek	Grayson	New	20	
Big Horse Creek	Grayson	New	18	
Big Laurel Creek	Carroll	New	11	
Big Reed Island Creek	Carroll	New	20	
Big Stony Creek	Giles	New	20	
Bournes Branch	Grayson	New	16	
Brannon Branch	Carroll	New	N/S	7 BT collected in last 2 samples
Buffalo Branch	Floyd	New	17	
Cabin Creek	Grayson	New	20	
Cherry Creek	Carroll	New	0	
Chestnut Creek	Grayson	New	17	
Chisholm Creek	Carroll	New	12	
EF Cove Creek	Tazewell	New	14	
Crooked Creek	Carroll	New	15	
EF Crooked Creek	Carroll	New	20	
Daniel Branch	Carroll	New	N/S	12 BT collected in last 2 samples
Ding Branch	Bland	New	26	
Dismal Branch	Giles	New	0	
Dismal Creek	Giles	New	N/S	3 BT collected in last 3 samples
Dixon Branch	Giles	New	N/S	Inaccessible
Dobbins Creek	Floyd	New	0	
WF Dodd Creek	Floyd	New	0	
Dry Run	Wythe	New	N/S	4 BT collected in last 3 samples
EF Dry Run	Wythe	New	20	
Upper WF Dry Run	Wythe	New	10	
WF Dry Run	Wythe	New	19	
NB Elk Creek	Grayson	New	15	
Elkhorn Creek	Carroll	New	10	
WP Furnace Creek	Floyd	New	17	
Grassy Creek	Carroll	New	9	
Greasy Creek	Floyd	New	N/S	5 BT collected in last 4 samples
Greens Creek	Carroll	New	0	

Table 1. - Continued

Stream	County	Drainage	No. Sampled	Comment
Howell Creek	Floyd	New	20	
Jones Creek	Wythe	New	N/S	12 BT collected in last 4 samples
Kinser Creek	Wythe	New	N/S	Inaccessible
Laurel Creek	Bland	New	N/S	11 BT collected in last 3 samples
Laurel Creek	Giles	New	20	
Laurel Branch	Giles	New	22	
Laurel Creek	Grayson	New	10	
Little Cranberry Creek	Carroll	New	N/S	16 BT collected in last 3 samples
Little Indian Creek	Floyd	New	20	
EF Little Reed Island Creek	Carroll	New	11	
WF Little Reed Island Creek	Carroll	New	0	
Little Snake Creek	Carroll	New	8	
Little Stony Creek	Giles	New	14	
Little Wilson Creek	Grayson	New	19	
Meadow Creek	Floyd	New	0	
Meadow View Run	Carroll	New	0	
Meredith Branch	Giles	New	N/S	Inaccessible
Middle Fox Creek	Grayson	New	17	
Mill Creek	Carroll	New	12	
Mill Creek	Grayson	New	0	
Mine Branch	Carroll	New	N/S	10 BT collected in last 3 samples
Mira Fork	Floyd	New	0	
Nettle Hollow Creek	Giles	New	N/S	3 BT collected in last 3 samples
No Business Creek	Giles	New	20	
Oldfield Creek	Floyd	New	12	
Opossum Creek	Grayson	New	17	
Payne Creek	Floyd	New	0	
Pearis Thompson Branch	Giles	New	17	
Pine Creek I	Carroll	New	N/S	5 BT collected in last 4 samples
Pine Creek	Carroll	New	0	
Piney Creek	Giles	New	0	
Pipestem Branch	Carroll	New	N/S	3 BT collected in last 3 samples
Ripshin Creek	Grayson	New	10	
Road Creek	Carroll	New	0	
Roads Creek	Carroll	New	11	
Silverleaf Branch	Floyd	New	0	
Snake Creek	Carroll	New	20	
Spurlock Creek	Floyd	New	0	
Standrock Branch	Giles	New	20	
Staunton Branch	Carroll	New	0	
Stone Mountain Creek	Carroll	New	N/S	5 BT collected in last 3 samples
NF Stony Creek	Giles	New	22	
Sulfur Springs Branch	Carroll	New	11	
Tory Creek	Carroll	New	19	
Whitetop Creek	Grayson	New	13	
Little Fisher Creek	Carroll	Yadkin	0	
Little Paul’s Creek	Carroll	Yadkin	N/S	18 BT collected in last 3 samples
Paul’s Creek	Carroll	Yadkin	20	
SF Stewarts Creek	Carroll	Yadkin	13	

Table 2. - Allele frequencies at four polymorphic loci in wild brook trout populations in 56 southwest Virginia streams, grouped by drainage. Charles Creek, a known southern-form population, was included as a southern-form reference group. Individuals from Paint Bank Hatchery, which cultures the northern form, were included as a northern-form reference group.

		CK-A2*		***G3PDH****		***GPI-A****			***MDH-B1,2****	
	N	*78	*100	*45	*100	*87	*100	*115	*100	*145
Controls										
Charles Creek, NC	5		1.00		1.00		1.00		1.00	
Paint Bank Hatchery	16	1.00		0.44	0.56		1.00		1.00	
Holston River drainage										
Grassy Branch	12		1.00		1.00		1.00		1.00	
Henshew Branch	20	1.00		0.45	0.55		1.00		1.00	
Parks Creek	10	0.05	0.95		1.00		1.00		1.00	
Pennington Branch	12	0.08	0.92		1.00		1.00		1.00	
Roaring Fork	8	0.56	0.44		1.00	0.69	0.31		1.00	
Sturgill Branch	16	0.19	0.81		1.00		1.00		0.75	0.25
James River drainage										
Barbours Creek	20	1.00		0.08	0.93		1.00		1.00	
Ewins Run	20	1.00			1.00		1.00		1.00	
Pickles Branch	20	1.00			1.00		1.00		1.00	
New River drainage										
Bear Creek	23	0.02	0.98		1.00		1.00		1.00	
Big Horse Creek	18		1.00		1.00		1.00		1.00	
Big Laurel Creek	11	0.05	0.95	0.09	0.91		1.00		1.00	
Big Reed Island Cr	20	0.08	0.93		1.00		1.00		0.95	0.05
Bournes Branch	16	0.03	0.97		1.00		1.00		1.00	
Buffalo Branch	16	0.06	0.94		1.00		0.97	0.03	1.00	
Cabin Creek	20	0.05	0.95		1.00		1.00		1.00	
Chestnut Creek	17	0.12	0.88		1.00		1.00		1.00	
Chisholm Creek	12		1.00		1.00		1.00		0.96	0.04
Crooked Creek	15		1.00		1.00		1.00		1.00	
Ding Branch	26	0.25	0.75	0.02	0.98		1.00		0.94	0.06
EF Cove Creek	14	0.11	0.89		1.00		0.93	0.07	1.00	
EF Crooked Creek	20	0.03	0.98		1.00		0.98	0.02	1.00	
EF Dry Run	20		1.00		1.00		1.00		1.00	
EF Little Reed Island	10		1.00		1.00		1.00		1.00	
Elkhorn Creek	10		1.00		1.00		1.00		0.95	0.05
Fox Creek	20	0.18	0.83		1.00		0.95	0.05	0.88	0.12
Grassy Creek	9		1.00		1.00		1.00		1.00	
Howell Creek	20	0.05	0.95		1.00		1.00		0.98	0.02
Laurel Branch	22	0.23	0.77		1.00		1.00		0.98	0.02
Laurel Creek	10		1.00		1.00		1.00		1.00	
Laurel Creek	20	0.10	0.90		1.00		0.98	0.02	1.00	
Little Indian Creek	19	0.79	0.21		1.00		1.00		0.95	0.05
Little Snake Creek	8		1.00		1.00		1.00		1.00	
Little Stony Creek	14	0.11	0.89		1.00		0.96	0.04	1.00	
Little Wilson Creek	19	0.21	0.79	0.03	0.97		1.00		0.82	0.18
Middle Fox Creek	12	0.04	0.96		1.00	0.04	0.96		0.58	0.42
Mill Creek	17	0.12	0.88		1.00		1.00		0.82	0.18
NB Elk Creek	14	0.25	0.75		1.00		1.00		1.00	
NF Stony Creek	21	0.02	0.98		1.00		0.98	0.02	1.00	
No Business Creek	20	0.20	0.80	0.03	0.98		1.00		0.90	0.10

Table 2. - Continued

	N	*CK-A2**		*G3PDH**		*GPI-A**			*MDH-B1,2**	
		**78*	**100*	**45*	**100*	**87*	**100*	**115*	**100*	**145*
New River cont.										
Oldfield Creek	12		1.00		1.00		1.00		1.00	
Opossum Creek	17	0.03	0.97		1.00		1.00		0.72	0.28
Pearis Thompson Br	17	1.00		0.15	0.85		1.00		0.91	0.09
Ripshin Creek	10	0.15	0.85		1.00		1.00		0.75	0.25
Roads Creek	11		1.00		1.00		0.95	0.05	1.00	
Snake Creek	20		1.00		1.00		1.00		0.98	0.02
Standrock Branch	20		1.00		1.00		1.00		1.00	
Stony Creek	20	0.18	0.83	0.03	0.98		1.00		0.95	0.05
Sulfur Springs Br	10	0.30	0.70		1.00		1.00		1.00	
Tory Creek	19		1.00		1.00		1.00		1.00	
Upper WF Dry Run	10		1.00		1.00		1.00		1.00	
WF Dry Run	19		1.00		1.00		1.00		1.00	
Whitetop Creek	12	0.13	0.88		1.00		1.00		1.00	
WP Furnace Creek	17	0.12	0.88		1.00		1.00		0.97	0.03
Yadkin River drainage										
Pauls Creek	20		1.00		1.00		1.00		1.00	
SF Stewarts Creek	24		1.00		1.00		1.00		1.00	

Table 3. - Allele frequencies at two pairs of isoloci in wild brook trout populations in 56 southwest Virginia streams, grouped by drainage. Charles Creek, a known southern-form population, was included as a southern-form reference group. Individuals from Paint Bank Hatchery, which cultures the northern form, were included as a northern-form reference group.

		sAAT-1,2[a]				***GPI-B1,2***[b]					
	N	**100*	**118*	**100/118*	**118/136*	*30	*40	*50	*70	*100	*120
Controls											
Charles Creek, NC	5		1.00				0.30		0.70		
Paint Bank Hatchery	16	0.13		0.88			0.38		0.48	0.14	
Holston River drainage											
Grassy Branch	12		1.00				0.46		0.50	0.04	
Henshew Branch	20	0.15		0.85			0.50		0.50		
Parks Creek	10		0.90	0.10			0.30		0.53	0.18	
Pennington Branch	12		0.58	0.42			0.50		0.50		
Roaring Fork	8	0.13	0.13	0.75			0.63		0.38		
Sturgill Branch	16		1.00				0.19		0.59	0.22	
James River drainage											
Barbours Creek	20		0.90	0.10			0.50		0.50		
Ewins Run	20		0.65	0.35			0.50		0.50		
Pickles Branch	20		1.00				0.50		0.50		
New River drainage											
Bear Creek	23		0.91	0.09			0.07		0.57	0.37	
Big Horse Creek	18		0.94	0.06			0.01		0.75	0.24	
Big Laurel Creek	11		0.64	0.36			0.05		0.45	0.48	0.02
Big Reed Island Cr	20		0.70	0.30			0.05		0.50	0.45	
Bournes Branch	16		1.00				0.03		0.52	0.45	
Buffalo Branch	16		0.81	0.19					0.50	0.50	
Cabin Creek	20		0.95	0.05			0.03		0.50	0.48	
Chestnut Creek	17		0.81	0.19			0.03		0.65	0.32	
Chisholm Creek	12		0.83	0.17			0.04		0.56	0.40	
Crooked Creek	15		0.87	0.13					0.75	0.25	
Ding Branch	26		0.50	0.50			0.12	0.01	0.52	0.36	
EF Cove Creek	14		0.86	0.14			0.04	0.09	0.63	0.25	
EF Crooked Creek	20		0.95	0.05					0.58	0.43	
EF Dry Run	20		1.00						0.75	0.25	

Table 3. - Continued

	N	*sAAT-1,2*[a]				*GPI-B1,2*[b]					
		**100*	**118*	**100/118*	**118/136*	*30	*40	*50	*70	*100	*120
New River cont.											
EF Little Reed Island	10		0.80	0.20			0.08		0.50	0.43	
Elkhorn Creek	10		0.90	0.10			0.05		0.70	0.25	
Fox Creek	20		0.65	0.35			0.10	0.01	0.64	0.25	
Grassy Creek	9		1.00			0.06			0.53	0.42	
Howell Creek	20		0.60	0.40			0.04		0.50	0.46	
Laurel Branch	22		0.32	0.68			0.38		0.50	0.13	
Laurel Creek	10		1.00						0.75	0.25	
Laurel Creek	20		0.55	0.45					0.73	0.28	
Little Indian Creek	19	0.11		0.89			0.30		0.47	0.22	
Little Snake Creek	8		0.88	0.13			0.03		0.50	0.47	
Little Stony Creek	14		1.00						0.57	0.43	
Little Wilson Creek	19		0.47	0.53			0.11		0.54	0.36	
Middle Fox Creek	12		0.33	0.67			0.15		0.62	0.24	
Mill Creek	17		0.47	0.53			0.02		0.65	0.33	
NB Elk Creek	14		0.57	0.43			0.07		0.50	0.43	
NF Stony Creek	21		0.48	0.52			0.01		0.67	0.32	
No Business Creek	20		0.50	0.50			0.14		0.54	0.33	
Oldfield Creek	12		0.83	0.17			0.02		0.71	0.27	
Opossum Creek	17		0.94	0.06			0.03	0.01	0.75	0.21	
Pearis Thompson Br	17		0.18	0.82			0.50		0.50		
Ripshin Creek	10		0.50	0.50			0.03		0.73	0.25	
Roads Creek	11		1.00				0.05		0.55	0.41	
Snake Creek	20		0.65	0.35			0.03		0.51	0.46	
Standrock Branch	20		0.85	0.15			0.06	0.14	0.49	0.31	
Stony Creek	20		0.45	0.55					0.50	0.50	
Sulfur Springs Br	10		0.60	0.40				0.15	0.58	0.28	
Tory Creek	19		0.79	0.21			0.04		0.57	0.39	
Upper WF Dry Run	10		1.00						0.50	0.50	
WF Dry Run	19		0.37	0.05	0.58				0.70	0.30	
Whitetop Creek	12		0.08	0.92			0.04		0.69	0.27	
WP Furnace Creek	17		0.88	0.12		0.01	0.06		0.68	0.25	
Yadkin River drainage											
Pauls Creek	20		1.00						0.65	0.35	
SF Stewarts Creek	24		1.00						0.48	0.52	

[a] Phenotype frequencies; genotypes among heterozygous individuals could not be determined for *sAAT-1,2**.

[b] *GPI-B1,2** constitute isoloci and were treated as a single tetraploid locus in order to estimate allele frequencies.

Table 4. - Genetic diversity of populations of brook trout from 56 streams, based on analysis at four polymorphic allozyme loci (*CK-A2*, G3PDH*, GPI-A*, sMDH-B1,2**). Abbreviations include: number of individuals analyzed (*N*), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (*Ho*), and observed heterozygosity (*He*).

Population	N	P	A	H_o	H_e
Holston River drainage					
Grassy Branch	12	0.00	1.0	0.000	0.000
Henshew Branch	20	0.25	1.3	0.125	0.127
Parks Creek	10	0.25	1.3	0.025	0.025
Pennington Branch	12	0.25	1.3	0.042	0.040
Roaring Fork	8	0.50	1.5	0.188	0.246
Sturgill Branch	16	0.50	1.5	0.219	0.175
James River drainage					
Barbours Creek	20	0.25	1.3	0.021	0.036
Ewins Run	20	0.00	1.0	0.000	0.000
Pickles Branch	20	0.00	1.0	0.000	0.000
New River drainage					
Bournes Branch	16	0.25	1.3	0.016	0.016
Bear Creek	23	0.25	1.3	0.011	0.011
Big Horse Creek	18	0.00	1.0	0.000	0.000
Big Laurel Creek	11	0.50	1.5	0.068	0.066
Big Reed Island Cr	20	0.50	1.5	0.063	0.061
Stony Creek	20	0.75	1.8	0.125	0.111
Buffalo Branch	16	0.50	1.5	0.047	0.046
Cabin Creek	20	0.25	1.3	0.000	0.024
Chisholm Creek	12	0.25	1.3	0.021	0.021
Chestnut Creek	17	0.25	1.3	0.059	0.053
Crooked Creek	15	0.00	1.0	0.000	0.000
Ding Branch	26	0.75	1.8	0.145	0.133
EF Cove Creek	14	0.50	1.5	0.089	0.084
EF Crooked Creek	20	0.50	1.5	0.025	0.025
EF Dry Run	20	0.00	1.0	0.000	0.000
NB Elk Creek	14	0.25	1.3	0.125	0.097
Elkhorn Creek	10	0.25	1.3	0.025	0.025
Fox Creek	20	0.75	1.8	0.150	0.154
Grassy Creek	9	0.00	1.0	0.000	0.000
Howell Creek	20	0.50	1.5	0.038	0.037
Laurel Creek-Grayson	10	0.00	1.0	0.000	0.000
Laurel Creek-Giles	20	0.50	1.5	0.063	0.059
Laurel Branch	22	0.50	1.5	0.125	0.101
Little Indian Creek	19	0.50	1.5	0.132	0.111
EF Little Reed Island	10	0.00	1.0	0.000	0.000
Little Stony Creek	14	0.50	1.5	0.071	0.067
Little Snake Creek	8	0.00	1.0	0.000	0.000
Little Wilson Creek	19	0.75	1.8	0.184	0.176
Middle Fox Creek	12	0.75	1.8	0.250	0.168
Mill Creek	17	0.50	1.5	0.147	0.128
NF Stony Creek	21	0.50	1.5	0.024	0.024
No Business Creek	20	0.75	1.8	0.163	0.141
Oldfield Creek	12	0.00	1.0	0.000	0.000
Opossum Creek	17	0.50	1.5	0.155	0.119
Pearis Thompson Branch	17	0.50	1.5	0.118	0.106
Roads Creek	11	0.25	1.3	0.023	0.023

Table 4. - Continued

Population	***N***	***P***	***A***	H_o	H_e
New River drainage cont.					
Ripshin Creek	10	0.50	1.5	0.200	0.166
Snake Creek	20	0.25	1.3	0.013	0.013
Sulfur Springs Br	10	0.25	1.3	0.100	0.111
Standrock Branch	20	0.00	1.0	0.000	0.000
Tory Creek	19	0.00	1.0	0.000	0.000
Upper WF Dry Run	10	0.00	1.0	0.000	0.000
Whitetop Creek	12	0.25	1.3	0.063	0.057
WF Dry Run	19	0.00	1.0	0.000	0.000
WP Furnace Creek	17	0.50	1.5	0.044	0.068
Yadkin River drainage					
Pauls Creek	20	0.00	1.0	0.000	0.000
SF Stewarts Creek	24	0.00	1.0	0.000	0.000

Table 5. - Genetic diversity of brook trout populations, grouped by drainage, genetic origin, and geographic location relative to the eastern continental divide. Based on analysis at four polymorphic allozyme loci (*CK-A2**, *G3PDH**, *GPI-A**, *sMDH-B1,2**). Abbreviations include: number of populations per group (*N*), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (*Ho*), and observed heterozygosity (*He*).

Group	***N***	***P***	***A***	H_o	H_e
Holston River drainage	6	0.29	1.3	0.100	0.102
James River drainage	3	0.08	1.1	0.007	0.012
New River drainage	45	0.34	1.4	0.064	0.058
Yadkin River drainage	2	0.00	1.0	0.000	0.000
Southern form	19	0.05	1.1	0.004	0.004
Northern form	5	0.20	1.2	0.053	0.036
Introgressed	32	0.48	1.5	0.099	0.091
Atlantic drainages	5	0.05	1.1	0.004	0.007
Gulf of Mexico drainages	51	0.33	1.4	0.068	0.063

Table 6. - Genetic characterization at the *CK-A2** locus for brook trout populations in southwest Virginia that were not sampled in this study. Data were compiled from all known published and unpublished data sources.

Stream	**River Drainage**	**County**	***n***	**% Southern Allele**	**Citation**
Green Cove Creek	Holston	Washington	19	95	Palmer and Hallerman 2000
Grindstone Branch	Holston	Smyth	16	97	Guffey 1998
Houndshell Branch	Holston	Smyth	12	100	Guffey 1998
Jerry Creek	Holston	Smyth	11	100	Guffey 1998
Little Laurel Creek	Holston	Smyth	16	100	Guffey 1998
Johns Creek	James	Giles	23	0	Guffey 1998
Shawvers Run	James	Giles	23	0	Guffey 1998
Spy Run	James	Augusta	21	0	Guffey 1998
Valley Branch	James	Craig	15	0	Guffey 1998
Burks Fork	New	Floyd	15	67	Palmer and Hallerman 2000
Cox Branch	New	Tazewell	15	53	Palmer and Hallerman 2000
Dry Creek	New	Smyth	24	100	Guffey 1998
Hanks/EF Chestnut Creek	New	Grayson	10	70	Guffey 1998
Helton Creek	New	Grayson	21	79	Palmer and Hallerman 2000
Jerry Creek	New	Grayson	15	67	Palmer and Hallerman 2000
Killinger Creek	New	Smyth	12	88	Guffey 1998
Laurel Branch	New	Floyd	15	97	Guffey 1998
Laurel Fork	New	Floyd	7	79	Shull and Walker 1995
Lewis Fork	New	Grayson	21	79	Palmer and Hallerman 2000
Middle Fork Helton	New	Grayson	20	100	Guffey 1998
NF Elk Creek	New	Grayson	19	100	Guffey 1998
NP Buckhorn Creek	New	Carroll	25	100	Guffey 1998
Wilburn Branch	New	Grayson	21	75	Palmer and Hallerman 2000
Big Stony Creek	Roanoke	Bedford	10	0	Shull and Walker 1995
Little Stony Creek	Roanoke	Bedford	6	0	Shull and Walker 1995
Rock Castle Creek	Roanoke	Patrick	25	36	Guffey 1998
Turkey Creek	Yadkin	Carroll	15	47	Palmer and Hallerman 2000

Table 7. - Genetic characterization of brook trout populations in major river drainages, based on the frequency of the *CK-A2*100* allele using data gathered from all available published and unpublished studies.

River drainage	State	Position relative to continental divide	# of streams	# of individuals	% southern[1]	Data source[2]
Susquehanna	PA/MD	East	4	145	0	1,2
Ohio	MD	West	3	110	0	2
Gunpowder	MD	East	1	40	0	2
Patapsco	MD	East	1	40	0	2
Potomac	MD/VA	East	6	190	0	2, 8
James	VA	East	7	142	0	8,12
Rappahannock	VA	East	1	25	0	8
Roanoke	VA	East	3	41	22	6, 8
New	VA/NC	West	111	1999	85	8, 9, 11, 12
Yadkin	VA/NC	East	37	691	58	3, 6, 9, 11,12
Holston	VA/TN	West	24	320	91	3, 5, 8, 12
Nolichucky	NC/TN	West	51	1058	64	1, 3, 5, 11
French Broad	NC/TN	West	80	1281	73	3, 5, 10, 11
Little Tennessee	NC/TN	West	49	886	82	3, 7, 11
Watauga	NC/TN	West	44	691	88	3, 5, 11
Broad	NC	East	3	41	29	11
Hiwassee	NC	West	6	146	76	11
Cheoah	NC	West	10	210	80	11
Little	TN	West	8	90	80	3, 5
Tellico	TN	West	5	64	42	5
Savannah	NC/GA	East	27	533	63	4, 10, 11
Chattahoochee	GA	West	1	21	31	4
Tennessee	GA	West	7	145	93	4
Coosa	GA	West	1	12	100	4

[1] Allele frequency based on number of individuals analyzed per stream and averaged across all populations in each drainage.
[2] 1) Stoneking et al. 1981, 2) Morgan and Baker 1991, 3) McCracken et al. 1993, 4) Dunham et al. 1994, 5) Kriegler et al. 1995, 6) Shull and Walker 1995, 7) Hayes et al. 1996, 8) Guffey 1998, 9) Palmer and Hallerman 2000, 10) Galbreath 2002, 11) NCWRC 2006, 12) current study.

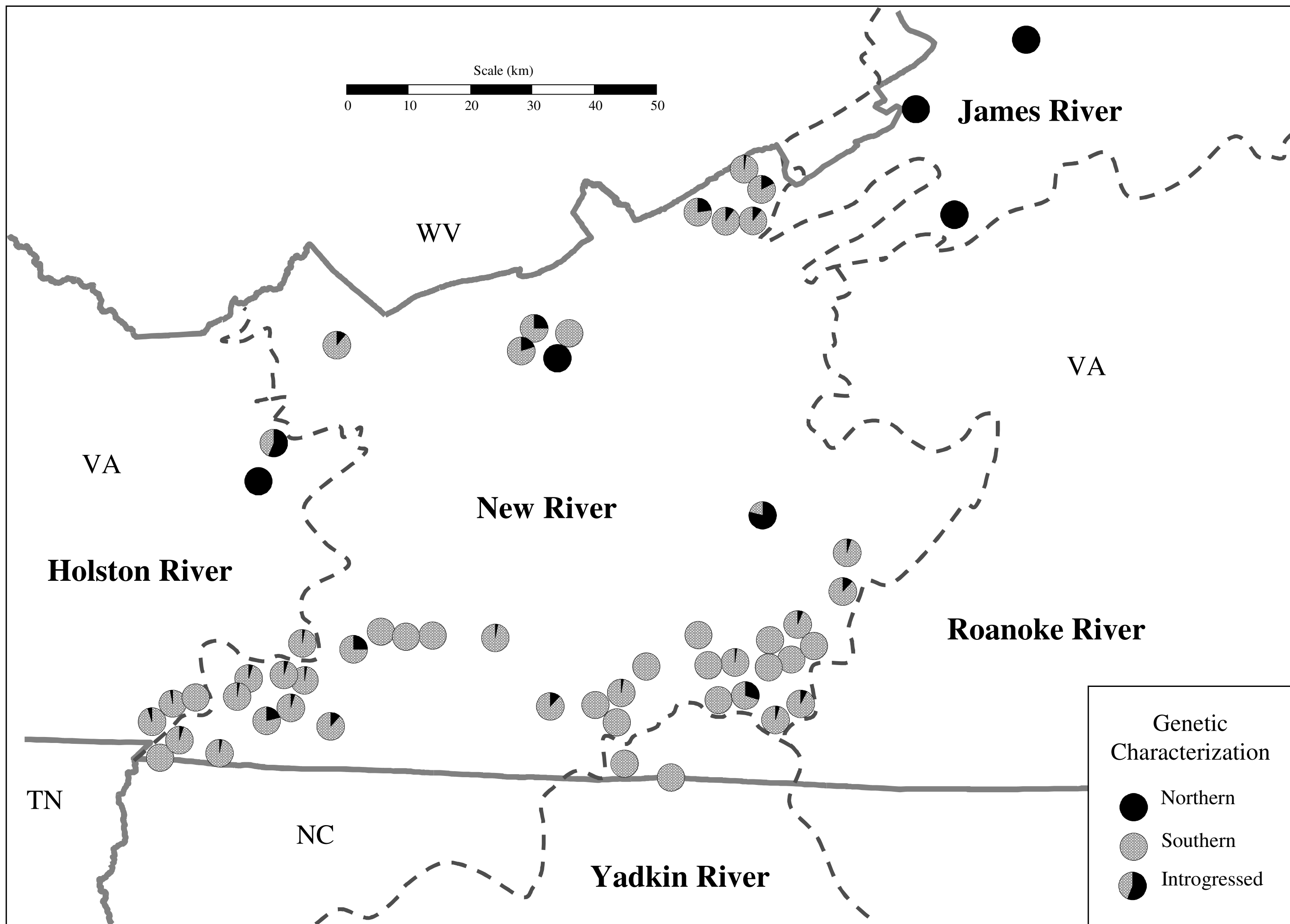


Figure 1. - Genetic characterization at the *CK-A2** locus for 56 wild brook trout populations in southwest Virginia. See Table 2 for details.

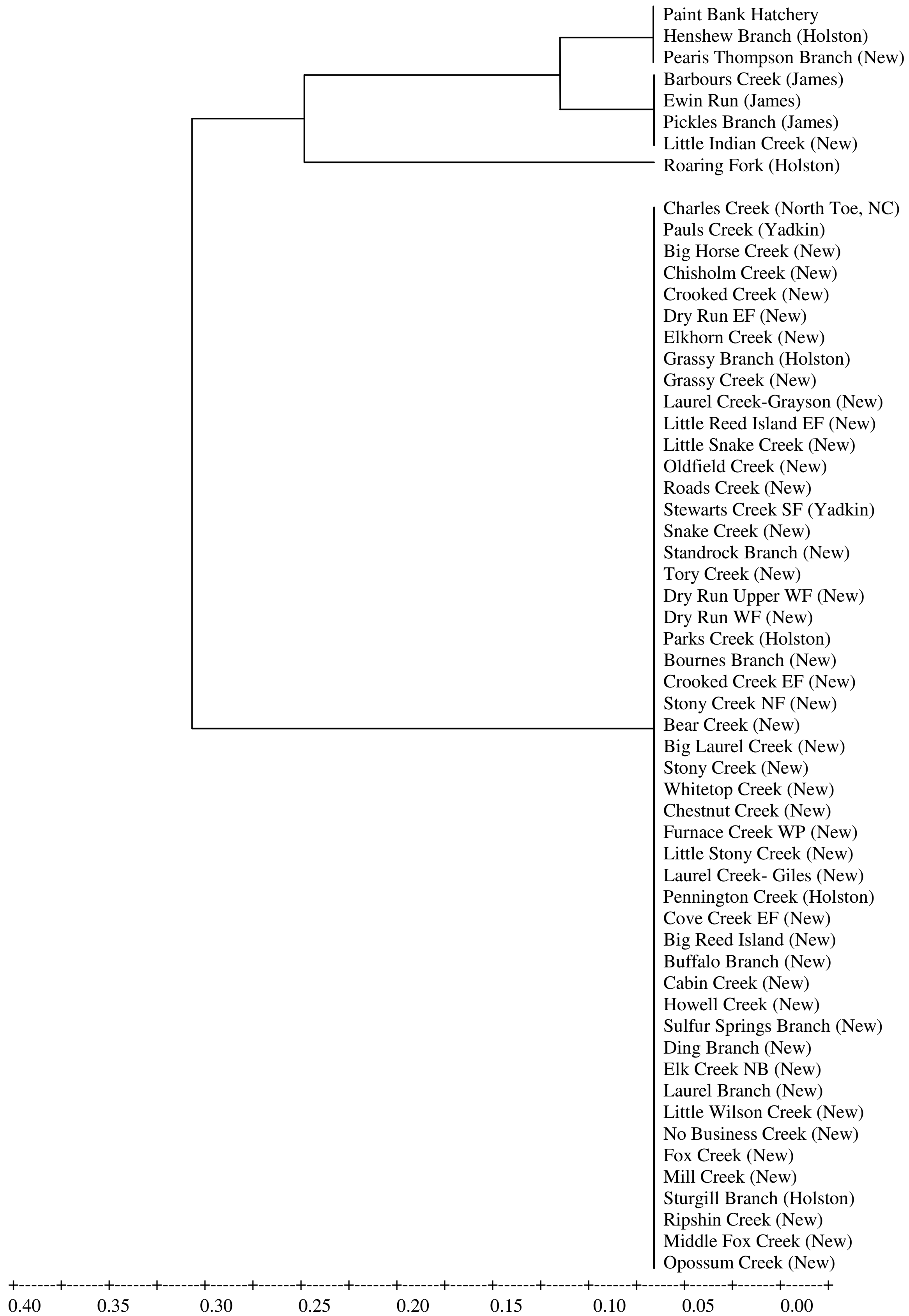


Figure 2. - UPGMA cluster analysis of genetic distance (Nei 1978) among brook trout populations in southwest Virginia. The major river drainages are shown in parentheses.

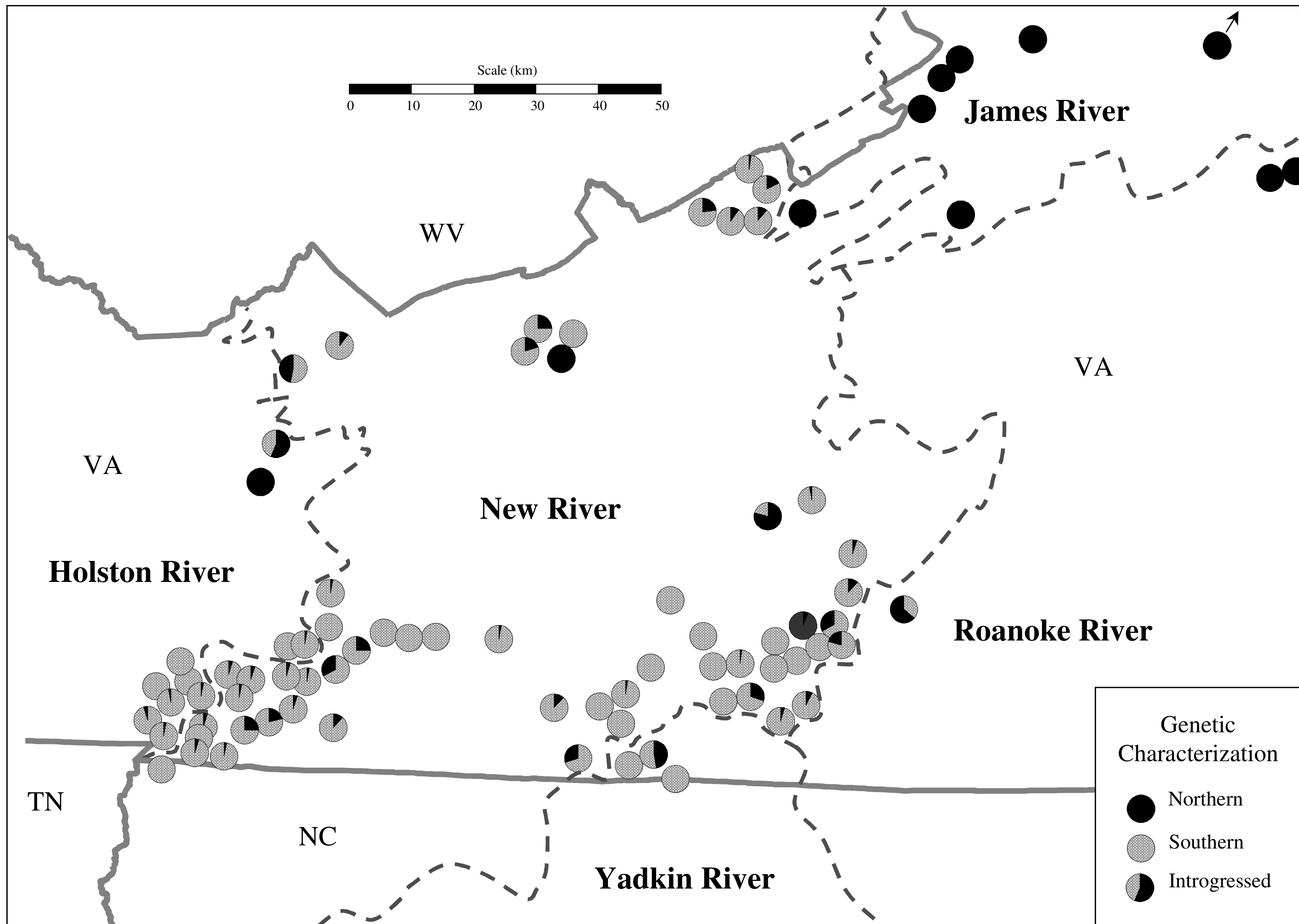


Figure 3. - Genetic characterization at the *CK-A2** locus for 83 wild brook trout populations in SW Virginia, including populations characterized in this study and populations characterized previously. Spy Run (James River drainage, Augusta County) was out of the range of this map. See Tables 1 and 6 for details.

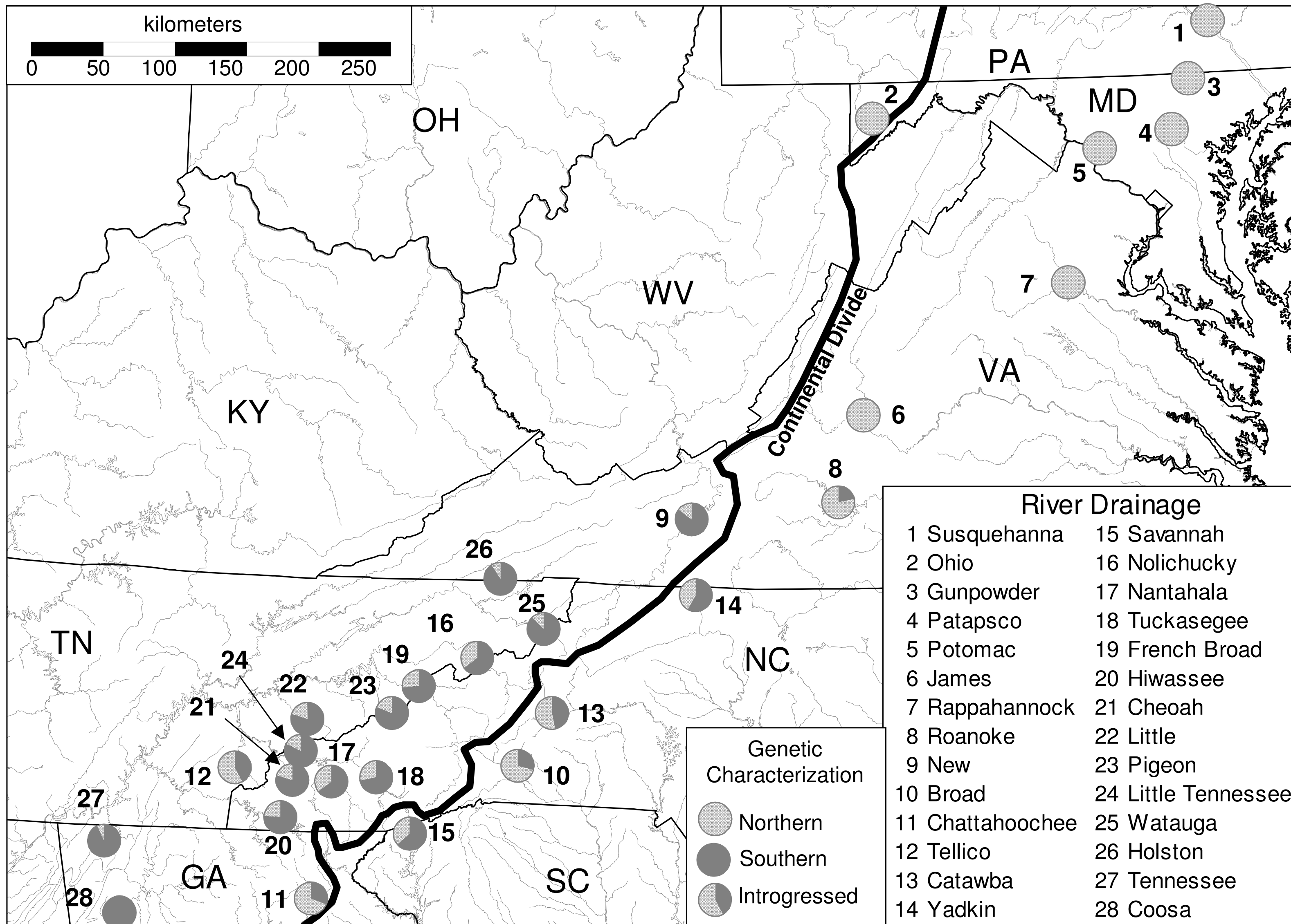


Figure 4. - Genetic characterization of brook trout populations in major river drainages, based on the *CK-A2** locus, using data compiled from all known genetic studies of brook trout populations throughout the native range. See Table 7 for details.

APPENDIX A

Protocol for the non-lethal collection and storage of brook trout muscle tissue

Materials needed :

- Dry ice (~ 1 lb) and air-tight cooler
- Cryovials (Nalgene Cryoware #5000-0020)
- Biopsy devise (Bard Monopty #121410)
- Clove oil (10% solution) and 1 ml plastic pipette
- 95% ethanol
- Scissors and coin envelopes
- 20 L bucket (2x)
- Live well/net

1) Using a 1 ml plastic pipette, dispense 2 ml of 10% clove oil into a bucket containing approximately 10 L of stream water. Swirl with hand or stick to ensure mixing.

2) Place fish in bucket and allow approximately 5 min for the anesthetic to work. Fish that do not respond to touch and float side/belly-up are fully anesthetized. The amount of anesthetic may have to be altered depending on the water condition and size of fish - too much anesthetic will kill the fish, and too little will stress them.

3) Label tubes and envelopes while waiting for anesthetic to work.

4) (Requires 2 people)
Person #1: Remove one anesthetized fish (>120mm TL) from bucket and hold firmly with one hand while injecting the primed biopsy devise using other hand. Needle should be inserted obliquely from back to front at a shallow depth into muscle tissue just below dorsal fin.
Person #2: Steady biopsy device and carefully press sample ejection button. Remove and retract needle. Quickly scrape tissue sample into tube and immediately place on dry ice. If necessary, repeat and place second sample into same tube.

5) Cut a corner of the caudal fin using sterile scissors and place fin-clip in labeled coin envelope. Fin-clip can be kept at room temperature until completely dry before placing at -20°C for long-term storage.

6) Place fish in live-well or bucket of fresh water until fully revived.

7) Release fish as close to capture-point as possible.

8) Dip biopsy needle and scissors in ethanol between each fish.

9) Muscle tissue should be placed at -80°C for long-term storage.

APPENDIX B

Protocol for cellulose-acetate gel electrophoresis of enzymatic proteins

Materials needed:

- Methodologies for allozyme analysis reference manual (Hebert and Beaton 1989)
- Gel electrophoresis tanks with wicks (Helena Laboratories Titan gel box)
- D.C. power supply
- Cellulose acetate gel plates (Helena Laboratories Titan III)
- Applicator kit (Helena Laboratories Super Z-12)
- 1.5 ml microcentrifuge tubes
- Plastic pestles to fit microcentrifuge tubes
- Tweezers, scalpel, marker, scissors, 95% ethanol
- Appropriate buffers (see Appendix D)

1) Cut corner of gel for orientation purposes. Label back-side (shiny mylar surface) with marker and soak in appropriate buffer for >20 minutes.

2) Label 1.5 ml microcentrifuge tubes in sets of 11, including at least one control individual of known origin. Place on ice in large, shallow tray. Add 150 µl cold 0.09M Tris-HCL buffer, pH 8.0 to each tube.

3) Remove samples from freezer and place in ice bath.

4) Remove one tissue biopsy plug with clean tweezers and place in tube with buffer. If whole young-of-year fish are provided, cut an equivalent piece with clean scalpel.

5) Grind tissue well using plastic pestle. Pulse vortex and spin down briefly (a few seconds).

6) Add 11 µl of supernatant to loading tray from right to left (skip space 12 and start with 11).

7) Remove gel from buffer and gently dab surface with paper towel to remove excess liquid. Load samples onto gel surface using applicator and place gel face-down on wicks in gel chamber containing appropriate buffer.

8) Secure chamber lid and attach electrodes from chamber to voltage meter. Run at 200 volts for 40 minutes.

APPENDIX C

Protocol for histochemical staining of cellulose acetate gels for the visualization of enzymatic proteins

Materials needed:

- 1.5% agarose in 7-10 ml aliquots, stored at 65°C
- Appropriate stains (see Appendix D)
- Small, flat tray for pouring stain onto gel
- 37°C incubator
- Digital camera to photograph gels
- Standard data collection sheets
- Sheet protectors to store/protect gels

1) Prepare stains containing stable ingredients in bulk and divide into 3 ml aliquots – store at -20°C. Defrost prior to use.

2) A few minutes before electrophoresis is complete, pour stain into small beaker and add labile ingredients. Mix.

3) When electrophoresis is complete, remove gel and place face-up in tray.

4) Remove aliquot of agarose from oven. Quickly pour into beaker with stain, mix and pour over gel.

5) Allow a minute to solidify and place at 37°C for 10-30 minutes (until bands are visible).

6) Rinse gel gently with cold water to remove agarose overlay.

7) Estimate migration distance of the bands relative to the control.

8) Record date, stain and population/individual ID on standard data collection sheet. Tape gel to paper and photograph.

9) Store within a sheet protector in a binder.

APPENDIX D

Buffer and stain recipes for each enzyme system

Modified from Hebert and Beaton (1989) and Galbreath et al. (2001)

Buffers

Tris Glycine (TG) buffer

30 g tris

144 g glycine

- Make up to 1 liter with water
- Dilute 1:9 and adjust to appropriate pH with 12N HCl

Sodium Phosphate buffer [0.1M]

30.5 ml Na_2HPO_4 [0.2M]

19.5 ml NaH_2PO_4 [0.2M]

- Make up to 100 ml with water and adjust to pH 7.0

Stains used with TG, pH 8.0 buffer system

Creatine Kinase (CK)

80 ml Tris-HCl [0.1M], pH 7.1

120 mg phosphocreatine

288 mg ADP

800 mg glucose

32 mg $MgCl_2$

16 ml β-NAD [2 mg/ml]

- Divide into 9-ml aliquots, wrap with foil, and freeze
- Immediately before use add:

750 μl MTT [10mg/ml]

52.5 μl hexokinase [1 unit/μl]

26 μl G_6PDH [1 unit/μl]

375 μl PMS [2 mg/ml]

7 ml agar

Glucose-6-Phosphate Isomerase (GPI)

18 ml Tris-HCl [0.09M], pH 8.0

27 ml β-NAD [2 mg/ml]

3.6 ml fructose-6-phosphate [20 mg/ml]

3.6 ml MTT [10 mg/ml]

- Divide into 4 ml aliquots, wrap with foil, and freeze
- Immediately before use add:

7 μl G_6PDH [1 unit/μl]

800 μl PMS [2 mg/ml]

4 ml agar

APPENDIX D continued

Stains used with TG, pH 7.5 buffer system

Aspartate Amino Transferase (AAT)

67 ml sodium phosphate buffer, pH 7.0
3.3 mg pyridoxal-5-phosphate
153 mg L-aspartic acid
87 mg α-ketoglutaric acid

- Adjust to pH 7.4 with ~100 drops 1M tris base
- Divide into 4-ml aliquots, wrap with foil, and freeze
- Immediately before use add:

3 ml fast blue [5 mg/ml]
4 ml agar

Malate Dehydrogenase (MDH)

15 ml Tris-HCl [0.09M], pH 8.0
22.5 ml β-NAD [2 mg/ml]
6 ml MTT [10 mg/ml]
7.8 ml malic substrate*

*Malic substrate

180 ml water
20 ml tris-HCl [0.2M], pH 9.0
3.68 g L-Malic acid
- Adjust to pH 8.0

- Divide into 4-ml aliquots, wrap with foil, and freeze
- Immediately before use add:

800 µl PMS [2 mg/ml]
4 ml agar

Glycerol-3-Phosphate Dehydrogenase (G3PDH)

9 ml Tris-HCl [0.09M], pH 8.0
22.5 ml β-NAD [2 mg/ml]
6 ml MTT [10 mg'ml]
13.2 ml DL-α-glycerophosphate [40 mg/ml]

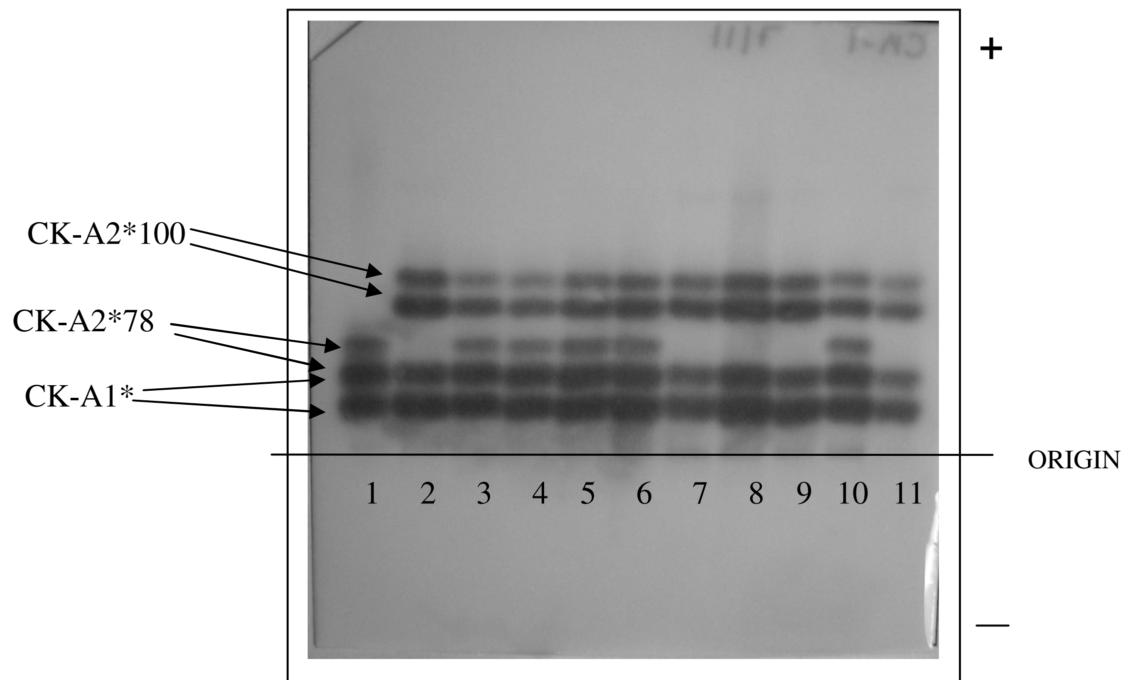
- Divide into 4-ml aliquots, wrap with foil, and freeze
- Immediately before use add:

800 µl PMS [2 mg/ml]
4 ml agar

APPENDIX E

Representative gel images for each enzyme system

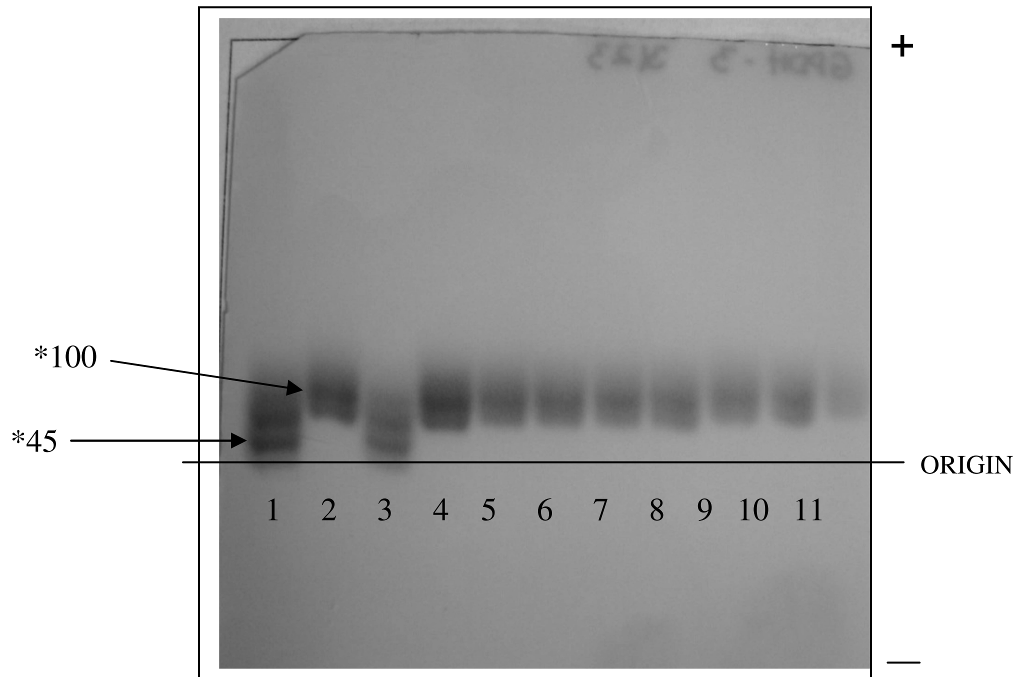
Creatine kinase (*CK-A2**)



Individual # (left to right)	Genotype *CK-A2**
1	78/78
2	100/100
3	78/100
4	78/100
5	78/100
6	78/100
7	100/100
8	100/100
9	100/100
10	78/100
11	100/100

Notes: The banding pattern for creatine kinase is not typical for a dimeric enzyme with two loci. There are no detectable heterodimers between the two loci, *CK-A1** and *CK-A2**, and each allele is represented by two bands, possibly the consequence of posttranslational modification of a single polypeptide unit (Richardson et al. 1986). The lower band for the CK-A2*78 allele overlaps with the upper band for the monomorphic *CK-A1** locus.

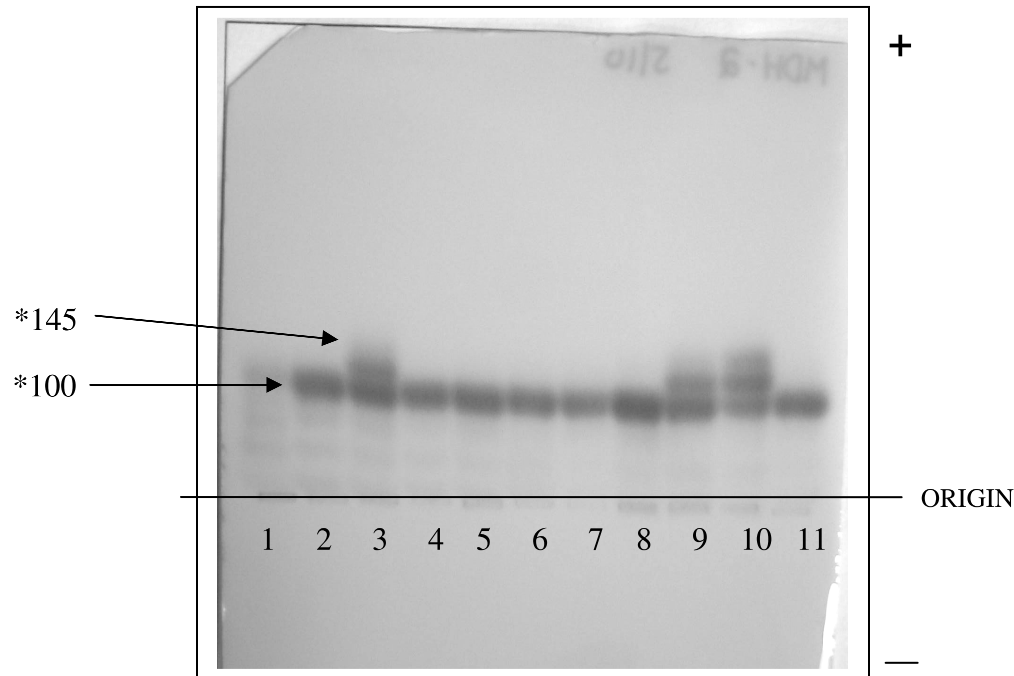
Glycerol-3-phosphate dehydrogenase (*G3PDH**)



Individual # (left to right)	Genotype *G3PDH**
1	45/100
2	100/100
3	45/100
4	100/100
5	100/100
6	100/100
7	100/100
8	100/100
9	100/100
10	100/100
11	100/100

Notes: This locus is often presented as a smear, so caution and consistency should be exercised when scoring alleles.

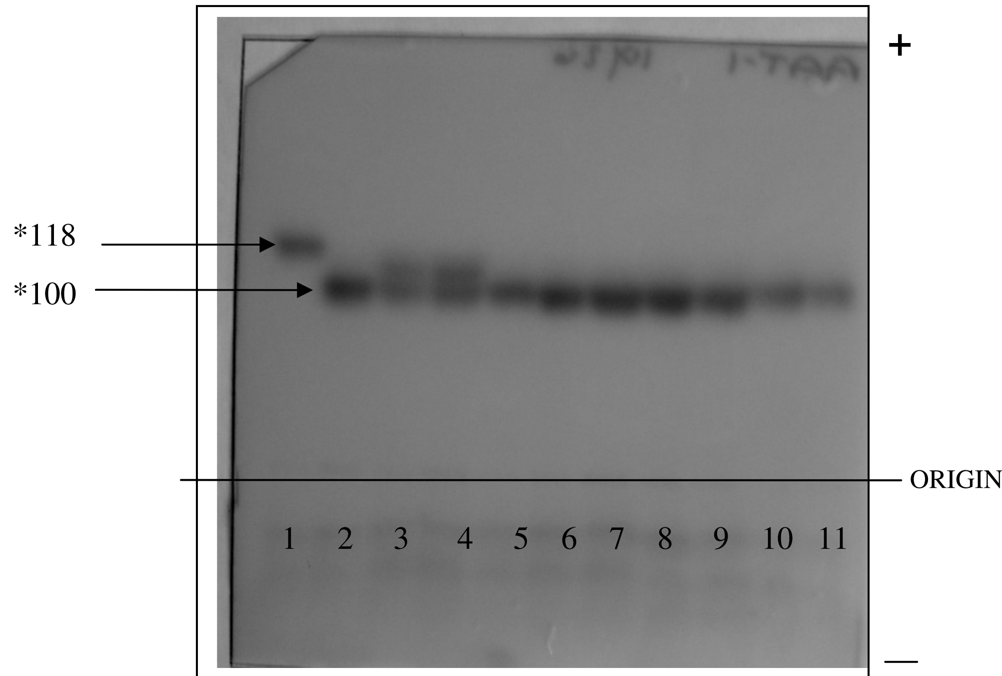
Malate dehydrogenase (*sMDH-B1,2**)



Individual # (left to right)	Genotype *sMDH-B1,2**
1	too faint to score
2	100_4
3	$100_3/145$
4	100_4
5	100_4
6	100_4
7	100_4
8	100_4
9	$100_3/145$
10	$100_2/145_2$
11	100_4

Notes: The fact that *sMDH-B1,2** are isoloci is evident by the differences in dosage. Individuals 3 and 9 have three copies of the *100 allele and one copy of *145, whereas individual #10 has two copies of both alleles.

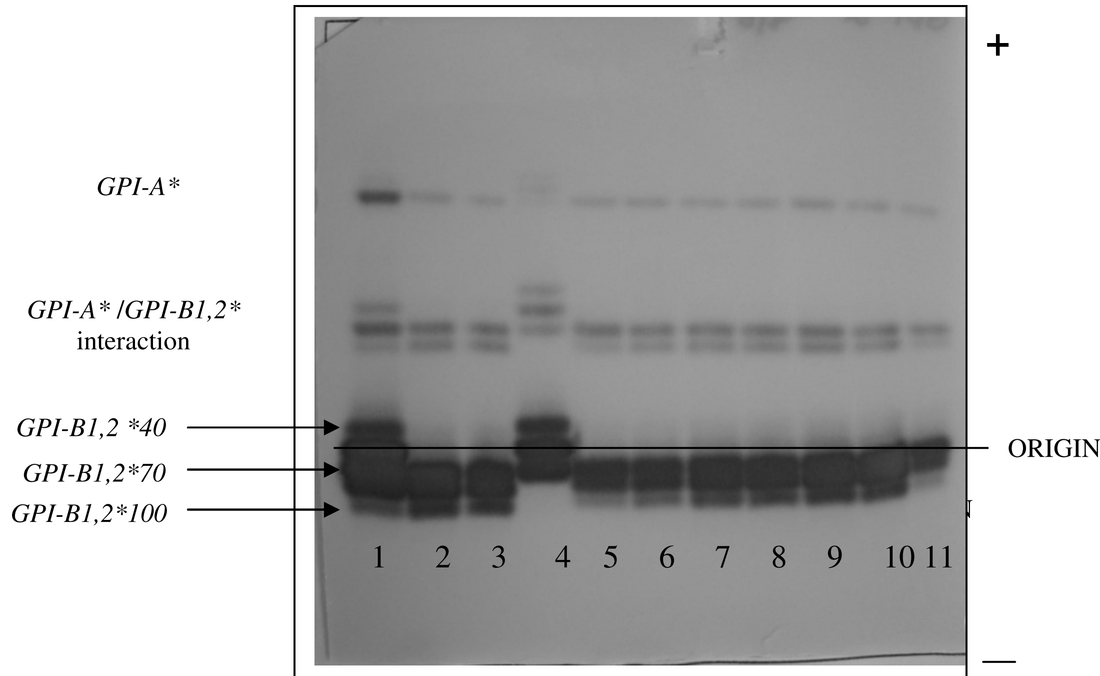
Aspartate aminotransferase (*sAAT-1,2**)



Individual # (left to right)	Genotype *sAAT-1,2**
1	118_4
2	100_4
3	$100_2/118_2$
4	$100_2/118_2$
5	100_4
6	100_4
7	100_4
8	100_4
9	100_4
10	100_4
11	100_4

Notes: Estimating dosage for isoloci can prove arbitrary, as can be seen with individuals 3 and 4; it is difficult to know if there are two copies of both alleles, or three copies of *100, and one copy of *118.

Glucose-6-phosphate isomerase (*GPI-A** and *GPI-B1,2**)



Individual # (left to right)	Genotype *GPI-A**	Genotype *GPI-B1,2**
1	100/100	$40/70_2/100$
2	100/100	$70_2/100_2$
3	100/100	$70_2/100_2$
4	100/100	$40_2/70_2$
5	100/130	$70_2/100_2$
6	100/100	$70_2/100_2$
7	100/100	$70_2/100_2$
8	100/100	$70_2/100_2$
9	100/100	$70_2/100_2$
10	100/100	$70_2/100_2$
11	100/100	$70_3/100$

Notes: Notice the location of the origin; some alleles are migrating anodally and others are migrating cathodally, which makes naming the alleles difficult. To reduce confusion, I treated all of the alleles from *GPI-B1,2** as if they were migrating cathodally and eliminated the "-" notation (*40 just above origin, *70 just below the origin, and *100 is the most cathodal). Also, estimating dosage can prove arbitrary, as can be seen with individuals 5 through 11; it is difficult to know if there are two copies of both alleles, or three copies of *70, and one copy of *1.