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Conservation Concerns for the Candy Darter (*Etheostoma osburni*) with Implications Related to Hybridization

Isaac Gibson

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**Conservation concerns for the Candy Darter (*Etheostoma osburni*) with
implications related to hybridization**

Isaac Gibson

**Thesis submitted to the
Davis College of Agriculture, Natural Resources, and Design
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Masters of Science in
Wildlife and Fisheries Resources**

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Division of Forestry and Natural Resources

**Morgantown, WV
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Greenbrier, Distinct Population Segment, DPS**

ABSTRACT

Conservation concerns for the Candy Darter (*Etheostoma osburni*) with implications related to hybridization

Isaac Gibson

Candy Darters *Etheostoma osburni* and Variegate Darters *E. variatum* are both native to streams of West Virginia and Virginia. The geographic ranges of these two species were historically separated by Kanawha Falls, a natural barrier to fish dispersal located at Glen Ferris, WV. Habitat degradation may have reduced the geographic range of Candy Darters, a species of concern in West Virginia and Virginia as well as a federal species of concern. Currently, the species is undergoing a review for listing under the U.S. Endangered Species Act. In the early 1980s, Variegate Darters or putative hybrids were first collected at locations upstream of Kanawha Falls, and have since undergone range expansion. Genetic and morphologic data were examined for individuals from the New, Gauley, and Greenbrier river drainages. Individuals were genotyped using a suite of 5 microsatellite loci to investigate potential hybridization. There was strong evidence for the existence of Distinct Population Segments (DPS) of the Candy Darter. Widespread hybridization, however, was found throughout populations of the Candy Darter. A geographic hybrid zone was estimated with the highest levels of introgression representing the kernel of the hybrid swarm and the locations of F1 hybrids delineating the periphery. The F1 hybrid was meristically evaluated as intermediate within and across characters for parental species. Introgressive hybridization threatens the genetic integrity of the Candy Darter, and may lead to population extirpation and/or extinction.

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TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF APPENDICES	vii
<i>Chapter 1: Introduction and Literature Review .....</i>	<i>1</i>
Candy Darter (<i>Etheostoma osburni</i>)	1
Variegate Darter (<i>Etheostoma variatum</i>).....	2
History of <i>E. variatum</i> in New River Drainage	2
Hybridization (<i>E. osburni</i> X <i>E. variatum</i>)	3
Conservation Issues	3
<i>Chapter 2: Hybridization between <i>Etheostoma osburni</i> and <i>Etheostoma variatum</i>.....</i>	<i>6</i>
Introduction.....	6
Materials and Methods.....	8
Results.....	13
Discussion	16
Conclusions.....	19
Management Implications.....	19
<i>Chapter 3: Comparison of <i>Etheostoma osburni</i> between the Gauley and Greenbrier river drainages .....</i>	<i>21</i>
Introduction.....	21
Materials and Methods.....	22
Results.....	28
Discussion	31
Conclusions.....	34
Management Implications.....	34
LITERATURE CITED	35
TABLES	40
FIGURES	54
APPENDICES	67

LIST OF TABLES

Table 1. List of collection sites enumerated to correspond with map of study area. Total number of specimens, number of pure <i>E. osburni</i> , and percentage of <i>E. variatum</i> alleles of that sample is given. All coordinates are in Zone 17.....	40
Table 2. List of sites visited for this study that produced no specimens of either <i>E. osburni</i> or of hybrids. All coordinates are in Zone 17.....	41
Table 3. Meristic characters evaluated for F1 hybrids and for <i>E. osburni</i> (data for Gauley and Greenbrier populations of <i>E. osburni</i> is combined). Data for <i>E. variatum</i> is from Page (1983) and Page and Burr (2011).	42
Table 4. Sites represented in each sample used in STRUCTURE analysis, construction of a neighbor-joining tree, and for pairwise comparison of F_{ST}	43
Table 5. Morphometric distances used in shape analysis of <i>E. osburni</i> . Variable enumeration corresponds to Figures 15 and 16. Box truss distance measurements, head length, outer-pelvic width, and caudal length (1 - 23, 26, 31) are presented as thousandths of standard length. Measurements taken on the head (24, 25, 27 - 30) are presented as thousandths of head length. .	44
Table 6. Meristic characters evaluated for Gauley and Greenbrier populations of <i>E. osburni</i> . Data for <i>E. osburni</i> from Page (1983) and Page and Burr (2011) is given.	46
Table 7. Results of HWE calculation for each population of <i>E. osburni</i> per locus and across all loci using Fisher's Method (Fisher 1935). Weir and Cockerham (1984) estimate of F_{IS} is given.	47
Table 8. Results of HWE calculation for each sample of <i>E. osburni</i> (as described in Table 4) per locus and across all loci using Fisher's Method (Fisher 1935). Weir and Cockerham (1984) estimate of F_{IS} is given.	48
Table 9. Pairwise comparison of F_{ST} among samples (as described in Table 4; above the diagonal) with P-values obtained after 900 permutations (below the diagonal). Indicative adjusted nominal level (5%) for multiple comparisons is 0.001. Significant values are listed in bold font.	50
Table 10. Allelic frequency for the Gauley and Greenbrier populations of <i>E. osburni</i> ($n_{Gauley} = 67$; $n_{Greenbrier} = 144$; $n_{Total} = 211$). The status as a private allele is indicated.	51
Table 11. Summary of allelic variation of Gauley and Greenbrier populations of <i>E. osburni</i> including number of alleles, allelic size range, allelic richness, observed heterozygosity (H_o), and expected heterozygosity (H_e).	52
Table 12. Summary of 20 repeated measurements on a single individual for error quantification of morphometric distances. All values are presented in millimeters.	53

LIST OF FIGURES

Figure 1. Distribution records of <i>E. osburni</i> in West Virginia from 1931 - 2015 (WVDNR unpublished data).	54
Figure 2. Distribution records of <i>E. variatum</i> morphometrically identified in the New River drainage, West Virginia from 1982 – 2014 (WVDNR unpublished data). These data do not include genetic detection of <i>E. variatum</i> alleles of the current study.	55
Figure 3. Collection sites for materials used in this study. Site labels correspond with Table 1.	56
Figure 4. Photograph depicting typical cephalic squamation of <i>Etheostoma variatum</i> . One scale patch is shown on the opercle. Two scale patches are shown in the supraopercular region. <i>Etheostoma osburni</i> X <i>Etheostoma variatum</i> may possess any or none of these scale patches.	57
Figure 5. STRUCTURE diagram depicting posterior probability of assignment (vertical axis) for all individuals collected at K = 2. Samples 1 – 10 are comprised of pure <i>E. osburni</i> . Horizontal axis is sample location number.	58
Figure 6. STRUCTURE diagram depicting posterior probability of assignment (vertical axis) for <i>E. osburni</i> at K = 2. Samples 3 – 8 are from the Gauley River drainage. Samples 1, 2, 9, and 10 are from the Greenbrier River drainage. Horizontal axis is sample location number.....	58
Figure 7. Posterior probability of assignment (vertical axis) to six classifications: <i>E. osburni</i> , <i>E. variatum</i> , F1 hybrid, F2 hybrid, backcross with <i>E. osburni</i> , and backcross with <i>E. variatum</i> . Site numbers (horizontal axis) correspond to Table 1. Elevation in each drainage increases with distance from the dashed line. The relative location of Summersville Dam is indicated.	59
Figure 8. <i>Etheostoma variatum</i> alleles detected during this study (open diamonds). Solid circles indicate the presence of pure <i>E. osburni</i> in a sample. Open circles indicate the collection location of first generation (F1) hybrids of <i>E. osburni</i> X <i>E. variatum</i> . The proportion of <i>E. variatum</i> alleles within each sample where <i>E. variatum</i> alleles were detected is labeled.....	60
Figure 9. <i>Etheostoma osburni</i> X <i>Etheostoma variatum</i> . 71 mm SL male, Deer Creek (Site 19), Pocahontas Co., WV, 7 March 2017. Photograph by Dr. Stuart A. Welsh.....	61
Figure 10. <i>Etheostoma osburni</i> X <i>Etheostoma variatum</i> . 68 mm SL female, Deer Creek (Site 19), Pocahontas Co., WV, 7 March 2017. This individual was gravid at the time of collection. Photograph by Dr. Stuart A. Welsh.	61
Figure 11. <i>Etheostoma osburni</i> . 78 mm SL male, East Fork Greenbrier River, Pocahontas Co., WV, 4 April 2016. Photograph by Dr. Stuart A. Welsh.....	62
Figure 12. <i>Etheostoma osburni</i> . 65 mm SL female, Little River East Fork Greenbrier River, Pocahontas Co., WV, 28 March 2017. Photograph by Dr. Stuart A. Welsh.....	62

Figure 13. <i>Etheostoma variatum</i> . 66 mm SL male, Twentymile Creek, Nicholas Co., WV, 19 April 2017. Photograph by Dr. Stuart A. Welsh.	63
Figure 14. <i>Etheostoma variatum</i> . 57 mm SL female, Twentymile Creek, Nicholas Co., WV, 19 April 2017. Photograph by Dr. Stuart A. Welsh.	63
Figure 15. Box truss network comprised of 23 distances for morphometric analysis of <i>E. osburni</i> and <i>E. osburni</i> X <i>E. variatum</i> . Numeric labels correspond to Table 5.	64
Figure 16. Supplemental distances for morphometric analysis of <i>E. osburni</i> and <i>E. osburni</i> X <i>E. variatum</i> . Labels correspond to Table 5.....	64
Figure 17. Neighbor-joining tree based on 1000 generated trees. Values presented are percents of 1000 generated trees that group the samples together. The drainage to which each sample belongs is labeled in parentheses. Members of each sample are described in detail in Table 4.	65
Figure 18. Plots of the sheared second and third principal components (SPC2 and SPC3) that depict shape differences between the Gauley (triangles) and Greenbrier (circles) populations of <i>E. osburni</i>	66

LIST OF APPENDICES

Appendix 1. Posterior probability of assignment to six species/hybrid classifications. Site numbers that are listed correspond to Table 1. Each row represents an individual (n = 335).	67

Chapter 1: Introduction and Literature Review

The closely related Candy Darter (*Etheostoma osburni*; Hubbs and Trautman 1932) and Variegate Darter (*E. variatum*; Kirtland 1840) are native to West Virginia and endemic to the Ohio River drainage of North America. The geographic ranges of these two species were historically separated by Kanawha Falls, a natural barrier to fish dispersal located at Glen Ferris, WV (Hocutt and Wiley 1986; Stauffer 1995; Page and Burr 2011). This biogeographic barrier, which blocked upstream dispersal of *E. variatum*, is thought to have allowed the two populations of darters to form the extant populations of *E. osburni* and *E. variatum* through allopatric speciation.

Etheostoma variatum was introduced upstream of Kanawha Falls which created an additional conservation concern for *E. osburni* (Cincotta et al. 1999; Wellman 2004; Burns 2007). Most likely, *E. variatum* was introduced from one or many anglers' bait buckets. Bait bucket introduction is a common progenitor of invasive fish and other aquatic organisms (Litvak and Mandrak 1993; Ludwig and Leitch 1996; Lintermans 2004; Rahel 2007; Loughman and Welsh 2010). Hybridization of these two species has been documented (Switzer et al. 2004; Switzer et al. 2007). This hybridization and subsequent introgression may lead to the extirpation and/or extinction of *E. osburni* (Switzer et al. 2007).

Candy Darter (*Etheostoma osburni*)

Etheostoma osburni is endemic to the upper Kanawha River system upstream of Kanawha Falls. Its range includes the Gauley and Greenbrier rivers of West Virginia and the New River within West Virginia and Virginia (Jenkins and Burkhead 1994; Stauffer et al. 1995). Within West Virginia, historic distribution records have documented this species throughout the Gauley, Greenbrier, and Bluestone rivers, and Indian Creek of the New River drainage (Figure 1). *Etheostoma osburni* is a brilliantly colored, benthic fish of the family Percidae. It is found in small streams and midsize rivers. This darter primarily occupies the loose rubble and boulder substrate of silt free riffle habitat (Chipps et al. 1994; Dunn and Angermeier 2016). *Etheostoma osburni* feeds opportunistically on benthic macroinvertebrates. Mayflies (Ephemeroptera) comprise the majority of their diet (Schoolcraft et al. 2007). Schoolcraft and

Tarter (2002) estimated April 21 as the approximate peak spawning time of *E. osburni* in the South Fork of the Cherry River. Jenkins and Burkhead (1994) documented a significant reduction in the range of *E. osburni* in Virginia in recent history. Due to limited distribution and population reduction, *E. osburni* is listed as a species of concern in West Virginia, a species of special concern in Virginia, and a federal species of concern (USFWS 1989; Jelks et al. 2008).

Variegate Darter (*Etheostoma variatum*)

Etheostoma variatum is closely related to *E. osburni*. They both belong to the subgenus *Etheostoma* and are members of the *variatum* complex (Hubbs and Black 1940). In addition to *E. osburni* and *E. variatum*, the *variatum* complex also includes: *Etheostoma euzonum* (Hubbs and Black 1940), *E. erythrozonum* (Switzer and Wood 2009), *E. kanawhae* (Raney 1941), and *E. tetrazonum* (Hubbs and Black 1940). *Etheostoma variatum* commonly occurs over a relatively large area of the Ohio River drainage including the Kanawha River drainage below Kanawha Falls (Stauffer et al. 1995) and excluding the Wabash and Tennessee River systems (Hubbs and Black 1940); within this range it occurs in Indiana, Kentucky, New York, Ohio, Pennsylvania, and West Virginia. Like *E. osburni*, the habitat of this darter is primarily comprised of the rubble and boulder substrate of riffle habitat in small streams and midsize rivers. *Etheostoma variatum* subsists on a diet of benthic macroinvertebrates. May (1969) estimated the approximate spawning activity of *E. variatum* to occur during the period of April 15 to May 10.

History of *E. variatum* in New River Drainage

In 1982, a specimen of *E. variatum* was collected from New River at Sandstone Falls (D. Cincotta, pers. comm.). This was the earliest known record of *E. variatum* collected upstream of Kanawha Falls (i. e. in the New River drainage; Figure 2). In the 1990s, specimens of *E. variatum* were collected from Anthony Creek, a tributary to the Greenbrier River (D. Cincotta, pers. comm.). At this time, both species were sympatric at this location in Anthony Creek. Wellman et al. (2004) collected *E. variatum* while surveying the ichthyofauna of the New River. Switzer (2004) found evidence that *E. osburni* and *E. variatum* were potentially hybridizing in the New River drainage. Switzer et al. (2007) found strong evidence of hybridization between these two species and found no first generation (F1)

hybrids or *E. osburni* at Anthony Creek implying that *E. osburni* had been swamped out, or replaced, by hybridization with *E. variatum* at this site (Avisé 2004). This study indicated Anthony Creek as the upstream-most extent of hybridization in the Greenbrier River drainage; no evidence of hybridization upstream of Anthony Creek was found. Switzer et al. (2007) also found no evidence of hybridization in the Gauley River drainage upstream of Summersville Dam.

Hybridization (*E. osburni* X *E. variatum*)

Etheostoma variatum is known to hybridize with *E. osburni* (Switzer et al. 2007). Hybridization, in this context, occurs when two different species mate and produce offspring that possess genes from both parental species. *Etheostoma variatum* or hybrid (*E. variatum* X *E. osburni*) individuals have been identified at locations within the range of *E. osburni* (Cincotta et al. 1999; Wellman 2004). Switzer et al. (2007) found genetic evidence of introgression of *E. variatum* within populations of *E. osburni* in the New River drainage. Introgression is the incorporation of genes from one population into another population through progressive hybridization and backcrossing (Harrison and Larson 2014). Introgression is possible when the product of interspecific hybridization is viable offspring. The viable offspring are able to mate with other hybrids or parental species which allows the movement of genes between populations. Introgressive hybridization seems to be replacing populations of *E. osburni* with populations of *E. variatum* hybrids (Rhymer and Simberloff 1996; Matthews et al. 2016). This progressive genetic swamping is a primary threat to all *E. osburni* populations.

Conservation Issues

Etheostoma osburni has a limited national distribution and its range is shrinking (Chipps et al. 1993; Jenkins and Burkhead 1994). Due to its limited range, the species has been designated as a Species of Concern in West Virginia, a Species of Special Concern in Virginia, and a Federal Species of Concern. *Etheostoma osburni* is a candidate for listing under the U.S. Endangered Species Act (Douglas, pers. comm.). Portions of the New, Gauley, and Greenbrier river drainages have been invaded by *E. variatum*. A successful conservation strategy for the imperiled *E. osburni* requires a thorough understanding of the dynamics of hybridization between these two species.

Hybridization with introgression is one mechanism that can cause the extinction of native species (Rhymer and Simberloff 1996). Introgressive hybridization between *E. osburni* and *E. variatum* is occurring throughout the Gauley (below Summersville Dam), Greenbrier, and New (below Bluestone Dam) river drainages in West Virginia. All of the populations of *E. osburni* within this distribution have become or are threatened with imminent genetic extinction. The populations of *E. osburni* in these drainages that have not already been lost will most likely disappear due to introgression with *E. variatum*.

Hybrids of Candy and Variegate darters have not been thoroughly examined. It is important to be able to identify individuals of hybrid origin based on visual inspection, providing a valuable marker in determining the zone of hybridization and detecting this occurrence in other regions. Due to the varying degrees of hybridization (first generation, second generation, backcrosses, etc.) affecting the identification of these two closely related species, relying on morphologic identification alone can be problematic. It was necessary to, first, utilize genetic techniques to thoroughly assess populations of the subject fishes and identify individuals with hybrid ancestry for morphological analysis and to identify pure *E. osburni* for evaluation. The current geographic extent of hybridization needed to be surveyed. A genetic assay of hybridization was performed by Switzer et al. (2007), which identified Anthony Creek as the upstream extent of introgression with *E. variatum* in the Greenbrier River drainage. Individuals collected upstream of Anthony Creek did not exhibit evidence of hybridization in 2004. Switzer et al. (2007) further indicated the importance of monitoring above Anthony Creek for upstream evidence of introgression. Increasing sample sizes rather than increasing the number of genetic markers increases the probability of detecting non-native alleles (Della Croce et al. 2017). It is important to identify where unaffected populations of *E. osburni* occur so that conservation efforts may focus on those areas. Chapter 2 of this thesis reports on the current extent of hybridization between these two species and discusses meristic characters of the F1 hybrid. Switzer et al. (2007) found evidence of genetic divergence between the Gauley and Greenbrier river drainage populations of *E. osburni*. Due to genetic differences between populations, a loss of one population will result in the loss of genetic diversity for the species as a whole. Chapter 3 emphasizes the genetic and morphometric differences between the populations of *E. osburni* in

West Virginia. This study focuses on the populations of *E. osburni* in West Virginia. It should be noted that *E. variatum* has yet to be reported in the New River drainage of Virginia where other investigations of *E. osburni* have been conducted by Leftwich et al. (1994); Angermeier (1995); Leftwich et al. (1996); Angermeier and Pinder (2015); and Dunn and Angermeier (2016).

Chapter 2: Hybridization between *Etheostoma osburni* and *Etheostoma variatum*

Introduction

In recent studies, *E. variatum* was found to have been introduced within the range of *E. osburni* (Cincotta et al. 1999; Wellman 2004; Burns 2007). The most likely method of introduction is the release of *E. variatum* individuals from one or many anglers' bait buckets. Bait bucket introduction is a common progenitor of invasive fish and other aquatic organisms (Litvak and Mandrak 1993; Ludwig and Leitch 1996; Lintermans 2004; Rahel 2007; Loughman and Welsh 2010). Hybridization of these two species in the New River drainage has been documented (Switzer et al. 2007). This hybridization and subsequent introgression may lead to population extirpation and/or extinction of *E. osburni* (Switzer et al. 2007).

In 1982, a specimen of *E. variatum* was collected from New River at Sandstone Falls (D. Cincotta, pers. comm.). This was the earliest known record of *E. variatum* collected upstream of Kanawha Falls (i. e. in the New River drainage). In the 1990s, specimens of *E. variatum* were collected from Anthony Creek, a tributary to the Greenbrier River (D. Cincotta, pers. comm.). At this time, both species were sympatric at this location in Anthony Creek. *E. variatum* individuals continued to be collected from New River in 1997 (Cincotta et al. 1999). Wellman et al. (2004) collected *E. variatum* while surveying the ichthyofauna of the New River.

Switzer (2004) found evidence that *E. osburni* and *E. variatum* were potentially hybridizing in the New River drainage. Subsequently, Switzer et al. (2007) found strong evidence of hybridization between these two species. That study found no first generation (F1) hybrids or *E. osburni* at Anthony Creek implying that *E. osburni* had been swamped out, or replaced, by hybridization with *E. variatum* at this site. Switzer et al. (2007) indicated Anthony Creek as the upstream-most extent of hybridization in the Greenbrier River drainage; no evidence of hybridization above Anthony Creek was found. They also found no evidence of hybridization in the Gauley River drainage upstream of Summersville Dam.

Hybridization with introgression is one mechanism that can cause the extinction of native species (Rhymer and Simberloff 1996). The breakdown of species boundaries through introgressive hybridization resulting in a loss of species diversity has been reported in a variety of fish families such as

Cichlidae, Gasterosteidae, Salmonidae, and Cyprinidae (Seehausen et al. 1997; Taylor 2006; Muhlfeld et al. 2009; Almodóvar et al. 2012). Introgression as a cause of loss of genetic and species diversity has been observed in other darters (Percidae: *Etheostoma*; Halas and Simons 2014; Matthews et al. 2016). This erosion of species boundary and attrition of biodiversity is occurring throughout the Gauley and Greenbrier populations of *E. osburni*. The populations of *E. osburni* within this distribution have become or are threatened with imminent genetic extinction. The populations in these drainages that have not already disappeared will most likely disappear due to introgression with *E. variatum*.

Etheostoma osburni has a limited national distribution and its range is shrinking (Chipps et al. 1993; Jenkins and Burkhead 1994; WVDNR unpublished data). Due to its limited range, the species has been designated as a Species of Concern in West Virginia, a Species of Special Concern in Virginia, and a Federal Species of Concern. *Etheostoma osburni* is a candidate for listing under the U.S. Endangered Species Act (Douglas, pers. comm.). A successful conservation strategy for the imperiled *E. osburni* requires a thorough understanding of the dynamics of hybridization between these two species.

Hybrids of Candy and Variegate darters have not been thoroughly examined. It is important to be able to identify individuals of hybrid origin based on visual inspection, providing a valuable marker in determining the zone of hybridization and detecting this occurrence in other regions. Due to the varying degrees of hybridization (first generation, second generation, backcrosses, etc.) affecting the identification of these two closely related species, relying on morphologic identification alone can be problematic. It was necessary to, first, utilize genetic techniques to thoroughly assess populations of the subject fishes and identify individuals with hybrid ancestry for morphological analysis and to identify pure *E. osburni* for evaluation. The current geographic extent of hybridization needed to be surveyed. A genetic assay of hybridization was performed by Switzer et al. (2007), which identified Anthony Creek as the upstream extent of introgression with *E. variatum* in the Greenbrier River drainage. Individuals collected upstream of Anthony Creek did not exhibit evidence of hybridization in 2004. That study indicated the importance of monitoring above Anthony Creek for upstream evidence of introgression. Increasing sample sizes rather than increasing the number of genetic markers increases the probability of detecting non-native

alleles (Della Croce et al. 2017). This study utilized large sample sizes distributed over a broad range within the Gauley and Greenbrier river drainages to delineate where unaffected populations of *E. osburni* occur within West Virginia. These data are important to conservation efforts that will attempt to preserve this species. Hence, the objectives of this study were to 1) synthesize and map distributional data for *E. variatum* introgression in the Gauley and Greenbrier populations of *E. osburni* and 2) describe meristic characters of *E. osburni* X *E. variatum* hybrids

Materials and Methods

Collection and Preservation

Tissue samples and potential morphometric specimens (n = 335) were collected from 22 of 45 sampling locations (Table 1; Figure 3). Sites that did not produce specimens are listed in Table 2. For sampling, a field technician, outfitted with a backpack electrofishing unit and two forward-facing wands, shocked in a downstream fashion towards a seine net (5 ft x 10 ft x 3/16 in; 1.24 m x 3.05 m x 4.76 mm). The seine net was placed approximately 5 m below the technician. Two additional field technicians flanked the electrofisher to disrupt the substrate. A series of intertransects that were parallel to the direction of the stream current were followed until nearly full coverage of the riffle habitat for that reach was attained or a minimum of 30 individuals was obtained, whichever occurred first. Multiple riffles in some extended reaches were sampled to increase sample size when *E. osburni* was sparse.

A tissue sample was taken from the right pelvic fin from each individual for genetic analysis. Fin clips were placed in 99% ethyl alcohol, and then the entire fish was placed in 10% formalin. Specimen labels were created to identify individuals and link genetic data to morphometric specimens. Formalin in specimen containers was discarded after at least six months and replaced with water. The water was replaced with fresh water once per day for four consecutive days after which the containers were filled with 45% isopropyl alcohol.

Genetic Characterization

Switzer et al. (2008) developed a set of 15 polymorphic microsatellite DNA markers for examining genetic variation within populations and potential hybridization between *E. osburni* and *E. variatum*. Five of these microsatellite loci (*Eos-C2*, *Eos-C3*, *Eos-C6*, *Eos-C112*, and *Eos-C117*) were found to be diagnostic in the differentiation between the two species. This set of diagnostic loci was instrumental in detecting hybridization and verifying the purity of species of individuals. Primers for these loci were manufactured by and obtained from Integrated DNA Technologies.

Total genomic DNA was extracted with the Promega SV 96 Genomic DNA Purification System following a modified version of the “Animal Tissues” protocol (Promega Technical Bulletin Part #TB303). Approximately 4 µg ($\pm$ 2µg; or approximately 1 mm X 2 mm) of pelvic fin tissue per specimen was prepared in each well of a 96-well plate for a 16 hour digestion at 55 °C. The elution process consisted of two 50 µL elutions (for a total elution volume of 100 µL) and was performed with a combination of vacuum and centrifugation. Concentrations of extracted DNA were quantified with a NanoDrop Lite spectrophotometer. A dilution (10 ng/µL) of each extraction product was amplified using polymerase chain reaction (PCR).

Conditions of microsatellite PCR consisted of 20 ng of genomic DNA, 0.4X QIAGEN Multiplex PCR Master Mix, and 0.2 µM of each primer (with the forward primer fluorescently labeled) in a 10-µL final reaction volume. A C1000 Touch (BioRad) thermal cycler was used to conduct the PCR reaction that consisted of an initial denaturation at 95 °C for 15 min; 25 cycles of 94 °C denaturation for 30 s, 57 °C annealing for 90 s, 72 °C extension for 60 s; and a 60 °C final extension for 30 min.

Capillary electrophoresis of PCR product (2 µL per reaction) was conducted on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter) with 400 bp size standard (0.5 µL per reaction) and sample loading solution (27.5 µL per reaction). The associated software, GenomeLab GeXP Series Software Suite, was used to visualize analyzed fragments and manually score alleles to generate genotype data for each individual. ALLELOGRAM v2.2 (Manaster 2009) was used to normalize and bin alleles utilizing a positive control common to all capillary electrophoresis runs.

Error of laboratory practices was evaluated. Ten percent of the tissue samples were selected with a random number generator. Each of these samples was extracted, amplified, visualized, and scored again according to the aforementioned practices. These data were compared to those of the original series to identify inconsistencies between allele scoring. Error rates were summarized as the number of inconsistently scored alleles divided by the total number of alleles.

Error of allele scoring was evaluated. A random number generator was used to select 47 (14%) of the original capillary electrophoresis results. The corresponding electropherograms were cross-read by another researcher to independently score alleles. These data were compared to the original data to identify inconsistencies between allele scoring. A discrepancy between original and independently scored alleles was interpreted as potential error and summed. Error rates were summarized as the number of inconsistently scored alleles divided by the total number of alleles.

The potential for null alleles was investigated with MICROCHECKER v2.2.3 (Van Oosterhout et al. 2003). Default settings were used with a maximum expected allele size of 500 bp, a 95% confidence interval, and 1000 randomizations. Each population identified during the STRUCTURE analysis was investigated independently. The software GENEPOP v4.2 (Raymond and Rousset 1995) was used to determine the status of Hardy-Weinberg equilibrium (HWE) of populations defined during the STRUCTURE analysis. A probability test for each locus in the population was conducted to estimate exact P-values with the Markov chain method. The following Markov chain parameters were used: Dememorization number = 1000; 100 batches; and 1000 iterations per batch. The significance level was set at $\alpha = 0.05$.

The purity and admixture of each specimen was manually verified by examining the five diagnostic loci. If an individual possessed *E. variatum* alleles in any quantity, then it was considered to be a hybrid. It is possible that an individual may have accumulated only *E. variatum* alleles at each of the diagnostic loci through progressive introgression. Switzer et al. (2007) discovered individuals that possessed only *E. variatum* alleles at the diagnostic loci but possessed a haplotype of the cytochrome b gene that indicated hybrid ancestry. One of the main intentions of this study was to identify pure *E.*

osburni by reasonably ruling out the possibility of hybridization and, so, there were considered to be two categories of fish, pure *E. osburni* and non-pure *E. osburni*. Hence, fully *E. variatum* nuclear genotypes that may have arisen through a history of hybrid ancestry were referred to as hybrids in this study. Individuals that possessed one *E. osburni* allele and one *E. variatum* allele at each of the 5 diagnostic loci were considered to be F1 hybrids.

The number of independently operating genetic clusters or populations (K) was inferred with STRUCTURE v2.3.4 (Pritchard et al. 2000). The initial analysis was conducted on the entire microsatellite data set representing 335 individuals. Ten independent runs for each K from K = 1 to K = 13 with 100,000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in period of 100,000 generations were performed. The default settings which included correlated allele frequencies, assumed admixture, and no prior information were used. STRUCTURE HARVESTER v0.6.94 was used to visualize the STRUCTURE results and implement the ΔK method of Evanno et al. (2005) to determine the number of K at the highest level of genetic partitioning (Earl and vonHoldt 2012). The composition of each population was determined by assigning individuals to populations based on affinity for the cluster representing hybrids. If an individual had an admixture proportion $q \geq 0.994$ for the cluster representing *E. osburni* then it was assigned to that cluster; otherwise, it was assigned to the cluster representing hybrids. This admixture proportion was selected because it was found, upon manual inspection of the diagnostic loci, that individuals with lower admixture proportions ($q < 0.994$) possessed *E. variatum* alleles. Individuals that comprised the clusters were manually corroborated with microsatellite data to search for the presence of *E. variatum* alleles. The initially identified population of hybrids was not investigated for substructure. A recursive, hierarchical approach to investigate substructure, or deeper delineation of populations, was implemented. A subsequent STRUCTURE analysis with 10 independent runs for each K from K = 1 to K = 12 with 100,000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in period of 100,000 generations was conducted on the *E. osburni* cluster. Each subsequently identified population was investigated in the same fashion.

NewHybrids v1.1 Beta 3 (Anderson and Thompson 2002) was used to estimate the posterior probability of the classification of genotyped individuals. Classifications were defined as *E. osburni*, *E. variatum*, F1 hybrid, F2 hybrid, backcross with *E. osburni*, and backcross with *E. variatum*. An individual was required to have a posterior probability of classification of $P \geq 0.998$ for *E. osburni*; otherwise, it was assigned to the classification with the next highest posterior probability. This value was used because a natural break in the continuity of posterior probabilities occurs at $P = 0.998$ and because every individual with a probability $P < 0.998$ for *E. osburni* classification was discovered to have at least one *E. variatum* allele upon manual inspection of the genotype. Individuals were required to have a posterior probability of classification of $P \geq 0.650$ for F1 hybrid; otherwise, it was assigned to the next highest probability of classification. The individuals with strong assignment ($P \geq 0.680$) to backcross with *E. osburni* were considered to be the result of F1 hybrid backcross with *E. osburni* whereas individuals with weak assignment ($P \leq 0.230$) were considered to be the result of multigenerational backcrossing with *E. osburni*. This method was designed by corroboration with manual inspection of genotypes, follows natural breaks in the continuity of posterior probabilities, and imposes the strictest rules for assignment of backcrosses with *E. osburni* and assignment of F1 hybrids.

Meristics

Meristic data from F1 hybrids (two males and two females) were compared with those of 41 individuals of pure *E. osburni* males. The F1 specimens represent one male and one female each from the Gauley and Greenbrier river drainages. The *E. osburni* individuals were collected from 12 sites in the Gauley and Greenbrier river drainages for a concurrent study (Table 3). Sex was determined by examining genital papillae and supported by male nuptial characters such as thickened fins, enlarged scale margins, and concentrations of pigment. Data for 14 meristic variables were recorded: dorsal-fin spines, dorsal-fin rays, pectoral-fin rays, pelvic-fin rays, pelvic-fin spines, anal-fin rays, anal-fin spines, scales above lateral line, scales below lateral line, scales along lateral line (left), scales along lateral line (right), circumpeduncle scales, dark lateral bars, and dorsolateral saddles. Presence-absence data were recorded for five variables: dorsal-fin saddle, breast squamation (embedded), breast squamation (exposed), opercle

squamation, and supraopercular squamation. The dorsolateral saddle that occurs beneath the first dorsal fin was counted separate from the other saddles. Supraopercular squamation may occur in the dorsolateral region of the head above the opercle (Figure 4). The minimum, maximum, and mode values for meristic variables are reported.

Results

Genetic Characterization

Ten percent of the original tissue samples were randomly selected and extracted, amplified, visualized, and scored again for comparison to original scores. The validation process yielded results that were identical to the corresponding original data; therefore, an error rate of zero was attributed to laboratory practices.

Fourteen percent (47 of the total 335) of the electropherograms were cross-read for error quantification of allele scoring. Of these data, 6 of 470 (0.013) alleles were scored differently by an independent researcher. These differences represented four alleles at two loci in three individuals. By locus, there were no allelic discrepancies found at *Eos-C2*, *Eos-C3*, and *Eos-C6*; *Eos-C6* yielded an error rate of 0.021; and *Eos117* yielded an error rate of 0.043. At this error rate, we would expect approximately 43 possible allelic discrepancies within the total of 3350 alleles. None of these discrepancies resulted in scoring alleles that would misassign an individual to species or hybrid class nor did any of the discrepancies result in novel alleles for that sample. This error rate was considered to be acceptable.

The MICROCHECKER analysis found no evidence for scoring error due to stuttering, no evidence for large allele dropout, and no evidence of null alleles at any of the loci in the Gauley and Greenbrier populations. There was a general excess of homozygotes found in the population of hybrids at *Eos-C3* (exp. = 29.895, obs. = 50), *Eos-C112* (exp. = 80.798, obs. = 95), and *Eos-C2* (exp. = 50.048, obs. = 62). All of the loci that comprised the population of hybrids were significantly out of HWE: *Eos-*

C2 ($F_{IS} = 0.1656$, $P = 0.001$), *Eos-C3* ($F_{IS} = 0.2175$, $P < 0.000$), *Eos-C6* ($F_{IS} = 0.0662$, $P = 0.005$), *Eos-C112* ($F_{IS} = 0.3323$, $P < 0.000$), and *Eos-C117* ($F_{IS} = 0.1045$, $P < 0.000$).

A total of 335 individuals from the Gauley and Greenbrier river drainages were genotyped. Of this total, 124 genotypes included alleles within the size range of *E. variatum* discovered during manual inspection. The initial STRUCTURE run revealed $K = 2$ at the highest level of genetic partitioning which represented *E. osburni* and the cluster of hybrids that comprised the hybrid swarm ($n = 124$) (Figure 5). A secondary run that included individuals assigned to the population of *E. osburni* revealed substructuring within this species (Figure 6). It was found that $K = 2$ within *E. osburni* which represented a distinction between the two populations of the Gauley and Greenbrier river drainages. No additional substructuring was found within these two populations of *E. osburni*. This phenomenon is discussed in more detail in Chapter 3. Substructuring was not investigated for the population of hybrids.

Posterior probability of assignment to six classes for each individual was calculated (Figure 7; Appendix 1). Three individuals from the Gauley River drainage upstream of Summersville Dam (Sites 4 - 11) had probabilities of assignment to backcross with *E. osburni* ($0.227 \leq P \leq 0.180$). All other individuals from the Gauley River drainage above Summersville Dam assigned to *E. osburni* ($P \geq 0.998$). All individuals from Gauley River (Site 2), Bells Creek (Site 1), Greenbrier River (Site 12), and Anthony Creek (Sites 13, 14, and 15) assigned to *E. variatum* or backcross with *E. variatum* ($P > 0.500$) except for three individuals that assigned to F1 hybrid ($P > 0.650$; Site 2) and another individual that assigned to backcross with *E. osburni* ($P = 0.321$). One individual from Knapp Creek (Site 16) assigned to F1 hybrid ($P = 0.660$); one individual assigned to *E. osburni* ($P = 0.997$); two individuals assigned to backcross with *E. osburni* ($P \geq 0.776$); and one individual assigned to *E. variatum* ($P = 0.994$). All individuals from Knapp Creek (Site 17) assigned to *E. osburni* ($P \geq 0.999$) except one individual that assigned to backcross with *E. osburni* ($P = 0.146$). All individuals from Sitlington Creek (Site 18) assigned to *E. osburni* ($P \geq 0.998$) except one individual that assigned to backcross with *E. osburni* ($P = 0.149$). One individual from Deer Creek (Site 19) assigned to F1 hybrid; 2 individuals had strong assignment to backcross with *E. osburni* ($P \geq 0.827$); one individual assigned to backcross with *E. osburni* ($P = 0.181$); and eight

individuals assigned to *E. osburni* ($P \geq 0.999$). All individuals from East Fork Greenbrier River (Site 20) assigned to *E. osburni* ($P \geq 0.998$). All individuals from Little River East Fork Greenbrier River assigned to *E. osburni* ($P \geq 0.998$) except two individuals that assigned to backcross with *E. osburni* ($P \geq 0.054$). All individuals from West Fork Greenbrier River assigned to *E. osburni* ($P \geq 0.998$) except one that assigned to backcross with *E. osburni* ($P = 0.893$).

Geographic Distribution of Hybrids

Locations of *E. variatum* alleles, % *E. variatum* alleles, numbers of specimens collected, and numbers of pure *E. osburni* collected at each site were determined (Table 1; Figure 8). *E. variatum* alleles were detected at two locations in the Gauley River drainage above Summersville Dam (Sites 4 and 9, which are Cherry and Gauley rivers, respectively). One individual at Site 9 possessed one *E. variatum* allele and two individuals at Site 4 each possessed one *E. variatum* allele. All other individuals sampled from above Summersville Dam possessed only *E. osburni* alleles at the five diagnostic loci (i. e. pure *E. osburni*). The site immediately below Summersville Dam (Site 3) produced six individuals each with one *E. variatum* allele. Anthony Creek, Gauley River at the town of Swiss, and all sites between (Sites 1, 2, and 12 - 15) were characterized by populations comprised of at least 84.6 % *E. variatum* alleles. None of these sites had any individuals that possessed only *E. osburni* alleles at each of the five diagnostic loci. F1 hybrid genotypes with a posterior probability of assignment ($P \geq 0.650$) as F1 hybrids were discovered at Sites 2, 16, and 19. The samples of darters at these three sites had 9.17%, 84.55%, and 45.71% *E. variatum* alleles, respectively. One *E. variatum* allele in one individual was discovered in the sample of Sitlington Creek (Site 18). One individual sampled from Knapp Creek (Site 17) had one *E. variatum* allele; all other individuals possessed only *E. osburni* alleles. No *E. variatum* alleles were found in the sample of the East Fork Greenbrier River (Site 20). Two individuals from Little River East Fork Greenbrier River each had one *E. variatum* allele; all other individuals possessed only *E. osburni* alleles. One individual from West Fork Greenbrier River (Site 22) had three *E. variatum* alleles; all other individuals possessed only *E. osburni* alleles.

Meristic Characterization of F1 Hybrid

Two male and two female F1 hybrids were meristically evaluated. This set of specimens represented a male and a female from each of the Gauley and Greenbrier river drainages. Meristic characters evaluated for the F1 hybrid were dorsal-fin spines, dorsal-fin rays, pectoral-fin rays, pelvic-fin rays, pelvic fin spines, anal-fin rays, anal-fin spines, scales above lateral line, scales below lateral line, scales along lateral line, circumpeduncle scales, dark lateral bars, and dorsolateral saddles, dorsal-fin saddle, embedded breast squamation, exposed breast squamation, opercle squamation, supraopercular squamation (Table 3). The F1 hybrid lateral line scale count range was above the mode for *E. variatum* and below the mode for *E. osburni*. All F1 hybrids possessed characters of both parental species. Photographs of individuals that express hybrid characters are shown in Figures 9 and 10. Photographs of *E. osburni* and *E. variatum* are given in Figures 11 through 14.

Discussion

The percentage of *E. variatum* alleles present at each site can be used to monitor changes in introgression rates over time. This metric can be used to estimate the intensity of genetic swamping experienced by *E. osburni* and monitor expansion of the hybrid swarm. Another method to observe development of the hybrid swarm is to delineate it by the presence of samples containing F1 hybrids and/or backcrosses with F1 hybrids. These samples could also conceivably contain both *E. osburni* and *E. variatum* parental species genotypes. Important sites in this study that produced these genotypes that can be used as current landmarks for the delineation of the hybrid swarm are Gauley River (Site 2), Knapp Creek (Site 16), and Deer Creek (Site 19). These three sites can be considered inflection points where the population of hybrids meets populations of *E. osburni*. While F1 hybrids were collected at Site 2, pure *E. osburni* were not collected here and so the true inflection point in the Gauley River probably lies slightly upstream of this site. Within the bounds of these landmarks, the populations and individuals are composed largely of *E. variatum* alleles. Beyond these landmarks, populations are characterized by members of *E. osburni* of pure descent and a few multi-generational backcrosses with *E. osburni*.

Our data suggested a spread of the hybrid swarm over the time period of 2004 to 2014. *E. variatum* alleles were detected at the majority of the upstream-most sample sites in the Greenbrier River drainage such as Sitlington Creek (Site 18), West Fork Greenbrier River (Site 22), and Little River East Fork Greenbrier River (Site 21). *E. variatum* alleles were also found at the upstream-most sample site in the Gauley River drainage below Summersville Dam (Site 3). Switzer et al. (2007) found no evidence of hybridization in the Greenbrier River drainage upstream of Anthony Creek. The current study found a substantial presence of *E. variatum* alleles throughout the drainage upstream of Anthony Creek. The collection of F1 hybrids during this study indicates contemporary hybridization at these sites and the presence of an active hybrid zone. Switzer et al. (2007) found no evidence of hybridization in the Gauley River drainage above Summersville Dam but the current study detected *E. variatum* alleles at two sites in this area (Sites 4 and 9).

The hybrid swarm could spread naturally from a single introduced nucleus or could be facilitated by anthropogenic introductions of *E. variatum* alleles. There may be continual or many introductions of *E. variatum* (e. g. bait bucket, aquarium release). These introductions may be occurring repeatedly at the same location or sporadically throughout the drainage. *Etheostoma variatum* alleles may also be transported by human aided movement of hybrid individuals. It seems more likely, though, that the current state of the hybrid zone is a natural expansion of an established hybrid swarm.

Introgressive hybridization is one mechanism that facilitates the dispersal of invasive fish species (Hitt et al. 2003; Walters et al. 2008; Ward et al. 2012). Expansion, following introduction and establishment, is characteristic of some hybrid swarms (Kolar and Lodge 2001). *Etheostoma osburni* alleles were detected in the sample from Bells Creek where there are no records for *E. osburni*. The presence of *E. osburni* alleles in this sample suggests the possible natural dispersal of hybrid individuals to this area from a population of invaded *E. osburni*. A substantial quantity of visually identified *E. variatum* was collected in New River (Wellman et al. 2004). The presence of *E. variatum* and hybrids in the drainage, the thoroughness of genetic swamping of *E. osburni* by *E. variatum* within the hybrid zone, the presence of hybrids where *E. osburni* was previously absent, and the pattern of species alleles along a

concentration continuum suggest a natural dispersion of the hybrid swarm in the Greenbrier River drainage. If dispersion of the hybrid swarm was to rely solely on widespread anthropogenic introductions, it may be expected to appear as many concentrated populations of hybrids or *E. variatum* rather than a single, dendritic continuum. This does not preclude the possibility of past, contemporary, or future *E. variatum* introductions simultaneous to natural dispersion and does not explain the presence of *E. variatum* alleles in the Gauley River drainage which were likely the product of additional and separate introduction events.

The fitness consequences of introgression between *E. osburni* and *E. variatum* are unknown. One possible consequence is outbreeding depression. This would occur when hybrid individuals exhibit lower fitness in the *E. osburni* range than the parental species. Another potential outcome is heterosis, or hybrid vigor, which is the production of hybrid offspring that exhibit fitness traits that are superior to those of the parental species. Given the widespread dispersal of hybrids in the New, Gauley, and Greenbrier river drainages, outbreeding depression is likely not the result of introgression. *Etheostoma osburni* is found in colder headwater streams than *E. variatum*. Cold headwater stream temperatures may favor *E. osburni* and decrease the rate of *E. variatum* introgression, however, it is not a complete barrier to *E. variatum* gene flow as evidenced by the discovery of *E. variatum* alleles in all of the headwater locations sampled in the Greenbrier River drainage.

The F1 hybrid phenotype exhibits a range of characters that overlaps with both parental species. All of the meristic character ranges of the F1 hybrid overlapped with ranges for *E. osburni* generated for a concurrent study as well as overlapping with published ranges for *E. variatum* (Page 1983; Page and Burr 2011). A notable character that aided in the identification of a hybrid was the presence of cephalic squamation. Although Page (1983) lists the opercular squamation as variable in *E. osburni*, this study found that it, in conjunction with squamation of the supraopercular region, was a useful character for distinguishing hybrids (Figure 4). F1 hybrid individuals were found to express combinations of characters of both parental species as well as express intermediacy within single characters. This was observed in meristics and pigmentation.

There are limitations to using only microsatellite loci to detect hybridization. It is possible for a specimen to possess nuclear genotypes from one parental species but have mitochondrial haplotypes from another parental species (Wilson and Bernatchez 1998; Vilà et al. 2003; Switzer et al. 2007). Another study observed a greater range of introgression with non-neutral single nucleotide polymorphisms compared to neutral microsatellite loci (Fitzpatrick et al. 2009). A more sensitive approach to detecting introgression should include mitochondrial and non-neutral markers in addition to diagnostic microsatellite loci.

Conclusions

The detrimental effects of human-induced introductions of an invasive species are apparent in the results of this research. This study found strong evidence that *E. osburni* in the Greenbrier River drainage are threatened with genetic swamping by *E. variatum*. The influence of the hybrid swarm in this drainage is pervasive. Evidence of *E. variatum* introduction into the Gauley River drainage above Summersville Dam was detected in low concentrations; however, the presence of a hybrid swarm has yet to be observed in the upper drainage. A loss of the Greenbrier population of *E. osburni* would leave a substantial gap in the geographic range and genetic diversity of this species.

Management Implications

Non-native aquatic taxa are abundant and widespread in the upper Kanawha River system. Invasive species are one of the largest factors that reduce biodiversity and threaten native species (Vitousek et al. 1997). The prevention of further introductions should be a primary goal for aquatic conservation. *Etheostoma variatum* was most likely introduced to the New River drainage through one or many anglers' bait buckets. It is recommended that only a selected set of particular species should be listed and clearly posted as legal aquatic organisms allowed as bait for each body of water to reduce the potential for introductions. Advertisement in the form of signs and in fishing regulations that concisely explain that it is illegal to move all other species of aquatic organisms (e. g. fish, crayfish, etc.) should be

considered. While a complete ban on using aquatic organisms for fishing bait is likely to be met with fierce resistance, it should be strongly considered as a management component to protect the Gauley population of *E. osburni* and in other waters where imperiled species of aquatic taxa occur. Future studies should monitor changes in the presence of *E. variatum* in the Gauley and Greenbrier river drainages.

Chapter 3: Comparison of *Etheostoma osburni* between the Gauley and Greenbrier river drainages

Introduction

The Candy Darter (*Etheostoma osburni*; Hubbs and Trautman 1932) is a brilliantly colored species of darter in the perch family (Percidae: Etheostomatini). *Etheostoma osburni* is endemic to the upper Kanawha River system above Kanawha Falls. Its range includes the Gauley and Greenbrier rivers of West Virginia and the New River within West Virginia and Virginia (Jenkins and Burkhead 1994; Stauffer et al. 1995). Within West Virginia, it has been historically distributed throughout the Gauley River, the Greenbrier River, the Bluestone River, and Indian Creek of the New River drainage. This darter primarily occupies the rubble and boulder substrate of riffle habitat of small streams and midsize rivers (Chipps et al. 1994). *Etheostoma osburni* feeds opportunistically on benthic macroinvertebrates. Mayflies (Ephemeroptera) comprise the majority of their diet (Schoolcraft et al. 2007). Schoolcraft and Tarter (2002) estimated the peak spawning time of *E. osburni* in the South Fork of the Cherry River to be approximately April 21. Jenkins and Burkhead (1994) documented a significant reduction in the range of *E. osburni* in Virginia in recent history. Due to limited distribution and population reduction, *E. osburni* is listed as a Species of Concern in West Virginia, a Species of Special Concern in Virginia, and a Federal Species of Concern (USFWS 1989; Jelks et al. 2008).

Portions of the New, Gauley, and Greenbrier river drainages have been invaded by *E. variatum*, which is genetically swamping populations of *E. osburni*. Hybridization with introgression is one mechanism that can cause the extinction of native species (Rhymer and Simberloff 1996). This is occurring throughout the Gauley River (below Summersville Dam), New River (below Bluestone Dam), and Greenbrier River drainages. All of the populations of *E. osburni* within this distribution have become or are threatened with imminent genetic extinction. The populations in these drainages that have not disappeared due to habitat loss will most likely disappear due to introgression with *E. variatum*. Summersville and Bluestone Dams are the only barriers preventing the genetic extinction of *E. osburni*.

A study by Switzer et al. (2007) found evidence of genetic divergence between the Gauley and Greenbrier populations of *E. osburni*. Switzer et al. (2007) stated that it appeared to be a longstanding allopatric fragmentation of the Gauley and Greenbrier populations that resulted in relatively high levels of differentiation. They also stated that the two populations appear to have experienced different demographic events such as a bottleneck followed by range expansion in the Gauley population. This is in contrast to the Greenbrier River drainage with an apparent larger population of *E. osburni* and greater stability over time (Switzer et al. 2007). Due to genetic differences between populations, a loss of one population would result in the loss of genetic diversity for the species as a whole.

Gene diversity between geographic clusters is generally greater than between localities within clusters (Gyllensten 1985). Species that inhabit headwater stream habitat may have high levels of genetic divergence at small spatial scales (Turner and Robison 2006). Another species of darter, the Ashy Darter (*Etheostoma cinereum*) has a fragmented range, the fragmented populations have genetically diverged, and three populations showed morphologic variation that supported the recognition of distinct management units (Powers et al. 2004). The Cumberland River population of *E. cinereum* has been described as *E. maydeni* (Powers et al. 2012).

This chapter identifies genetic and morphometric differences between the populations of *E. osburni* in West Virginia. The objectives of this study were to evaluate *E. osburni* of the Gauley and Greenbrier river drainages to 1) estimate the number of distinct populations (K) of *E. osburni* in West Virginia and measure genetic differentiation (F_{ST}) among the populations, and 2) evaluate these populations with morphometric techniques to identify morphological differences.

Materials and Methods

Collection and Preservation

Tissue samples and potential morphometric specimens (n = 335) were collected from 22 locations (Table 1). A total of 45 locations were visited for this study. Sites that did not produce specimens are listed in Table 2. A field technician was outfitted with a backpack electrofishing unit and two forward-

facing wands to shock in a downstream fashion towards a seine net (5 ft x 10 ft x 3/16 in; 1.24 m x 3.05 m x 4.76 mm). The seine net was placed approximately 5 m below the technician. Two additional field technicians flanked the electrofisher to disrupt the substrate. A series of intertransects that were parallel to the direction of the stream current were followed until nearly full coverage of the riffle habitat for that reach was attained or a minimum of 30 individuals was obtained, whichever occurred first. Multiple riffles in some extended reaches were sampled to increase sample size when *E. osburni* was sparse.

A tissue sample was taken from the right pelvic fin from each individual for genetic analysis. Fin clips were placed in 99% ethyl alcohol, and then the entire fish was placed in 10% formalin. Labels for each specimen were created to identify individuals and to link genetic data to morphometric specimens. The formalin was discarded after at least six months and replaced with water. The water was replaced with fresh water once per day for four consecutive days after which the containers were filled with 45% isopropyl alcohol.

Morphometric data were recorded from adult males. The minimum standard length was 57.8 mm. *Etheostoma osburni* is sexually dimorphic and so it was important to compare measurements within a particular sex. Sex was determined by examining genital papillae and supported by male nuptial characters such as thickened fins, enlarged scale margins, and concentrations of pigment. In addition to restricting morphometric data collection to adult males, it was important to verify that specimens were of pure *E. osburni* ancestry due to the presence of potential introgression with an expanding hybrid swarm.

Genetic Characterization

Switzer et al. (2008) developed a set of 15 polymorphic microsatellite DNA markers for examining genetic variation within populations and potential hybridization between *E. osburni* and *E. variatum*. Five of these microsatellite loci (*Eos-C2*, *Eos-C3*, *Eos-C6*, *Eos-C112*, and *Eos-C117*) were found to be diagnostic in the differentiation between the two species. This set of diagnostic loci was instrumental in detecting hybridization and verifying the purity of species of individuals. Primers for these loci were manufactured by and obtained from Integrated DNA Technologies. Data collected for these five diagnostic loci for a concurrent study of hybridization was used for evaluating the population

structure of *E. osburni* in this study. The purity and admixture of each specimen was manually verified by examining the five diagnostic loci. Individuals that possessed *E. variatum* alleles in any quantity at the diagnostic loci were excluded from genetic and morphometric analytical comparison between populations of *E. osburni*.

Total genomic DNA was extracted with the Promega SV 96 Genomic DNA Purification System following a modified version of the “Animal Tissues” protocol (Promega Technical Bulletin Part #TB303). Approximately 4 µg ($\pm$ 2µg; or approximately 1 mm X 2 mm) of pelvic fin tissue per specimen was prepared in each well of a 96-well plate for a 16 hour digestion at 55 °C. The elution process consisted of two 50 µL elutions (for a total elution volume of 100 µL) and was performed with a combination of vacuum and centrifugation. Concentrations of extracted DNA were quantified with a NanoDrop Lite spectrophotometer. A dilution (10 ng/µL) of each extraction product was created and amplified using the polymerase chain reaction (PCR).

Five microsatellite loci were amplified (*Eos-C2*, *Eos-C3*, *Eos-C6*, *Eos-C112*, and *Eos-C117*; Switzer et al. 2008). Conditions of microsatellite PCR consisted of 20 ng of genomic DNA, 0.4X QIAGEN Multiplex PCR master Mix, and 0.2 µM of each primer (with the forward primer fluorescently labeled) in a 10-µL final reaction volume. A C1000 Touch (BioRad) thermal cycler was used to conduct the PCR reaction that consisted of an initial denaturation at 95 °C for 15 min; 25 cycles of 94 °C denaturation for 30 s, 57 °C annealing for 90 s, 72 °C extension for 60 s; and a 60 °C final extension for 30 min.

Capillary electrophoresis of PCR product (2 µL per reaction) was conducted on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter) with 400 bp size standard (0.5 µL per reaction) and sample loading solution (27.5 µL per reaction). The associated software, GenomeLab GeXP Series Software Suite, was used to visualize analyzed fragments and manually score alleles to generate genotype data for each individual. ALLELOGRAM v2.2 (Manaster 2009) was used to normalize and bin alleles utilizing a positive control common to all capillary electrophoresis runs.

Error of laboratory practices was evaluated. Ten percent of the tissue samples were selected with a random number generator. Each of these samples was extracted, amplified, visualized, and scored again according to the aforementioned practices. These data were compared to those of the original series to identify inconsistencies between allele scoring. Error rates were summarized as the number of inconsistently scored alleles divided by the total number of alleles.

Error of allele scoring was evaluated. A random number generator was used to select 47 (14%) of the original capillary electrophoresis results. The corresponding electropherograms were cross-read by another researcher to independently score alleles. These data were compared to the original data to identify inconsistencies between allele scoring. A discrepancy between original and independently scored alleles was interpreted as potential error and summed. Error rates were summarized as the number of inconsistently scored alleles divided by the total number of alleles.

The potential for null alleles was investigated with MICROCHECKER v2.2.3 (Van Oosterhout et al. 2003). Default settings were used with a maximum expected allele size of 500 bp, a 95% confidence interval, and 1000 randomizations. Each population identified during the STRUCTURE analysis was investigated independently. The software GENEPOP v4.2 (Raymond and Rousset 1995) was used to determine the status of Hardy-Weinberg equilibrium (HWE) of populations defined during the STRUCTURE analysis and of the 10 samples defined in Table 4 that comprise the Gauley and Greenbrier populations. A probability test for each locus in the population or sample was conducted to estimate exact P-values with the Markov chain method. The following Markov chain parameters were used: Dememorization number = 1000; 100 batches; and 1000 iterations per batch. The significance level was set at $\alpha = 0.05$.

The number of independently operating genetic clusters or populations (K) was inferred with STRUCTURE v2.3.4 (Pritchard et al. 2000). The initial analysis was conducted on the entire microsatellite data set representing 335 individuals. Ten independent runs for each K from K = 1 to K = 13 with 100,000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in period of 100,000 generations were performed. The default settings which included correlated allele frequencies, assumed

admixture, and no prior information were used. STRUCTURE HARVESTER v0.6.94 was used to visualize the STRUCTURE results and implement the ΔK method of Evanno et al. (2005) to determine the number of K at the highest level of genetic partitioning (Earl and vonHoldt 2012). A recursive, hierarchical approach to investigate substructure, or deeper delineation of populations, was implemented. A subsequent STRUCTURE analysis with 10 independent runs for each K from $K = 1$ to $K = 12$ with 100,000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in period of 100,000 generations was conducted on the *E. osburni* cluster. Each subsequently identified population was investigated in the same fashion.

To measure genetic differentiation, pairwise tests of fixation index (F_{ST}) among sample locations and between the Gauley and Greenbrier populations were conducted with FSTAT v2.9.3.2 (Goudet 2002). The indicative adjusted nominal level for multiple comparisons was set at $\alpha = 0.05$. An analysis of genetic distance was performed with the PHYLIP v3.695 software package (Felsenstein 2009). Individuals ($n = 211$) of pure *E. osburni* from 16 sites were used for this analysis (Table 4). Some of these sites yielded low ($n < 10$) numbers of pure *E. osburni* and were therefore combined with geographically adjacent sites to create larger sample sizes for a total of 10 samples in this analysis as described in Table 4. One thousand replicated samples were generated and a neighbor-joining tree was constructed based on the Cavalli-Sforza chord measure (Cavalli-Sforza and Edwards 1967).

To measure genetic diversity, private alleles in the Gauley and Greenbrier populations were identified with GENEPOP v4.2 (Raymond and Rousset 1995). The proportion of polymorphic loci in the two populations was calculated. FSTAT v2.9.3.2 (Goudet 2002) was used to calculate allelic richness per locus. Observed and expected heterozygosities for each locus of each population were calculated with ARLEQUIN v3.5.2.2 (Excoffier and Lischer 2010). Differences of observed heterozygosity and allelic richness between populations were normally distributed as determined with a Shapiro-Wilk test. A paired t-test was used to test significance of difference between the Gauley and Greenbrier populations for observed heterozygosity and allelic richness per locus.

Morphometrics

Morphometric and meristic data were taken from 41 adult male individuals of pure *E. osburni* descent. These fish were collected from 12 sites in the Gauley ($n_{\text{specimens}} = 16$; $n_{\text{sites}} = 5$) and Greenbrier ($n_{\text{specimens}} = 25$; $n_{\text{sites}} = 5$) river drainages. These metrics are enumerated and described in Tables 5 and 6. The box truss network system, modified from Strauss and Bookstein (1982) in the style of Welsh and Wood (2008), was used to produce morphometric data. A series of 11 midsagittal landmarks was selected and 23 distances were measured between the landmarks to create a box truss network (Figure 15). Seven distances that characterize head geometry (including gape and eye width) were measured. Three additional distances (standard length, outerpelvic width, and caudal fin length) were taken for a total of 33 straight-line (point to point) distances measured for each individual (Figure 16). Digital calipers were used to measure distances and record values to the hundredth of a millimeter. Data for 14 meristic variables were recorded: dorsal-fin spines, dorsal-fin rays, pectoral-fin rays, pelvic-fin rays, pelvic-fin spines, anal-fin rays, anal-fin spines, scales above lateral line, scales below lateral line, scales along lateral line (left), scales along lateral line (right), circumpeduncle scales, dark lateral bars, and dorsolateral saddles. Presence-absence data were recorded for five variables: dorsal-fin saddle, breast squamation (embedded), breast squamation (exposed), opercle squamation, and supraopercular squamation. The dorsolateral saddle that occurs beneath the first dorsal fin was counted separate from the other saddles. Supraopercular squamation may occur in the dorsolateral region of the head above the opercle. The minimum, maximum, and mode values for meristic variables are reported. Sheared principal components analysis (SPCA) was used to analyze morphometric data. SPCA was implemented with SAS code (D. Swofford, Statistical Analysis Systems Institute, Inc., Carry, NC; SAS Institute 1990). Error of measured morphometric distances was assessed. Morphometric evaluation of a single individual was repeated 20 times. This series of measurements was taken according to the aforementioned practices to produce a separate set of data. Error of morphometric data is reported as variance within each variable.

Results

Genetic Characterization

Ten percent of the original tissue samples were randomly selected and extracted, amplified, visualized, and scored again for comparison to original scores. The validation process yielded results that were identical to the corresponding original data; therefore, an error rate of zero was attributed to laboratory practices.

Fourteen percent (47 of the total 335) of the electropherograms were cross-read for error quantification of allele scoring. Of these data, 6 of 470 (0.013) alleles were scored differently by an independent researcher. These differences represented four alleles at two loci in three individuals. By locus, there were no allelic discrepancies found at *Eos-C2*, *Eos-C3*, and *Eos-C6*; *Eos-C6* yielded an error rate of 0.021; and *Eos117* yielded an error rate of 0.043. At this error rate, we would expect approximately 43 possible allelic discrepancies within the total of 3350 alleles. None of these discrepancies resulted in scoring alleles that would misassign an individual to species or hybrid class nor did any of the discrepancies result in novel alleles for that sample. This error rate was considered to be acceptable.

The MICROCHECKER analysis found no evidence for scoring error due to stuttering, no evidence for large allele dropout, and no evidence of null alleles at any of the loci in the Gauley and Greenbrier populations. All loci in the Gauley and Greenbrier populations of *E. osburni* were in HWE (Tables 7 and 8). The Gauley population, including each sample that comprises this population, is fixed for a single allele at *Eos-C3*. Each sample that comprised the Gauley and Greenbrier populations was in HWE.

A total of 335 individuals from the Gauley and Greenbrier river drainages were genotyped. Of this total, 124 genotypes included alleles within the size range of *E. variatum* and, therefore, were excluded from analysis of or comparison between populations of *E. osburni*. This resulted in sample sizes of 144 and 67 for the Gauley and Greenbrier populations of *E. osburni*, respectively.

A STRUCTURE analysis that included individuals assigned to the initial population of *E. osburni* identified in Chapter 2 revealed substructuring within this species (Figure 6). It was found that $K = 2$ within *E. osburni*, which represented a distinction between the two populations of the Gauley and Greenbrier river drainages. No additional substructuring was found within these two populations of *E. osburni*.

The Gauley and Greenbrier populations of *E. osburni* were highly differentiated. Differentiation between the two populations was calculated as $F_{ST} = 0.144$ ($P = 0.017$). A pairwise calculation of F_{ST} among the 10 samples described in Table 4 resulted in $-0.011 \geq F_{ST} \geq 0.205$ (Table 9). The largest value ($F_{ST} = 0.205$) was observed between Upper Gauley River and Mid-Greenbrier River. All pairwise calculations of F_{ST} among samples between the Gauley and Greenbrier river drainages were significant at the indicative adjusted nominal level (5%) for multiple comparisons ($P = 0.001$). One calculation of F_{ST} among samples within the Gauley River drainage was significant at this level; this was between Upper Gauley River and Laurel Creek.

Another method to observe genetic differences between the two populations of *E. osburni* is through the construction of a neighbor-joining tree. As seen in Figure 17, all samples within each of the Gauley and Greenbrier populations group together in 98.5 % of 1000 generated trees. This demonstrates a marked distinction between the two populations. With one exception noted below, there were levels of consensus greater than 50 % (52.1 % - 91.2 %) for all of the within drainage positions of the tree. The Upper and Lower Gauley river samples were indicated as neighbors in 46.4 % of 1000 generated trees.

A summary of allelic diversity, frequency, and variation per locus for each population is given in Tables 10 and 11. The number of alleles per locus ranged from 1 – 7 in the Gauley population and 2 – 10 in the Greenbrier population. There were a total of 18 alleles across all loci in the Gauley population, 29 in the Greenbrier population, and an overall total of 30. The number of private alleles was 1 in the Gauley population and 12 in the Greenbrier population. The proportion of polymorphic loci was 0.8 in the Gauley population and 1.0 in the Greenbrier population. Observed heterozygosity (H_o) and expected heterozygosity (H_e) mean values for all loci in the Gauley population were 0.557 and 0.552, respectively.

H_o and H_e mean values for all loci in the Greenbrier population were 0.442 and 0.436, respectively. H_o did not differ significantly between populations ($P = 0.973$). Average allelic richness was 3.324 and 5.800 for the Gauley and Greenbrier populations, respectively, and did not differ significantly between populations but approaches significance ($P = 0.059$). This lack of significance may be due to a low number ($n = 5$) of loci used.

Morphometric Characterization

Error of measured morphometric distances was assessed with a series of measurements that was repeated 20 times for a single individual. A summary of these measurements and associated variances are reported in Table 12. The overall minimum, maximum, and mean variance was 0.01 mm, 0.29 mm, and 0.08 mm, respectively. The minimum variance was obtained with depth at caudal flexure, eye width, anterior internasal width, posterior internasal width, interorbital width, gape width, and outerpelvic base width, equivalently as 0.01. The maximum variance was obtained with caudal-fin length. This level of error was considered acceptable.

The Greenbrier population had greater morphometric ranges in 18 of 32 distance variables measured. As such, the Gauley population morphometry was a subset of that of the Greenbrier population for these variables. The Gauley population portrayed higher maximum values for 13 of the other variables and a lower minimum for 1. The Gauley population exhibited a wider range of values than the Greenbrier population for only one variable. This general wider range of morphometric values for the Greenbrier population may be an artifact of a larger sample size. All of the ranges for all of the characters show overlap. Nonetheless, plots of SPC2 by SPC3 show considerable separation along the SPC2 axis, although, without complete separation.

Plots of the sheared second and third principal components (SPC2 and SPC3) depict shape differences between the Gauley and Greenbrier populations of *E. osburni* (Figure 18). Interorbital width (0.78), followed by snout–branchiostegal connection (-0.26), loaded highest on the SPC2 axis. Snout–branchiostegal connection (0.42), branchiostegal connection–midpoint between pelvic-fin bases (0.30), snout–anterior margin of eye (0.26), occiput–first dorsal-fin spine (-0.46), base of anal fin (-0.27), and

first anal-fin ray–last dorsal-fin ray (-0.24) loaded highest on the SPC3 axis. 21.9% and 18.4% of the variation was explained along the SPC2 and SPC3 axis, respectively. Morphometric distances that were used in SPCA of the Gauley and Greenbrier populations of *E. osburni* were summarized (Table 5).

The meristics for all variable ranges overlapped between the Gauley and Greenbrier drainages. While there was not complete separation of ranges for any of the variables, many differed modally (Table 6). The number of pectoral fin rays, anal fin rays, scales below the lateral line, scales along the lateral line, and scales around the caudal peduncle differed, modally, between populations. One of the largest meristic differences between populations was that of embedded breast squamation. The Greenbrier population had a 64 % rate of individuals that possessed embedded breast squamation compared to 12.5 % of the Gauley population. Furthermore, Greenbrier population members that exhibited embedded breast squamation typically possessed larger numbers of scales compared to those of the Gauley population. The means of all standard meristic variables (i. e. non-presence/absence variables) differed between populations by less than one standard deviation.

Typically, when the dorsolateral saddle count was 4, rather than 5, it was because the caudal peduncle saddles were partially or entirely joined. The dorsal-fin saddle was not always readily distinguishable. At times it was obscured by dark background pigment and at other times it may have been faded in a fixed specimen but, perhaps, visible in a live specimen. The variability of these two characters (dorsal-fin saddle and caudal peduncle saddle) is why they are considered to be a separate meristic in this study. Excluding the variability of the dorsal-fin saddle allowed for a more meaningful overall meristic for the other dorsolateral saddles.

Discussion

This study found that the Gauley and Greenbrier populations are highly differentiated with a fixation index of $F_{ST} = 0.144$ ($P = 0.017$). Values of F_{ST} range from 0.0 to 1.0. A value of $F_{ST} = 0.05$ is considered to be moderately differentiated and a value of $F_{ST} = 0.15$ is considered to be highly

differentiated. All of the pairwise comparisons of samples between the Gauley and Greenbrier populations yielded significant F_{ST} values which ranged from 0.103 – 0.205 ($P = 0.001$). With one exception, all of the comparisons of samples within populations yielded non-significant F_{ST} values. Switzer et al. (2007) also found that the Gauley and Greenbrier populations were significantly differentiated when compared with AMOVA using F-statistics, R-statistics, and Φ -statistics. Different demographic events, different results of genetic drift, and mutations that are not shared between populations affect allelic frequencies of these populations independently. In recognition of these F_{ST} values, the Gauley and Greenbrier populations of *E. osburni* appear to have experienced long-term allopatric separation.

Most of the comparisons between samples within populations resulted in low values of F_{ST} . Two comparisons yielded extremely low values. An index of $F_{ST} = -0.005$, interpreted as $F_{ST} = 0$, between Lower Gauley River and South Fork Cherry River implies complete panmixia. While this may have been the case historically, Summersville Dam is a major geographical barrier that prevents random mating between these two clusters of fish. For the same reason, an equivalently obscure index of $F_{ST} = 0.000$ was obtained with a comparison between Lower Gauley River and North Fork Cherry River. Relatively new geographical barriers such as dams can cause genetic divergence in fish, including rapid speciation in darters, between separated populations (Near and Benard 2004; Roberts et al. 2013). Summersville Dam, however, seems to have had little influence on differentiation compared to historical events. The low F_{ST} values for above and below Summersville Dam within the Gauley River drainage imply that the genetic differentiation between the Gauley and Greenbrier populations of *E. osburni* are due to reasons, such as natural divergence, other than the dam.

This study and a prior study (Switzer et al. 2007) have found strong evidence of the existence of Distinct Population Segments (DPS). There are two criteria for designation as a DPS: 1) discreteness; and 2) significance. The populations are discrete as evidenced by high genetic differentiation. There was a presence of a high number of private alleles in the Greenbrier population found in this study and a high rate of private alleles in both populations found by Switzer et al. (2007) using the full set of microsatellite

loci. There were high F_{ST} indices among all pairwise comparisons of samples between the drainages. Furthermore, the two populations are discrete because they are physically separated by distance. Switzer et al. (2007) discussed how the Gauley and Greenbrier populations are separated by over 600 km of river distance, have a long standing history of separation, and have experienced different demographic events. That study postulated that the Greenbrier population has maintained a consistently larger population over time compared to a possible bottleneck that the Gauley population may have experienced. Hence, the two populations have 1) discreteness due to both marked genetic and physical separation. The two populations have significance because 2) the loss of any extant population, including either the Gauley or Greenbrier population, of *E. osburni* would result in a significant gap in the range of this species. In addition, the Gauley and Greenbrier populations differ markedly in genetic characteristics. For these two reasons, the two populations meet the significance criterion. A concurrent study found that a hybrid swarm is in the active process of expansion in the Greenbrier River drainage (detailed in Chapter 2). This is causing a rapid loss of *E. osburni* in this drainage and will probably result in a complete loss of this population. Evidence of a low level of hybridization was also found in the Gauley population but a hybrid swarm was not detected. This population will face the same threat if *E. variatum* is successfully established in the Gauley River drainage. The imperiled status of these populations and this species as a whole would likely fulfill the status requirement for listing of the DPS as endangered or threatened. Although this study did not investigate *E. osburni* of Virginia, the same components of discreteness, significance, and status likely apply when including this cluster of *E. osburni*. Each population of *E. osburni* merits strong consideration for designation as a DPS.

This study was performed in tandem with another study that investigated the current geographic extent of an expanding hybrid swarm and, therefore, used the same genotype data set that was generated for that study. That study focused on identifying hybrid individuals with five diagnostic loci. Switzer et al. (2008) identified and evaluated 14 microsatellite loci for *E. osburni* which included 9 loci in addition to the diagnostic loci used for this study. The scope of the results and conclusions of this study are within the context of this suite of five diagnostic loci.

Conclusions

The Greenbrier and Gauley populations of *E. osburni* are genetically distinct. They are reproductively operating as separate populations and exhibit characteristic allelic diversities. They have a high level of population differentiation. These two populations exhibit a large quantity of morphometric overlap but have subtle differences in character ranges. The two populations appear to be in the process of speciation but is early in their independent trajectories

Management Implications

Regulations should be considered for reducing further introductions of *E. variatum* to the Gauley River drainage, as suggested in Chapter 2. Genetic diversity between the Gauley, Greenbrier, and Virginia populations of *E. osburni* can be investigated with the 9 additional microsatellite loci that were identified by Switzer et al. (2008) for a total of 14 loci. If it is determined that the Greenbrier and Virginia populations of *E. osburni* are not substantially differentiated, then pure *E. osburni* individuals from the Greenbrier population could be used in a reintroduction program to rescue this lineage. Former *E. osburni* range such as Bluestone River and Indian Creek drainages of West Virginia are reintroduction location options. Bluestone Dam may limit dispersal of *E. variatum* into this system. If the Greenbrier and Virginia populations are substantially differentiated, then risk evaluation may be prudent as introgression between these two populations of *E. osburni* may create further conservation concerns. The Gauley, Greenbrier, and Virginia populations of *E. osburni* should be considered for designation as Distinct Population Segments (DPS). *Etheostoma osburni* should be considered for listing as endangered under the U.S. Endangered Species Act. Furthermore, the biodiversity of West Virginia may benefit from the existence of a State Endangered Species Act for more effective conservation of native imperiled species.

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TABLES

Table 1. List of collection sites enumerated to correspond with map of study area. Total number of specimens, number of pure *E. osburni*, and percentage of *E. variatum* alleles of that sample is given. All coordinates are in Zone 17.

Site	Stream	Location Description	Drainage	n Total	n <i>E. osburni</i>	% <i>E. variatum</i> alleles	UTM N	UTM E
1	Bells C.	1.1 road miles from junction CR 16 X CR 39	Gauley	17	0	89.41	4232887	483116
2	Gauley R.	Swiss; Upstream 0.25 mile on Swiss Road	Gauley	33	0	84.55	4231525	488817
3	Gauley R.	Downstream of Summersville Dam at USGS gauging station	Gauley	31	25	1.94	4229683	509794
4	Cherry R.	Rt 20 bridge in Holcomb	Gauley	35	33	0.57	4234356	534874
5	Laurel C.	Namo Chapel; Just above mouth of McMillion Creek	Gauley	17	17	0.00	4223493	536452
6	N. F. Cherry R.	In Richwood at Johnstown Road bridge; Behind mill	Gauley	17	17	0.00	4231341	541739
7	S. F. Cherry R.	~1.05 air miles upstream of mouth/confluence with North Fork Cherry River	Gauley	16	16	0.00	4229809	543197
8	Gauley R.	Rt 20; 1.1 Road miles upstream Camp Caesar	Gauley	7	7	0.00	4250657	546185
9	Gauley R.	Bolair; 2.0 miles south of Rt 20 from junction CR 42	Gauley	15	14	0.67	4252624	546783
10	Gauley R.	On CR 42 between Rt 20 and site 9.0 miles above Rt 20	Gauley	2	2	0.00	4253707	553975
11	Gauley R.	9.0 miles above Rt 20 on CR 42	Gauley	13	13	0.00	4253288	559195
12	Greenbrier R.	Riffle above Rt 219 bridge in Ronceverte	Greenbrier	31	0	98.06	4177571	547282
13	Anthony C.	0.2 air miles above mouth	Greenbrier	9	0	98.89	4193987	559556
14	Anthony C.	At primitive campsite 0.9 miles below Blue Bend camp entrance	Greenbrier	9	0	91.11	4196407	562946
15	Anthony C.	16/2 bridge; near Bluebend National Recreation Area	Greenbrier	1	0	100.00	4197112	565084
16	Knapp C.	Above mouth of Browns Creek	Greenbrier	7	1	45.71	4228162	585845
17	Knapp C.	Above first bridge south of Frost on CR 92	Greenbrier	5	4	2.00	4235260	596196
18	Sitlington C.	Just below confluence of Galford and Shock runs	Greenbrier	13	12	0.77	4244680	600001
19	Deer C.	Above Rt 66 bridge just west of CR 92	Greenbrier	12	8	9.17	4251247	598885
20	E. F. Greenbrier R.	CR 28/19 bridge	Greenbrier	5	5	0.00	4267423	607928
21	Little R. E. F. Greenbrier R.	0.63 miles from intersection Rt 250 X Rt 28 near Thornwood	Greenbrier	31	29	0.65	4267486	610633
22	W. F. Greenbrier R.	75 m section above FR312	Greenbrier	9	8	3.33	4273165	602606
TOTAL				335	211			

Table 2. List of sites visited for this study that produced no specimens of either *E. osburni* or of hybrids. All coordinates are in Zone 17.

Stream	Location Description	Drainage	UTM N	UTM E
Anglins Creek	Rt. 41 bridge	Gauley	4221131	510002
Anthony Creek	Just below Blue Bend swimming area	Greenbrier	4197386	564545
Anthony Creek	Neola. Below mouth of meadow creek. Below CR14 bridge.	Greenbrier	4202498	576267
Becky Run	Just upstream of mouth	Gauley	4222640	547705
Dry Run	CR 17 bridge near hatchery	Greenbrier	4236281	578176
East Fork Greenbrier River	Above East Fork Greenbrier R. bridge in Durbin	Greenbrier	4266872	602886
Gauley River	Immediately downstream of Bolair	Gauley	4250259	544576
Gauley River	Just downstream of Summersville Dam	Gauley	4230002	509719
Greenbrier River	Below Rt. 219 bridge in Ronceverte	Greenbrier	4177647	546701
Griffith Creek	1.1 air miles west of Greenbrier River	Greenbrier	4175907	526618
Knapp Creek	2.0 road miles from CR 39 X Greenbrier River bridge in Marlinton	Greenbrier	4229820	581451
Knapp Creek	In Marlinton. Above 39/2 (5th Avenue) bridge	Greenbrier	4230537	579296
Laurel Creek	First crossing (19/25) just above Swiss	Gauley	4230728	489701
Leatherbark Run	By CR 1. Above impoundment at Cass Scenic Railroad Workshop	Greenbrier	4251123	594956
Meadow River	Old railroad trestle, now a footbridge	Gauley	4214920	509435
North Fork Deer Creek	Above CR 6 bridge	Greenbrier	4253331	604078
Rich Creek	~150m upstream mouth above first bridge to abandoned RR bridge	Gauley	4230814	486695
Rosen Run	First bridge upstream confluence with North Fork Deer Creek	Greenbrier	4252137	601988
Second Creek	.2 miles above Rt. 219 at CR 219/1 bridge	Greenbrier	4170760	548030
Sitlington Creek	CR 28/26 halfway between each terminus at Rt. 28	Greenbrier	4246726	597894
South Fork Cherry River	Above mouth of Little Blizzard Run	Gauley	4220967	550909
Swago Creek	228 m below US 219 bridge	Greenbrier	4227590	575685
Williams River	~1 mile above bridge on Woodrow Rd near Handley.	Gauley	4238848	569372

Table 3. Meristic characters evaluated for F1 hybrids and for *E. osburni* (data for Gauley and Greenbrier populations of *E. osburni* is combined). Data for *E. variatum* is from Page (1983) and Page and Burr (2011).

Character	<i>E. osburni</i> (n = 41)					F1 Hybrid (n = 4)					<i>E. variatum</i>
	Min	Max	Mean	SD	Mode	Min	Max	Mean	SD	Mode	
Dorsal-fin spines	11	13	12.2	0.44	12	12	14	12.75	0.96	NA	11 - 13
Dorsal-fin rays	12	15	13.4	0.70	13	13	13	13.00	0.00	13	12 - 16 (13)
Pectoral-fin rays	13	16	14.6	0.60	15	14	15	14.75	0.50	NA	14 - 16 (15)
Pelvic-fin rays	5	6	5.07	0.26	5	5	5	5.00	0.00	5	
Pelvic-fin spines	1	1	1.00	0.00	1	1	1	1.00	0.00	1	
Anal-fin rays	8	11	9.44	0.65	9	8	10	9.00	1.15	NA	8 - 10 (9 - 10)
Anal-fin spines	2	2	2	0.00	2	2	2	2.00	0.00	2	2
Scales above lateral line	6	9	7.51	0.64	7	7	8	7.25	0.50	NA	6 - 8 (6 - 7)
Scales below lateral line	8	11	9.37	0.77	9	8	10	9.00	0.82	NA	7 - 10 (8 - 9)
Scales along lateral line (left)	58	71	64.44	2.90	64	55	63	60.25	3.59	NA	48 - 60 (50 - 56)
Scales along lateral line (right)	59	71	64.39	2.72	68	54	62	58.75	3.4	NA	48 - 60 (50 - 56)
Circumpeduncle scales	21	27	23.59	1.12	23	21	24	22.75	1.26	NA	20 - 25 (21 - 23)
Dark lateral bars	10	14	11.73	1.19	12	7	10	8.33	1.53	NA	
Dorsolateral saddles	4	5	4.88	0.33	5	4	5	4.33	0.58	NA	4
Dorsal-fin saddle		95.1 % of specimens					0 % of specimens				
Breast squamation (embedded)		43.9 % of specimens					25 % of specimens				
Breast squamation (exposed)		unscaled					0 % of specimens				
Opercle squamation		unscaled					25 % of specimens				
Supraopercular squamation		unscaled					75 % of specimens				

Table 4. Sites represented in each sample used in STRUCTURE analysis, construction of a neighbor-joining tree, and for pairwise comparison of F_{ST} .

Sample	n _{Sample}	Drainage	No.	Stream	Location Description	n _{Site}
Little R. E. Fk. Greenbrier R.	29	Greenbrier	21	Little River East Fork Greenbrier River	.63 miles from intersection Rt 250 X Rt 28 near Thornwood	29
Upper Greenbrier R.	13	Greenbrier	20	East Fork Greenbrier River	CR 28/19 bridge	5
			22	West Fork Greenbrier River	75m section above FR 312	8
Sitlington C.	12	Greenbrier	18	Sitlington Creek	Just below confluence of Galford Run and Shock Run	12
Mid-Greenbrier R.	13	Greenbrier	16	Knapp Creek	Above mouth of Browns Creek	1
			17	Knapp Creek	Above first bridge south of Frost on CR 92	4
			19	Deer Creek	Above Rt 66 Bridge just West of 92	8
Laurel C.	17	Gauley	5	Laurel Creek	Namo Chapel	17
Cherry R.	33	Gauley	4	Cherry River	Rt 20 Bridge in Holcomb	33
N. Fk. Cherry R.	17	Gauley	6	North Fork Cherry River	In Richwood at Johnstown Road Bridge. Behind...	17
S. Fk. Cherry R.	16	Gauley	7	South Fork Cherry River	~1.05 air miles above mouth/confluence with...	16
Upper Gauley R.	36	Gauley	9	Gauley River	Bolair. 2.0 miles south of Rt 20 from junction CR 4	14
			10	Gauley River	On CR 42 between SR 20 and the site which was 9 miles from SR 20	2
			8	Gauley River	Rt 20; 1.1 Road miles upstream Camp Caesar	7
			11	Gauley River	9 miles above SR 20 on CR 42	13
Lower Gauley R.	25	Gauley	3	Gauley River	USGS Gauging Station just below Summersville Dam	25
TOTAL	211					

Table 5. Morphometric distances used in shape analysis of *E. osburni*. Variable enumeration corresponds to Figures 15 and 16. Box truss distance measurements, head length, outer-pelvic width, and caudal length (1 - 23, 26, 31) are presented as thousandths of standard length. Measurements taken on the head (24, 25, 27 - 30) are presented as thousandths of head length.

No.	Distance	Gauley (n = 16)				Greenbrier (n = 25)			
		Min	Max	Mean	SD	Min	Max	Mean	SD
SL	Standard length (mm)	63.1	85.8	74.2	4.9	57.8	84.2	70.3	7.1
1	Snout – occiput	200	221	212	6.1	198	221	210	6.6
2	Occiput – first dorsal-fin spine	105	145	124	10.7	104	145	130	10.9
3	Base of first dorsal fin	294	343	320	12.1	273	349	315	17.1
4	Base of second dorsal fin	184	230	205	10.8	194	240	215	11.3
5	Last dorsal fin ray – dorsal caudal flexure	166	192	176	6.8	152	193	171	10.7
6	Depth at caudal flexure	113	150	127	9.6	113	144	124	6.0
7	Last anal-fin ray – ventral caudal flexure	208	243	227	9.8	203	241	218	9.0
8	Base of anal fin	140	171	158	8.5	139	182	162	10.4
9	Mid-point between pelvic-fin bases – first anal-fin ray	287	340	314	13.0	291	344	318	14.4
10	Branchiostegal connection – midpoint between pelvic-fin bases	202	228	215	6.0	189	236	202	12.3
11	Snout – branchiostegal connection	91	111	101	5.3	92	136	104	8.2
12	Branchiostegal connection – occiput	164	188	175	5.6	150	181	167	8.3
13	Branchiostegal connection – first dorsal-fin spine	250	289	273	10.2	246	290	269	11.3
14	Occiput – mid-point between pelvic fin bases	210	240	219	7.7	194	233	211	10.2
15	Mid-point between pelvic fin bases – first dorsal-fin spine	184	217	200	9.9	180	228	201	10.9
16	Mid-point between pelvic fin bases – second dorsal-fin origin	352	388	361	10.6	317	392	361	17.0
17	First dorsal fin spine – anal-fin origin	366	398	383	8.3	352	397	377	13.2
18	Anal-fin origin – second dorsal-fin origin	179	214	198	8.8	173	212	190	10.3
19	Anal-fin origin – last dorsal-fin ray	233	270	253	8.9	222	271	251	12.7

20	Second dorsal-fin origin – last anal-fin ray	224	259	236	9.4	222	256	239	9.0
21	Last dorsal-fin ray – last anal-fin ray	130	149	136	5.0	114	138	125	5.9
22	Last anal-fin ray – dorsal caudal flexure	248	284	264	9.2	231	271	252	10.4
23	Last dorsal-fin ray – ventral caudal flexure	203	234	220	9.0	189	227	211	9.1
24	Snout – anterior margin of eye	338	381	358	13.7	314	397	356	19.0
25	Eye width	207	236	221	8.3	213	251	230	8.2
26	Snout – opercle spine tip	230	256	243	6.4	226	258	238	7.2
27	Anterior internasal width	151	182	166	10.0	147	187	166	9.1
28	Posterior internasal width	144	196	165	11.7	145	183	168	10.1
29	Interorbital width	122	172	150	13.5	104	159	137	14.9
30	Gape width	270	324	294	13.5	239	339	285	24.3
31	Outerpelvic base width	86	115	101	5.8	94	107	100	3.7
32	Caudal-fin length	175	194	186	5.4	97	193	171	17.7

Table 6. Meristic characters evaluated for Gauley and Greenbrier populations of *E. osburni*. Data for *E. osburni* from Page (1983) and Page and Burr (2011) is given.

Character	Page (1983); Page and Burr (2011)	Gauley (n = 16)					Greenbrier (n = 25)				
		Min	Max	Mean	SD	Mode	Min	Max	Mean	SD	Mode
Dorsal-fin spines	10 - 14 (12 - 13)	12	13	12.31	0.48	12	11	13	12.12	0.44	12
Dorsal-fin rays	12 - 16 (13 - 14)	12	14	13.06	0.68	13	13	15	13.64	0.70	13
Pectoral-fin rays		13	15	14.25	0.68	14	13	16	14.76	0.60	15
Pelvic-fin rays	14 - 15 (15)	5	6	5.19	0.40	5	5	5	5.00	0.00	5
Pelvic-fin spines		1	1	1.00	0.00	1	1	1	1.00	0.00	1
Anal-fin rays	8 - 11 (9 - 10)	9	10	9.25	0.45	9	8	11	9.56	0.65	10
Anal-fin spines	2	2	2	2.00	0.00	2	2	2	2.00	0.00	2
Scales above lateral line	6 - 9 (8)	7	9	7.75	0.86	7	6	8	7.36	0.64	7
Scales below lateral line	8 - 13 (9 - 10)	8	10	9.25	0.77	10	8	12	9.44	0.77	9
Scales along lateral line (Left)	58 - 70 (62 - 66)	60	66	63.31	1.78	64	58	71	65.16	2.90	62
Scales along lateral line (Right)	58 - 70	59	68	63.38	2.78	63	60	71	65.04	2.72	68
Circumpeduncle scales	21 - 25 (22 - 24)	22	27	23.81	1.11	24	21	25	23.44	1.12	23
Dark lateral bars		11	13	12.25	0.58	12	10	14	11.40	1.19	12
Dorsolateral saddles	5	4	5	4.88	0.34	5	4	5	4.88	0.33	5
Dorsal-fin saddle		100 % of specimens					92.0 % of specimens				
Breast squamation (embedded)		12.5 % of specimens					64 % of specimens				
Breast squamation (exposed)	unscaled	unscaled					unscaled				
Opercle squamation	variable	unscaled					unscaled				
Supraopercular squamation		unscaled					unscaled				

Table 7. Results of HWE calculation for each population of *E. osburni* per locus and across all loci using Fisher's Method (Fisher 1935). Weir and Cockerham (1984) estimate of F_{IS} is given.

Population	Locus	P-value	S.E.	F_{IS}
Greenbrier	<i>Eos-C2</i>	1.000	0.000	-0.091
	<i>Eos-C112</i>	0.273	0.021	0.062
	<i>Eos-C3</i>	1.000	0.000	-0.023
	<i>Eos-C6</i>	0.454	0.024	-0.003
	<i>Eos-C117</i>	0.757	0.014	-0.134
	All	0.908		
Gauley	<i>Eos-C2</i>	0.179	0.005	-0.122
	<i>Eos-C112</i>	0.244	0.018	0.058
	<i>Eos-C3</i>	No information		
	<i>Eos-C6</i>	0.641	0.009	0.025
	<i>Eos-C117</i>	0.760	0.008	-0.010
	All	0.463		

Table 8. Results of HWE calculation for each sample of *E. osburni* (as described in Table 4) per locus and across all loci using Fisher's Method (Fisher 1935). Weir and Cockerham (1984) estimate of F_{IS} is given.

Population	Locus	P-value	S.E.	F_{IS}
Little R. E. Fk. Greenbrier R.	<i>Eos-C2</i>	1.000	0.000	-0.037
	<i>Eos-C112</i>	0.271	0.013	0.136
	<i>Eos-C3</i>	1.000	0.000	-0.037
	<i>Eos-C6</i>	0.362	0.015	0.023
	<i>Eos-C117</i>	0.631	0.008	-0.243
	All	0.850		
Upper Greenbrier R.	<i>Eos-C2</i>	1.000	0.000	-0.044
	<i>Eos-C112</i>	0.352	0.016	0.140
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.254	0.009	0.247
	<i>Eos-C117</i>	0.329	0.009	0.032
	All	0.531		
Sitlington C.	<i>Eos-C2</i>	1.000	0.000	-0.044
	<i>Eos-C112</i>	0.521	0.011	0.036
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.564	0.009	-0.200
	<i>Eos-C117</i>	0.861	0.005	-0.061
	All	0.949		
Mid-Greenbrier R.	<i>Eos-C2</i>	1.000	0.000	-0.044
	<i>Eos-C112</i>	0.085	0.006	-0.136
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	1.000	0.000	-0.169
	<i>Eos-C117</i>	0.277	0.007	0.203
	All	0.483		
Laurel C.	<i>Eos-C2</i>	0.729	0.002	-0.085
	<i>Eos-C112</i>	0.071	0.008	0.197
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.589	0.006	0.073
	<i>Eos-C117</i>	0.172	0.005	-0.147
	All	0.231		

Cherry R.	<i>Eos-C2</i>	1.000	0.000	-0.044
	<i>Eos-C112</i>	0.662	0.005	0.103
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.474	0.006	0.202
	<i>Eos-C117</i>	0.510	0.005	0.135
	All	0.886		
N. Fk. Cherry R.	<i>Eos-C2</i>	0.309	0.003	-0.353
	<i>Eos-C112</i>	0.366	0.009	0.236
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.232	0.005	0.052
	<i>Eos-C117</i>	0.626	0.005	-0.200
	All	0.413		
S. Fk. Cherry R.	<i>Eos-C2</i>	0.518	0.003	0.125
	<i>Eos-C112</i>	0.515	0.016	-0.100
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.587	0.006	-0.067
	<i>Eos-C117</i>	1.000	0.000	-0.048
	All	0.883		
Upper Gauley R.	<i>Eos-C2</i>	0.052	0.001	-0.426
	<i>Eos-C112</i>	0.767	0.006	-0.056
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.544	0.006	-0.058
	<i>Eos-C117</i>	1.000	0.000	-0.095
	All	0.469		
Lower Gauley R.	<i>Eos-C2</i>	1.000	0.000	-0.222
	<i>Eos-C112</i>	0.974	0.003	-0.158
	<i>Eos-C3</i>	No Information		
	<i>Eos-C6</i>	0.725	0.011	-0.158
	<i>Eos-C117</i>	1.000	0.000	-0.100
	All	1.000		

Table 9. Pairwise comparison of F_{ST} among samples (as described in Table 4; above the diagonal) with P-values obtained after 900 permutations (below the diagonal). Indicative adjusted nominal level (5%) for multiple comparisons is 0.001. Significant values are listed in bold font.

Sample		Greenbrier River drainage				Gauley River drainage					
		Little R. E. F. Greenbrier R.	Upper Greenbrier R.	Sitlington C.	Mid-Greenbrier R.	Laurel C.	Cherry R.	N. F. Cherry R.	S. F. Cherry R.	Upper Gauley R.	Lower Gauley R.
Greenbrier River drainage	Little R. E. F. Greenbrier R.	0.000	-0.008	0.001	-0.008	0.186	0.145	0.160	0.162	0.201	0.136
	Upper Greenbrier R.	0.251	0.000	0.008	-0.004	0.142	0.121	0.134	0.122	0.179	0.104
	Sitlington C.	0.026	0.051	0.000	-0.005	0.147	0.110	0.122	0.123	0.161	0.103
	Mid-Greenbrier R.	0.644	0.242	0.307	0.000	0.159	0.123	0.147	0.150	0.205	0.130
Gauley River drainage	Laurel C.	0.001	0.001	0.001	0.001	0.000	0.012 ₄	0.017	-0.006	0.085	0.026
	Cherry R.	0.001	0.001	0.001	0.001	0.084	0.000	-0.011	0.012	0.046	0.011
	N. F. Cherry R.	0.001	0.001	0.001	0.001	0.354	0.619	0.000	-0.002	0.009	0.000
	S. F. Cherry R.	0.001	0.001	0.001	0.001	0.710	0.342	0.708	0.000	0.036	-0.005
	Upper Gauley R.	0.001	0.001	0.001	0.001	0.001	0.002	0.0578	0.018	0.000	0.012
	Lower Gauley R.	0.001	0.001	0.001	0.001	0.011	0.398	0.226	0.527	0.143	0.000

Table 10. Allelic frequency for the Gauley and Greenbrier populations of *E. osburni* ($n_{\text{Gauley}} = 67$; $n_{\text{Greenbrier}} = 144$; $n_{\text{Total}} = 211$). The status as a private allele is indicated.

Locus	Size (bp)	Greenbrier	Gauley	Overall	Private
<i>Eos-C2</i>	108	0.090	0.490	0.363	
<i>Eos-C2</i>	112	0.910	0.510	0.637	
<i>Eos-C112</i>	149	0.090	0.215	0.175	
<i>Eos-C112</i>	161	0.015	0.010	0.012	
<i>Eos-C112</i>	165	0.119	0.000	0.038	Greenbrier
<i>Eos-C112</i>	169	0.015	0.004	0.007	
<i>Eos-C112</i>	173	0.179	0.063	0.100	
<i>Eos-C112</i>	177	0.239	0.000	0.076	Greenbrier
<i>Eos-C112</i>	181	0.082	0.000	0.026	Greenbrier
<i>Eos-C112</i>	185	0.246	0.670	0.536	
<i>Eos-C112</i>	189	0.008	0.035	0.026	
<i>Eos-C112</i>	193	0.008	0.004	0.005	
<i>Eos-C3</i>	209	0.970	1.000	0.991	
<i>Eos-C3</i>	213	0.030	0.000	0.010	Greenbrier
<i>Eos-C6</i>	244	0.022	0.000	0.007	Greenbrier
<i>Eos-C6</i>	248	0.276	0.285	0.282	
<i>Eos-C6</i>	252	0.515	0.288	0.360	
<i>Eos-C6</i>	256	0.060	0.316	0.235	
<i>Eos-C6</i>	260	0.060	0.111	0.095	
<i>Eos-C6</i>	268	0.015	0.000	0.005	Greenbrier
<i>Eos-C6</i>	272	0.022	0.000	0.007	Greenbrier
<i>Eos-C6</i>	276	0.022	0.000	0.007	Greenbrier
<i>Eos-C6</i>	280	0.008	0.000	0.002	Greenbrier
<i>Eos-C117</i>	245	0.008	0.000	0.002	Greenbrier
<i>Eos-C117</i>	253	0.008	0.000	0.002	Greenbrier
<i>Eos-C117</i>	257	0.097	0.000	0.031	Greenbrier
<i>Eos-C117</i>	261	0.702	0.653	0.668	
<i>Eos-C117</i>	265	0.164	0.306	0.261	
<i>Eos-C117</i>	269	0.022	0.031	0.028	
<i>Eos-C117</i>	273	0.000	0.010	0.007	Gauley

Table 11. Summary of allelic variation of Gauley and Greenbrier populations of *E. osburni* including number of alleles, allelic size range, allelic richness, observed heterozygosity (H_o), and expected heterozygosity (H_e).

Population	Locus	No. Alleles	Size Range (bp)	Allelic Richness	H_o	H_e
Gauley n = 144	<i>Eos-C2</i>	2	108 - 112	2.000	0.563	0.502
	<i>Eos-C112</i>	7	149 - 193	5.777	0.472	0.501
	<i>Eos-C3</i>	1	209	1.000	0.000	-
	<i>Eos-C6</i>	4	248 - 260	4.000	0.708	0.726
	<i>Eos-C117</i>	4	261 - 273	3.845	0.486	0.481
	Mean			3.324	0.557	0.552
	SD			1.864	0.108	0.116
Greenbrier n = 67	<i>Eos-C2</i>	2	108 - 112	2.000	0.179	0.164
	<i>Eos-C112</i>	10	149 - 193	10.000	0.776	0.827
	<i>Eos-C3</i>	2	209 - 213	2.000	0.060	0.058
	<i>Eos-C6</i>	9	244 - 280	9.000	0.657	0.655
	<i>Eos-C117</i>	6	245 - 269	6.000	0.537	0.474
	Mean			5.800	0.442	0.436
	SD			3.768	0.309	0.323
Total n = 211	<i>Eos-C2</i>	2	108 - 112	2.000	-	0.463
	<i>Eos-C112</i>	10	149 - 193	9.041	-	0.665
	<i>Eos-C3</i>	2	209 - 213	1.785	-	0.019
	<i>Eos-C6</i>	9	244 - 280	6.902	-	0.728
	<i>Eos-C117</i>	7	245 - 273	5.302	-	0.485
	Mean			5.006	-	0.472
	SD			3.137	-	0.278

Table 12. Summary of 20 repeated measurements on a single individual for error quantification of morphometric distances. All values are presented in millimeters.

No.	Distance	(mm)			
		Min	Max	Mean	s ²
SL	Standard length	75.72	76.81	76.46	0.08
1	Snout – occiput	15.97	16.51	16.17	0.02
2	Occiput – first dorsal-fin spine	9.01	9.92	9.50	0.06
3	Base of first dorsal fin	24.12	25.21	24.59	0.10
4	Base of second dorsal fin	16.12	17.24	16.77	0.07
5	Last dorsal fin ray – dorsal caudal flexure	10.74	12.03	11.48	0.10
6	Depth at caudal flexure	9.57	9.83	9.70	0.01
7	Last anal-fin ray – ventral caudal flexure	15.61	17.29	16.48	0.17
8	Base of anal fin	12.06	13.56	12.55	0.14
9	Mid-point between pelvic-fin bases – first anal-fin ray	25.46	26.34	25.87	0.07
10	Branchiostegal connection – midpoint between pelvic-fin bases	14.54	15.58	15.19	0.09
11	Snout – branchiostegal connection	7.04	8.08	7.51	0.06
12	Branchiostegal connection – occiput	13.40	13.86	13.57	0.02
13	Branchiostegal connection – first dorsal-fin spine	20.08	21.61	20.90	0.12
14	Occiput – mid-point between pelvic fin bases	15.48	16.49	16.20	0.07
15	Mid-point between pelvic fin bases – first dorsal-fin spine	15.07	15.89	15.52	0.07
16	Mid-point between pelvic fin bases – second dorsal-fin origin	27.80	29.81	28.43	0.21
17	First dorsal fin spine – anal-fin origin	28.72	29.82	29.19	0.08
18	Anal-fin origin – second dorsal-fin origin	14.02	15.31	14.42	0.11
19	Anal-fin origin – last dorsal-fin ray	19.09	20.73	19.79	0.17
20	Second dorsal-fin origin – last anal-fin ray	17.78	18.50	18.09	0.04
21	Last dorsal-fin ray – last anal-fin ray	9.53	10.57	10.01	0.10
22	Last anal-fin ray – dorsal caudal flexure	17.70	19.86	19.26	0.24
23	Last dorsal-fin ray – ventral caudal flexure	14.44	15.78	15.13	0.13
24	Snout – anterior margin of eye	5.57	6.78	6.40	0.07
25	Eye width	4.15	4.44	4.26	0.01
26	Snout – opercle spine tip	18.01	18.55	18.28	0.02
27	Anterior internasal width	2.72	3.18	3.03	0.01
28	Posterior internasal width	3.00	3.37	3.16	0.01
29	Interorbital width	2.41	2.76	2.60	0.01
30	Gape width	5.58	6.02	5.77	0.01
31	Outerpelvic base width	7.35	7.73	7.47	0.01
32	Caudal-fin length	13.43	14.89	14.25	0.29

FIGURES

Figure 1. Distribution records of *E. osburni* in West Virginia from 1931 - 2015 (WVDNR unpublished data).

Figure 2. Distribution records of *E. variatum* morphometrically identified in the New River drainage, West Virginia from 1982 – 2014 (WVDNR unpublished data). These data do not include genetic detection of *E. variatum* alleles of the current study.

Figure 3. Collection sites for materials used in this study. Site labels correspond with Table 1.

Figure 4. Photograph depicting typical cephalic squamation of *Etheostoma variatum*. One scale patch is shown on the opercle. Two scale patches are shown in the supraopercular region. *Etheostoma osburni* X *Etheostoma variatum* may possess any or none of these scale patches.

Figure 5. STRUCTURE diagram depicting posterior probability of assignment (vertical axis) for all individuals collected at $K = 2$. Samples 1 – 10 are comprised of pure *E. osburni*. Horizontal axis is sample location number.

Figure 6. STRUCTURE diagram depicting posterior probability of assignment (vertical axis) for *E. osburni* at $K = 2$. Samples 3 – 8 are from the Gauley River drainage. Samples 1, 2, 9, and 10 are from the Greenbrier River drainage. Horizontal axis is sample location number.

Figure 7. Posterior probability of assignment (vertical axis) to six classifications: *E. osburni*, *E. variatum*, F1 hybrid, F2 hybrid, backcross with *E. osburni*, and backcross with *E. variatum*. Site numbers (horizontal axis) correspond to Table 1. Elevation in each drainage increases with distance from the dashed line. The relative location of Summersville Dam is indicated.

Figure 8. *Etheostoma variatum* alleles detected during this study (open diamonds). Solid circles indicate the presence of pure *E. osburni* in a sample. Open circles indicate the collection location of first generation (F1) hybrids of *E. osburni* X *E. variatum*. The proportion of *E. variatum* alleles within each sample where *E. variatum* alleles were detected is labeled.

Figure 9. *Etheostoma osburni* X *Etheostoma variatum*. 71 mm SL male, Deer Creek (Site 19), Pocahontas Co., WV, 7 March 2017. Photograph by Dr. Stuart A. Welsh.

Figure 10. *Etheostoma osburni* X *Etheostoma variatum*. 68 mm SL female, Deer Creek (Site 19), Pocahontas Co., WV, 7 March 2017. This individual was gravid at the time of collection. Photograph by Dr. Stuart A. Welsh.

Figure 11. *Etheostoma osburni*. 78 mm SL male, East Fork Greenbrier River, Pocahontas Co., WV, 4 April 2016. Photograph by Dr. Stuart A. Welsh.

Figure 12. *Etheostoma osburni*. 65 mm SL female, Little River East Fork Greenbrier River, Pocahontas Co., WV, 28 March 2017. Photograph by Dr. Stuart A. Welsh.

Figure 13. *Etheostoma variatum*. 66 mm SL male, Twentymile Creek, Nicholas Co., WV, 19 April 2017. Photograph by Dr. Stuart A. Welsh.

Figure 14. *Etheostoma variatum*. 57 mm SL female, Twentymile Creek, Nicholas Co., WV, 19 April 2017. Photograph by Dr. Stuart A. Welsh.

Figure 15. Box truss network comprised of 23 distances for morphometric analysis of *E. osburni* and *E. osburni* X *E. variatum*. Numeric labels correspond to Table 5.

Figure 16. Supplemental distances for morphometric analysis of *E. osburni* and *E. osburni* X *E. variatum*. Labels correspond to Table 5.

Figure 17. Neighbor-joining tree based on 1000 generated trees. Values presented are percents of 1000 generated trees that group the samples together. The drainage to which each sample belongs is labeled in parentheses. Members of each sample are described in detail in Table 4.

Figure 18. Plots of the sheared second and third principal components (SPC2 and SPC3) that depict shape differences between the Gauley (triangles) and Greenbrier (circles) populations of *E. osburni*.

APPENDICES

Appendix 1. Posterior probability of assignment to six species/hybrid classifications. Site numbers that are listed correspond to Table 1. Each row represents an individual (n = 335).

Site	<i>E. osburni</i>	<i>E. variatum</i>	F1 Hybrid	F2 Hybrid	Backcross w/ <i>E. osburni</i>	Backcross w/ <i>E. variatum</i>
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.002	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.002	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
5	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.767	0.000	0.000	0.006	0.227	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000
8, 9, 10, 11	0.999	0.000	0.000	0.000	0.001	0.000

[illegible]

[illegible]

[illegible]

Site	<i>E. osburni</i>	<i>E. variatum</i>	F1 Hybrid	F2 Hybrid	Backcross w/ <i>E. osburni</i>	Backcross w/ <i>E. variatum</i>
2	0.000	0.978	0.000	0.000	0.000	0.022
2	0.000	0.000	0.004	0.166	0.002	0.828
2	0.000	0.000	0.511	0.052	0.321	0.116
2	0.000	0.977	0.000	0.000	0.000	0.023
2	0.000	0.994	0.000	0.000	0.000	0.006
2	0.000	0.977	0.000	0.000	0.000	0.023
2	0.000	0.190	0.000	0.010	0.000	0.801
2	0.000	0.994	0.000	0.000	0.000	0.006
2	0.000	0.978	0.000	0.000	0.000	0.022
2	0.000	0.994	0.000	0.000	0.000	0.006
2	0.000	0.849	0.000	0.001	0.000	0.150
2	0.000	0.967	0.000	0.000	0.000	0.033
2	0.000	0.019	0.000	0.023	0.000	0.958
2	0.000	0.000	0.655	0.026	0.077	0.243
2	0.000	0.993	0.000	0.000	0.000	0.007
2	0.000	0.994	0.000	0.000	0.000	0.006
2	0.000	0.000	0.701	0.024	0.089	0.186
2	0.000	0.305	0.000	0.022	0.000	0.674
2	0.000	0.994	0.000	0.000	0.000	0.006
2	0.000	0.966	0.000	0.000	0.000	0.033
2	0.000	0.978	0.000	0.000	0.000	0.022
1	0.000	0.978	0.000	0.000	0.000	0.022
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.072	0.000	0.034	0.000	0.894
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.975	0.000	0.000	0.000	0.025
1	0.000	0.880	0.000	0.001	0.000	0.119
1	0.000	0.883	0.000	0.002	0.000	0.116
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.373	0.000	0.012	0.000	0.616
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.881	0.000	0.001	0.000	0.118
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.176	0.000	0.026	0.000	0.798
1	0.000	0.586	0.000	0.009	0.000	0.405
1	0.000	0.513	0.000	0.005	0.000	0.482
1	0.000	0.994	0.000	0.000	0.000	0.006
1	0.000	0.978	0.000	0.000	0.000	0.022
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.978	0.000	0.000	0.000	0.022
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.967	0.000	0.000	0.000	0.033

Site	<i>E. osburni</i>	<i>E. variatum</i>	F1 Hybrid	F2 Hybrid	Backcross w/ <i>E. osburni</i>	Backcross w/ <i>E. variatum</i>
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.849	0.000	0.001	0.000	0.150
12	0.000	0.978	0.000	0.000	0.000	0.022
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.978	0.000	0.000	0.000	0.022
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.379	0.000	0.007	0.000	0.614
12	0.000	0.994	0.000	0.000	0.000	0.006
12	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.978	0.000	0.000	0.000	0.022
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.978	0.000	0.000	0.000	0.022
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.881	0.000	0.001	0.000	0.119
13, 14, 15	0.000	0.836	0.000	0.002	0.000	0.162
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
13, 14, 15	0.000	0.961	0.000	0.001	0.000	0.037
13, 14, 15	0.000	0.978	0.000	0.000	0.000	0.022
13, 14, 15	0.000	0.880	0.000	0.001	0.000	0.119

Site	<i>E. osburni</i>	<i>E. variatum</i>	F1 Hybrid	F2 Hybrid	Backcross w/ <i>E. osburni</i>	Backcross w/ <i>E. variatum</i>
13, 14, 15	0.000	0.881	0.000	0.001	0.000	0.118
13, 14, 15	0.000	0.994	0.000	0.000	0.000	0.006
16	0.000	0.000	0.038	0.067	0.003	0.893
16	0.000	0.000	0.061	0.151	0.029	0.759
16	0.999	0.000	0.000	0.000	0.001	0.000
16	0.138	0.000	0.001	0.049	0.812	0.000
16	0.000	0.000	0.660	0.026	0.079	0.236
16	0.000	0.000	0.068	0.141	0.776	0.016
16	0.000	0.994	0.000	0.000	0.000	0.006
17	0.999	0.000	0.000	0.000	0.001	0.000
17	0.851	0.000	0.000	0.003	0.146	0.000
17	0.999	0.000	0.000	0.000	0.001	0.000
17	0.999	0.000	0.000	0.000	0.001	0.000
17	0.999	0.000	0.000	0.000	0.002	0.000
18	0.848	0.000	0.000	0.003	0.149	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.999	0.000	0.000	0.000	0.002	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.998	0.000	0.000	0.000	0.002	0.000
18	0.999	0.000	0.000	0.000	0.002	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.999	0.000	0.000	0.000	0.002	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
18	0.999	0.000	0.000	0.000	0.002	0.000
18	0.999	0.000	0.000	0.000	0.001	0.000
19	0.999	0.000	0.000	0.000	0.002	0.000
19	0.000	0.000	0.652	0.026	0.076	0.246
19	0.999	0.000	0.000	0.000	0.001	0.000
19	0.999	0.000	0.000	0.000	0.002	0.000
19	0.999	0.000	0.000	0.000	0.001	0.000
19	0.999	0.000	0.000	0.000	0.002	0.000
19	0.815	0.000	0.000	0.004	0.181	0.000
19	0.999	0.000	0.000	0.000	0.001	0.000
19	0.999	0.000	0.000	0.000	0.001	0.000
19	0.118	0.000	0.000	0.055	0.827	0.000
19	0.000	0.000	0.001	0.104	0.895	0.000
19	0.999	0.000	0.000	0.000	0.002	0.000
20	0.999	0.000	0.000	0.000	0.002	0.000
20	0.999	0.000	0.000	0.000	0.001	0.000
20	0.999	0.000	0.000	0.000	0.001	0.000
20	0.999	0.000	0.000	0.000	0.001	0.000

Site	<i>E. osburni</i>	<i>E. variatum</i>	F1 Hybrid	F2 Hybrid	Backcross w/ <i>E. osburni</i>	Backcross w/ <i>E. variatum</i>
20	0.999	0.000	0.000	0.000	0.002	0.000
21	0.998	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.945	0.000	0.000	0.002	0.054	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.853	0.000	0.000	0.003	0.144	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.998	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.998	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
21	0.999	0.000	0.000	0.000	0.002	0.000
21	0.999	0.000	0.000	0.000	0.001	0.000
22	0.999	0.000	0.000	0.000	0.001	0.000
22	0.999	0.000	0.000	0.000	0.001	0.000
22	0.998	0.000	0.000	0.000	0.002	0.000
22	0.000	0.000	0.006	0.101	0.893	0.001
22	0.998	0.000	0.000	0.000	0.002	0.000
22	0.999	0.000	0.000	0.000	0.001	0.000
22	0.999	0.000	0.000	0.000	0.002	0.000
22	0.998	0.000	0.000	0.000	0.002	0.000
22	0.998	0.000	0.000	0.000	0.002	0.000