

## **CHAPTER II**

### **MATERIALS AND METHODS**

#### *Study Design*

Twenty-two streams containing naturally reproducing populations of brook trout were sampled during 2000 to provide data from all major New Jersey drainages known to contain wild brook trout (Figure 7). Study streams were generally small, first or second order streams that were primarily located in the headwaters of larger river systems routinely stocked with catchable-size cultured trout (Table 1). Nineteen of these streams, considered to have high potential for harboring indigenous brook trout populations, were selected using the following criteria: (1) no documented trout stocking history, and (2) absence of reproducing populations of brown and/or rainbow trout (which indicate prior salmonid stocking). Streams having natural barriers that could genetically isolate brook trout populations and prevent interactions with cultured trout stocked downstream were considered ideal candidates, but only one stream selected (Crooked Brook tributary) was able to meet this additional criterion. Subsequent to sampling it was learned that one of the 19 streams selected, Hacklebarney Brook, was stocked with trout in the past by NJDFW, and Cresskill Brook may have

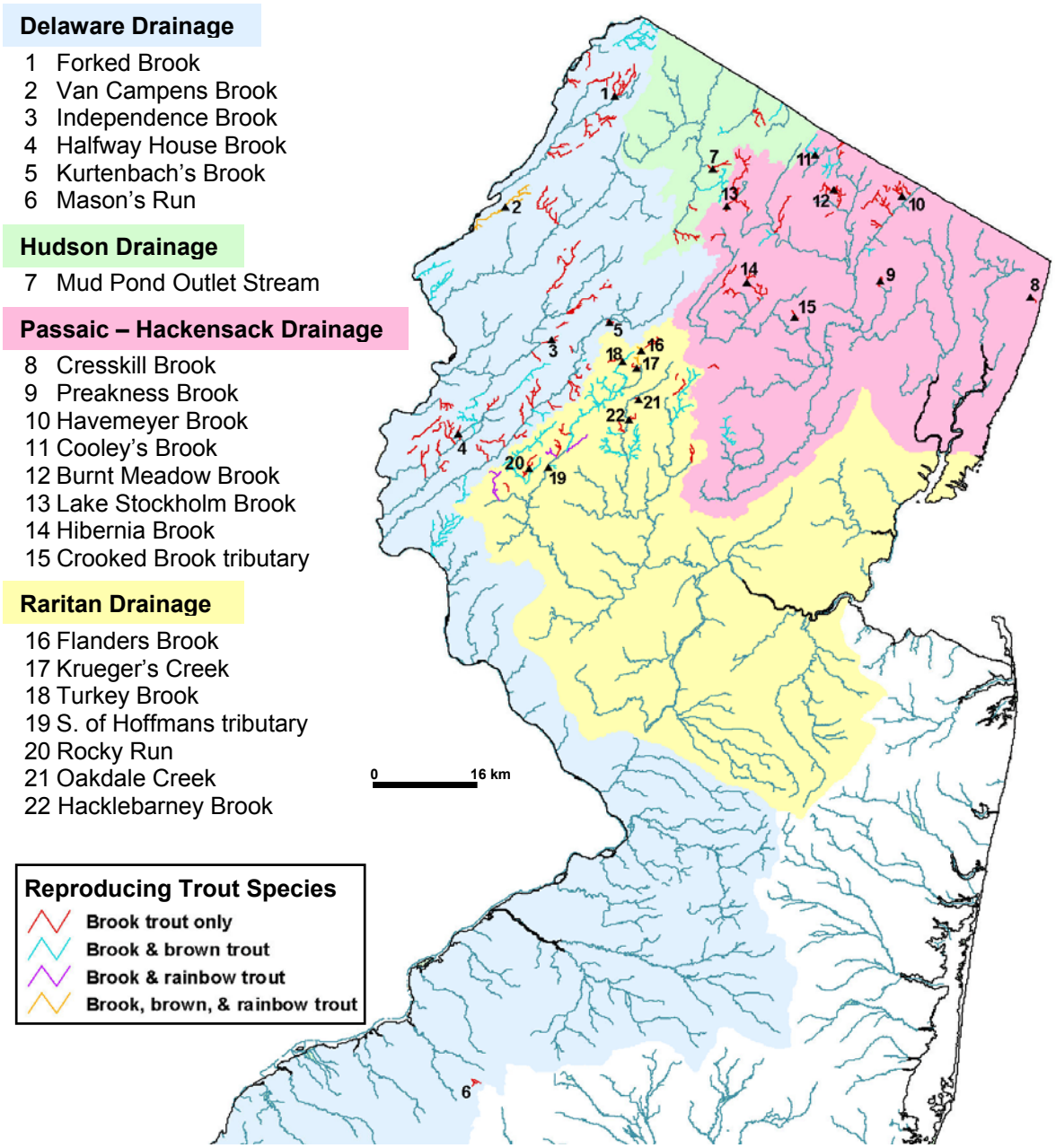


FIGURE 7.— Map indicating the location of 22 sites in New Jersey where brook trout, *Salvelinus fontinalis*, were collected in 2000.

TABLE 1.—Location and trout stocking history information for 23 brook trout collection sites in New Jersey. Trout stocking history information was obtained from NJ Division of Fish and Wildlife records, unless otherwise noted (M = mainstem stream; T = tributary to mainstem stream).

Site code	Drainage	Mainstem stream	Tributary	Latitude Longitude	Trout stocking history
FOR	Delaware	Big Flat Brook	Forked Brook	41°14'24.40"N 74°44'48.30"W	M - stocked annually 40+ yrs T - no record of stocking
VCB	Delaware	Delaware River	Van Campens Brook	41°04'36.00"N 74°57'30.59"W	M - generally not stocked along NJ/PA T - stocked extensively prior to 1979
IND	Delaware	Pequest River	Independence Creek	40°53'01.60"N 74°51'54.60"W	M - stocked annually 40+ yrs T - no record of stocking
HWH	Delaware	Pohatcong Creek	Halfway House Brook	40°44'44.67"N 75°02'46.34"W	M - stocked annually 40+ yrs T - no record of stocking
KUR	Delaware	Musconetcong River	Kurtenbach's Brook	40°54'33.24"N 74°45'17.61"W	M - stocked annually 40+ yrs T - no record of stocking
MAS	Delaware	Big Timber Creek	Masons Run	39°47'13.10"N 75°00'04.50"W	M - not stocked, but several off-stream impoundments stocked regularly T - no record of stocking
MPO	Hudson	Wallkill River	Mud Pond Outlet Stream	41°08'00.00"N 74°33'18.90"W	M - stocked annually 40+ yrs T - no record of stocking
CRE	Newark Bay	Hackensack River	Cresskill Brook	40°56'43.20"N 73°56'30.40"W	M - stocked annually 40+ yrs T - no record of stocking
PRE	Passaic	Passaic River	Preakness Brook	40°58'10.81"N 74°13'52.90"W	M - not stocked extensively T - onstream impundment ? km downstream of sample site stocked (Barbours Pond)
HAV	Passaic	Ramapoo River	Havemeyer Brook	41°05'39.60"N 74°11'23.10"W	M - stocked annually 40+ yrs T - no record of stocking
COO	Passaic	Wanaque River	Cooleys Brook	41°09'18.37"N 74°21'25.13"W	M - stocked annually 40+ yrs T - stocked extensively prior to 1990

TABLE 1.—Continued.

Site code	Drainage	Mainstem stream	Tributary	Latitude Longitude	Trout stocking history
BMB	Passaic	Wanaque River	Burnt Meadow Brook	41°06'10.73"N 74°19'20.05"W	M - stocked annually 40+ yrs T - no record of stocking
LSB	Passaic	Pequannock River	Lake Stockholm Brook	41°04'48.25"N 74°31'39.17"W	M - stocked annually 40+ yrs T - no record of stocking
HIB	Passaic	Rockaway River	Hibernia Brook	40°58'04.12"N 74°29'26.59"W	M - stocked annually 40+ yrs T - stocked downstream of sample site, below on-stream impoundment (???)
CBT	Passaic	Rockaway River	Crooked Brook tributary	40°55'04.50"N 74°23'49.02"W	M - stocked annually since ????? T - no record of stocking
FLA	Raritan	S/Br. Raritan River	Flanders Brook	40°52'02.62"N 74°41'41.20"W	M - stocked annually 40+ yrs T - stocked annually prior to 1990
KRU	Raritan	S/Br. Raritan River	Krueger's Creek	40°50'29.89"N 74°42'07.97"W	M - stocked annually 40+ yrs T - no record of stocking
TUR	Raritan	S/Br. Raritan River	Turkey Brook	40°51'04.55"N 74°43'48.14"W	M - stocked annually 40+ yrs T - no record of stocking
SOH	Raritan	S/Br. Raritan River	S. of Hoffmans tributary	40°41'46.00"N 74°52'16.33"W	M - stocked annually 40+ yrs T - no record of stocking
ROC	Raritan	S/Br. Raritan River	Rocky Run	40°41'42.54"N 74°54'35.41"W	M - stocked annually 40+ yrs T - no record of stocking
OAC	Raritan	Lamington River	Oakdale Creek	40°47'48.13"N 74°41'51.57"W	M - stocked annually 40+ yrs T - no record of stocking
HAC	Raritan	Lamington River	Hacklebarney Brook	40°46'02.42"N 74°43'03.31"W	M - stocked annually 40+ yrs T - stocked annually prior to 19??
PTH	-	-	Pequest Trout Hatchery	-	Brook trout eggs obtained from the North Attleboro National Fish Hatchery in Massachusetts (Nashua strain) when hatchery production commenced in 1982.

been privately stocked with trout (anecdotal information provided by a caretaker of property bordering the brook when the stream was electrofished). Three additional streams, each from a different major drainage and having a long history of trout stocking (but not recently stocked), were also sampled. For comparison purposes, samples were taken from cultured brook trout reared at the NJDFW Pequest Trout Hatchery.

### ***Sample Collection***

Brook trout were collected from study streams using pulsed direct current backpack electrofishers (Smith-Root Model Type VII or 12-B) (Figure 8). A sample size of 10 – 15 fish (>10 cm) was targeted, though fewer were collected from streams with low population densities. The distance sampled therefore varied from stream to stream, and generally ranged from 100 – 300 m.



Figure 8.—Collection of brook trout using a backpack electrofisher.



Figure 9.—Cardiac puncture technique used to obtain blood samples.

Fish were anesthetized with tricaine methanesulfonate (Finquel) and approximately 100- $\mu$ L of blood was taken by cardiac puncture using a 28-gauge insulin syringe (B&D) (Figure 9). Anesthetized fish were returned to the stream immediately following this procedure and monitored until they recovered sufficiently to swim away. Blood was initially stored in vacutainers containing EDTA and immediately placed on ice. Within 24 hours of

collection, samples were transferred to microcentrifuge tubes and frozen and stored at -55°C until DNA extraction was performed.

### ***DNA Extraction***

Genomic DNA was isolated from 247 blood samples using one of two protocols. Most extractions (193 samples) were performed at East Stroudsburg University using a commercially available DNA extraction kit (Biorad InstaGene™ Whole Blood Kit). The manufacturer's guidelines were followed, using 10-μL of blood. Extraction success was visually confirmed with electrophoresis on a 1% agarose gel stained with ethidium

bromide, using 8-μL of the supernatant containing the extracted DNA, and 2-μL dye (Figure 10).

Deer DNA was run in one lane for quality control purposes. Gels were photo-documented with Polaroid 667 film. The extraction process was repeated for failures until successful or the

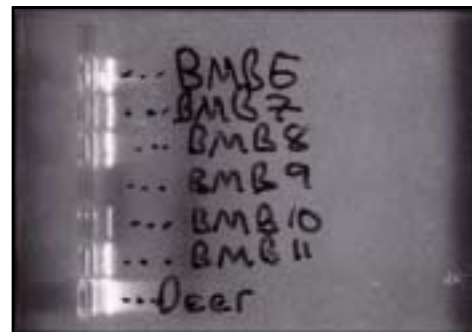


Figure 10.—DNA extraction success was confirmed electrophoretically and photo-documented

sample supply exhausted. Supernatants were placed in microcentrifuge tubes and stored at -55°C. For the remaining 53 samples, blood was placed on FTA® cards, air-dried, and sent to the USGS, Leetown Science Center, Kearneysville, WV for DNA extraction. For DNA extractions performed by USGS, the Puregene DNA extraction kit (Gentra Systems, Minneapolis, Minnesota; Buccal Cell Protocol used, p. 32 in Puregene instruction manual) was followed.

### ***Microsatellite DNA Amplification***

PCR was used to amplify 13 microsatellite loci using primer pairs designed specifically for brook trout (*SfoB52*, *SfoC24*, *SfoC28*, *SfoC38*, *SfoC79*, *SfoC86*, *SfoC88*, *SfoC113*, *SfoC115*, *SfoC129*, *SfoD75*, *SfoD91*, *SfoD100*; T. L. King, USGS, unpublished). The forward primers were fluorescently labeled with HEX, FAM, or NED dye (Applied Biosystems). Supernatants from DNA extractions were diluted 10:1 with deionized water, thoroughly mixed, and used for the DNA template. Reactions were generally successful using this dilution, therefore, DNA was not quantified prior to PCR. Reaction failures were repeated using undiluted supernatant for the template. Amplifications for each sample were carried out in three 15- $\mu$ L reaction solutions, each containing a different set of four or five primer pairs. The components of each master mix solution are given in Table 2. The amplification cycle typically consisted of a 2-min initial denaturation at 94°C, followed by 35 cycles of 94°C denaturing for 45 s, 56°C annealing for 45 s, and a 72°C extension for 2-min. Cycling concluded with a 10-min extension at 72°C. PCR failures were repeated using single-locus reactions. Amplifications were carried out on either a PTC-200 or PTC-225 Thermal Cycler (MJ Research). All aspects of PCR were performed by the USGS.

### ***Fragment Analysis***

Fragment analysis (using fluorescently labeled DNA fragments obtained through PCR) was performed on an Applied Biosystems (Foster City, CA, USA) ABI 3100 Genetic Analyzer, as described in King et al. (2001). Genescan™ 3.7 Analysis software and Genotyper™ 3.6 Fragment Analysis software (Applied Biosystems) was used to

TABLE 2.—Three master mixes used to amplify 13 microsatellite loci in 23 brook trout collections from New Jersey. Forward primers are labeled with fluorescent dye (*fam*, *hex*, or *ned*). Stock concentrations used: 10 mM trisHCl [pH 8.3] buffer, 25 mM MgCl<sub>2</sub>, 10 mM dNTPs, 5 mM, *Taq* DNA polymerase.

Master Mix A		Master Mix B		Master Mix C	
Quantity (μL)	Reagent concentration	Quantity (μL)	Reagent concentration	Quantity (μL)	Reagent concentration
3.96	dH2O	3.39	dH2O	2.34	dH2O
2.625	0.875 1X bufer	2.625	0.875 1X buffer	2.625	0.875 1X buffer
2.25	3.75 mM MgCl <sub>2</sub>	2.25	3.75 mM MgCl <sub>2</sub>	2.25	3.75 mM MgCl <sub>2</sub>
1.905	0.3175 mM dNTPs	1.905	0.3175 mM dNTPs	1.905	0.3175 mM dNTPs
0.225	0.075 uM <i>SfoC24 fam</i>	0.24	0.08 uM <i>SfoC86 hex</i>	0.42	0.14 uM <i>SfoC113 fam</i>
0.225	0.075 uM <i>SfoC24</i>	0.24	0.08 uM <i>SfoC86</i>	0.42	0.14 uM <i>SfoC113</i>
0.36	0.12 uM <i>SfoB52 fam</i>	0.27	0.09 uM <i>SfoC88 hex</i>	0.48	0.16 uM <i>SfoC115 fam</i>
0.36	0.12 uM <i>SfoB52</i>	0.27	0.09 uM <i>SfoC88</i>	0.48	0.16 uM <i>SfoC115</i>
0.15	0.05 uM <i>SfoD100 hex</i>	0.33	0.11 uM <i>SfoC129 hex</i>	0.42	0.14 uM <i>SfoC79 hex</i>
0.15	0.05 uM <i>SfoD100</i>	0.33	0.11 uM <i>SfoC129</i>	0.42	0.14 uM <i>SfoC79</i>
0.33	0.011 uM <i>SfoC38 ned</i>	0.69	0.23 uM <i>SfoC28 ned</i>	0.75	0.25 uM <i>SfoD91a hex</i>
0.33	0.011 uM <i>SfoC38</i>	0.69	0.23 uM <i>SfoC28</i>	0.75	0.25 uM <i>SfoD91a</i>
0.18	0.06 uM <i>SfoD75 ned</i>		-		-
0.18	0.06 uM <i>SfoD75</i>		-		-
0.27	0.09 units/uL <i>Taq</i>	0.27	0.09 units/uL <i>Taq</i>	0.24	0.09 units/uL <i>Taq</i>
1.5	DNA template	1.5	DNA template	1.5	DNA template
15	Total	15	Total	15	Total



score, bin, and output allelic (and genotypic) data. All aspects of the fragment analysis were performed by the USGS.

### ***Data Analysis***

The allelic data generated for 240 individuals were initially examined using Microsatellite Toolkit (Parks 2001), an add-in utility for Microsoft® Excel (Windows versions, Excel 97 or later) that contains tools for population geneticists working with microsatellites. Toolkit was used to identify data entry errors and detect genetically identical samples. Once the data set was finalized (Appendix, Table A1), Toolkit was used to bring the data into input file format for further analysis with other population genetics software. In this study, a null (nonamplifying) homozygote was detected at one locus (*SfoD91*) in one collection (Lake Stockholm Brook, LSB) (Table A1, Appendix). This locus was retained in subsequent analyses, unless otherwise noted, to maximize the number of independent alleles and reduce the coefficient of variation of estimates of genetic distance (Kalinowski 2002).

Genetic diversity within 23 collections was quantified using BIOSYS-1 (Swofford and Selander 1981) by calculating allelic frequencies, number of alleles per loci, loci polymorphism, observed heterozygosity ( $H_O$ ), and expected heterozygosity ( $H_E$ ). Corrected estimates of allelic diversity based upon the smallest sample size ( $n = 7$  for collections and  $n = 4$  for drainages) and Wright's (1969) inbreeding coefficient ( $F_{IS}$ ) were estimated for each collection using FSTAT (Goudet 1995). Thirteen loci were used to derive all values for each collection except for the Lake Stockholm Brook collection

(12 loci used; *SfoD91* excluded). The number of unique alleles, by collection and drainage, was determined using GenAIEx (Peakall and Smouse 2006).

The genotypes at each locus for each collection were tested for conformity to Hardy–Weinberg equilibrium (HWE) by comparing the observed genotype frequencies with the frequencies expected for an ideal population (large, randomly mating population of diploid organisms that reproduce sexually, have nonoverlapping generations, where the effects of mutation, migration, and selection are negligible). This test was performed in GenePop 3.1 (Raymond and Rousset 1995) using the Markov chain randomization test of Guo and Thompson (1992). Though not common, microsatellites can be clustered in the genome and therefore linkage disequilibrium should always be tested (Selkoe and Toonen 2006). To assess if loci assorted independently (i.e. not transmitted to offspring as a pair), linkage disequilibrium (LD) was tested for all pairs of loci using the randomization method of Raymond and Rousset (1995) in GenePop 3.1 with 10,000 dememorizations, 100 batches, and 5,000 iterations per batch. Significance levels for HWE and LD, and all other multiple comparison tests, were adjusted using sequential Bonferroni methods (Rice 1989) with an initial  $\alpha$  level of  $0.05/k$ ,  $k$  being the number of tests.

The statistical significance of allele frequency differences between each pair of samples was tested by means of the genetic differentiation randomization test in GenePop. Results were combined over loci using Fisher's method (Sokal and Rohlf 1994) and adjusted for multiple tests with the sequential Bonferroni method. To test for genetic differentiation among the brook trout collections, pairwise  $F_{ST}$  values were obtained with GenePop 3.4. Pairwise  $R_{ST}$  values among collections were also calculated

using GenePop 3.4 and are provided for comparison purposes with  $F_{ST}$  values.  $F_{ST}$  assumes allelic diversity results from migration and gene drift, while  $R_{ST}$  also measures mutational differences between alleles (King et al. 2006).

Several techniques were used to describe the genetic relationships among collections and drainages. The population genetic structure was quantified at several levels using an analysis of molecular variance (AMOVA) (Excoffier et al. 1992), performed in GenAlEx using pairwise  $R_{ST}$  values. To determine how much of the variation is due to differences among populations versus drainages, the total amount of genetic variation was partitioned into (1) the proportion due to genetic differences among collections, both within and between drainages and (2) the proportion due to genetic variation within and among drainages, with collections within drainages pooled.

To transform the allelic frequency data into a distance matrix, genetic distance estimates for all pairwise collection comparisons were determined using the chord distance measure of Cavalli-Sforza and Edwards (1967), implemented by FSTAT (Goudet 1995). This metric measures the distance as though the collections were on a multidimensional sphere. It is based on the infinite allele model of mutation which assumes that most new mutations arise in a stepwise fashion by the gain or loss of repeated units (Shaklee and Currens 2003). This metric is generally considered more appropriate than the logarithmic-derived genetic distance metric developed by Nei (1972, 1978) when random genetic drift, rather than mutation, is the primary force of divergence (Shaklee and Currens 2003).

An unrooted phylogenetic tree was fitted using the distance matrix and the neighbor-joining algorithm implemented by PHYLIP (Felsenstein 1992), a package of

computer programs for inferring phylogenies. TreeView (Page 1996), a program for displaying and printing phylogenies, was used to visualize the tree. Maximum likelihood assignment tests (Paetkau et al. 1995) used to determine the likelihood of each individual's multilocus genotype being found in the population and drainage from which it was sampled, were conducted using GeneClass 1.0.02 (Cornuet et al. 1999) with the Bayesian method ("leave one out" procedure). In the event of null frequencies, a constant likelihood of 0.01 was assumed. The *SfoD91* locus was not included in the AMOVA and assignment tests, due to the presence of null alleles in all animals from one collection (Lake Stockholm Brook, LSB).