# Current Status of the Bluestripe Shiner, *Cyprinella callitaenia*, in Alabama tributaries of the Chattahoochee River

By

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## Abstract

The imperilment of many freshwater fish species has become increasingly evident across the Southeastern United States in the last half-century. Increases in water usage, urbanization, and habitat alteration, as well as decreases in flow and habitat connectivity have all contributed to the imperilment and extirpation of many populations of Southeastern fishes. The impacts of these man-made changes are especially hard on lotic species with specific life history needs and historically high population connectivity such as the Bluestripe Shiner, *Cyprinella callitaenia*. Populations of this species have been steadily declining across its range within the Chattahoochee, Flint, and Apalachicola rivers since the species was described in 1957. There is evidence that this decline has been greater in Alabama tributaries of the Chattahoochee river, than in other areas of its native range in Georgia and Florida. To better understand this decline and determine potential catalysts, we collected both eDNA samples and physical specimens from across the specie's range in Alabama, Georgia, and Florida. From these, we determined the current distribution of the species within Alabama and the genetic integrity of several populations within the ACF drainage. We believe that the Bluestripe Shiner has been extirpated from most of its native range within Alabama. Furthermore, the species does not seem to be currently hybridizing with its sister species, the Blacktail Shiner, but is merely unable to adapt to increasing habitat degradation, increasing population isolation, and decreasing water availability. Alternatively, past hybridization could have occurred long enough ago or so rapidly that Bluestripe Shiner DNA has been eliminated from Blacktail Shiner populations over time, leaving no trace of the event. This analysis offers greater insight into the effects humans have had on

specialized aquatic species within the ACF, and will hopefully aid in the management and protection of sensitive aquatic communities throughout the Southeastern United States.

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## List of Abbreviations

| | |
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| ACF | The Apalachicola, Chattahoochee, and Flint rivers |
| DArT | Diversity Array Technology |
| eDNA | Environmental DNA |
| GIS | Geographic Information Systems |
| NGS | Next Generation Sequencing |
| PCoA | Principle Coordinate Analysis |
| PCR | Polymerase Chain Reaction |
| SNPs | Single Nucleotide Polymorphisms |
| Fts | Pairwise Fixation Indexes |

## Introduction

Freshwater ecosystems provide a unique home to some of the most diverse, rare, and sensitive fish species on earth (Walsh et al. 2011). Human exploitation of these systems and the surrounding biota has generated many negative pressures upon native species, sending diversity into a downward spiral. Over the last century, increases in urbanization, impoundments, water usage, non-native species introductions, agriculture, pollution, sedimentation, overharvesting, and land use change have all contributed to a large decline in freshwater species across the globe (Warren et al. 2000; Strayer and Dudgeon 2010). While some fish species inhabit large geographic ranges, many are very limited in their distribution, only residing in a few systems or even a single drainage. Having such a limited range can result in high endemism and high turnover for populations or species, especially when an outside pressure is introduced (Strayer and Dudgeon 2010). Therefore, studying fish species on a regional scale and evaluating historical and current threats to species diversity is necessary to protect and preserve imperiled species, endemic populations, and native communities (Jelks et al. 2008).

One region of great conservation concern is the Southeastern United States. This region is the most biologically diverse freshwater ecosystem in North America, supporting hundreds of native and endemic species, which comprise about 47% of freshwater fishes in the U.S. (Warren et al. 1997; Warren et al. 2000). Of the ten states with the highest freshwater fish diversity, seven are in the Southeast. These include Alabama, Tennessee, and Georgia which each contain more than 300 native fish species, with both Alabama and Tennessee supporting approximately one third of freshwater fish species native to the U.S. (Warren and Burr 1994; Boschung and Mayden 2004). This richness of species makes tributaries and drainages in the Southeast, especially those

running through Tennessee, Alabama, and Georgia, some of the most diverse waterways in the world (Jelks et al. 2008).

Of the 650 native species found in the region, over a quarter are considered imperiled (Warren et al. 2000). Many Southeastern fish assemblages have been heavily altered by impoundments, increasing urbanization, and land-use change (Walters et al. 2005; Warren et al. 2011). Land use change from riparian forestland to urban developments or agricultural use along these waterways has led to increased sedimentation, increased water temperatures, and decreased water availability in many large streams and rivers in the Southeast (Warren et al. 1997; Jelks et al. 2008; Warren et al. 2011). Since many fishes in the Southeast are endemic or have populations that are becoming increasingly isolated, even slight alterations in local habitat quality and water availability can extirpate a species (Warren and Burr 1994; Warren et al. 2000). Species specialized for benthic habitats, spring habitats, or riverine habitats are at high risk for imperilment and extirpation (Warren et al. 2011; Dowling 2012). These specialists have evolved a life history strategy that is dependent upon a heterogenous environment containing several types of habitat and various flow regimes (Maceina et al. 2007). Once this habitat is covered by sand, silt, or debris, or no longer accessible due to flow restrictions, the area may no longer be habitable for the species, and populations can become isolated from source populations, which eliminates geneflow and increases inbreeding depression, or extirpated entirely from a system (Walsh et al. 2011; Taylor and Peterson 2014). Unfortunately, almost all large river systems in the United States are now dammed or restricted which has led to reduced spawning runs of anadromous species, decreased lotic habitat, increased population isolation, and increased species imperilment (Warren et al. 2000; Taylor et al. 2018).

Increasing habitat degradation and population isolation can increase the risk of competition with other native and non-native species and can also increase the likelihood of hybridization (Schönhuth and Mayden 2009; Dakin et al 2015). These species are either already adapted to the new altered habitat, or they are able to quickly adapt to the rapid decline in habitat and water quality (DeVivo 1995; Johnston and Maceina 2008). These "generalists' usually exhibit behavioral attributes such as broad feeding tendencies, non-specific habitat preferences, and opportunistic reproductive strategies which can increase the likelihood of successful establishment (Ward 2012; Lawson and Johnston 2015). This can happen within an isolated population or even within a population with initially high abundances (Mooney and Cleland 2001). The Blacktail Shiner, *Cyprinella venusta*, is one such species that has been shown to be able to alter its life history parameters to survive in conditions that other sympatric species cannot (Casten and Johnston 2008). This success in survival can lead species like the Blacktail Shiner to become ubiquitous in areas where they historically were not a dominant species or have become introduced, leading to competition and even genetic mixing with congeneric species that are unable to adapt.

Another contributing factor to freshwater fish imperilment is hybridization, which is known to occur in Actinopterygii more than any other class of vertebrates (Scribner et al. 2001; Broughton et al. 2011; Higgins et al. 2015). Factors such as external fertilization, the lack of behavioral isolating mechanisms, limited spawning habitat, the increasing prevalence of non-native congeneric species, increases in habitat disturbance, and increased habitat homogeneity all contribute to the high rates of hybridization within fishes (Scribner et al. 2001; Dakin et al. 2015). Natural hybridization can be contained within a small geographic area known as hybrid zones (Ward et al. 2012). These zones can separate two parental populations that remain pure

and do not mix. However, mixing can continue to occur often rapidly until a hybrid swarm is formed. This can flood the system with introgressed individuals and can lead to the extirpation of one or both parental populations (Scribner and Page 2001; Broughton et al. 2011; Ward et al. 2012). Hybridization drives rare taxa to extirpation or extinction mainly through genetic swamping, where the rare species is replaced by a breeding population of hybrids effectively turning two species into one (Broughton et al. 2011). Hybridization can also hinder a species through demographic swamping, which affects population growth rates by allocating energy towards the production of maladaptive hybrids instead of viable offspring (Todesco et al. 2016).

Genetic swamping is especially prevalent when a non-native congeneric species is introduced into a system (Koppelman 2015; Ward et al. 2012; Taylor et al. 2018). This is usually due to a lack of reproductive isolation mechanisms usually present between sympatric congeneric species (Higgins et al. 2015; Ward et al. 2012). Non-native fish introductions can happen through accidental release or through the intentional stocking of non-natives. Hybridization events from non-native species introductions have been documented in the Southeast in several species including within the genus *Cyprinella* (DeVivo et al. 1995; Broughton et al. 2011; Taylor et al. 2018). Non-native introductions can also produce hybrid swarms which can quickly inundate a system with introgressive hybridization (Scribner and Page 2001; Broughton et al. 2011).

Two studies from the Coosa river in Alabama and Georgia, found widespread introgression between two species of *Cyprinella* due to the formation of a hybrid swarm (Walters and Blum 2008; Ward et al. 2012). The invasive Red Shiner, *C. lutrensis*, seemed to have a dominate phenotype over the Blacktail Shiner, producing hybrids closely resembling Red Shiner in size and coloration. This could be the reason that Red Shiner have remained prevalent in the

system as hybrids began to preferentially mate with individuals most closely resembling maternal parental taxa (Ward et al. 2012). There is a possibility that these hybrid swarms can advance so rapidly that they go undetected and can lead to species erosion being mistaken for displacement from factors like competition instead of hybridization (Walters and Blum 2008; Ward et al. 2012).

Interspecific introgression and the formation of hybrid swarms are not limited to non-native introductions and have been seen to occur between naturally sympatric species. This is often brought on by human caused disturbances which can breakdown former reproductive isolation mechanisms that separated two species (Hasselman et al. 2014). This phenomenon has been coined reverse-speciation as two divergent species that have evolved barriers to genetic mixing begin to exhibit admixture and introgression (Seehausen 2006; Taylor et al. 2006; Hasselman et al. 2014). Examples of reverse-speciation have been seen in many species of freshwater fish. Precipitated by the loss of habitat heterogeneity, several populations of species of three-spine sticklebacks in British Columbia began admixing and developed hybrid swarms (Taylor et al. 2006). Another study determined that habitat alteration created hybrid swarms between two sympatric species of landlocked alewife in a Northeaster U.S. reservoir. These swarms were not present within populations of the species that remained anadromous (Hasselman et al. 2014).

Pressures from competition and hybridization along with habitat degradation have led to several species becoming locally extirpated from sites that they historically inhabited within the Apalachicola, Chattahoochee, and Flint river systems, ACF (Shepard et al. 1995; Johnston and Farmer 2004; Taylor and Peterson 2014). The Shoal Bass is a species of *Micropterus* that has been studied extensively in the last decade. Shoal Bass are considered habitat specialists and

prefer medium to large sized rivers (Williams and Burgess 1999). They also depend upon habitat connectivity and habitat heterogeneity which have both been shown to be key factors in Shoal Bass life history (Johnston and Kennon 2007). Several extirpations of this species have been recorded, including all populations from within Alabama tributaries, which are believed to be functionally extirpated (Katechis 2015; Taylor et al. 2018). Factors attributed to the loss of Shoal Bass from Alabama include habitat loss, water loss, population isolation, and competition and introgression with native and non-native congeners (Taylor et al. 2018).

The Bluestripe Shiner, *Cyprinella callitaenia*, is another specialist species in decline along its native range in the ACF basin in Alabama, Georgia, and Florida. First described in 1956 by Bailey and Gibbs, the Bluestripe Shiner was named *Notropis callitaenia* and placed within what is now the genus *Cyprinella*. *Cyprinella* is one of the most widespread genera in the family Cyprinidae, consisting of 30 species from central and eastern North America (Schonhuth and Mayeden 2010). Many species of *Cyprinella* are found in abundance within the Southeastern United States, including the ACF basin. The Bluestripe Shiner is distinguished from the sympatric Blacktail Shiner, *Cyprinella venusta*, by its prominent steel blue mid-lateral band, uniformed tubercle arrangement, lack of caudal spot, and other morphological characteristics. Historically, it was believed that the Bluestripe Shiner was the sister species of the Ocmulgee Shiner (*Cyprinella callisema*) based on similar osteological characters (Bailey and Gibbs 1956). More recently, the Bluestripe Shiner has been placed in the Southern Appalachian clade of *Cyprinella*, which includes Ocmulgee Shiner, and is sister species with the Bannerfin Shiner, *Cyprinella leedsi*, found in the Altamaha Drainage of South Carolina and Georgia (Schonhuth and Mayden 2009).

The Bluestripe Shiner is believed to spawn from April through August, utilizing rock crevices as spawning sites. Females are known to hold between 88 and 230 mature eggs within their ovaries but are believed to be able to deposit many more throughout the breeding season by utilizing fractional spawning (Wallace Ramsey 1981). Other life-history aspects of the species have not been studied (Shepard et al. 1995). This species is found both above and below the Fall line in medium to large size streams over sandy or rocky substrate with no vegetation, and strong to moderate flow compared the Blacktail Shiner's more ubiquitous habitat selection (Bailey and Gibbs 1956; Shepard et al. 1995; Casten and Johnston 2009).

Populations of Bluestripe Shiner have been historically found throughout larger Alabama tributaries of the Chattahoochee River (Fig. 2). The species has also been sporadically found in reservoirs in Alabama, with one population historically known to exist in the holding pond at Farley Nuclear Power Plant in Houston County (Shepard et al. 1995). According to records provided by both ADCNR and Georgia DNR, individuals have been collected from at least 28 localities within Alabama between 1957 and 2013, including localities from 12 Alabama tributaries of the Chattahoochee (Appendix 1, Figure 1).

Since its description, the species has been observed to hybridize with other species of *Cyprinella.* Several hybrid individuals of Bluestripe x Blacktail shiner have been observed within Alabama streams (Bailey and Gibbs 1957; Maceina et al. 2007). As well as one individual believed to be a cross between Bluestripe Shiner and Red Shiner, *Cyprinella lutensis,* (Wallace and Ramsey 1982). In all three instances, individuals were identified as hybrid based on morphological characteristics. Hybridization, though fairly common within this genus, is often only locally occurring and complete integration between species is not common (Schonhuth and Mayden 2009; Higgens et al. 2015). While Blacktail Shiner and Bluestripe Shiner both remain in

Alabama, the invasive Red Shiner does not seem to have become established within this portion of the Chattahoochee and populations of Red Shiner are not believed to be currently residing in this area (C. Johnston, Personal Communication, September 2019). So far, no genetic analysis has been conducted to determine the genetic integrity of Alabama Bluestripe Shiner populations or if hybridization is continuing to occur between the Bluestripe Shiner and other congeneric species.

The widespread impoundment of sections of the Chattahoochee River within Alabama has been occurring frequently since before the 1950s (Shepard 1995; Williams and Burgess 1999; Taylor et al 2018). The Chattahoochee River was historically free flowing along its expanse in Alabama, approximately 170 miles. This would have connected all of Alabama's Chattahoochee tributaries, allowing for widespread geneflow across the Bluestripe Shiner's range. Its main channel is now disrupted by ten impoundments within Alabama alone. Within these impoundments, once lotic, well-oxygenated habitat is now lentic and anoxic. Walter F, George reservoir encompasses 70.6 square miles, and has turned tributaries that were historically connected directly to the Chattahoochee into lentic systems.

Fish assemblage declines have been recently documented in major Chattahoochee River tributaries in Alabama (Johnston and Maceina 2008; Dowling 2012; Lawson and Johnston 2015). These declines are usually due to the disappearance of specialist species, like the Bluestripe Shiner, and the emergence of generalist species (Johnston and Farmer 2004; Maceina et al 2007). At some sites, fish assemblages have become dominated by Blacktail Shiner, which has increased in abundance and range in these streams (Casten and Johnston 2008; Lawson and Johnston 2015). It is possible that hybridization has resulted as this species encounters Bluestripe Shiner, or it has outcompeted the species for degraded habitat.

Bluestripe Shiner populations have been noticeably declining in Alabama since the species was first described in 1957 (Wallace and Ramsey 1981; Shepard et al. 1995; Maceina et al. 2007). Several historical populations are now believed to be extirpated and the status of the species in Alabama is currently unknown (Shepard et al. 1995; Johnston and Farmer 2004). Since 2006 the State of Georgia has listed the status of the Bluestripe Shiner as Rare along with the State of Florida which lists the species as Rare and Imperiled. The species has not been given a special status by the U. S. Fish and Wildlife Service (USFWS), or by the State of Alabama, but was elevated to a conservation status of Highest Conservation Concern by the Alabama Nongame Fish Committee in 2012. With such low numbers of individuals believed to be remaining within Alabama waterways, traditional sampling methods may not be enough to detect the presence of the species at historical locations. Therefore, a method that would allow for the detection of rare species or small populations would be needed to fully determine if viable populations of Bluestripe Shiner are still extant.

Environmental DNA, or eDNA, is a forensic technique that has been proven to work in detecting rare and endangered species without the need of a physical specimen (Janosik and Johnston 2015). If a species is present near a targeted area, it could leave behind scales, feces, and slime which, through a simple water sample, can be detected. eDNA sampling has been found to be far superior to traditional sampling in both success rate and sampling time. One study found that traditional detection of the imperiled Slackwater Darter only produced individuals at one out of the 49 sites samples compared to eDNA sampling which detected 23 positive locations for the species (Janosik and Johnston 2015).

Due to the relatively rapid degradation and dilution of DNA within waterways, the positive detection of a species leads to the conclusion that the species is present in close

proximity to the sample site (Barnes et al. 2014; Shollenberger 2019). While stream size can affect detection probability (Jane et al. 2015), several studies have successfully detected rare and previously non occurring species in large river systems. The Alabama Sturgeon was thought to be extinct or so rare that detection was not possible using traditional methods. Using eDNA, the sturgeon has been detected in several sites along the mainstem Alabama River (Pfleger et al. 2016). The Snail Darter, a species much smaller in body size than the Alabama Sturgeon, was previously not thought to occur in several stretches and tributaries of the Tennessee River. Using eDNA, it has now been detected in over 30 sites in both the Tennessee mainstem and tributaries within Alabama and Tennessee (Shollenberger 2019).

While eDNA can detect the presence or absence of a species based on the creation of a species-specific primer, determining if the number of individuals detected and whether they are pure or genetic hybrids is not possible through eDNA alone (Thomsen and Willerslev 2015). Therefore, conducting empirical sampling at positive sites for the targeted species and collecting individuals is necessary to determine the status of a population. While traditionally species were identified based on phenotypic characteristics and hybridization was noted when an individual possessed traits ascribed to two different species, it has been shown that hybrids can exhibit phenotypic traits favoring one parental phenotype over another, effectively masking the ability to detect hybridization through phenotypic identification alone (Ward et al. 2012; Todesco et al. 2016). The use of genetic markers can provide powerful descriptive and predictive insight into instances of fish hybridization (Scribner and Page 2001), but conducting a genetic analysis on many individuals from various populations of several *Cyprinella* species can be time consuming and costly. An efficient and cost-effective technique will be needed to investigate hybridization within our populations.

Next generation sequencing, NGS, is a technique that can generate large amounts of sequence data rapidly and can provide thousands of genetic markers without the need for prior genetic sequencing (Ekblom and Galindo 2011; Melville et al. 2017). There are many methods of NGS sequencing, some of which utilize restriction enzymes to produce a representation of an individual's genome. DArTseq, is one such method which combines the genome complexity reduction power of DArT, Diversity Array Technology, with NGS platforms (Melville et al. 2017). While DArTseq was originally utilized for applications in plant species, it has recently been applied to vertebrate studies, including ichthyofauna (Nguyen et al. 2018; Hamilton et al. 2019), and has been used for applications such as the study of inter- and intra-species genetic diversity and relationships (Melville et al. 2017).

DArTseq reduces genome complexity by using a combination of endonucleases which targets low-copy DNA fragments, allowing for the detection of a high number of informative genetic single nucleotide polymorphisms, SNPs, across the genome. This provides a 'representation' across individuals within a data set. Through NGS, these 'representations' produce a small sequence of DNA and compare each individual's genetic state to all others as either one of two forms of homozygous states or as heterozygous (Melville et al. 2017; Hamilton et al. 2018). Data generated through DArTseq can be processed and analyzed using various software platforms including R programming through the package dartR. The processing power of DArTseq in reducing genome complexity, the low cost per sample, and the ability to reveal relationships within and between species makes this application ideal for determining the genetic status of Bluestripe Shiner populations and to determine if populations in Alabama and Georgia are interbreeding with other congeneric species.

The decline of the Bluestripe Shiner in Alabama has not been thoroughly studied. Few surveys have been conducted for the species since its description, with the last statewide survey being over two decades old. Therefore, there is not enough recent data to determine a current distribution of the Bluestripe Shiner within Alabama. A genetic analysis of imperiled populations of Bluestripe Shiner has also not been conducted. This analysis could determine if hybridization with sympatric species of *Cyprinella* is playing a role in the disappearance of the species along with anthropogenic impacts.

We believe that this species has been extirpated from most, if not all of its historical range in Alabama. We also believe that hybridization with congeneric species could be playing a role in this extirpation. Our goal is to utilize eDNA along with empirical sampling techniques and DArTseq to determine a current distribution for and the genetic integrity of Bluestripe Shiner populations in Alabama. This study seeks to answer three main questions

1. What is the current distribution of the Bluestripe Shiner in Alabama?
2. What is the current genetic integrity of the Bluestripe Shiner populations within Alabama?
3. What are possible catalysts contributing to the extirpation of the Bluestripe Shiner in Alabama?

## Materials and Methods

### *Site Selection*

In recent decades, several studies have conducted surveys for Bluestripe Shiner in Alabama (Shepard et al. 1995; Johnston and Farmer 2004; Johnston and Maceina 2008; Dowling 2012). The most extensive survey for the species includes 48 localities within Alabama, sampled

during the summers of 1994 and 1995 (Shepard et al. 1995). Another survey, conducted to determine the status of several imperiled species, includes thirty-four sites within the Uchee watershed (Johnston and Farmer 2004). Site coordinates from these studies along with other historic sites where Bluestripe Shiner individuals had been captured (ADCNR) were compiled into a data set and imported into Arc Map for possible use for this study. Some sites were not used because they were no longer accessible, or they were unsuitable for sampling. Additional sites were added during this survey based on perceived viable habitat and ease of access for sampling. Several sites were also surveyed more than once because of the successful capture of Bluestripe Shiner individuals during the study, positive eDNA detection, or historical collection success. In total, 39 sites were chosen for empirical and eDNA sampling in Alabama (Appendix 2). These sites were would allow for an extensive statewide survey that would provide a thorough assessment of the status of the species within Alabama. Eight sites in Georgia, two on the Chattahoochee and Six along the Flint River, were also chosen for empirical sampling to supplement Bluestripe Shiner DNA for genetic analysis. While most of our sites were both empirically sampled and sampled for eDNA, some sites were either too deep for seining/backpack electroshocking or surveyed after our eDNA survey was completed, leaving some sites sampled only by empirical sampling or eDNA and not both (Appendix 2).

*eDNA Sampling and PCR Analysis*

Thirty-three water samples were collected from Alabama tributaries of the Chattahoochee and Chattahoochee Reservoirs in Alabama (Appendix 2). No sites were sampled more than once. At each site, eDNA was collected using three 15 mL water samples. Immediately after collection, each sample was added to 1.5 mL of 3M sodium acetate and 33 mL of 95% ethanol and stored at room temperature until DNA extraction. A control consisting of the 3M sodium

acetate, ethanol, and 15 ml of deionized water was included with our samples to ensure that there was no contamination during collection and transportation.

All DNA extractions for our eDNA samples were conducted at the University of West Florida, UWF. Water samples were centrifuged at 3500 RPMS for 30 minutes at 4°C. After the supernatant was discarded, the leftover pellet transferred to a 1.5 mL tube and centrifuged at 14000 RPM for 3 minutes. Next, DNA extraction was completed using a DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Extraction controls were included to ensure that there was no contamination of the samples during extraction. All DNA extractions were run through the OneStep™ Inhibitor Kit (ZYMO RESEARCH) after each extraction was completed.

Following DNA extraction, A species-specific primer was designed in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for use in a Polymerase Chain reaction, PCR, to amplify any Bluestripe Shiner DNA that could be contained in our samples. The results of the PCR were visualized using gel electrophoresis. A 203-base pair segment of the cytochrome-b subunit of mitochondrial DNA was targeted. Primers (L: 5' AAG AGG GGT TCT GGC ACT AT 3'; R: 5' TTG AAG CGA CTT GTC CAA TG 3') were designed using Primer 3 (Untergasser et al., 2012) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). These primers were then compared against sequence data from Blacktail Shiner, Tallapoosa Shiner, Red Shiner, and any available sequence data from other closely related species potentially present in the system, in order to reduce the risk of amplification of the wrong species. Primers were further tested for accuracy using tissue from both Blacktail and Bluestripe shiner individuals to ensure that only Bluestripe Shiner DNA was amplified.

All PCRs were conducted using 2.5µL of DreamTaq™ (10X, ThermoFisher Scientific) buffer, 0.5µL of dNTP, 1 µL of each forward and reverse primer, 0.3 µL of DreamTaq™

(ThermoFisher Scientific), 18.7µL of Milli-Q® $H_2O$, and 1µL of extracted DNA from the sample for a final volume of 25 µL. PCR conditions were as follows: initial incubation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 seconds, 59.1 °C for 30 seconds, 72 °C for 1 min and 30 seconds, with a final extension at 72 °C for 7 min. PCR results were visualized using Gel electrophoresis on an agarose gel stained with ethidium bromide. All samples were independently run three times using the same protocol to reduce the risk of false negatives and to increase detection probability (Yamamoto et al. 2017; Ficetola et al. 2015). Positive samples were selected and purified for sequencing and bidirectionally sequenced using Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific) to ensure species specificity. Sequences were edited using Sequencher 5.4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) and confirmed using nucleotide BLAST (Basic Local Alignment Search Tool, blast.ncbi.nlm.nih.gov). Confirmed positive detections of Bluestripe Shiner DNA indicate that individuals were present within the site at the time of eDNA collection. Due to rapid DNA degradation, dilution from water flow, limited transport distance, and the likely low density of individuals, we believe that any positive samples originated from a source close in proximity to our collection site. Positive sites were used to conduct new empirical sampling efforts or to repeat empirical sampling for Bluestripe Shiner individuals at those locations.

*Empirical Sampling*

Empirical sampling was conducted during the summers of 2018 and 2019 using several techniques based on site conditions and the number of crewman available for that site (Appendix 2). In total, 35 Alabama sites and eight sites from Georgia were surveyed using empirical sampling methods. Forms of empirical sampling included backpack electroshocking, shoreline seining, cast netting, and boat electroshocking.

Backpack electroshocking was conducted using a Smith-Root LR-24 Electrofisher by shocking into a 10 ft seine with 1/8$^{th}$ inch nylon mesh at 225 to 300 volts depending on water conductivity. This technique was used for a majority of our sites. The 10-foot seine was set within a deep run and shocking would begin approximately 10 feet upstream of the seine. The shocker would drive fish into the seine which was lifted and taken to shore once the shocking probe reached the lead line. The backpack electrofisher was also used in conjunction with a dipnet in areas of low flow to stun fish for potential capture by a dip netter. Once fish were captured, they were identified by species and *Cyprinella* individuals were placed in a 5-gallon bucket.

In streams with no flow and at reservoir sites, shoreline seining was conducted using 20 ft and 30 ft bag seines. The seine was pulled parallel to the shore for approx. 30 feet, then the seiner closest to the shore would plant their brail-pole while the seiner in the middle of the stream or reservoir would turn in towards the shore, capturing fish that had been driven in front of the seine. The seine was then pulled up to the shore where the lead line was lifted, driving all fish in the net into the seine purse. From shore, individuals were identified by species and captured *Cyprinella* transferred to a 5-gallon bucket.

In areas with high flow and large boulders, or at sites where only one surveyor was available, cast netting was conducted with a 6 ft diameter 3/8-inch nylon mesh cast net. The caster would stand on shore or over a large run in the middle of the stream and cast the net into an area of slack-water like an eddy or a pool. The cast-net would then be hauled in and any fish caught would be identified by species, transferring any *Cyprinella* individuals to a 5-gallon bucket. While this method routinely produced specimens of both Bluestripe and Blacktail shiner, it was not without bias. Only large males would remain in the net as the mesh was too large to

capture small males and female individuals. This could have contributed to low detection at sites where cast netting was the only sampling method used.

At the mouths of tributaries along the Chattahoochee where stream depth was too deep for wading, boat electroshocking was conducted using a 14-foot flat bottom aluminum boat with a Smith Root 2.5 GPP electrofisher set at 5 amps. All Fish were captured by two dip netters standing at the bow of the boat. All captured individuals were identified by species and any *Cyprinella* caught were transferred to a 5-gallon bucket.

Throughout all sampling forms, all captured *Cyprinella* individuals were transferred to a five-gallon bucket for holding. If an individual was identified as Bluestripe Shiner, it was immediately separated to decrease risk of cross contamination with Blacktail Shiner individuals and preserved in 95% ethanol at room temperature (25°C) for later genetic analysis. Individuals of Blacktail Shiner and Tallapoosa Shiner were further studied for signs of hybridization, then fin clips were taken and stored in 95% ethanol at room temperature (25°C), and specimens were euthanized and stored in formalin for voucher purposes. In the case of Blacktail Shiner, only the first individual caught at a site and every fifth individual caught after were used for genetic analysis in order to prevent bias and increase the likelihood of getting a full genetic representation of the site. Only species of *Cyprinella* were collected and recorded during this survey.

*Genetic Analysis*

DNA extraction of our tissue samples was completed using a DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). All tissue extractions were completed at the University of West Alabama, UWA. After extraction, 91 samples were sent to Diversity Arrays Technology

Pty Ltd at the University of Canberra Bruce in Australia, in a single 96-well plate for DArTseq assays. These samples included Bluestripe, Blacktail, and Tallapoosa shiner individuals caught in Alabama as well as Bluestripe and Blacktail shiner individuals from both the Chattahoochee and the Flint rivers in Georgia. Individuals were taken from Georgia because of our lack of specimens from Alabama. If hybridization was occurring with Georgia populations of Bluestripe Shiner, it could shed light on hybridization within Alabama. One Blacktail Shiner individual from the Tallapoosa drainage, which is outside of the range of the Bluestripe Shiner, was also added to the plate to compare against Blacktail Shiner from the ACF. All DNA samples were extracted from individual fin clips. Sequencing was completed using an Illumina HiSeq 2500 sequencing system.

Analysis of all sequenced data was conducted in R programming using the package dartR v. 1.1.11 (Gruber et al. 2019). In order to read our SNPs data, a genlight object was created named glcc for "GL object of Cyprinella Callitaenia". Next, all monomorphic loci were removed. All SNPs with missing loci were removed using a missing data threshold of .90. SNPs with inconsistent calls were also removed using a threshold of .95. Individuals were removed that did not meet a call rate of .85, which removed both Tallapoosa Shiner individuals from the analysis. In instances where there was more than one locus per sequence tag, all but one locus where removed. We visualized the genetic differences of our individuals and populations using a Principle Coordinates Analysis, PCoA, with five factors.

From here, our SNPs were analyzed using fastSTRUCTURE and converted to geno format using the LEA package v. 2.8 (Frinchot and Francois 2014). We chose a range of 1-10 for K (number of populations) to be analyzed by fastSTRUCTURE, as well as additional parameters. The K that best fit our analysis was determined based on the lowest cross entropy

score (Frinchot et al. 2014). A membership coefficient (Q), was calculated for each individual formeach of the three population clusters (Appendix 4). The membership coefficient defines the mean probability that an individual belongs to any one of the populations (K). The sum of membership coefficients for each individual is equal to 1. An input file was generated for fastSTRUCTURE and then converted from its STRUCTURE format to allow processing in LEA. Individual membership coefficients were then written into excel and displayed as a stacked bar graph. Bluestripe Shiner individuals were also analyzed separately from Blacktail Shiner individuals using fastStructure. After determining K, and analyzing our stacked bar graph for Bluestripe Shiner, Fixation Indexes, fst, were calculated using a bootstrap of 50 at 95% confidence to determine if our populations contained a significant difference in allele frequency.

Finally, historical water data from USGS was used to create a graph of water withdrawals by county from 1985 to 2015 for each county containing one or more of our study sites. Historical water gauge data from USGS was also used to graph the average yearly discharge of Uchee Creek from 1948 to present.

## Results

### *eDNA Detection*

Bluestripe Shiner eDNA was detected at 1/33 (3%) of our sites in Alabama (Figure 2, Figure 3, Appendix 2). Site 38, Wehadkee Creek, was the only site where Bluestripe Shiner DNA was positively detected. This site has historically produced Bluestripe Shiner individuals, but none have been collected since 1995 (Appendix 1). The species was not detected at any other historical locations. The Farley Nuclear Power Plant service water pond, which was believed to hold the highest density of Bluestripe Shiner in Alabama (Shepard et al. 1995), did not produce a

positive sample. While such a low detection rate does not indicate that Bluestripe Shiner are absent from our sites, when paired with empirical sampling this finding could support our hypothesis that the species has been mostly extirpated from Alabama tributaries of the Chattahoochee.

*Empirical Sampling*

Only one out of 39 sites sampled in Alabama produced a Bluestripe Shiner individual. Site 29 on Halawakee Creek produced a single male Bluestripe Shiner with pronounced tubercles in three distinct rows, a vibrant mid-lateral blue stripe, and little fin pigmentation except for white at the base of its dorsal rays and white along dorsal, caudal, anal, and pelvic margins. Based on phenotypic features, this individual was identified as pure. This individual was caught while backpack electroshocking within a deep run just above Lake Harding reservoir (Figure 2, Figure 3).

More Bluestripe Shiner individuals were sampled outside of Alabama tributaries to supplement a lack of data for our genetic analysis. Several Bluestripe Shiner individuals were collected in Georgia within the mainstem of the Chattahoochee River below Riverview dam using a cast net. This method provided the highest success rate for the capture of Bluestripe Shiner individuals during this study, especially large males. Bluestripe Shiner was the dominant *Cyprinella* species at this site with no Blacktail Shiner observed during multiple sampling trips. Both males and females were caught at this site. All males had similar features to the single male from site 29, but they also displayed orange coloration in both their caudal and dorsal rays as well as more pronounced white coloration on dorsal rays and fin margins. Females were gray in color with a slight mid-lateral black stripe which ran all the way to the base of the caudal fin. Both males and females were also collected from two sites along the Flint river using a seine.

Individual males were either not mature or not in breeding colors and had few tubercles with little to no fin coloration. Females were similar to those caught in the Chattahoochee.

In total, 34 Bluestripe Shiner individuals were caught and identified during this study. Of those, 20 individuals were used for genetic analysis. Two individuals identified as Tallapoosa Shiner were caught in Wehadkee Creek in Alabama and processed for genetic analysis. Blacktail Shiner individuals were present in a majority of the sites sampled. 60 individuals from Alabama tributaries of the Chattahoochee were used for genetic analysis along with four individuals from the Flint river in Georgia, and one individual from the Tallapoosa in Alabama (Appendix 2). No Red Shiner individuals were caught or observed during this survey.

*Genetic Analysis*

At total of 62007 SNPs were obtained from 82 individuals. Our PCoA determined that there were three distinct population clusters of *Cyprinella* based on our SNPs. Of these, 2551 (4.1%) contained no missing values. Here, we present the results from the 85% call rate dataset which contained 11570 loci. Both Tallapoosa Shiner individuals were removed as call rates were <85%. Average trimmed sequence length of our 11570 loci was 66.2bp. After secondaries were removed, we were left with 79 individuals (Appendix 3), represented by a total of 9843 loci with an average trimmed sequence length of 66.1bp. A PCoA exhibited strong differentiation between three distinct genetic clusters (Figure 4) The first two principal coordinates of our PCoA accounted for 80% and 5.6% of the variance, respectively, and jointly accounted for 85.6% of the total variation in our data set. Our single Bluestripe Shiner from Alabama (clip #74) along with Bluestripe Shiner individuals from both the Flint and the Chattahoochee in Georgia were far removed along PC1 compared to Blacktail Shiner individuals. Blacktail Shiner individuals were differentiated from each other along PC2 with a distinct cluster of individuals from the Flint and

lower Alabama Chattahoochee tributaries below a less distinct cluster of individuals from upper Chattahoochee tributaries and our single Blacktail Shiner individual from the Tallapoosa.

Based on our fastSTRUCTURE analysis, it was determined that K= 3 was the best fit our analysis, suggesting we had three distinct genetic clusters (Frinchot et al. 2014). This supported our PCoA results. Our Stacked Bar Graph (Figure 5,), reveals that all of our Bluestripe Shiner individuals fall within a distinct population, with 16 Bluestripe Shiner individuals identified as pure Q>99% and one individual, 44, that could not be classified as either pure or admixed Q >95% Q<5% for the Bluestripe Shiner cluster and Chatt/Flint Blacktail Shiner population cluster respectively (Melville et al. 2017) (Figure 6, Appendix 4). This could indicate gene-flow within the system, but is more than likely the result of retained ancestral polymorphisms, which have been noted within several species of *Cyprinella* and can often be mistaken for evidence of hybridization (Schonhuth and Mayden 2009). Interestingly, six Blacktail Shiner individuals from several upper Alabama tributaries of the Chattahoochee exhibited a Q>98% for the Talla/Chatt Blacktail Shiner population cluster along with our Blacktail Shiner individual from the Tallapoosa (Figure 7). Five Blacktail Shiner individuals were determined to be admixtures between the Talla/Chatt Blacktail Shiner population and the Chatt/Flint Blacktail Shiner population $0.90 > Q \geq 0.10$ (Melville et al. 2017). All other Blacktail Shiner individuals from lower Alabama and the Flint exhibited a Q>95% for the Chatt/Flint Blacktail Shiner cluster.

A final fastSTRUCTURE analysis was conducted on 16 Bluestripe Shiner individuals (Figure 8), after Blacktail Shiner individuals were removed from the data set. From this, it was determined that a population of K=2 best fit our analysis and that our Bluestripe Shiner individuals fell into either the Chattahoochee Drainage population, or the Flint Drainage population (Figure 9). Two individuals were determined to be admixture between both

populations based on individual population percentage. The Fts between our Chattahoochee and Flint Bluestripe Shiner populations was .0695 (p-value= 0, C.I. 95%), showing a significant difference in allele frequency between the two populations.

Finally, our water usage data from Alabama counties containing our sites along with our annual discharge data from Uchee creek, support several recent studies that water loss has continued to increase within Alabama Chattahoochee tributaries since the 1950s (Dowling 2012; Lawson and Johnston 2015).

**Discussion**

The findings from this study suggest that although some Bluestripe Shiner populations remain in Alabama tributaries, they have been largely extirpated from historical locations and may soon be extirpated statewide. The use of both empirical sampling and eDNA should have detected the species, even if they were present in low numbers. However, our results suggest that Bluestripe Shiner persists at only two localities in Alabama. The statewide survey for Bluestripe Shiner in Alabama during the mid-1990s noted that the species had been extirpated from several localities and that numbers were noticeably declining statewide. The author believed that key factors in the reduction in Bluestripe Shiner statewide included the increase in impoundments along the Chattahoochee, and modifications to habitat along systems like Uchee Creek (Shepard et al. 1995). While Bluestripe Shiner populations evolutionarily exist in much higher numbers than Shoal Bass, populations could have suffered the same fate within Alabama tributaries as geneflow was completely halted and habitat connectivity was removed by water loss and impoundment creation.

Both Halawakee and Wehadkee creeks provide viable habitat for Bluestripe Shiner along their expanse, although these habitat patches are small in size and separated by stretches of

unsuitable habitat. Both sites are also cut off from potential geneflow with the mainstem Chattahoochee River by the reservoirs that they flow into. At site 33 in Halawakee Creek large predators feeding directly below the last shoal were collected during backpack electroshocking. Several large Hybrid Striped Bass, Largemouth Bass, and Channel catfish were collected during sampling efforts, suggesting that Bluestripe Shiner might experience high predation at the small site. Thick vegetation was seen on most of the bedrock and boulders at the site, which would provide little spawning habitat for the species who naturally spawn over rocky crevices. While Bluestipe Shiner individuals remain at both sites, their numbers are low based on our eDNA and empirical detection rates. Extirpation from these two sites could be imminent if viable habitat continues to decline.

While several historical studies have noted examples of hybrid individuals Alabama systems, our SNPs data from Alabama as well as Georgia *Cyprinella* species shows no evidence of hybridization or introgression between Bluestripe Shiner and their congeners. While our results do not show current introgression between Bluestripe Shiner populations or signs of past introgression within Blacktail Shiner populations, we cannot currently prove or disprove that hybridization was not a factor in the extirpation of Bluestripe Shiner populations in Alabama. The hybridization timeline could have occurred well before our study, especially in the case of a hybrid swarm which can swamp out a parental population through genetic homogenization or competition in as little as five years (Mooney and Cleland 2001; Ward et al 2012).

Our fastSTRUCTURE ancestry analysis reveals two significant populations of Bluestripe Shiner based on individual population proportions. These populations line up directly with individuals from the Chattahoochee Drainage in Georgia and Alabama, and Individuals from the Flint Drainage based on individual Q-values. Our Fixation Index confirms this, showing a

significant difference in allele frequency between the two populations. This could be the result of natural genetic drift, or it could be the result of loss of genetic flow, especially for the Chattahoochee population, as inbreeding can lead to reduced fitness through increased homozygosity decreasing effective population size. This along with the effects of random genetic drift can begin to increasingly alter allele frequencies within a species (Hallerman 2003). Further genetic analysis of individuals within the Chattahoochee, Flint, and Apalachicola rivers could yield further evidence of distinct populations of Bluestripe Shiner within each drainage. Interestingly, Blacktail Shiner individuals sampled during this study showed genetic similarities with those from the Tallapoosa River population. Several Blacktail Shiner individuals show high population proportions for our Tallapoosa drainage population, based on our Tallapoosa individual. Evidence of a stream capture event has been documented in Wehadkee Creek, and our results support this hypothesis (Shepard et al. 1995; Jarrett et al. 2017).

Previous findings indicate widespread fish assemblage changes and population isolation within several Alabama tributaries of the Chattahoochee (Johnston and Farmer 2004; Dowling 2012; Lawson and Johnston 2015). In particular, these studies documented a significant increase in distributional range and abundance of Blacktail Shiner. These studies determined increased water loss and significant land use change within and along several Alabama Chattahoochee tributaries have led to the decreased diversity of several stream assemblages as well as the disappearance of several species from historic locations. Increases in pine monoculture and urbanization have been noted as major factors in the loss of water along streams like Uchee creek (Shepard et al. 1995; Dowling 2012). Our examination of water usage supports this finding as water withdrawals have increased for several of our observed counties since 1985, and annual water discharge from Uchee Creek has declined since 1948 (Figure 10, Figure 11).

As crevice spawners, Bluestripe Shiner reproduction is impacted heavily by decreased water availability. Not only are fewer crevices available for spawning but decreases in flow can also lead to excessive sedimentation and reduced oxygen availability for the developing embryos, which can increase time of development and decrease embryo survival (Rombough 1988, Vives 1993). Along with decreased reproduction, Bluestripe Shiners naturally occur in medium to large river systems with high connectivity. As Alabama's tributaries continue to decline, systems like Uchee Creek which historically had sustained flow and habitat connectivity now no longer exhibit those characteristics as water is lost from the system and lentic environments increase (Maceina et al 2008; Dowling 2012). These creeks are no longer suitable for Bluestripe Shiner populations based on observed life history parameters for the species, and populations have either slowly moved back to the mainstem Chattahoochee or have been slowly extirpated overtime.

Our results do not support our hypothesis that Bluestripe Shiner populations in Alabama may have been genetically swamped out by Blacktail Shiner. The catalyst for their apparent decline may be associated with the habitat use and corresponding water loss associated with previously documented fish assemblage changes. Conservation efforts aimed at protecting water and land use in Chattahoochee may prevent further decline of Bluestripe Shiner as well as other Chattahoochee drainage fishes in Alabama. Additionally, a more intensive empirical and genetic survey of both Halawakee and Wehadkee creeks could help shed more light on both populations and how they continue to survive when others have been extirpated. This could also provide more insight into the extirpation of other populations like the Uchee Creek population and populations in tributaries within and below Walter F. George Reservoir. Surveying within the lotic stretches of the Chattahoochee mainstem along Alabama's border is also needed to

determine if source populations still exist there, if they have become sink populations, or if they too have been extirpated.

Around the world Freshwater fish species are in decline as a result of human induced pressures. Fish assemblages in the Southeastern United States, in particular Alabama, have not been immune to this rapid decline in diversity. Alabama's high diversity of both native and endemic freshwater fish makes the conservation of the State's waterways paramount in preserving North American freshwater fish diversity. The famous phrase, "think globally, act locally," perfectly encapsulates the role of freshwater conservation. By studying, identifying, and fixing impacts threatening imperiled populations of aquatic organisms at the local level, we can protect, preserve, and increase biodiversity on a global level.

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Figure 1. Overview of historic Bluestripe collections from Alabama 1949-Present

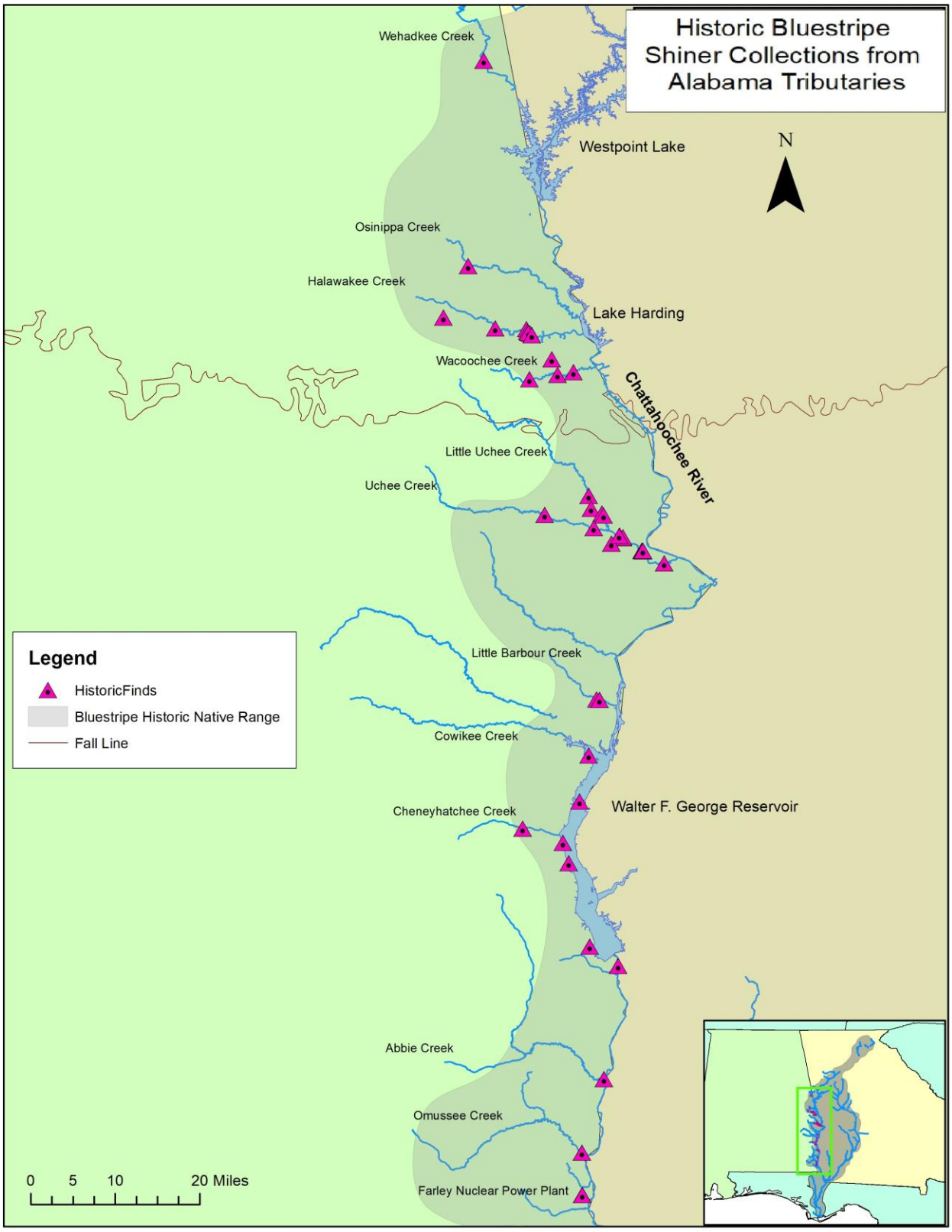


Figure 2: Overview of Alabama sampling sites and eDNA and empirical sampling success.

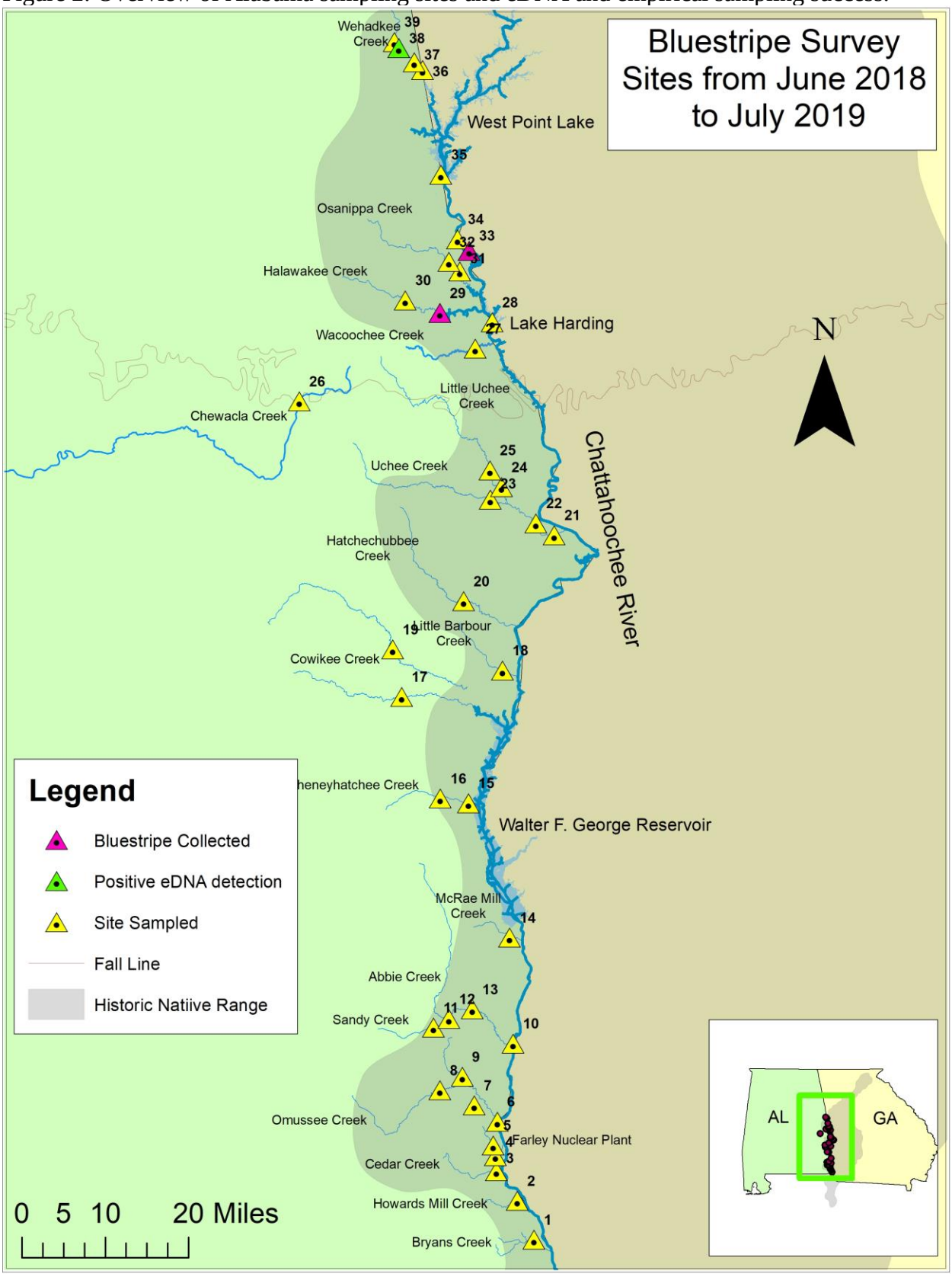


Figure 3: Sites 26-39 sampled above the Fall line in Alabama.

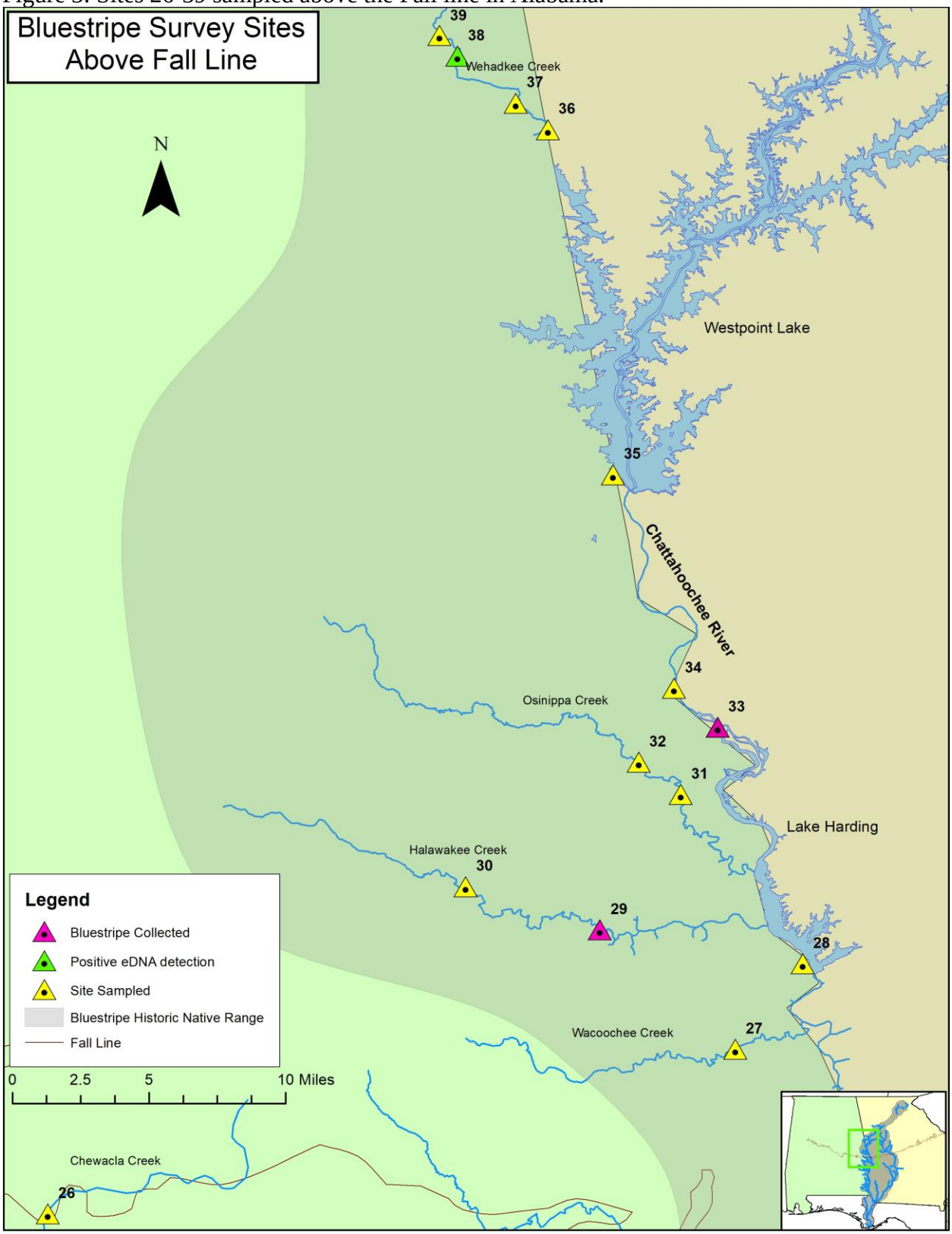


Figure 4: PCoA plot generated from a genetic analyses of 9843 SNPs for 79 Bluestripe Shiner and Blacktail Shiner individuals. Each of the three population clusters is colored to match those in Figure 5.

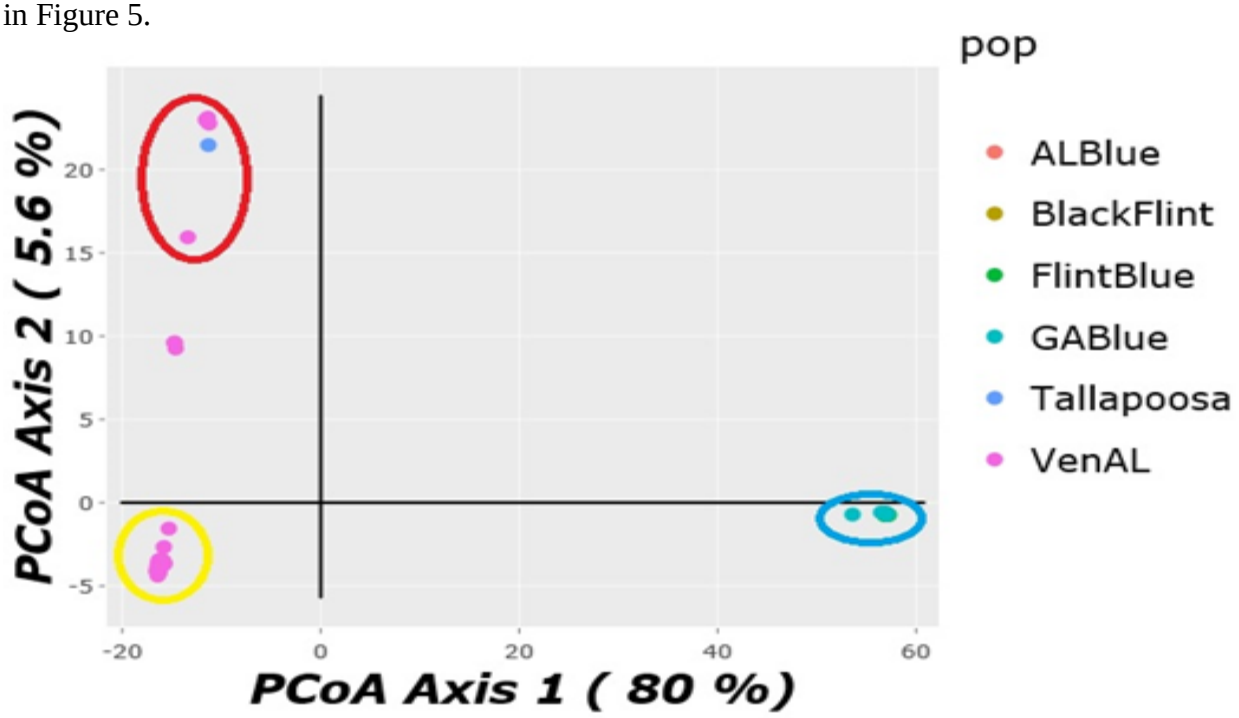
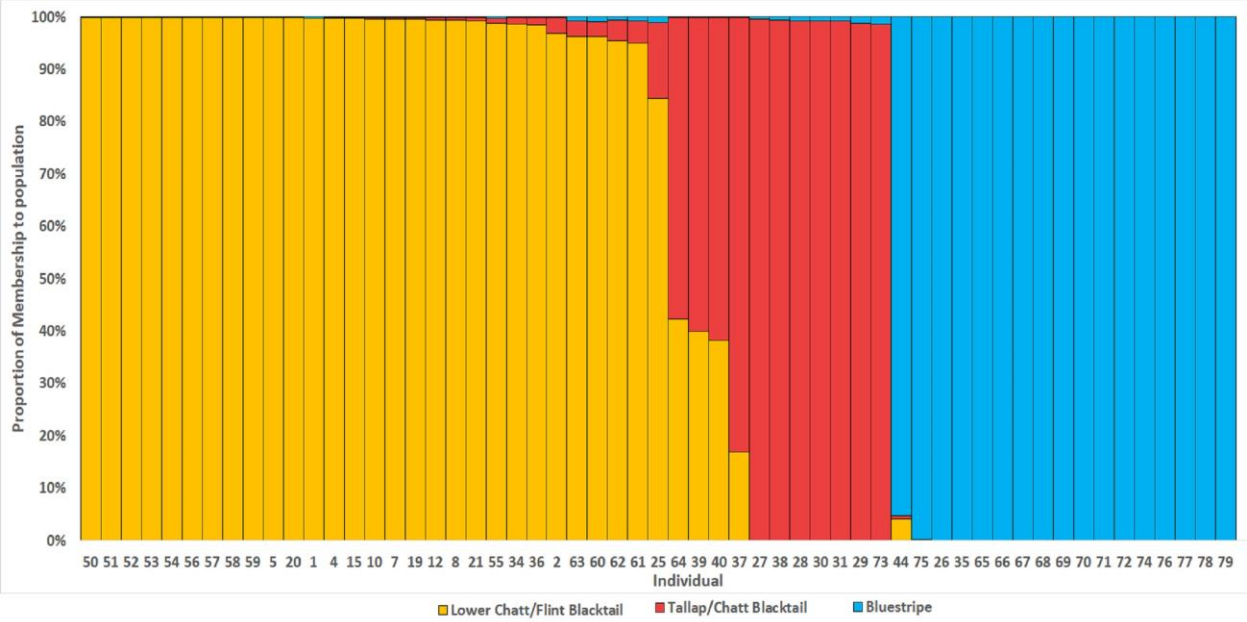



Figure 5: fastSTRUCTURE plot at K=3 of 9843 SNPs for 79 Bluestripe Shiner and Blacktail Shiner individuals. Each color corresponds to a population cluster from Figure 4.




*Several pure individuals from the Lower Chatt/Flint Blacktail population cluster were removed to allow for a cleaner plot. These individuals are still present in Figure 5 and can also be found in Appendix 4.

Figure 6: Overview of population cluster proportion and sample location for 79 *Cyprinella* individuals. Pie Chart colors correspond to population cluster colors in Figure 5. Appendix 1.

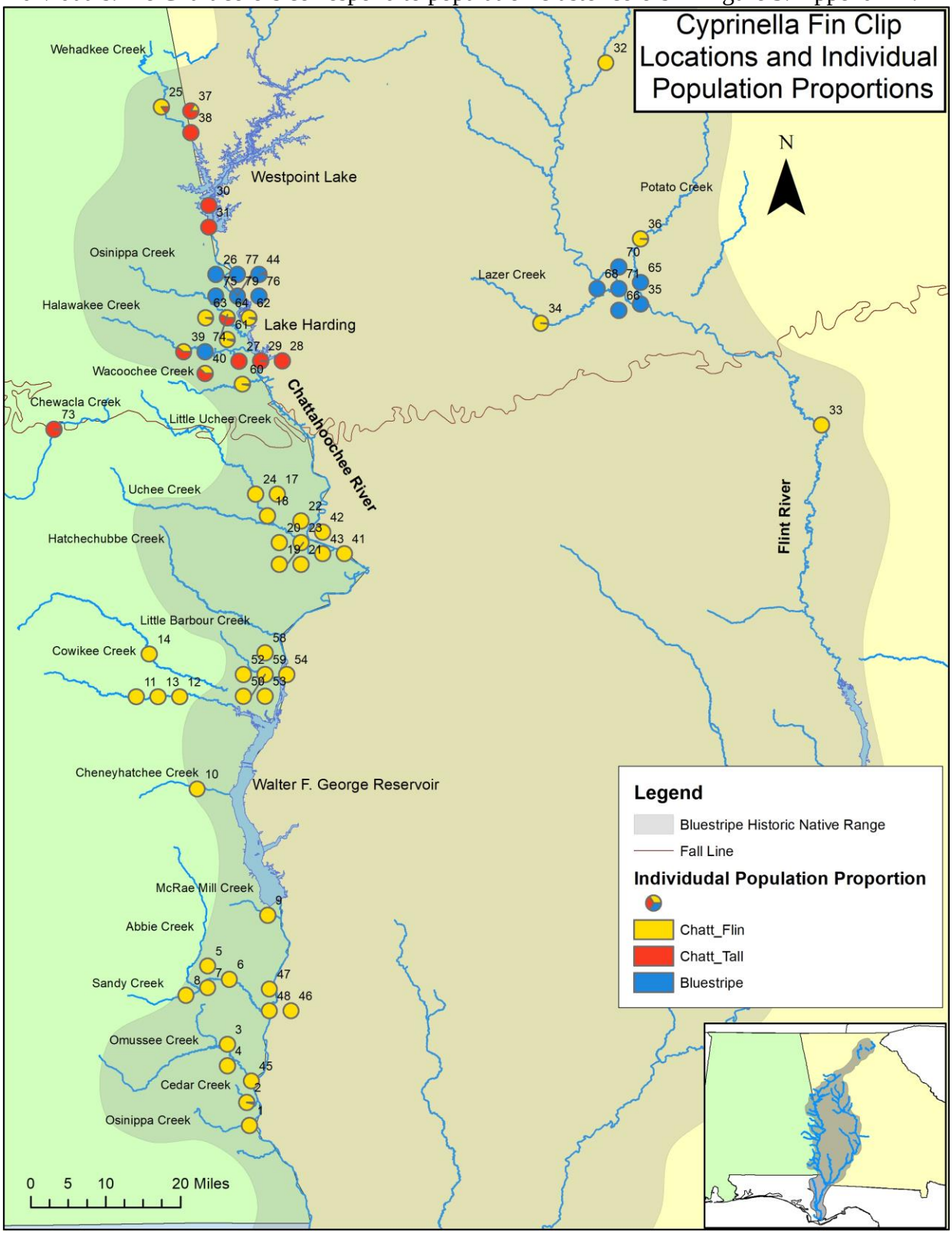


Figure 7: Population cluster proportion and sample location for *Cyprinella* individuals sampled above the fall line in Alabama. Pie Chart colors correspond to population cluster colors in Figure 5.

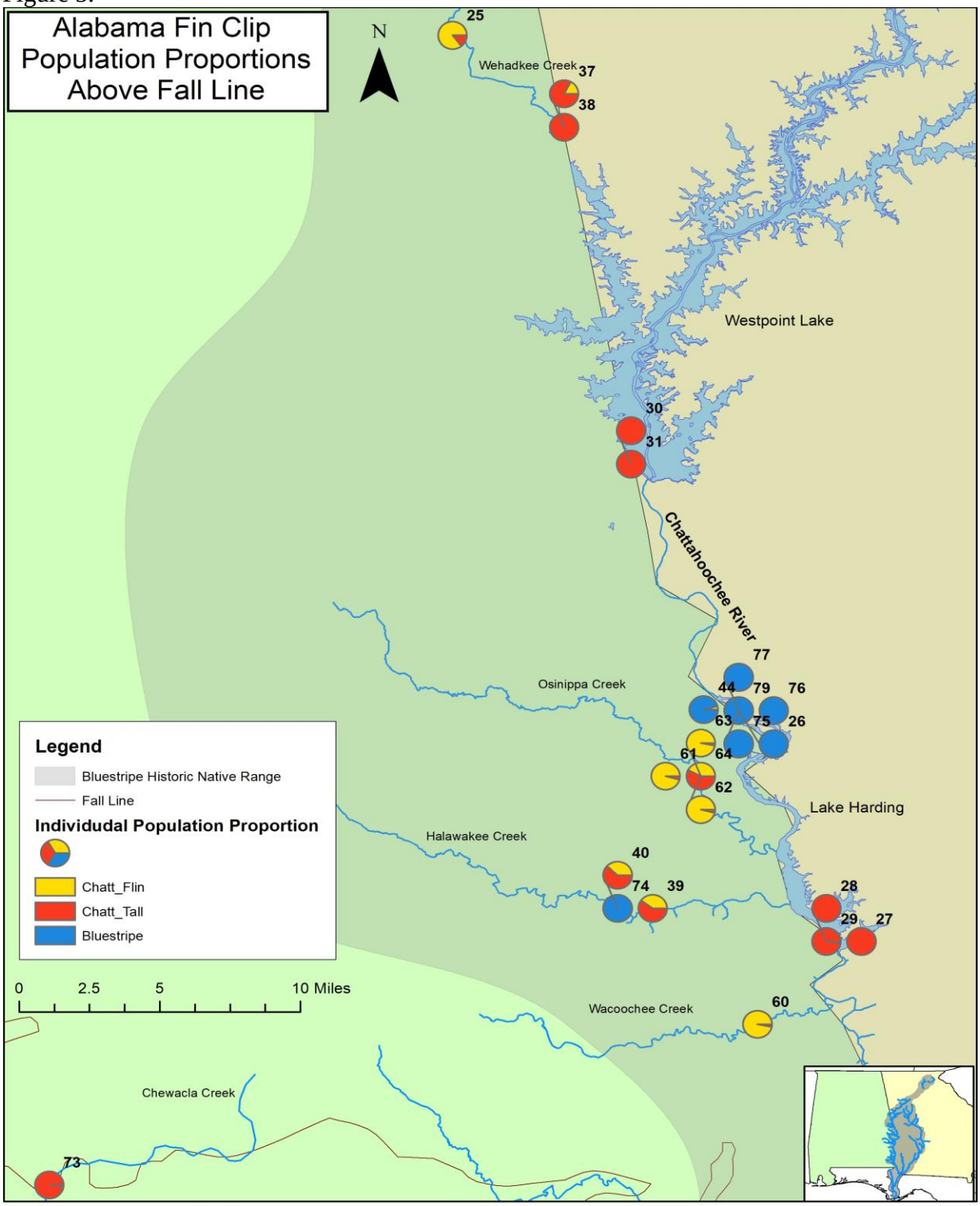


Figure 8: fastSTRUCTURE plot of 16 Bluestripe Shiner individuals at K=2 based on the Chattahoochee and Flint river drainages. Colors correspond to Figure 9.

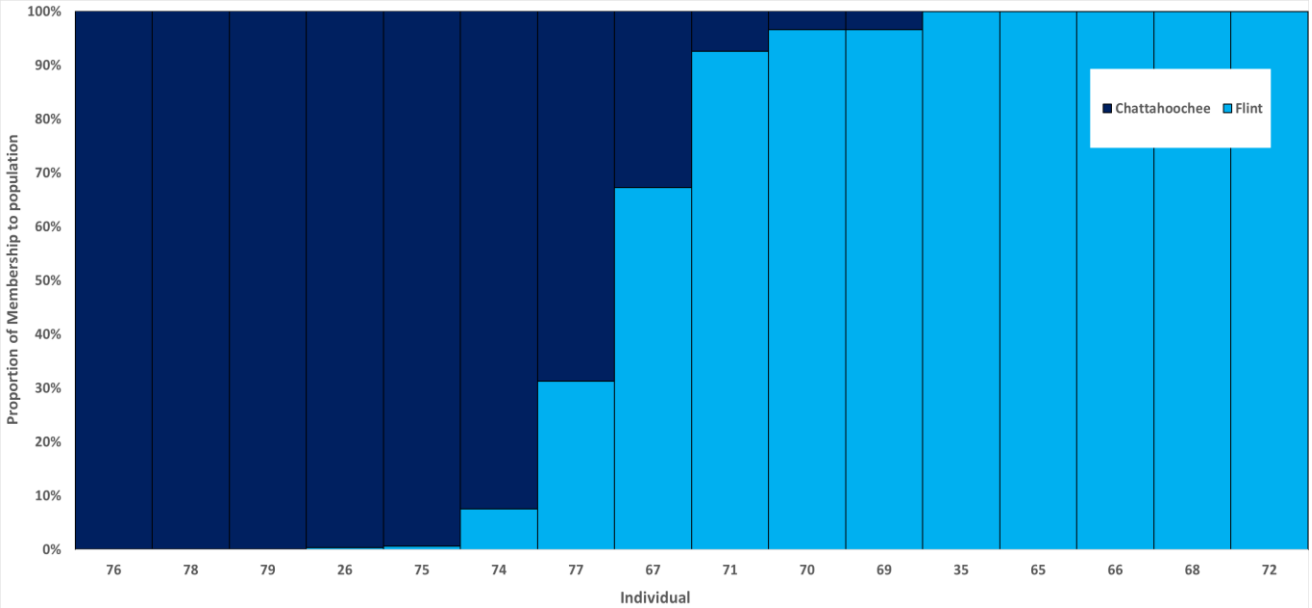


Figure 9: Population cluster proportion and sample location for Bluestripe Shiner individuals sampled. Pie Chart colors correspond to stacked bar population colors

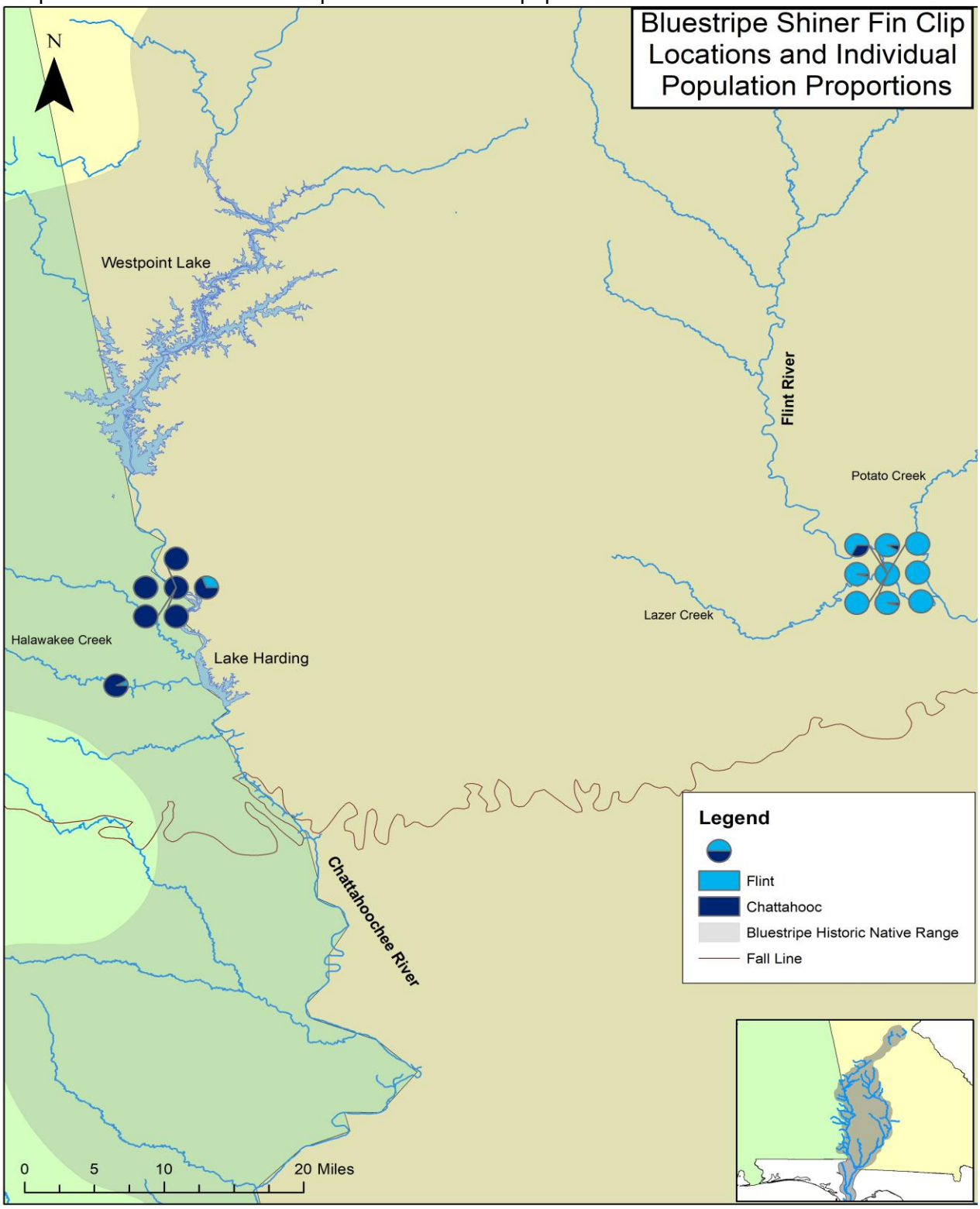


Figure 9: Public water withdrawals from 1985-2015 in Alabama counties that contain one or more of our study sites. Data from USGS.

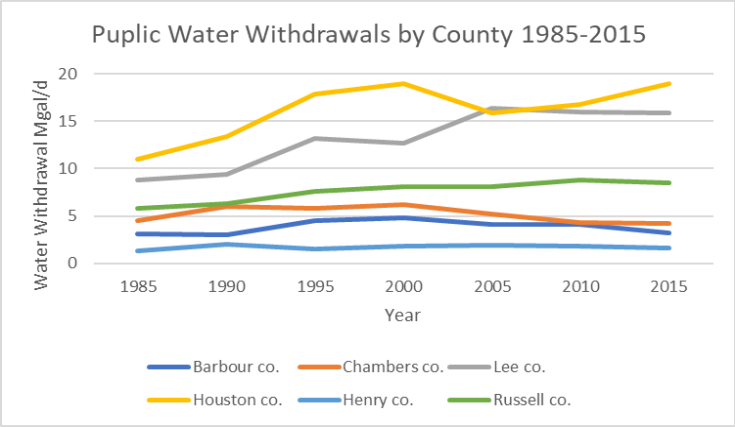
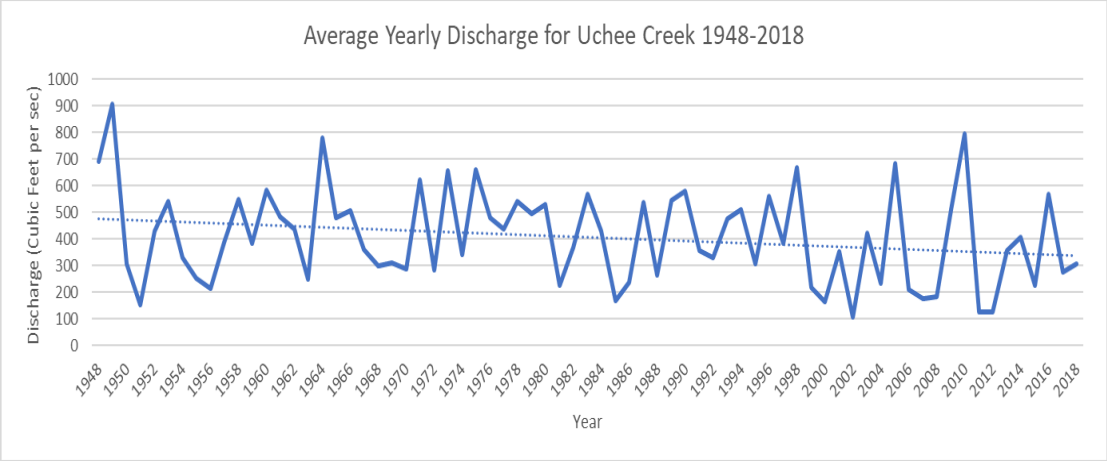



Figure 10: Average yearly discharge for Uchee Creek 1948-2018 from USGS site 02342500.



Appenxix 1. Historic collection data for Bluestripe Shiner in Alabama 1949-2009. Sites displayed in Figure 2

| Location | County | Date | # Collected | Technique | Latitude | Longitude | Collectors |
|---|---|---|---|---|---|---|---|
| Uchee Creek 9.2 mi. South of Phoenix City | Russell | 12-Jun-49 | 2 | Seine | 32.34221 | -85.05355 | R. D. Suttkus, C. F. Cole, R. H. Gibbs |
| Chattahoochee River near Eufaula | Barbour | 11-Sep-54 | 11 | Seine | 31.88746 | -85.12256 | R. M. Gibbs, D.M. Bailey |
| Uchee Creek Nucholls Rd. 3.5 mi. SE U. S. Hwy. 241 | Russell | 2-Oct-54 | 15 | Seine | 32.34221 | -85.05355 | J. S. Dendy |
| Uchee River, ca.2 mi.below confluence with Little Uchee Creek | Russell | 2-Oct-54 | *1 | Unknown | 32.33861 | -85.04806 | J.S. Dendy, Class |
| Uchee Creek 5.5 air miles south of Crawford Highway 169 | Russell | 1-Oct-55 | 1 | Unknown | 32.35572 | -85.09772 | J. S. Dendy |
| Uchee Cr., 3.1 airmi W of Fort Mitchell, below RR bridge | Russell | 8-Oct-55 | *1 | Unknown | 32.34028 | -85.04806 | J.S. Dendy, Class |
| Cowpin Creek Tributary of Uchee Creek | Russell | 24-May-64 | 3 | Unknown | 32.32901 | -85.06788 | W. Smith-Vaniz and W. Rogers |
| Uchee Cr., 7.6 mi N.Cottonton on HWY 165 | Russell | 1-Sep-67 | *1 | Unknown | 32.31576 | -85.01295 | R.J. Gilbert and L.A. Johnson |
| Little Barbour Creek, 6.7 miles S of Cottonton on Hwy 165 | Barbour County | 17-Apr-68 | *1 | Unknown | 32.06222 | -85.09306 | R.J. Gilbert and C Clement |
| Uchee Cr., 6.2 mi S.of Crawford, Hwy.169 | Russell | 4-Nov-68 | *1 | Unknown | 32.37861 | -85.18194 | R.J. Gilbert, P Naftel, C Clement |
| Tributary to Halawakee Creek; T20N R28E S33E | Lee | 28-May-70 | *1 | Unknown | 32.71615 | -85.35548 | Mathur, Naftel |
| Halawakee Cr., 2.2 airmi SW of Beulah, below covered bridge | Lee | 29-Jul-70 | *1 | Unknown | 32.645 | -85.17 | Mathur, H. Wahlquist |
| Halawakee Creek, Below Beans Mill | Lee | 29-Jan-71 | *1 | Unknown | 32.6974 | -85.26691 | Mathur, Wahlquest |
| Halawakee Cr., 2.1 airmi SSW of Beulah, at covered bridge | Lee | 22-Oct-71 | *1 | Unknown | 32.61083 | -85.20833 | J.S. Ramsey, L.A. Barclay, Ichthyology Class |
| Little Uchee Creek 5.6 Airmiles WSW from Brickyard, Alabama | Russell | 24-Oct-71 | 28 | Unknown | 32.41067 | -85.10666 | P. Kirk et al. |
| Little Uchee Cr., 5.6 airmi WSW of Brickyard; T16N R29E S11SW | Russell | 24-Oct-71 | *1 | Unknown | 32.38861 | -85.10278 | Kirk, Wisdom, Cox, Moon |
| Little Uchee Creek 4.6 Airmiles WSW from Brickyard, Alabama | Russell | 31-Oct-71 | 27 | Unknown | 32.38091 | -85.08392 | R. Busch et al. |
| Little Uchee Creek, 4.6 miles WSW of Brickyard, US 431 bridge | Russell | 31-Oct-71 | *1 | Unknown | 32.37722 | -85.08111 | Busch, Moon, Kirk |
| Halawakee Creek at Covered Bridge Road | Lee | 2-Nov-72 | 37 | Unknown | 32.68594 | -85.20403 | Barclay et al. |
| Halawakee Creek, Creek just above Lake Harding backwater, 2.1 airmi SSW Beulah co.rd | Lee | 19-May-75 | *1 | Unknown | 32.69639 | -85.21333 | J.S. Ramsey, Ichthyology Class |
| Halawakee Cr., 2.2 airmi.SW of Beulah; T20N R28E S35NE | Lee | 5-May-76 | *1 | Unknown | 32.69167 | -85.21194 | J.S. Ramsey, Ichthyology Class |

*Indicates specimens that are found in museum collections. The number of individuals collected at that site is unknown.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Halawakee Creek at Covered Bridge Road | Lee | 17-Jun-76 | 2 | Unknown | 32.68594 | -85.20403 | J.S. Ramsey and R. K. Wallace |
| Walter F. George Resivoir shoreline below Cheneyhatchee Creek | Barbour | 17-Aug-76 | 5 | Unknown | 31.827379, | -85.155 | F. Scott |
| Shoreline of W. F. George Reservoir, Chattahoochee River 5.3 miles S of Eufala | Barbour | 17-Aug-76 | *1 | Unknown | 31.81636 | -85.15078 | T. Scott, GA DNR, et al |
| Halawakee Cr., just above confl.with Harding backwater, 2.1 airmi SW of Beulah | Lee | 9-Apr-77 | *1 | Unknown | 32.68889 | -85.20722 | T.J. Timmons, K Wallace |
| Uchee Cr., 7.5 airmiles SSE Phenix City, co.rt.39 | Russell | 23-Apr-77 | *1 | Unknown | 32.3425 | -85.05444 | Wallace, Wallace |
| Halawakee Cr., 2.2 airmi.SW of Beulah | Lee | 7-May-77 | *1 | Unknown | 32.69028 | -85.21 | K Wallace, T. Timmons |
| Uchee Cr., 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 15-May-77 | *1 | Unknown | 32.31667 | -85.01528 | K Wallace, Wallace |
| Uchee Cr., 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 8-Jun-77 | *1 | Unknown | 32.31667 | -85.01528 | K Wallace, B Wallace |
| Uchee Creek, 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 9-Jun-77 | *1 | Unknown | 32.31667 | -85.01528 | K Wallace, B Wallace |
| Uchee Cr., 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 4-Jul-77 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, B Wallace |
| Uchee Creek, 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 14-Jul-77 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, B Wallace |
| Uchee Creek, 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 8-Aug-77 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, J.M. Pierson |
| Uchee Cr., 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 9-Aug-77 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, J.M. Pierson |
| Uchee Creek, 1.9 airmi.S of Fort Mitchell | Russell | 6-Sep-77 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, W. Seesock |
| Uchee Creek, 1.9 airmi.S of Fort Mitchell | Russell | 10-Feb-78 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, W. Wieland |
| Uchee Cr., 1.9 airmi.S of Fort Mitchell, Hwy 165 | Russell | 28-Mar-78 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, D. Darr |
| Uchee Cr., 1.9 airmi S of Fort Mitchell, Hwy 165 | Russell | 17-Apr-78 | 2 | Unknown | 32.31639 | -85.01528 | J.S. Ramsey, K Wallace, W. Wieland |
| Uchee Creek, 9.8 airmi.S of Phenix | Russell | 17-Apr-78 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, J.S. Ramsey |
| Uchee Cr.1.9 airmi S of Fort Hwy 165 | Russell | 20-Jun-78 | *1 | Unknown | 32.31639 | -85.01528 | K Wallace, W. Wieland |
| Farley Nuclear Powerplant Service Pond | Houston | 28-Sep-83 | 1 | Boat Electrofish | 31.2134 | -85.11767 | Alabama Power Company |
| Farley Nuclear Powerplant Service Pond | Houston | 8-Nov-84 | 15 | Seine | 31.2134 | -85.11767 | M. E. Peirson and M. P. Tyberghein |
| Farley Nuclear Powerplant Service Pond | Houston | 29-Apr-86 | 7 | Seine | 31.2134 | -85.11767 | Collector: M. Pierson and E. Tyberghein. |
| Uchee Creek at Highway 431 | Russell | 19-May-89 | 1 | Unknown | 32.35922 | -85.10718 | Geological Survey of Alabama |

*Indicates specimens that are found in museum collections. The number of individuals collected at that site is unknown.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Uchee Creek at 101st Airborn Division road | Russell | 17-Dec-91 | 8 | Unknown | 32.29526 | -84.97733 | W. S. Burkhead and M. Wallace |
| Farley Nuclear Powerplant Service Pond | Houston | 10-May-94 | 16 | Seine | 31.2134 | -85.11767 | T. E. Shepard et al. |
| Halawakee Creek at Covered Bridge Road | Lee | 13-Jun-94 | 2 | Seine | 32.68594 | -85.20403 | T. E. Shepard et al. |
| Little Barbour Creek at Alabama Highway 165 | Babour | 18-Apr-95 | 4 | Seine | 32.06136 | -85.08834 | T. E. Shepard et al. |
| Wehadkee Creek .5 miles downstream of Rock Mills | Randolph | 19-Apr-95 | 2 | Seine | 33.15649 | -85.28663 | T. E. Shepard et al. |
| Chattahoochee River downstream of Walter F. George Dam | Henry | 20-Jun-95 | 1 | Boat Electrofish | 31.60595 | -85.05603 | T. E. Shepard et al. |
| Omussee Creek from Alabama Highway 52 to mouth | Houston | 21-Jun-95 | 6 | Boat Electrofish | 31.28596 | -85.11799 | T. E. Shepard et al. |
| Abbie Creek at mouth | Henry | 21-Jun-95 | 8 | Boat Electrofish | 31.41187 | -85.08073 | T. E. Shepard et al. |
| Walter F. George Reservoir Upstream of White Oak Creek | Barbour | 12-Jul-95 | **(14) | Shoreline Rotenone | 31.78165 | -85.14105 | ADCNR |
| Cowikee Creek opposite Lakepoint State Park | Barbour | 13-Jul-95 | **(14) | Shoreline Rotenone | 31.96623 | -85.10652 | ADCNR |
| Walter F. George Reservoir shoreline near Hardridge Creek | Henry | 17-Aug-95 | **(14) | Shoreline Rotenone | 31.63901 | -85.10483 | ADCNR |
| Farley Nuclear Powerplant Service Pond | Houston | 3-Jun-98 | 1 | Seine | 31.2134 | -85.11767 | B.R. Kuhajda, R.L. Mayden, and P.M. Harris |
| Wacoochee Creek | Lee | 7-Jun-99 | 12 | Unknown | 32.62278 | -85.13272 | M. Hill |
| Wacoochee Creek, 2.5 miles N of Bleeker | Lee | 10-Jul-06 | 2 | Seine | 32.6183 | -85.1598 | R.A. Kennon, N.R. Ozburn, A.R. Henderson, D.E. Holt |
| Cheynehatchee Creek at Hwy 32 | Barbour | 4-Jun-08 | 1 | Backpack E. Shock/ Seine | 31.84073 | -85.21985 | Geological Survey of Alabama |
| Osanippa Creek at Chambers County Road 83 | Chambers | 6-Jul-09 | 2 | Backpack E. Shock/ Seine | 32.80422 | -85.31309 | ADCNR |
| **Indicates 14 individuals from three sites that were grouped into a single collection. The specific number from each site is unknown. | | | | | | | |

Appenxix 2. Collection site appendeix, eDNA results, empirical sampling technique, and *Cyprinella* caught by species.

| Location | Latitude | Longitude | Site # | Date/s Surveyed | Drainage | eDNA Result | Emperical Sampling Technique | Cyprinella Collected |
|---|---|---|---|---|---|---|---|---|
| Bryans Creek at AL 95 | 31.069496 | -85.045001 | 1 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |
| Howards Mill Creek at AL 95 | 31.137525 | -85.077362 | 2 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |
| Cedar Creek at AL 95 | 31.18968 | -85.118802 | 3 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |
| Farley Nuclear Plant | 31.216378 | -85.1197713 | 4 | 9/14/2018 | Chattahoochee | NEG | Shoreline Seine/eDNA | none |
| Wilson Creek at AL 95 | 31.234753 | -85.124132 | 5 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Omussee Creek Mouth | 31.276147 | -85.115201 | 6 | 4/15/2019 | Chattahoochee | NA | Boat Shock | *C. venusta* |
| Hurricane Creek at Cr 22 | 31.305015 | -85.161533 | 7 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Omusee Creek at Cr 55 | 31.332225 | -85.230207 | 8 | 6/18/2018 | Chattahoochee | NEG | eDNA | NA |
| Stevenson Creek at Cr 134 | 31.355145 | -85.184725 | 9 | 6/19/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |
| Abbie Creek Mouth | 31.411727 | -85.080348 | 10 | 4/16/2019 | Chattahoochee | NA | Boat Shock | *C. venusta* |
| Sandy Creek at CR 56 | 31.441586 | -85.241578 | 11 | 6/20/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Abbie Creek at Hwy 53 | 31.456413 | -85.209692 | 12 | 6/20/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Abbie Creek at AL 95 | 31.472343 | -85.162186 | 13 | 6/20/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| McRae Mill Creek at AL 10 | 31.596162 | -85.083415 | 14 | 6/20/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Eufaula at 431 | 31.831611 | -85.162661 | 15 | 7/24/2018 | Chattahoochee | NEG | Shoreline Seine/eDNA | *C. venusta* |
| Cheneyhatchee Creek at CR 32 | 31.84052 | -85.220011 | 16 | 6/20/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| South Fork Cowikee Creek at CR 79 | 32.018733 | -85.295798 | 17 | 7/2/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | 7/23/2018, 4/16/2019 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Middle Fork Cowikee CR49 | 32.101429 | -85.312535 | 19 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Hatchechubbee Creek at Pittsview | 32.183194 | -85.165522 | 20 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Uchee Creek at Fort Benning | 32.29535 | -84.977246 | 21 | 4/15/2019 | Chattahoochee | NA | Shoreline Seine | *C. venusta* |
| Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Uchee Creek at Hwy 431 | 32.359257 | -85.107366 | 23 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Little Uchee Creek at Hwy 431 | 32.380869 | -85.083845 | 24 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Little Uchee Creek at Hwy 28 | 32.410625 | -85.106858 | 25 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Chewacla Creek | 32.535748 | -85.49686 | 26 | 7/24/2018 | Tallapoosa | NEG | Cast Net/eDNA | C. venusta |
| Wacoochee Creek at Cr 379 | 32.62279 | -85.132716 | 27 | 7/24/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Lake Harding | 32.668065 | -85.096975 | 28 | 7/25/2018 | Chattahoochee | NEG | Shoreline Seine/eDNA | *C. venusta* |
| Halawakee at Covered Bridge Road | 32.685995 | -85.20438 | 29 | 6/22/2018, 7/24/2018, 4/15/2019 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta, C. callitaenia(1)* |
| Halwakee Creek | 32.708927 | -85.275557 | 30 | 7/24/2018 | Chattahoochee | NEG | eDNA | NA |
| Osinippa Creek at Hopewell Road | 32.757605 | -85.161609 | 31 | 7/24/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta* |
| Osinippa Creek | 32.774689 | -85.183866 | 32 | 7/24/2018 | Chattahoochee | NEG | eDNA | NA |
| Chattahochee River at Riverview Dam | -85.142085 | 32.793192 | 33 | 8/24/2018, 5/22/2019 | Chattahoochee | NA | Shoreline Seine/Cast net | *C. callitaenia* |
| Chatt below Langdale Dam | -85.165067 | 32.813831 | 34 | 8/15/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | none |
| West Point Lake | -85.197411 | 32.926782 | 35 | 7/24/2018 | Chattahoochee | NEG | Shoreline Seine/eDNA | *C. venusta* |
| Wehadkee Creek Last Shoal | -85.231974 | 33.109288 | 36 | 4/15/2019 | Chattahoochee | NA | Backpack Shock | *C. venusta* |
| Wehadkee at 684 | -85.248878 | 33.123264 | 37 | 7/24/2018 | Chattahoochee | NEG | Cast net/eDNA | none |
| Wehadkee at 686 | -85.27982 | 33.148123 | 38 | 7/24/2018 | Chattahoochee | **POS** | Cast Net/eDNA | none |
| Wehadkee Below Rock Mills | -85.28938 | 33.159039 | 39 | 7/23/2018 | Chattahoochee | NEG | Backpack Shock/eDNA | *C. venusta, C. gibbsi* |

Appendix 3. List of individuals used for PCoA and fastSTRUCTURE analyses, the location they were found, and their assigned population based on phenotypic characteristics and location found

| Clip # | Site Name | Latitude | Longitude | Site # | Drainage | Species | Pop | Date Collected |
|---|---|---|---|---|---|---|---|---|
| 1 | Cedar Creek | 31.18968 | -85.118802 | 3 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 2 | Wilson Creek at Al 95 | 31.234753 | -85.124132 | 5 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 3 | Hurricane Creek at Cr 22 | 31.305015 | -85.161533 | 7 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 4 | Hurricane Creek at Cr 22 | 31.305015 | -85.161533 | 7 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 5 | Abbie Creek at AL 95 | 31.472343 | -85.162186 | 13 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 6 | Abbie Creek at AL 95 | 31.472343 | -85.162186 | 13 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 7 | Abbie Creek at Hwy 53 | 31.456413 | -85.209692 | 12 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 8 | Sandy Creek at CR 56 | 31.441586 | -85.241578 | 11 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 9 | McRae Mill Creek at AL 10 | 31.596162 | -85.083415 | 14 | Chattahoochee | *C. venusta* | VenAL | 6/19/2018 |
| 10 | Cheneyhatchee Creek at CR 32 | 31.84052 | -85.220011 | 16 | Chattahoochee | *C. venusta* | VenAL | 6/20/2018 |
| 11 | South Fork Cowikee Creek at CR 79 | 32.018733 | -85.295798 | 17 | Chattahoochee | *C. venusta* | VenAL | 7/2/2018 |
| 12 | South Fork Cowikee Creek at CR 79 | 32.018733 | -85.295798 | 17 | Chattahoochee | *C. venusta* | VenAL | 7/2/2018 |
| 13 | South Fork Cowikee Creek at CR 79 | 32.018733 | -85.295798 | 17 | Chattahoochee | *C. venusta* | VenAL | 7/2/2018 |
| 14 | Middle Fork Cowikee Creek at CR 49 | 32.101429 | -85.312535 | 19 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 15 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 16 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 17 | Little Uchee Creek at Hwy 431 | 32.380869 | -85.083845 | 24 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 18 | Little Uchee Creek at Hwy 431 | 32.380869 | -85.083845 | 23 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 19 | Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 20 | Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 21 | Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 22 | Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 23 | Uchee Creek at Hwy 165 | 32.316719 | -85.014356 | 22 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 24 | Little Uchee Creek at Hwy 28 | 32.410625 | -85.106858 | 25 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 25 | Wehadkee at Rock Mills | 33.159039 | -85.28938 | 38 | Chattahoochee | *C. venusta* | VenAL | 7/23/2018 |
| 26 | Chattahochee River Below Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 8/24/2018 |
| 27 | Lake Harding | 32.668065 | -85.096975 | 28 | Chattahoochee | *C. venusta* | VenAL | 7/24/2018 |
| 28 | Lake Harding | 32.668065 | -85.096975 | 28 | Chattahoochee | *C. venusta* | VenAL | 7/24/2018 |
| 29 | Lake Harding | 32.668065 | -85.096975 | 28 | Chattahoochee | *C. venusta* | VenAL | 7/24/2018 |
| 30 | West Point Lake | 32.926782 | -85.197411 | 35 | Chattahoochee | *C. venusta* | VenAL | 7/24/2018 |
| 31 | West Point Lake | 32.926782 | -85.197411 | 35 | Chattahoochee | *C. venusta* | VenAL | 7/24/2018 |
| 32 | Flint Mainstem | 33.244717 | -84.429313 | NA | Flint | *C. venusta* | BlackFlint | 7/26/2018 |
| 33 | Flint Mainstem | 32.544032 | -84.0121 | NA | Flint | *C. venusta* | BlackFlint | 7/26/2018 |
| 34 | Lazer Creek Flint river | 32.741128 | -84.555174 | NA | Flint | *C. venusta* | BlackFlint | 7/27/2018 |
| 35 | Flint Po Biddy | 32.778002 | -84.369318 | NA | Flint | *C. callitaenia* | FlintBlue | 7/27/2018 |
| 36 | Potato Creek | 32.904239 | -84.36223 | NA | Flint | *C. venusta* | BlackFlint | 7/27/2018 |
| 37 | Wehadkee Last Shoal | 33.109288 | -85.231974 | 36 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 38 | Wehadkee Last Shoal | 33.109288 | -85.231974 | 36 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 39 | Halawakee Creek at Covered Bridge Road | 32.685995 | -85.20438 | 29 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 40 | Halawakee Creek at Covered Bridge Road | 32.685995 | -85.20438 | 29 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 41 | Uchee Fort Benning | 32.29535 | -84.977246 | 21 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 42 | Uchee Fort Benning | 32.29535 | -84.977246 | 21 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 43 | Uchee Fort Benning | 32.29535 | -84.977246 | 21 | Chattahoochee | *C. venusta* | VenAL | 4/15/2019 |
| 44 | Chattahochee River near Valley | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 8/24/2018 |
| 45 | Omussee Boat Mouth | 31.276147 | -85.115201 | 6 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 46 | Abbie Creek Mouth | 31.411727 | -85.080348 | 10 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 47 | Abbie Creek Mouth | 31.411727 | -85.080348 | 10 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 48 | Abbie Creek Mouth | 31.411727 | -85.080348 | 10 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 49 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 50 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 51 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 52 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 53 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 54 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 55 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 56 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 57 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 58 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 59 | Little Barbour Creek at AL 165 | 32.061587 | -85.088573 | 18 | Chattahoochee | *C. venusta* | VenAL | 4/16/2019 |
| 60 | Wacoochee Creek AL 379 | 32.623217 | -85.132406 | 27 | Chattahoochee | *C. venusta* | VenAL | 4/18/2019 |
| 61 | Osinippa Creek at Hopewell Road | 32.757605 | -85.161609 | 31 | Chattahoochee | *C. venusta* | VenAL | 4/18/2019 |
| 62 | Osinippa Creek at Hopewell Road | 32.757605 | -85.161609 | 31 | Chattahoochee | *C. venusta* | VenAL | 4/18/2019 |
| 63 | Osinippa Creek at Hopewell Road | 32.757605 | -85.161609 | 31 | Chattahoochee | *C. venusta* | VenAL | 4/18/2019 |
| 64 | Osinippa Creek at Hopewell Road | 32.757605 | -85.161609 | 31 | Chattahoochee | *C. venusta* | VenAL | 4/18/2019 |
| 65 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 66 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 67 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 68 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 69 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 70 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 71 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 72 | Lazer Creek Mouth | 32.807891 | -84.404251 | NA | Flint | *C. callitaenia* | FlintBlue | 8/14/2018 |
| 73 | Chewacla Creek | 32.535748 | -85.49686 | 26 | Tallapoosa | *C. venusta* | Tallapoosa | 2/12/2018 |
| 74 | Halawakee Creek at Covered Bridge Road | 32.685995 | -85.20438 | 29 | Chattahoochee | *C. callitaenia* | ALBlue | 6/22/2018 |
| 75 | Chattahochee River at Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 5/22/2019 |
| 76 | Chattahochee River at Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 5/22/2019 |
| 77 | Chattahochee River at Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 5/22/2019 |
| 78 | Chattahochee River at Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 5/22/2019 |
| 79 | Chattahochee River at Riverview Dam | 32.793192 | -85.142085 | 33 | Chattahoochee | *C. callitaenia* | GABlue | 5/22/2019 |

Appendix 4: List of individuals used for PCoA and fastSTRUCTURE analyses and their Q-value proportion for each population cluster. Populations correspond to Figure 7.

| Clip # | Q-Value by Population Cluster | | | Latitude | Longitude |
|---|---|---|---|---|---|
| | Chatt/Flint Blacktail | Tallap/Chatt Blacktail | Bluestripe | | |
| 1 | 0.998025 | 9.9991E-05 | 0.00187456 | 31.18968 | -85.118802 |
| 2 | 0.968996 | 0.029864 | 0.00113991 | 31.234753 | -85.124132 |
| 3 | 0.9998 | 0.00009999 | 0.00009999 | 31.305015 | -85.161533 |
| 4 | 0.997096 | 0.00280398 | 0.00009999 | 31.305015 | -85.161533 |
| 5 | 0.999784 | 0.00011627 | 9.9991E-05 | 31.472343 | -85.162186 |
| 6 | 0.9998 | 0.00009999 | 0.00009999 | 31.472343 | -85.162186 |
| 7 | 0.995703 | 0.00419745 | 0.00009999 | 31.456413 | -85.209692 |
| 8 | 0.993796 | 0.00610368 | 0.00009999 | 31.441586 | -85.241578 |
| 9 | 0.9998 | 0.00009999 | 0.00009999 | 31.596162 | -85.083415 |
| 10 | 0.996714 | 0.00318642 | 9.9991E-05 | 31.84052 | -85.220011 |
| 11 | 0.9998 | 0.00009999 | 0.00009999 | 32.018733 | -85.295798 |
| 12 | 0.994695 | 0.00520489 | 9.9991E-05 | 32.018733 | -85.295798 |
| 13 | 0.9998 | 0.00009999 | 0.00009999 | 32.018733 | -85.295798 |
| 14 | 0.9998 | 0.00009999 | 0.00009999 | 32.101429 | -85.312535 |
| 15 | 0.99703 | 0.00282785 | 0.00014255 | 32.061587 | -85.088573 |
| 16 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 17 | 0.9998 | 0.00009999 | 0.00009999 | 32.380869 | -85.083845 |
| 18 | 0.9998 | 0.00009999 | 0.00009999 | 32.380869 | -85.083845 |
| 19 | 0.995399 | 0.00450099 | 9.9991E-05 | 32.316719 | -85.014356 |
| 20 | 0.999097 | 9.9991E-05 | 0.00080351 | 32.316719 | -85.014356 |
| 21 | 0.992998 | 0.00690197 | 9.9991E-05 | 32.316719 | -85.014356 |
| 22 | 0.9998 | 0.00009999 | 0.00009999 | 32.316719 | -85.014356 |
| 23 | 0.9998 | 0.00009999 | 0.00009999 | 32.316719 | -85.014356 |
| 24 | 0.9998 | 0.00009999 | 0.00009999 | 32.410625 | -85.106858 |
| 25 | 0.844884 | 0.145272 | 0.00984404 | 33.159039 | -85.28938 |
| 26 | 0.00009999 | 0.00009999 | 0.9998 | 32.793192 | -85.142085 |
| 27 | 9.9991E-05 | 0.995274 | 0.0046259 | 32.668065 | -85.096975 |
| 28 | 9.9991E-05 | 0.993451 | 0.00644916 | 32.668065 | -85.096975 |
| 29 | 9.9991E-05 | 0.987414 | 0.0124857 | 32.668065 | -85.096975 |
| 30 | 9.9991E-05 | 0.993221 | 0.00667946 | 32.926782 | -85.197411 |
| 31 | 9.9991E-05 | 0.992481 | 0.00741949 | 32.926782 | -85.197411 |
| 32 | 0.9998 | 0.00009999 | 0.00009999 | 33.244717 | -84.429313 |

| | | | | | |
|---|---|---|---|---|---|
| 33 | 0.9998 | 0.00009999 | 0.00009999 | 32.544032 | -84.0121 |
| 34 | 0.985961 | 0.0139389 | 9.9991E-05 | 32.741128 | -84.555174 |
| 35 | 0.00009999 | 0.00009999 | 0.9998 | 32.778002 | -84.369318 |
| 36 | 0.985027 | 0.0145163 | 0.00045703 | 32.904239 | -84.36223 |
| 37 | 0.17002 | 0.82988 | 9.9991E-05 | 33.109288 | -85.231974 |
| 38 | 9.9991E-05 | 0.993728 | 0.00617231 | 33.109288 | -85.231974 |
| 39 | 0.399982 | 0.599918 | 9.9991E-05 | 32.685995 | -85.20438 |
| 40 | 0.38237 | 0.61753 | 9.9991E-05 | 32.685995 | -85.20438 |
| 41 | 0.9998 | 0.00009999 | 0.00009999 | 32.29535 | -84.977246 |
| 42 | 0.9998 | 0.00009999 | 0.00009999 | 32.29535 | -84.977246 |
| 43 | 0.9998 | 0.00009999 | 0.00009999 | 32.29535 | -84.977246 |
| 44 | 0.0418476 | 0.006096 | 0.952056 | 32.793192 | -85.142085 |
| 45 | 0.9998 | 0.00009999 | 0.00009999 | 31.276147 | -85.115201 |
| 46 | 0.9998 | 0.00009999 | 0.00009999 | 31.411727 | -85.080348 |
| 47 | 0.9998 | 0.00009999 | 0.00009999 | 31.411727 | -85.080348 |
| 48 | 0.9998 | 0.00009999 | 0.00009999 | 31.411727 | -85.080348 |
| 49 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 50 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 51 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 52 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 53 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 54 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 55 | 0.988229 | 0.010073 | 0.00169789 | 32.061587 | -85.088573 |
| 56 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 57 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 58 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 59 | 0.9998 | 0.00009999 | 0.00009999 | 32.061587 | -85.088573 |
| 60 | 0.962289 | 0.0288005 | 0.00891074 | 32.623217 | -85.132406 |
| 61 | 0.950475 | 0.0425496 | 0.00697521 | 32.757605 | -85.161609 |
| 62 | 0.955744 | 0.0387898 | 0.00546628 | 32.757605 | -85.161609 |
| 63 | 0.962435 | 0.0302207 | 0.00734382 | 32.757605 | -85.161609 |
| 64 | 0.423107 | 0.576793 | 9.9991E-05 | 32.757605 | -85.161609 |
| 65 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 66 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 67 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 68 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |

| | | | | | |
|---|---|---|---|---|---|
| 69 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 70 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 71 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 72 | 0.00009999 | 0.00009999 | 0.9998 | 32.807891 | -84.404251 |
| 73 | 9.9991E-05 | 0.986867 | 0.0130328 | 32.535748 | -85.49686 |
| 74 | 0.00009999 | 0.00009999 | 0.9998 | 32.685995 | -85.20438 |
| 75 | 9.9991E-05 | 0.00267134 | 0.997229 | 32.793192 | -85.142085 |
| 76 | 0.00009999 | 0.00009999 | 0.9998 | 32.793192 | -85.142085 |
| 77 | 0.00009999 | 0.00009999 | 0.9998 | 32.793192 | -85.142085 |
| 78 | 0.00009999 | 0.00009999 | 0.9998 | 32.793192 | -85.142085 |
| 79 | 0.00009999 | 0.00009999 | 0.9998 | 32.793192 | -85.142085 |

Appendix 5: List of individual Bluestripe Shiner used for PCoA and fastSTRUCTURE analyses and their Q-value proportion for each population. Populations Correspond to Figure 8.

| Clip# | Drainage | |
|---|---|---|
| | Flint | Chattahoochee |
| 76 | 1.00E-04 | 0.9998 |
| 78 | 1.00E-04 | 0.9998 |
| 79 | 1.00E-04 | 0.9998 |
| 26 | 0.002935 | 0.996965 |
| 75 | 0.005642 | 0.994258 |
| 74 | 0.075374 | 0.924526 |
| 77 | 0.312975 | 0.686925 |
| 67 | 0.672472 | 0.327428 |
| 71 | 0.925432 | 0.0744677 |
| 70 | 0.965603 | 0.0342965 |
| 69 | 0.966142 | 0.0337578 |
| 35 | 0.9998 | 1.00E-04 |
| 65 | 0.9998 | 1.00E-04 |
| 66 | 0.9998 | 1.00E-04 |
| 68 | 0.9998 | 1.00E-04 |
| 72 | 0.9998 | 1.00E-04 |