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Distribution, Hybridization, and Taxonomic Status of Two-lined Salamanders (*Eurycea bislineata* complex) in Virginia and West Virginia

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ABSTRACT

We used three diagnostic protein markers to examine salamanders of the *Eurycea bislineata* complex at 80 localities in Virginia and West Virginia. Two groups were strongly differentiated and met at a narrow contact zone. Rare hybridization was observed as well as limited introgression up to 5 km north and 10 km south of the contact zone. At the contact zone, 1% F_1, 2% F_2, 32% backcross, and 66% parental genotypes were observed. This pattern of parapatric distribution with limited hybridization and introgression argues for the recognition of *Eurycea bislineata* and *E. cirrigera* as separate species.

Keywords: *Eurycea bislineata* complex, electrophoresis, hybridization, speciation.

INTRODUCTION

A lively debate has surrounded the systematics of the *Eurycea bislineata* complex (*E. bislineata*, *E. cirrigera*, and *E. wilderae*) over the past several decades (Pauley & Watson, 2005; Sever, 2005). In a broadscale study using protein electrophoresis of 22 proteins and 66 populations, Jacobs (1987) elevated each of the previously recognized subspecies to full specific status. Petranka (1998), however, recommended the recognition of subspecies until such time as contact zones were examined and the degree of gene exchange occurring between genetic subgroups was quantified. Kozak & Montanucci (2001) subsequently studied the contact zone between *E. cirrigera* and *E. wilderae* in the Carolinas and found evidence of a parapatric distribution with very little gene exchange between the two groups. Camp et al. (2000) found *E. cirrigera* and *E. wilderae* together in a zone of sympatry approximately 15 km wide in Georgia. Guttman & Karlin (1986), in a paper described by Petranka (1998) as the strongest evidence that *E. bislineata* is a semispecies or superspecies complex, examined the boundary between *E. bislineat*a and *E. cirrigera* in Ohio and Indiana. They found evidence of a narrow hybrid zone where only occasional hybrids and backcross progeny occurred.

Several authors (Guttman & Karlin, 1986; Jacobs, 1987; Petranka, 1998; Kozak & Montanucci, 2001) commented on the need for additional genetic analyses of contact zones between each of the species pairs to provide further evidence for a taxonomy that accurately reflects the evolutionary history of the *E. bislineata* complex. We present a fine-scaled genetic analysis of the contact zone between *E. bislineata* and *E. cirrigera* in Virginia and West Virginia. This is the first genetic analysis of species boundaries in West Virginia (Sever, 2005) and a detailed analysis of the southern end of the same contact zone studied by Guttman & Karlin (1986). We include data on hybridization and introgression between these two taxa. Our data confirm the conclusions of Guttman & Karlin (1986) that limited hybridization occurs along a narrow and well-defined contact zone, and these taxa are sufficiently distinct to warrant specific status.

MATERIALS AND METHODS

We collected two-lined salamanders from throughout West Virginia and Virginia from 1987–1995. However, more extensive collections were made in central Virginia, both to delineate the location of the contact zone, and to determine its composition (Appendix 1). Animals were transported to the lab where they were sacrificed in 1,1,1-Trichloro-2-methyl-2-propanol. Whole body homogenates were prepared by grinding in a buffer

composed of .01M Tris-HCl, .25M Sucrose, .001M EDTA, pH = 7.0. Homogenates were individually centrifuged at 10,000 x G for 15 min and frozen at -80 ºC until electrophoresis, which was usually within one month of sample preparation.

We used diagnostic electrophoretic loci described by Guttman & Karlin (1986) and Jacobs (1987) to identify salamanders. Allozymes are encoded by nuclear genes which exhibit codominant inheritance. They can be used to identify species-specific genotypes, as well as various types of hybrids. The designation of genetic loci and alleles follows that of Jacobs (1987). Glutamic Oxaloacetic Transaminase (GOT-1, 2.6.1.1) was resolved using the lithium hydroxide pH 8.1 buffer of Selander et al. (1971). Six-Phosphogluconate Dehydrogenase (6-PGDH, 1.1.1.44) and Alanyl-leucyl Peptidase (PEP, 3.4.11) were resolved using the tris-citrate pH 8.0 buffer of Selander et al. (1971). We used standard starch-gel electrophoretic techniques with 12.5% hydrolyzed potato starch from Starch-Art®.

We did not use several of the diagnostic markers used by Guttman & Karlin (1986) and Jacobs (1987). GOT-2/AAT-2 was temperature labile and could not be consistently scored for all individuals from all populations and was therefore omitted from further consideration. Although we found variation at these loci, Malic Enzyme and Lactate Dehydrogenase (LDH-1) were not diagnostic in West Virginia and Virginia when compared with the other three markers. Jacobs (1987) also did not use LDH-1.

At each locus, electromorphs with the same mobility were considered homologous, and those with different mobilities were recorded as different alleles. Alleles with the greatest anodal mobility were designated as "a" and the others were assigned "b", "c", "d", or "e" in order of decreasing mobility from the gel origin. We established allelic homologies by simultaneously running representative samples from several populations on the same gel.

"Pure" species genotypes were those with electromorphs characteristic of only one of the species. Some individuals were heterozygous for all three of the protein markers and exhibited electromorphs characteristic of both parental species. These individuals could have been the result of a large variety of gametic combinations. However, the simplest and thus most probable interpretation, is that they resulted from an interspecific mating of "pure" species genotypes. We considered them F1 hybrids and, by inference, characterized a variety of other genotypes that resulted from interbreeding and backcrossing (Guttman & Karlin, 1986). F_2 hybrids were homozygous for one species at one or two markers and homozygous for the other species at the remaining markers. Backcross genotypes (backcross to *E. bislineata* or *E. cirrigera* possible) were heterozygous for one or two markers (but not all three) and homozygous for one of the parental species at the remaining markers.

RESULTS AND DISCUSSION

Allele frequencies for each of the three marker loci demonstrate that certain alleles are fixed or nearly so in each population sampled. The allelomorphs characterizing populations 1–33 differ from those in populations 36–80. Furthermore, there is an abrupt discontinuity in the frequency of these three diagnostic enzymes across a narrow geographic area. Populations 1–33 taken from the north and east portions of our study area, are characterized by the following *E. bislineata* genotypes (Guttman & Karlin, 1986): 6-PGDH^{c,d}; GOT-1^{c,d}; PEP^{c} (Appendix 1). Conversely, populations 36–80 taken from the south and west portions of our study area, are characterized by the following *E. cirrigera* genotypes (Guttman & Karlin, 1986): 6-PGDH^{a,b}; GOT-1^{a,b}; PEP^{a,b,d,e} (Appendix 1). Populations 34 and 35 contain both *E. bislineata* and *E. cirrigera* genotypes as well as F_1 and other hybrid genotypes (Appendix 1). Our *E. bislineata* populations correspond very closely to the Virginia/West Virginia portions of lineages A and B (forming a single clade on phylogram) from a phylogeographic study of *Eurycea* using mtDNA haplotypes (Kozak et al., 2006), whereas our *E. cirrigera* populations correspond with the Virginia/West Virginia portions of lineage E from that same study.

Including allelomorphic data from southeastern Ohio presented in Guttman & Karlin (1986), the contact zone between *E. bislineata* and *E. cirrigera* can be traced in general detail. In Ohio, *E. bislineata* is found north of central Ohio. In West Virginia, *E. bislineata* occurs in the east to approximately Tucker, Randolph, Webster, and Pocahontas counties. In Virginia, *E. bislineata* occurs north of an east/west line through the boundary between Greene and Albemarle counties, extending eastward to Stafford County (Fig. 1).

In Ohio, the contact zone follows the Wisconsin glacial boundary with *E. cirrigera* occurring along the boundary or to the south in unglaciated regions and *E. bislineata* to the north of these areas (Guttman & Karlin, 1986). The West Virginia distribution of *E. cirrigera* corresponds with the Allegheny Plateau physiographic province, whereas the distribution of *E. bislineata* corresponds to the Allegheny Mountain and Ridge and Valley provinces (Green & Pauley, 1987). In Virginia, the distribution of these species tends to cut across physiographic provinces rather than follow them. The distribution of *E. bislineata* corresponds roughly with the Shenandoah River drainage, following the northern

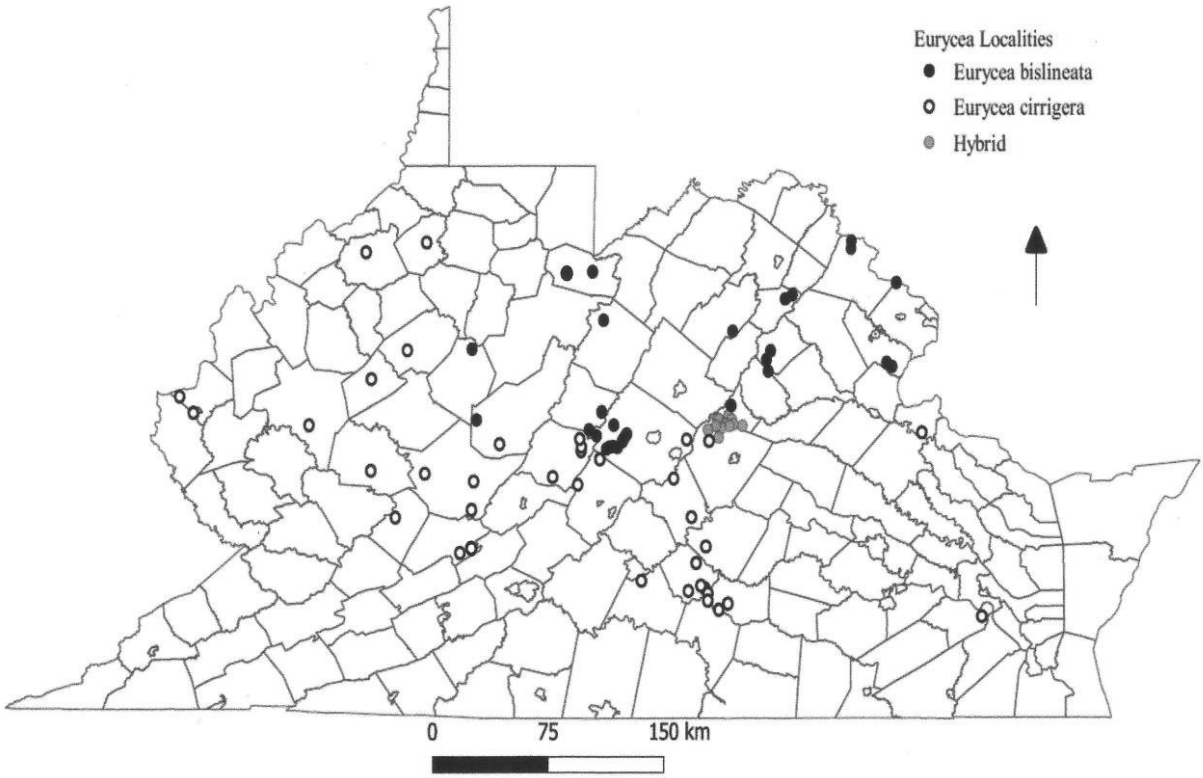

Fig. 1. Distribution of 80 populations of *Eurycea* sampled in Virginia and West Virginia. Black circles represent *E. bislineata*, white circles represent *E. cirrigera*, and gray circles represent a contact zone in Central Virginia including sympatric populations with F_1 hybrids (populations 34-35), as well as populations with F_2 hybrids and/or other backcross progeny (18-21, 38-45). See Appendix 1 for detailed locality information.

Allegheny, Shenandoah, and Blue Ridge mountains down to approximately Interstate Highway 64. *Eurycea cirrigera* occurs within the lower Potomac and James River drainages, and all points south and west within Virginia (Fig. 1).

The identity and status of two-lined salamanders on Virginia's Eastern Shore has long baffled state herpetologists. The Virginia Department of Game and Inland Fisheries (2016) reports "known" and "confirmed" occurrences of *E. cirrigera* from both Accomack and Northampton counties. There are also electrophoretically verified occurrences of *E. cirrigera* from Worcester County, the southernmost county on Maryland's Eastern Shore, southward onto Virginia's Eastern Shore (Gronert, 2018). In fact, Gronert (2018) cites unpublished data from Paul Sattler's lab (collected using the precise methods described in this paper) to verify these localities. Gronert (2018) also comments that the *E. cirrigera* from Worcester County are the northernmost records submitted to the Maryland Amphibian and Reptile Atlas. Given these data, and the fact that Gronert (2018) considers all two-lined salamanders north of Worcester County to be *E. bislineata*, the contact zone between *E. bislineata* and *E. cirrigera* probably occurs on Maryland's Eastern Shore, somewhere between Worcester and Caroline counties. The location of this contact zone is directly east, across the Chesapeake Bay, from the contact zone we report on Virginia's mainland in Stafford County (see Fig. 1), suggesting the current distribution was established sometime before the formation of the Chesapeake Bay. Kozak et al. (2006) suggest that present distributions in the *Eurycea bislineata* complex are more highly correlated with historical river drainage patterns than contemporary ones. Drainage patterns have changed over geological time but the geographic ranges of semiaquatic salamander species, such as *Eurycea,* have likely remained static or nearly so (Kozak et al., 2006). Our distribution data tend to confirm this concept.

Based on analyses of distributional and ecological patterns, several authors have suggested that *E. cirrigera* generally occurs in warmer and/or more southern streams that have a lowland character, whereas *E. bislineata* can

Table 1. Species composition (number of sampled individuals) of *Eurycea* populations in central Virginia contact zone, in an approximate northwest to southeast order.

Population	*E. bislineata*	Backcross to *E. bislineata*	F_1 Hybrid	Backcross to *E. cirrigera*	*E. cirrigera*	F_2 Hybrid
22	29					
21	14	6				
20	9	3				
19	15	7				
18	15	6				
34	10	13	1	3	2	1
35	2	1	1	6	3	
38				1	1	1
39					2	
40						1
41				9	11	1
42				1		
43				3	4	
44					4	
45				4	10	
46					3	
Total	**94**	**36**	**2**	**28**	**40**	**4**
% of Total	**46.1**	**17.6**	**1.0**	**13.7**	**19.6**	**2.0**

generally be found in colder and/or more northern streams (Guttman & Karlin, 1986; Camp et al., 2000; Kozak & Montanucci, 2001). These generalizations appear to hold true, at least in part, for Virginia and West Virginia as well. Some ecological literature suggests that *E. bislineata* is better adapted for survival in cold water streams as compared to *E. cirrigera* (Martof, 1955; Allen, 1963; Brooks & Sassaman, 1965). Additional ecological studies are necessary in areas of sympatry to determine how reproductive isolation is maintained between these two species (Sever, 2005).

The contact zone between *E. bislineata* and *E. cirrigera* is best described as parapatric with areas of local sympatry. The localities in central Virginia, which were examined in greater detail, are included in Table 1 in an approximate northwest-southeast order. Adjacent localities are separated by approximately 2–5 km. *Eurycea bislineata* and *E. cirrigera* maintained distinct gene pools in the contact zone. Only two localities (34 and 35) contained both parental genotypes and F_1 hybrids. As Guttman & Karlin (1986) reported for Ohio, even within sympatry only one species predominated at any locality (Table 1). This strict parapatric distribution severely limits contact and potential for hybridization. Our detailed analysis shows that only 2 of 204 (<1%) salamanders sampled from within the contact zone are F_1 hybrids (Table 1).

Like Guttman & Karlin (1986), our study found that F_1 hybrids are produced at a much lower frequency than subsequent backcross genotypes. In Ohio, 1.5% F_1 hybrids, 7.5% F_2 hybrids, 36% backcross, and 55% parental genotypes were reported in areas of sympatry. In Virginia, the corresponding values were 1%, 2%, 32%, and 66% (Table 1). Although the formation of F_1 hybrids is relatively rare, backcrosses are 25–30 times more frequent. The presence of F_2 hybrids and backcross genotypes indicates that F_1 hybrids may be viable and fertile. The four genotypes defined as F_2 hybrids in Table 1 are homozygous for an *E. bislineata* electromorph at one marker and homozygous *E. cirrigera* for at least one

other marker. These genotypes most likely represent advanced generation backcross genotypes, considering the low number of F_1 hybrids and the small potential for F_1 x F_1 crosses in these populations.

The area of sympatry within which both genotypes are found is no wider than several kilometers and probably much closer to several hundred meters. Guttman & Karlin (1986) reported that the northern end of the contact zone was no more than 15 km wide and that introgression occurred primarily from *E. bislineata* to *E. cirrigera*. In central Virginia, introgression was detected up to 5 km north and 10 km south of the contact zone. There are slightly more (18 vs. 14%) backcross genotypes with *E. bislineata* than *E. cirrigera.* However, *E. bislineata* is also more than twice as abundant (46 vs. 20%) as *E. cirrigera* in these same populations (Table 1). Therefore, while introgression is bidirectional, there is a higher proportion of *E. bislineata* alleles passed to *E. cirrigera* than in the opposite direction.

Our sample sizes are too small to test for Hardy-Weinberg equilibrium at the marker loci in these populations. Guttman & Karlin (1986) found equilibrium at these loci in backcrossed populations where only one parental species was present and at least some loci not at equilibrium when both parental species were present. When both parental genotypes were present, there was assortative mating within genotypes. When only one species was present, the hybrid and backcross genotypes exhibited random mating. Backcross genotypes had the same ability to obtain mates as parental genotypes.

This pattern of limited hybridization, coupled with Genetic Distance estimates of 0.346 (Guttman & Karlin, 1986) and 0.45 (Jacobs, 1987), provide evidence that *E. bislineata* and *E. cirrigera* maintain distinct gene pools. The picture is one of two species coming together with a parapatric distribution and maintaining genetic identity with only occasional hybridization. There is some introgression, but it is restricted to a narrow contact zone. Introgression may be limited by the low vagility of the salamanders, genetic incompatibility with a foreign genome, or natural selection eliminating foreign alleles not adapted to the local environment. Using the criteria of Mayr (1963) for the biological species concept, we agree with Guttman & Karlin (1986) and Jacobs (1987) that two species with separate gene pools are represented. The available evidence, derived from an analysis of the contact zone in both the northern and southern portions of the range, supports Jacobs' (1986) elevation of *E. bislineata* and *E. cirrigera* to full specific status.

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Appendix 1. Localities, sample sizes (N), and allelic frequencies (a-e) of three diagnostic proteins for 80 populations (Pop.) of the *Eurycea bislineata* complex from Virginia and West Virginia.

Pop.	N	Locality	6-PGDH				GOT-1				PEP				
			a	b	c	d	a	b	c	d	a	b	c	d	e
1	2	Gentle Falls Trail, Blackwater Falls State Park, near Davis, Tucker Co., WV (39° 06’ 43” N, 79° 29’ 44” W)			1.0				1.0				1.0		
2	1	Watershed 13, Fernow Experimental Forest, near Parsons, Tucker Co., WV (39° 05’ 27” N, 79° 41’ 11” W)			1.0				1.0				1.0		
3	3	South of Onego, US Rt. 33, Mexico Hollow below Lower Timber Ridge Rd., Pendleton Co., WV (38° 50’ 04” N, 79° 25’ 01” W)			1.0				1.0				1.0		
4	1	Laurel Fork River at Rt. 10, Holly River State Park, Webster Co., WV (38° 39’ 58” N, 80° 21’ 17” W)			1.0				1.0				1.0		
5	1	Cranberry Glades Backcountry, near North Fork of Cranberry River, Pocahontas Co., WV (38° 15’ 35” N, 80° 19’ 19” W)			1.0				1.0						
6	9	Unnamed tributary of Shaws Fork of Cowpasture River at Co. Rt. 616, 2.5 km S US Rt. 250, at FS 395.2, Highland Co., VA (38° 18’ 22” N, 79° 25’ 48” W)			1.0				1.0				1.0		
7	1	Unnamed tributary to Cowpasture River at FS 394, 13 km E Co. Rt. 627, Bath Co., VA (38° 12’ 09” N, 79° 31’ 13” W)			1.0								1.0		
8	13	Jerkemtight Creek at Hamilton Branch of Calfpasture River on FS 399, 1.1 km W Co. Rt. 629, Augusta Co., VA (38° 10’ 08” N, 79° 27’ 58” W)		.04	.96				.79	.21		.19	.69	.12	
9	10	West Dry Branch of Calfpasture River at Co. Rt. 688, 7.4 km E Co. Rt. 629, Augusta Co., VA (38° 13’ 49” N, 79° 20’ 41” W)		.17	.83				.89	.11			1.0		
10	2	Buffalo Branch, 2.25 km S Buffalo Gap on VA Rt. 42, Augusta Co., VA (38° 10’ 44” N, 79° 15’ 11” W)			1.0				.5	.5			1.0		
11	16	Buffalo Branch, 4.3 km S Buffalo Gap on VA Rt. 42, Augusta, Co., VA (38° 09’ 53” N, 79° 15’ 51” W)		.03	.97				.63	.37		.13	.87		
12	6	Little Calfpasture River, 8 km S Buffalo Gap on VA Rt. 42, Augusta Co., VA (38° 08’ 51” N, 79° 16’ 42” W)			1.0				.6	.4			1.0		

Appendix 1 (continued).

Pop.	N	Locality	6-PGDH				GOT-1				PEP				
			a	b	c	d	a	b	c	d	a	b	c	d	e
13	14	Little Calfpasture River, 10.5 km S Buffalo Gap on VA Rt. 42, Augusta Co., VA (38° 08' 03" N, 79° 17' 01" W)			.95	.05			.63	.37			.92	.08	
14	13	Smith Creek at Co. Rt. 601, 0.75 km S VA Rt. 42 at Augusta Springs, Augusta Co., VA (38° 06' 05" N, 79° 19' 05" W)			1.0				.69	.31			1.0		
15	2	Tributary of Little Calfpasture River at FS 382, 8.6 km N Co. Rt. 687, Augusta Co., VA (38° 05' 34" N, 79° 23' 44" W)			1.0				1.0				.5	.5	
16	14	Tributary of Little Calfpasture River at FS 382, 12.3 km N Co. Rt. 687, Augusta Co., VA (38° 06' 13" N, 79° 22' 25" W)			1.0				1.0				1.0		
17	2	Tributary of Little Calfpasture River at FS 382, 17.3 km N Co. Rt. 687, Augusta Co., VA (38° 06' 24" N, 79° 20' 50" W)			1.0				.5	.5			1.0		
18	22	Lynch River at Co. Rt. 628, 1 km E Co. Rt. 614, Greene Co., VA (38° 16' 01" N, 78° 36' 25" W)			1.0				.5	.5			1.0		
19	22	Lynch River W of Co. Rt. 614 and E of Co. Rt. 628, Greene Co., VA (38° 15' 42" N, 78° 35' 41" W)		.13	.85	.02		.02	.98			.02	.93	.05	
20	12	Roach River E of Co. Rt. 810, Greene Co., VA (38° 15' 18" N, 78° 32' 20" W)		.13	.83	.04			.83	.17		.06	.94		
21	20	Haneytown Creek, N of Co. Rt. 631, 0.8 km W Co. Rt. 630, Greene Co., VA (38° 17' 11" N, 78° 31' 40" W)		.11	.84	.05							.83	.17	
22	29	Swift Run along US Rt. 33, 0.6 km N Co. Rt. 625, Greene Co., VA (38° 20' 29" N, 78° 30' 33" W)			.98	.02			1.0				1.0		
23	2	Hughes River at Co. Rt. 231, Madison Co., VA (38° 32' 28" N, 78° 14' 38" W)			1.0				.5	.5			1.0		
24	5	Hazel River at Co. Rt. 600, 3.4 km S Co. Rt. 608, Rappahannock Co., VA (38° 36' 21" N, 78° 15' 23" W)			1.0				1.0				1.0		
25	1	Thornton River at Co. Rt. 522, Sperryville, Rappahannock Co., VA (38° 39' 28" N, 78° 13' 35" W)			1.0				1.0				1.0		
26	10	Tributary of Passage Creek at Co. Rt. 678, 0.2 km S Co. Rt. 675, Shenandoah Co., VA (38° 46' 09" N, 78° 29' 56" W)			1.0			.08	.84	.08			1.0		
27	16	Manassas Run at S shore of Shenandoah River at Co. Rt. 624, Warren Co., VA (38° 57' 24" N, 78° 07' 24" W)	.03	.28	.69		.09	.16	.75				.81	.16	.03
28	10	Dry Run at Co. Rt. 638, 0.2 km E Co. Rt. 603, Warren Co., VA (38° 58' 41" N, 78° 04' 26" W)			1.0				1.0				1.0		
29	1	Dutchman Creek at Co. Rt. 674, Loudoun Co., VA (39° 17' 17" N, 77° 39' 13" W)			1.0				1.0				1.0		
30	1	Milltown Creek just off Co. Rt. 691, 0.2 km W Co. Rt. 287, Loudoun Co., VA (39° 14' 44" N, 77° 39' 22" W)			1.0				1.0				1.0		
31	2	Tributary of Potomac River at Co. Rt. 602, 2.1 km N Co. Rt. 603, Fairfax Co., VA (39° 02' 57" N, 77° 19' 52" W)			1.0				1.0						
32	1	Tributary of Quantico Creek, Prince William Forest Park, Prince William Co., VA (38° 35' 20.9" N, 77° 23' 58.4" W)			1.0				1.0				1.0		
33	2	Mary Bird Branch, Prince William Forest Park, Prince William Co., VA (38° 34' 04" N, 77° 21' 39" W)			1.0				.5	.5			1.0		
34	30	Lynch River E of Co. Rt. 810, 0.4 km N Co. Rt. 633, Albemarle Co., VA (38° 14' 11" N, 78° 32' 33" W)		.26	.72	.02		.28	.70	.02		.26	.74		
35	13	Roach River at Co. Rt. 603 and 604, Greene Co., VA (38° 15' 02" N, 78° 30' 32" W)		.54	.33	.13		.69	.31			.83	.17		
36	1	A seep into Ginkoteague Creek at Port Conway, King George Co., VA (38° 11' 18" N, 77° 08' 58" W)		1.0				1.0							
37	3	Chippokes Plantation State Park, Surry Co., VA (37° 07' 57" N, 76° 43' 12" W)		1.0				1.0							
38	3	Parker Branch of Roach River at Co. Rt. 633, Greene Co., VA (38° 15' 41" N, 78° 30' 16" W)		.5	.5			1.0				.67	.33		
39	2	Welsh Run N of Co. Rt. 617, Greene Co., VA (38° 13' 35" N, 78° 25' 40" W)		1.0				1.0				1.0			
40	1	Roach River at Co. Rt. 648, Greene Co., VA (38° 14' 05" N, 78° 29' 36" W)		1.0				.5	.5			1.0			
41	21	Lynch River E of Co. Rt. 603, Albemarle Co., VA (38° 13' 29" N, 78° 31' 15" W)		.81	.09			.93	.07		.12	.71	.17		
42	1	Buck Mountain Creek at Co. Rt. 601, 0.5 km N Co. Rt. 671, Albemarle Co., VA (38° 12' 46" N, 78° 34' 49" W)		1.0				1.0				.5	.5		

Appendix 1 (continued).

Pop.	N	Locality	6-PGDH				GOT-1				PEP				
			a	b	c	d	a	b	c	d	a	b	c	d	e
43	7	Muddy Run on Co. Rt. 687, 1 km E Co. Rt. 810, Albemarle Co., VA (38° 13' 31" N, 78° 36' 59" W)		.86	.14			1.0			.14	.79	.07		
44	4	Doyles River at intersection of Co. Rts. 629 and 810, Albemarle Co., VA (38° 12' 11" N, 78° 40' 18" W)		1.0				1.0			.25	.75			
45	14	North Fork of Rocky Creek at Co. Rt. 671, 0.8 km E Co. Rt. 609, Albemarle Co., VA (38° 09' 33" N, 78° 35' 48" W)		1.0				1.0				.86	.11	.03	
46	3	Doyles River at intersection of Co. Rts. 674 and 810, Albemarle Co., VA (38° 08' 18" N, 78° 39' 51" W)		1.0				1.0			.17	.83			
47	5	Madison Run at Co. Rt. 663, Augusta Co., VA (38° 08' 43.9" N, 78° 49' 21.3" W)		1.0				1.0				1.0			
48	15	Stony Creek 0.8 km from end of Co. Rt. 751, Nelson Co., VA (37° 55' 22" N, 78° 54' 58" W)		1.0				1.0				.93	.07		
49	4	Tributary of Buffalo Creek at Co. Rt. 722, 0.8 km S VA Rt. 56, Nelson Co., VA (37° 42' 04" N, 78° 47' 16" W)		1.0				1.0				1.0			
50	7	Austin Creek at US Rt. 60, Buckingham Co., VA (37° 31' 52" N, 78° 41' 12" W)		1.0				1.0			.21			.43	.36
51	3	Right Hand Fork of Cub Creek at Co. Rt. 644, Appomattox Co., VA (37° 16' 30" N, 78° 48' 34" W)	.33	.67				1.0				.83		.17	
52	5	Bowler Creek at Co. Rt. 628, Appomattox Co., VA (37° 17' 27" N, 78° 41' 39" W)		1.0				1.0				.33		.67	
53	7	Suanee Creek at Co. Rt. 621, Appomattox Co., VA (37° 18' 23" N, 78° 43' 15" W)	.14	.86				1.0				.14		.79	.07
54	15	North Fork of David Creek at Co. Rt. 654, 1.9 km N US Rt. 24, Appomattox Co., VA (37° 26' 15" N, 78° 45' 18" W)		1.0				1.0				.2	.03	.43	.33
55	3	Tributary of Vaughns Creek, 1.5 km S US Rt. 460, Prince Edward Co., VA (37° 15' 44" N, 78° 40' 20" W)	.33	.67				1.0				.66	.17		.17
56	6	Brown's Branch of Buffalo Creek at Co. Rt. 664, 1.4 km S Co. Rt. 669, Prince Edward Co., VA (37° 10' 04" N, 78° 35' 43" W)	.08	.92				1.0				.25		.42	.33
57	3	Tributary of Buffalo Creek at Co. Rt. 686, 0.5 km E Co. Rt. 666, Prince Edward Co., VA (37° 12' 10" N, 78° 31' 53" W)		1.0				1.0						1.0	
58	3	South Fork of Spring Creek at Co. Rt. 757, 0.6 km S Co. Rt. 663, Prince Edward Co., VA (37° 13' 13" N, 78° 40' 21" W)		1.0				1.0						.17	.83
59	3	Opossum Creek at Co. Rt. 677, Campbell Co., VA (37° 20' 06" N, 79° 08' 57" W)		1.0				1.0				.5			.5
60	1	150 m E Co. Rt. 629, 2.25 km N Douthat State Park, Alleghany Co., VA (37° 55' 52" N, 79° 46' 48" W)		1.0				1.0						1.0	
61	6	Bratton Run at intersection of Co. Rts. 780 and 850, Rockbridge Co., VA (37° 53' 13" N, 79° 36' 06" W)		1.0				1.0						1.0	
62	3	Goshen Branch of Calfpasture River at VA Rt. 42, 8.25 km N Co. Rt. 780, 2.5 km S Co. Rt. 687, Rockbridge Co., VA (38° 02' 02" N, 79° 26' 33" W)		1.0				1.0							
63	16	Stuart Run at Co. Rt. 629, 0.2 km N Co. Rt. 633, Bath Co., VA (38° 04' 49" N, 79° 34' 38" W)		1.0				1.0						.67	.33
64	8	White Sulphur Spring Branch of Cowpasture River at Co. Rt. 672, 4.3 km S FS 394, Bath Co., VA (38° 06' 14" N, 79° 34' 31" W)		1.0				1.0						.67	.33
65	2	Unnamed tributary of Cowpasture River at FS 394, 0.2 km E Co. Rt. 627, Bath Co., VA (38° 08' 59" N, 79° 35' 15" W)		1.0				1.0						1.0	
66	4	Tributary of Greenbrier River, Brooks Memorial Arboretum, 3.4 km W Seebert entrance to Watoga State Park, Pocahontas Co., WV (38° 07' 11" N, 80° 09' 25" W)		1.0				1.0						.5	.5
67	5	Tributary of Greenbrier River, 4.2 km E intersection of US Rt. 219 and Anthony Rd, near Anthony, Greenbrier Co., WV (37° 54' 22" N, 80° 20' 32" W)		1.0				1.0						.7	.3
68	3	Tributary of Greenbrier River at Old Roads Trail, 1.4 km S Caldwell entrance to Greenbrier State Forest, Greenbrier Co., WV (37° 44' 34" N, 80° 21' 29" W)		1.0				1.0						1.0	
69	5	Potts Creek, 5.1 km E Co. Rt. 613 on WV Rt. 17, Monroe Co., WV (37° 27' 31" N, 80° 27' 58" W)		1.0				1.0						1.0	

Appendix 1 (continued).

Pop	N	Locality	6-PGDH				GOT-1				PEP				
			a	b	c	d	a	b	c	d	a	b	c	d	e
70	5	7.2 km S intersection of Co. Rt. 29 and 15, between Zenith and Waiteville, Monroe Co., WV (37° 29' 41" N, 80° 26' 24" W)		1.0				1.0						.5	.5
71	4	Trump Lilley Farm, 2.5 km NNW Hinton, Raleigh Co., WV (37° 41' 55" N, 80° 54' 04" W)		.87		.13	.13	.87			.25			.75	
72	1	0.8 km SW intersection of US Rt. 60 and Tommy Hall Rd, S of Rupert, Greenbrier Co., WV (37° 57' 02" N, 80° 41' 23" W)		1.0			.5	.5						1.0	
73	1	Rush Run at New River, near Thurmond, Fayette Co., WV (37° 58' 03" N, 81° 04' 35" W)		1.0				1.0			.5			.5	
74	2	Tributary of Kanawha River, Witcher Creek Rd., Belle, Kanawha Co., WV (38° 13' 46" N, 81° 30' 53" W)		1.0			.25	.75						.5	.5
75	5	Tributary of Beech Fork, 0.9 km SE intersection of Long Branch and Bowen Branch Rds., near Bowen, Wayne Co., WV (38° 18' 02" N, 82° 20' 21" W)	.1	.9			.5	.5						.2	.8
76	6	Tributary of Hisey Fork, 1.2 km SE intersection of US Rt. 52 and Miller Rd., Huntington Co., WV (38° 23' 33" N, 82° 26' 15" W)		1.0			.5	.5						.58	.42
77	1	Tributary of Elk River, 11.9 km SW intersection of WV Rts. 4 and 16 at Ivydale, Clay Co., WV (38° 29' 37" N, 81° 04' 23" W)		1.0			.5	.5						1.0	
78	2	Tributary of Elk River, 4.7 km SW intersection of WV Rt. 4 and Elk River at Gassaway, Braxton Co., WV (38° 39' 33" N, 80° 48' 51" W)		1.0			.5	.5						1.0	
79	2	Rail Trail State Park at intersection of Long Run Rd. and US Rt. 50, 6.5 km W Salem, Doddridge Co., WV (39° 16' 48" N, 80° 40' 39" W)		1.0			.25	.75.						.5	.5
80	2	Tributary of North Hughes River, Jughandle Campground, North Bend State Park, Ritchie Co., WV (39° 13' 23" N, 81° 06' 38" W)		1.0			.5	.5						1.0	