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Comparison of Cytochrome *b* variation and morphological features of isolated Banded Pygmy Sunfish (*Elassoma zonatum*) populations in the lower Green River and Coastal Plain systems in Western Kentucky

By

David Cravens

Thesis Approved:



Chair, Advisory Committee



Member, Advisory Committee



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Date

6/27/17

Comparison of Cytochrome b variation and morphological features of isolated Banded Pygmy Sunfish (*Elassoma zonatum*) populations in the lower Green River and Coastal Plain systems in Western Kentucky

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Submitted to the Faculty of the Graduate School of
Eastern Kentucky University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
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DEDICATION

This thesis is dedicated to my family and peers
for their unwavering support and countless assistance.

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Sherry Harrel, for her guidance and patience and allowing me take on such a daunting project. I would also like to thank the other committee members, Dr. David Hayes and Dr. Stephen Richter, for their comments and assistance over the past three years. Also, I wish to express my gratitude to Kelsey Rogers and Robert Jackson for their immense assistance in the lab. Without Robert, I would not have had any genetic data to report on. Finally, I express my thanks to my soon to be wife, Ashley, for her willingness to drop what she was doing and providing much needed field assistance. She encouraged me and supported me when I started to doubt myself.

ABSTRACT

The banded pygmy sunfish *Elassoma zonatum* is a wide-ranging species found throughout the Southeastern Coastal Plain of North America. In Kentucky, the populations are found above and below the inner boundary of the Coastal Plain. Due to their geographical separation comparisons in body size and morphological features along with genetic comparisons using mitochondrial cytochrome-b (Cyt-b) were conducted to test relationships between populations. Two sites, Cypress Creek in the Lower Green River system and Rose Creek in the Tradewater River drainage, were sampled from above the inner boundary and two additional sites, Bayou de Chien and Running Slough in the Mississippi River drainage, were sampled from below the boundary line. Collections from Rose Creek and Running Slough yielded no adult specimens for body measurement/morphological comparisons and were only used for genetic comparisons.

Using principal components analysis (PCA) of body measurements, minimal separation in body sizes was detected between drainages when males and females were combined (PC1 48.9%, PC2 16.7%). When separating sexes, complete separation was observed between both sites, PC1 62% and PC2 12.55% for females and PC1 48.19% and PC2 15.02% in males. PCA comparisons were also conducted using 12 morphological landmarks in geometric morphological analysis but yielded no distinct separation in drainages.

Cyt-b comparisons were conducted using sampled individuals in addition to 25 NCBI sequences of *Elassoma zonatum* collected from nearby drainages. Phylogenetic trees were constructed using maximum likelihood analysis and indicated that individuals from Rose Creek and Cypress Creek were nearly identical to each other and with individuals from another population collected above the fall line in Running Lake, Illinois and had low genetic diversity

while sampled individuals from Bayou de Chien and Running Slough had greater genetic diversity. Haplotype networks were constructed that indicated populations found above the Coastal Plain region were very similar to each other and could be indicative of a northward range expansion after the Wisconsin Glacial Episode.

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CHAPTER 1

INTRODUCTION

Phylogeography deals with the spatial arrangements of genetic lineages especially within and among closely related species (Avice, 2009). In aquatic organisms, phylogeographic patterns should give an indication of the geological history of the regions they occur in (Avice, 2000). In eastern North America, climatic changes during the Pleistocene likely played a significant role in shaping the current diversity and distributions of freshwater fishes in the region (Berendzen et al., 2003; Berendzen et al., 2008; Bossu et al., 2013; Mayden, 1988; Near et al., 2001).

Prior to the Pleistocene, river drainages in North America were quite different than present day. The major river systems during that period included the Old Ouachita, Arkansas, Missouri, Mississippi, Teays, Wabash, Ohio, Cumberland, Duck, and Tennessee rivers with many of the extant systems looking very different than their current form (Mayden, 1988). During this time the Central Highlands of eastern North America were one contiguous highland region that would be eventually fragmented by glacial cycles and form the Ozark and Ouachita highlands west of the Mississippi River and the Eastern Highlands located east of the Mississippi River (Thornbury, 1965).

During the Pliocene, the lower Ohio River was considerably smaller than its current form. The headwaters were located in present day southern Indiana and central Kentucky, including the Green and Tradewater rivers, and snaked across southern Illinois (Wabash River) to the present-day Cache River Valley. It continued south joining the Cumberland and Tennessee rivers prior to its confluence with the Mississippi (Burr and Warren, 1986). During

this period the present day Big Sandy, Licking, and Kentucky rivers were likely tributaries to the Teays River (Burr and Warren, 1986).

Mayden (1988) hypothesized that present-day species distributions in the Central Highlands were the result of glacial events during the Pleistocene that severed a physiographic province that consisted of widespread and speciose fauna into at least three disjunct areas referred to as the Eastern Highlands, Ozark Highlands, and Ouachita Highlands. This pre-Pleistocene vicariance hypothesis was supported by several studies that indicated an east-west Pliocene divergence of wide ranging fish species (e.g. Berendzen et al., 2003; Berendzen et al., 2008; Bossu et al., 2013; Near et al., 2001).

A key concept in evolutionary biology is that the divergent selective regime will often generate and maintain some type of phenotypic diversity (Langerhans et al., 2003). This divergent selection can lead to differences in phenotypic expression either by genetic differentiation or phenotypic plasticity (Levins, 1968; Orr and Smith, 1998; Robinson and Wilson, 1994; Schluter, 2000, cited in Langerhans et al., 2003; West-Eberhard, 1989). Such divergence is significant as it can influence microevolutionary changes and result in speciation (West-Eberhard, 1989). Mitochondrial DNA analysis can indicate whether populations have been separated for a long period of evolutionary time or if the separation has been much more recent (Hughes et al., 2009).

A common gene used in phylogenetic studies is the mitochondrial cytochrome-b (Cyt-b), due to it having a rate of base substitution that makes it suitable for comparisons between closely related species (Whitmore et al., 1994). Cyt-b has been considered one of the more useful genes for phylogenetic work, and is one of the more widely used, mainly because of the

structure and function of its protein product (Degli et al., 1993). Cyt-b gene contains slowly and rapidly evolving codon positions, and has been used for a diversity of systematic questions (Farias et al., 2001). Mitochondrial DNA (mtDNA) is passed via the female to offspring thus molecules from different families rarely recombine making mtDNA inheritance both haploid and asexual. Mutations alone normally account for the genetic variety of mtDNA that is observed in animal populations. MtDNA genotypes are referred to as haplotypes, that differ from each other by mutations. Due to the rapid pace of mtDNA evolution several haplotypes can be observed within a species (Awise, 2009)

The shape and size of a species of fish can vary across habitat types (Foster et al., 2015; Gaston et al., 2016; Hamel and Crispo, 2016). In pelagic forms, the individuals are usually adapted for feeding on zooplankton in open waters. Thus, those individuals usually have more streamlined, fusiform bodies. Benthic forms tend to be adapted to feeding on macroinvertebrates or other similar food items and have deeper bodies. Morphological diversity has been shown to be influenced by various ecological forces including resource use, predation, competition and water flow (Hamel and Crispo, 2016).

Quantifying meristic characteristics, such as number of fin rays, gill rakers, and scale rows have historically served as important methods for identifying fishes and can be used in statistical analysis, allowing for comparison of populations and sexes (Barlow, 1961 cited in Begg and Waldman, 1999). Meristic characteristics vary within and among species, making them useful for describing or identifying fishes (Strauss and Bond, 1990). Geometric morphometrics is the analysis of body shape and or shape of other morphological features (Begg and Waldman, 1999). Geometric morphometrics analysis utilizes data derived from discrete morphometric points, linear distances between points and the geometric relationships amongst points (Cadrin,

2005). Traditional systems of measurement, including calipers and measuring boards are commonly used in morphometric studies. However, digital imaging with accurate calibration provides superior data format and allows data to be stored as coordinates that can be analyzed using geometric methods. Also, digital images are easily archived and saved, allowing for one to reprocess and confirm the results (Cadrin, 2005).

The genus *Elassoma* encompasses seven recognized species: *E. zonatum* Jordan, *E. evergladei* Jordan, *E. okefenokee* Böhlke, *E. bohlke* Rohde and Arndt, *E. okatie* Rohde and Arndt, *E. alabamiae* Mayden, and *E. gilberti* Snelson, Krabbenhoft and Quattro. Four of these seven species (*E. alabamiae*, *E. boehlkei*, *E. gilberti*, and *E. okatie*) have only been described in the past 30 years (Rohde and Arndt 1987, Mayden 1993, Snelson et al., 2009). Five of the seven *Elassoma* species have restricted geographic distributions. Of these seven species, only the banded pygmy sunfish, *Elassoma zonatum*, occurs in Kentucky and was originally thought restricted to the Coastal Plain region (Clay, 1975). It is now known that *E. zonatum* populations in Kentucky inhabit three different drainages. The Coastal Plain population drains into the Mississippi River, while the Green and Tradewater rivers populations drain into the Ohio.

Elassoma zonatum was originally assigned a conservation status of special concern (Branson et al., 1981 cited in Burr and Warren, 1986). However, biological surveys conducted by the Kentucky State Nature Preserves Commission (KSNPC) in 1979–1980 discovered populations above the inner boundary of the Coastal Plain, often referred to as the fall line (Robison, 1986), in the Tradewater and Green River systems (Warren and Cicerello, 1982). Both populations represented new records for the state and extended the known Kentucky *Elassoma zonatum* range 160km east (Warren, 1980). With this range extension and new populations, Burr and

Warren (1986) suggested *E. zonatum* was more common than prior studies showed and should not be listed as a species of special concern.

Prior studies on the life history and habitat preference of *E. zonatum* in the Coastal Plain region of Kentucky indicated association in the summer with swampy areas among dense beds of aquatic vegetation supporting bald cypress (*Taxodium distichum*), tupelo (*Nyssa* spp.), oaks (*Quercus* spp.) and willows (*Salix* spp.) (Walsh and Burr, 1984). Barney and Anson (1920) noted typical habitat for *E. zonatum* in Cypress Bayou, Louisiana consisting of surface mats of vegetation with Coontail (*Ceratophyllum* spp.) growing underneath. With the distribution of *E. zonatum* in Kentucky paralleling cypress swamps, it has been hypothesized that suitable habitat was once found over the western third of the state and that isolated populations found in the Tradewater and Green River systems may be a result of historical wetland loss (Warren, 1980).

The objectives of this study were to utilize meristic counts and morphological measurements to provide a comparison of a disjunct *Elassoma zonatum* population found above the fall line in Cypress Creek, located in the Green River system, with those found below the fall line in Bayou de Chien in the Coastal Plain region. Genetic comparisons were conducted using Cyt-b analysis to look at genetic similarity/differences between populations collected above the fall line with those below the fall line, along with individuals collected in surrounding states. Such analysis could help answer if populations of Banded Pygmy Sunfish in Kentucky, found above the fall line are morphologically and genetically similar to other populations of *E. zonatum* found in Kentucky and in surrounding states, or if there is a distinct difference between populations, supporting the need for further studies to investigate potential causes.

CHAPTER 2

METHODS

Study Areas

Four populations of *Elassoma zonatum* were identified for this study. Two populations were collected from below the fall line in the Mississippi system. One site was located in a drainage ditch near Bayou de Chien in Hickman County. A second site was located in Running Slough also in Fulton County. Specimens above the fall line were collected from Cypress Creek State Nature Preserve in the Green River system in Muhlenberg County and from Rose Creek in the Tradewater drainage in Hopkins County ¹(Fig. 1).

Cypress Creek (Fig. 2), is a second order stream located in the Interior Low Plateaus Province; it is bordered by cypress swamps and flows into Pond River, which drains into the Green River, which eventually drains into the Ohio River. Bayou de Chien (Fig. 3), is a second order tributary to the Mississippi River located in the Coastal Plain region; it is surrounded by heavy agriculture resulting in many agricultural ditches draining into the stream. Rose Creek (Fig. 4), is a second order stream that meanders through Hopkins County before draining into retention ponds/wetlands bordering the Tradewater River; significant portions dry out during the summer months and serve as drainage for many agriculture fields in the area. Running Slough (Fig. 5) was the most western site and is a second order stream that has been mostly channelized to assist with field drainage. It eventually drains into Reelfoot Lake in the Upper Blue Basin in Tennessee. All streams have been heavily impacted over the past century due to agricultural practices resulting in straightened stream channels and increased siltation.

¹ All figures and tables are presented in appendices at end of thesis.

Morphological Comparison

Elassoma zonatum voucher specimens were procured from Cypress Creek and Bayou de Chien using ¼" (6mm) mesh dip nets during the summer of 2016. Collections yielded 24 individuals (12 males and 12 females) from Cypress Creek, and 25 individuals (9 males and 16 females) from ditches around Bayou de Chien. Specimens from Rose Creek and Running Slough were omitted from the morphological comparison due to no adult specimens being collected. Live specimens were brought back to Eastern Kentucky University (EKU) where photographs were taken using a camera mount to ensure same angle and lighting. A Fujifilm S9400W was used to photograph specimens. Specimens were photographed on a dissecting tray with dorsal and anal fins spread with inserted pins for better visualization of fin origins. A ruler was placed next to each specimen for scale and was necessary for geometric analysis. Photographs also allow visual comparison of body and fin coloration and assist with morphometric analysis and comparisons. Prior to photographing specimens for morphometric analysis, they were anesthetized with Tricaine Methanesulfonate (MS-222), and then preserved in 95% ethanol for use in genetic studies.

Software programs tpsDIG2 and tpsUTIL were used for landmark digitalization (Rohlf, 2010). Landmarks for morphological characters (Fig. 6) of individuals from both populations followed Armbruster (2012) guidelines. Meristic measurements were conducted adhering to Hubbs and Lagler (1974) and Rohde and Arndt (1987) guidelines to determine if populations followed usual identification methods for *E. zonatum*. Measurements of standard length, body depth and body width were taken to the nearest 0.1mm using dial calipers under a dissecting microscope. Body depth was measured vertically from the origin of the dorsal fin. Dorsal and anal fin length measurements were taken from the base of the first spine to the tip of the

longest ray. Eleven other proportional body measurements were taken using ImageJ software measurement function (<https://imagej.nih.gov/ij/>) and included pre-dorsal length, pre-pelvic length, pre-anal length, dorsal fin length, anal fin length, caudal peduncle length, caudal peduncle depth, head length, snout length, eye diameter and upper jaw length. All measurements were transformed to proportions expressed as thousandths of standard length. Linear regressions were conducted to test if any variables were correlated with standard length. Correlated values were removed and remaining measurements were compared using MANOVA with sex at each site as fixed factors. Pairwise Hotelling's tests were conducted post-hoc to determine differences among sexes between sites. Student's T-tests were then used to determine which measurements were significantly different between populations and sexes. In addition, all vertical trunk bars and preopercular pores were counted and compared between sexes and populations.

IMP 8 package including Coordgen8, and Twogroup8 (Sheets, 2014) was used to analyze landmark relationships and differences between means in Cypress Creek and Bayou de Chien populations. Coordgen8 was used to take digitized landmarks and save the data sets in a partial Procrustes file using an x1y1x2y2 format. Twogroup8 was used to conduct analytic test using Goodall's F test (Procrustes) to test for significant differences in shapes between two groups. Resampling test for F-test, Procrustes were conducted using 100 bootstraps. Partial Procrustes distances between the two means were calculated using Twogroup8 as well. Paleontological Statistics version 3.15 (PAST) (Hammer et al., 2001) was used to construct principal components analysis (PCA) from digitized landmark data and \log_{10} transformed body measurements prior to their transformations to proportions of standard length. Thin-plate spline transformations of

data were also constructed using PAST to help visualize relative warps. PAST was also used to create a confusion matrix from linear discriminant analysis (LDA)

Spawning coloration of males was assigned a value by using GNU Image Manipulation Program (GIMP) (available from <https://www.gimp.org/>). Each male specimen had three hue values taken using the color selector function with a sample average collected and with a radius setting of 5. One measurement was taken from the anterior third of the body around the lower jaw, a second measurement from the middle of the body around the trunk region below the origin of the dorsal fin. The final measurement was collected from the caudal peduncle after termination of the dorsal fin. The three measurements were then averaged together for an overall score for each male. Student's t-test was then used to compare the averages from each drainage to each other to detect if any difference in coloration could be observed.

Aging

Individuals were aged by removing three scales from the caudal peduncle region using dissecting scope and forceps to ensure that individuals of the same age class were being compared. Prior to removing scales, specimens had to be rehydrated with distilled water to allow for easy scale removal. Scales from 49 individuals collected from Cypress Creek n=24 and Bayou de Chien n=25 were slide mounted, with all three scales from individual specimens on one slide and labeled to match each specimen photograph. All three scales were observed using 60x magnification on a compound microscope. Any observed annuli were then recorded.

Molecular Techniques

In addition to the 24 adults collected from Cypress Creek and 25 adults from Bayou de Chien, 7 juveniles from Rose Creek and 17 juveniles from Running Slough were collected.

Extraction of whole genomic DNA from fin clips of preserved specimens was conducted via CTAB, following lab protocol from Saghai-Marroof et al. (1984). After CTAB extraction, specimen DNA was resuspended using 25µl of nuclease free water. Samples were checked with a Qubit fluorometer to verify quantity of DNA. Specimens that yielded less than 10ng/µl were reprocessed to ensure enough DNA was available to make working solutions. Once quantity was checked, a working solution was made using each specimen's DNA and nuclease free water to achieve a 50µl sample at 10ng/µl concentration. The samples with most genomic yield were used for amplification which represented 24 individuals from Bayou de Chien, 11 from Cypress Creek, 7 from Rose Creek and 16 from Running Slough. The complete Cyt-b gene was amplified using polymerase chain reaction (PCR) using a primer pair EZCytbL 5'-CCTGAAAACACCGTTGTAA - 3' and EZCytbH 5'-CAAGGCCGATGCTCTAACTC - 3' (Sandel, 2012). Twenty-five microlitre reactions were set up using 12.5µl GoTaq, 1.5µl upstream primer, 1.5µl downstream primer, 3µl DNA and 6.5µl of nuclease free water. Amplification conditions were 30 cycles of 95°C, 45 secs; 54°C, 30 secs; 72°C, 90 sec. After amplification, specimens were plated and sent off for Sanger sequencing by Eurofins Genomics (<http://www.eurofinsgenomics.com/en/home.aspx>). Prior to Sanger Sequencing purification of PCR products was conducted by Eurofins Genomics. After Sanger Sequencing, only 4 individuals from Cypress Creek, 7 from Rose Creek, 15 from Running Slough and 17 from Bayou de Chien yielded enough sequence data to be used in comparisons.

After analysis, forward and reverse sequences were joined and edited using DNA Dynamo. Consensus sequences were then imported into AliView (Larsson, 2014) and aligned using Muscle 3.8. Sequences for everglades pygmy sunfish, *Elassoma evergladei* and spotted sunfish, *Lepomis punctatus* were obtained from GenBank (Benson et al., 2005) and used as an outgroup.

Additional *E. zonatum* sequences from nearby drainages were pulled from GenBank that were used in Sandel, 2012 (Table 1), then aligned with sequences from this study to create a FastML tree (Price et al., 2009, Price et al., 2010), and then edited with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) to determine nearest neighbor sequences to include in analysis. A haplotype network using TCS networks (Clement et al., 2002) was constructed in PopArt (<http://popart.otago.ac.nz>) using all four study sites and additional neighbor sequences. CIPRES Science Gateway (Miller et al., 2010) was used to conduct jModelTest2 (Darriba et al., 2012; Guindon and Gascuel, 2003) and RAxML (Stamatakis, 2014) tests to determine best fit trees with *Elassoma evergladei* and *Lepomis punctatus* being used as outgroups. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (Kumar et al., 2015) was used to estimate evolutionary divergence between sequences (Tamura et al., 2004).

CHAPTER 3

RESULTS

Body Measurement Comparison

Linear regressions indicated that measurements of body depth ($r=-0.55$) and snout length ($r=-0.50$) were correlated to standard length and were removed prior to MANOVA. Individuals were sexed and MANOVA indicated that there was a statistical difference between sites based on sex, $F(36,101.2)=7.652$, $p<0.0005$; Wilk's $\Lambda=0.02058$. Post-hoc analysis using pairwise Hotelling's tests (Table 2) indicated significant differences between Cypress Creek females and Cypress Creek males ($p=0.0008$), Bayou de Chien males ($p=0.0079$) and Bayou de Chien females ($p=0.0004$). Males from Cypress Creek were significantly different from Bayou de Chien males ($p=0.0293$) and from Bayou de Chien females ($p<0.0001$). Males and females at Bayou de Chien were not statistically different from each other ($p=0.058343$).

Students t-test comparing differences of means in body measurements of sampled individuals from Bayou de Chien (BC) and Cypress Creek (CC) (Table 3) indicated significant differences in standard length BC (\bar{X} 30.22, SD 2.11) and CC (\bar{X} 26.64, SD 2.37); $t(47)=5.5908$, $p<0.0001$, body width BC (\bar{X} 116.88, SD 10.41), and CC (\bar{X} 156, SD 19.94); $t(47)=-8.6574$, $p<0.0001$, pre-pelvic length BC (\bar{X} 374.64, SD 17.71), and CC (\bar{X} 385.71, SD 5.22); $t(47)=-1.7658$, $p=0.04$, eye diameter BC (\bar{X} 78, SD 5.97), and CC (\bar{X} 72.25, SD 6.72); $t(47)=3.1692$, $p=0.0013$ and upper jaw length BC (\bar{X} 75.8, SD 7.8) and CC (\bar{X} 70.92, SD 7.25); $t(47)=2.2664$, $p=0.0149$.

Comparisons of males from sample sites (Table 4) indicated differences in standard length BC (\bar{X} 29.15, SD 2.32), and CC (\bar{X} 25.96, SD 1.84); $t(19)=1.7291$, $p=0.001$, body width BC (\bar{X} 113.78, SD 8.60) and CC (\bar{X} 149.06, SD 19.94); $t(19)=-4.9495$, $p<0.0001$, pre-dorsal length BC (\bar{X}

518.13, SD 45.06) and CC (\bar{X} 483.47, SD 43.28); $t(19)=1.7846$, $p=0.0452$, anal fin length BC (\bar{X} 245.85, SD 7.97), and CC (\bar{X} 250.32, SD 35.64); $t(19)=-10.3965$, $p<0.0001$, and upper jaw length BC (\bar{X} 79.84, SD 8.38) and CC (\bar{X} 74.13, SD 6.01); $t(19)=1.842$, $p=0.0419$.

Female comparisons (Table 5) showed significant differences in standard length BC (\bar{X} 30.22, SD 1.79), and CC (\bar{X} 27.32, SD 2.71); $t(26)=4.1187$, $p=0.0002$, body width BC (\bar{X} 118.68, SD 11.26), and CC (\bar{X} 162.75, SD 18.28); $t(26)=-7.8791$, $p<0.0001$, pre-pelvic length BC (\bar{X} 372.16, SD 19.04), and CC (\bar{X} 395.50, SD 24.02); $t(26)=2.8708$, $p=0.0040$, pre-anal length BC (\bar{X} 622.65, SD 31.20), and CC (\bar{X} 653.67, SD 32.73); $t(26)=-2.5497$, $p=0.0085$, caudal peduncle depth BC (\bar{X} 119.94, SD 7.73), and CC (\bar{X} 113.89, SD 7.81); $t(26)=2.0379$, $p=0.0259$, eye diameter BC (\bar{X} 76.82, SD 5.37) and CC (\bar{X} 71.70, SD 7.85); $t(26)=-20492$, $p=0.0253$ and upper jaw length BC (\bar{X} 73.49, SD 6.69) and CC (\bar{X} 67.78, SD 7.13); $t(26)=-2.1791$, $p=0.0191$).

PCA of \log_{10} transformed body measurements between Bayou de Chien and Cypress Creek indicated that Eigenvalues for PC1 and PC2 accounted for 65.64% of the variance (Fig. 7), which indicated minimal overlap between drainages and significant visual separation. Females from both drainages were compared as well. Eigenvalues for PC1 and PC2 accounted for 74.55% of the variance. The PCA plot (Fig. 8) showed complete separation by drainage. Male comparisons gave Eigenvalues that attributed 63.21% of variation to PC1 and PC2. The PCA plot showed significant drainage separation (Fig. 9).

Confusion matrix results computed from LDA indicated that the separate drainages (Table 6) were sufficient in identifying origin of individuals. Males (Table 7) and females (Table 8) both scored 100% correct assignment during analysis.

Age and Body Comparisons

No annuli were observed on any scale (Fig. 10), indicating all individuals were under 1 year of age. Preopercular pore counts were identical across both drainages with all individuals expressing 4 pores, paired as 2 and 2. Hue color comparisons of males from both drainages indicated a significant difference ($p=0.01$) between sites (Table 9). Males from Cypress Creek had darker shades of blue when compared to Bayou de Chien individuals.

Trunk bar counts (Table 10) between both drainages and between sexes had a mode of 11 bars. Bayou de Chien males and females ranged 9–11 bars while Cypress Creek males ranged 9–12 bar counts and females ranged 10–11.

Geometric Morphometric Analysis

Two groups analysis comparing both sites using landmark data resulted in a Procrustes F-test score of 2.36 with a significance level of $p=0.070$ with 100 bootstraps. Goodall's F-test resulted in $F=2.36$, $df=20$, $p=0.001$. Bootstrapped partial Procrustes distances resulted in a difference between means of 0.0205. Comparisons between females from both sites yielded Procrustes F-score of 3.96 $p=0.010$ with 100 bootstraps. Goodall's F-test analysis resulted in a $F=3.97$, $df=20.00$ $p<0.0001$. Bootstrapped partial Procrustes distances between means was 0.0298. Comparisons of males resulted in a F-score of 2.22, $p=0.0100$. Goodall's F-test resulted in $F=2.22$, $df 20$, $p=0.002$ with 100 bootstraps. For males, the bootstrapped partial Procrustes distances was 0.0021.

PCA comparisons of 12 Procrustes fitted landmarks for both drainages indicated that PC1 and PC2 explained 54.35% of the variation with PC3 explaining an additional 17.6%. The PCA plot (Fig. 11) did not show any distinct separation of drainages. Comparing males across

both drainages resulted in 57.5% of the variation being explained by PC1 and PC2. The plotted PCA (Fig. 12) showed little separation between groups. In females 60.26% of variation was explained by PC1 and PC2. The PCA plot (Fig. 13) showed some separation between drainages.

Thin-plate spline transformations using color coded comparison between drainages (Fig. 14) indicated that between drainages there was some enlarging of landmarks located at the occiput and around the eyes. In male comparisons (Fig. 15) there were only minor variations among landmarks. Females (Fig. 16) appear more variable with variations more evident in the head region and caudal peduncle landmarks.

Molecular

After conducting jModelTest2 a best fit consensus model was selected from 88 models. RAxML was then used to take that substitution model and generate a phylogenetic tree which showed individuals from Running Lake in Illinois falling out with individuals from Cypress Creek and Rose Creek indicating a grouping of individuals from above the Fall line while the majority of sequences from below the fall line grouped together (Fig. 17). A haplotype network was generated without outgroup sequences showing each sequenced individual's drainage (Fig. 18). Additional haplotype networks were constructed to show major drainage (Fig. 19) and location in relation to Fall Line (Fig. 20). Haplotype networks showed that individuals from above the Fall Line did share haplotypes, while an individual from below the Fall Line located in Lazare Louisiana had the most genetic variation from the rest of the sequenced individuals. Genetic differences from Mega 7 evolutionary divergence measurements (Table 11) indicated greatest pairwise distances was between all haplotypes to haplotype7 which represented one sequenced individual (OUACHT-LAZARE_LA_JQ514646).

CHAPTER 4

DISCUSSION

Comparison of measured individuals from this study to sampled *E. zonatum* populations from Mayden (1993) indicated that males in Cypress Creek fell into accepted ranges for known populations. However, mean body measurements of males from Cypress Creek were smaller than those measured by Mayden. Female measurements from Cypress Creek paralleled those seen in Mayden's comparison. However, females from Bayou de Chien were larger on average than females sampled by Mayden, while males were similar in measurements. Walsh and Burr (1984) noted that the maximum length of *E. zonatum* is usually 32-38mm but lacks definition as to whether those numbers refer to standard or total length. Some variation in size for such a wide-ranging species can be caused by more southern individuals growing larger due to earlier spawning periods and longer growing seasons (Walsh and Burr, 1984). Overall, it appears that individuals found above the fall line in Cypress Creek are of similar measurements to other sampled populations of *E. zonatum*.

Groupings were observed in the body measurement PCA analysis, with distinct separation of drainages observed. Such visual comparisons further indicate that there is separation between these drainages as far as morphological measurements are concerned. Of note though is that it appears that in the overall comparison of individuals from both drainages, Bayou de Chien individuals are mostly clustered together while Cypress Creek individuals seem to have greater variability. Body measurements were similar among males and females at Bayou de Chien, while measurements were more varied between sexes at Cypress Creek. With individuals seeming to fall out in respected drainages it may be possible to predict where unknown individuals of same age class may have originated from.

Geometric morphological data used in PCA plots yielded no defined separation of individuals between drainages. This lack of separation indicates that the 12 landmarks used simply do not relay enough variation in body size across the drainages to cause separation between sites. It appears likely that overall body shape between the sites is not being influenced by certain abiotic factors such as habitat or stream type.

One visual difference between sites was observed between males. Cypress Creek males appeared to have more blue coloration to their body while Bayou de Chien individuals appeared to be lighter overall. Assigning hue values and taking 3 measurements from each individual confirmed that there was a difference between sites. However, due to small sample sizes and restricted sampling sites it cannot be verified as a variable to separate drainages. Male specimens in Bayou de Chien were collected from very turbid drainage ditches while Cypress Creek individuals came from a clear swamp area where the bottom was easily visible. Turbidity has been noted to result in duller colored males as it can reduce the penetration of light resulting in reduced color signals from the color spectrum. With this reduced effectiveness of color, a subsequent relaxation of sexual selection occurs for color, with the result being the loss of nuptial coloration in males (Seehausen et al., 1997).

Using Cyt-b for genetic analysis revealed that there is little to no variation in individuals from Rose Creek and Cypress Creek. Both populations fall out with each other and have nearly identical sequences. However, an interesting observation was uncovered in the phylogenetic trees as two sequenced individuals from Running Lake in Illinois fell out with individuals collected in Cypress Creek and Rose Creek. This is an important observation, as all three sites are found above the fall line and could be indicative of founder effect. The Interior Low Plateaus of central Kentucky is noted to have sections such as the Shawnee Hills sharing similar aquatic

habitats to the Coastal Plain region (Burr and Warren, 1986). Thus, species found in the Coastal Plain region have dispersed to areas beyond the Coastal Plain. The Shawnee Hills region extends from Kentucky and into Illinois and contains the Tradewater River, Green River and Running Lake populations from this study.

The remaining sampled individuals showed greater genetic diversity. Running Slough individuals were observed to be closely aligned with individuals from Reelfoot Lake in Tennessee. Running Slough drains into Reelfoot Lake after traversing across the landscape for approximately 9 miles. Many of the Mississippi drainage individuals were similar to each other with only a specimen collected at Lazare Park near Ouachita River in Louisiana showing the greatest separation from all other individuals.

While the results indicate separation between individuals from above the fall Line to sites below the fall line, measurements of individuals from Cypress Creek and Bayou de Chien still fall within accepted measurements of *E. zonatum* throughout their entire range. Warren (1980) suggested that appropriate habitats for *E. zonatum* existed in the western third of Kentucky due to the parallel of *E. zonatum* distributions to bald cypress *Taxodium distichum*. Braun (1943), noted existence of *Taxodium distichum* in river swamps and sloughs that went as far east as Cypress Creek area. Warren and Cicerello (1982) concluded that the loss of wetland habitats has played a significant role in the sporadic distribution of *E. zonatum* above the fall line.

Elassoma zonatum and its sister species *E. evergladei*, and *E. okefenokee* have large overlapping geographic distributions that suggest a deeper divergence with current distributions representing postspeciation dispersal. In studies these species branched consistently from

deeper nodes across the mitochondrial and nuclear gene trees (Quattro et al., 2001). It is likely that during past glacial events *E. zonatum* suffered a range contraction. During the post-Wisconsinan deglaciation a northward expansion of *E. zonatum* was detected (Sandel, 2012). Due to the similarity of habitat between the Coastal Plain and Shawnee Hills region (Burr and Warren, 1986), the Shawnee Hills may have provided the northern limit of *E. zonatum* range expansion.

Further genetic testing should be conducted on populations found above the fall line to determine stability of populations. These populations are disjunct from other populations and may now be experiencing selective pressures. Biodiversity loss threatens to disrupt the function of ecosystems, with the potential of consequences for humans as well. Often this loss is measured by species extinction rates, but considerations should be made to include population diversity with measures including changes in size, number, distribution and genetic composition of population and potential implications those changes may have (Luck et al., 2003). Microsatellites have the potential to address some concerns of these population as they allow ecologists to estimate parameters such as migration rates, and relatedness of individuals (Selkoe and Toonen, 2006) but other molecular methods could be used by the researcher.

This study indicates the possibility of a range expansion of *Elassoma zonatum* after the Wisconsin glaciation. Populations found above the fall line are genetically similar to populations found below the fall line but do have their own unique haplotype that encompasses the Shawnee Hill region of Kentucky and Illinois. These populations are restricted in their range and future testing should be conducted to determine the genetic stability of said populations. Populations found in Kentucky are in areas of significant agriculture and mining activities although part of Cypress Creek is protected as a Kentucky State Nature Preserve property.

These activities have the potential to negatively impact those distributions. The Illinois population is located at Union County State Fish and Wildlife area and may see better protection than some of the Kentucky populations.

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APPENDICES

APPENDIX A
TABLES

Table 1. *Elassoma zonatum* and outgroup sequences acquired from NCBI from nearby drainages used for comparisons with sampled Kentucky populations. Two outgroups were used to construct phylogenetic tree. Outgroup1 was a *Elassoma evergladei* and Outgroup2 was *Lepomis punctatus*.

Drainage	Subdrainage	State	Latitude	Longitude	NCBI
White River	Little Red River	Arkansas	35.260700	-91.673700	JQ514577
White River	Little Red River	Arkansas	35.241217	-91.657383	JQ514578
White River	Little Red River	Arkansas	35.241217	-91.657383	JQ514579
Mississippi	Castor	Missouri	37.073300	-90.032500	JQ514596
Mississippi	Castor	Missouri	37.073300	-90.032500	JQ514597
Mississippi	St. John	Missouri	36.921050	-89.554980	JQ514598
Mississippi	Blue Ditch	Missouri	36.917680	-89.532530	JQ514599
Mississippi	Blue Ditch	Missouri	36.917680	-89.532530	JQ514600
Mississippi	Running Lake	Illinois	37.544530	-89.451170	JQ514601
Mississippi	Running Lake	Illinois	37.544530	-89.451170	JQ514602
Mississippi	Obion Creek	Kentucky	36.754560	-89.003200	JQ514603
Mississippi	Obion Creek	Kentucky	36.754560	-89.003200	JQ514604
Mississippi	Reelfoot	Tennessee	36.395760	-89.339970	JQ514605
Mississippi	Reelfoot	Tennessee	36.395760	-89.339970	JQ514606
Mississippi	Loosahatchie	Tennessee	35.418240	-89.523240	JQ514607
Mississippi	Loosahatchie	Tennessee	35.418240	-89.523240	JQ514608
Ouachita	Lazarre	Louisiana	32.483730	-92.117447	JQ514646
Ouachita	Lazarre	Louisiana	32.483730	-92.117447	JQ514647
Mississippi	Little Tallahatchie	Mississippi	34.519500	-89.364100	JQ514691
Mississippi	Little Tallahatchie	Mississippi	34.519500	-89.364100	JQ514692
Mississippi	Bay Creek	Illinois	37.392194	-88.745650	JQ514697
Mississippi	Grays Creek	Mississippi	34.968222	-89.273611	JQ514724
Mississippi	Grays Creek	Mississippi	34.968222	-89.273611	JQ514725
Mississippi	Missouri	Missouri	N/A	N/A	JQ514758
Mississippi	Missouri	Missouri	N/A	N/A	JQ514759
Outgroup1	N/A	N/A	N/A	N/A	KF746614
Outgroup2	N/A	N/A	N/A	N/A	AY828994

Table 2. Post-hoc analysis conducted using pairwise Hotelling's tests. Each value shown is the calculated p-value. A p-value <0.05 is considered significant.

	<i>CYPRESS CREEK FEMALES</i>	<i>BAYOU DE CHIEN FEMALES</i>	<i>CYPRESS CREEK MALES</i>	<i>BAYOU DE CHIEN MALES</i>
<i>CYPRESS CREEK FEMALES</i>		0.00042393	0.00085418	0.0078923
<i>BAYOU DE CHIEN FEMALES</i>	0.00042393		8.08E-06	0.058343
<i>CYPRESS CREEK MALE</i>	0.00085418	8.08E-06		0.0293
<i>BAYOU DE CHIEN MALES</i>	0.0078923	0.058343	0.0293	

Table 3. Proportional body measurement comparisons of sampled *Elassoma zonatum* individuals from Cypress Creek (n=24) and Bayou de Chien (n=25). A p value <0.05 is considered significant.

<i>Measurement</i>	<i>Bayou de Chien</i>			<i>Cypress Creek</i>			<i>t-value</i>	<i>p</i>
	<i>Range</i>	\bar{X}	<i>SD</i>	<i>Range</i>	\bar{X}	<i>SD</i>		
<i>Standard Length</i>	26.97-35.51	30.2208	2.11	22.93-31.96	26.64	2.37	5.5908	<0.0001
<i>Body Width</i>	101-139	116.88	10.41	115-194	156	19.94	-8.6574	<0.0001
<i>Body Depth</i>	215-280	244.72	17.66	223-308	268.79	22.57	-4.1683	<0.0001
<i>Predorsal Length</i>	432-578	505.04	38.58	438-586	500.29	42.59	0.4093	0.3421
<i>Prepelvic length</i>	327-411	374.64	17.71	312-421	385.71	5.22	-1.7658	0.0420
<i>Preanal length</i>	558-678	612.52	32.43	571-741	61.08	44.84	-1.6655	0.0512
<i>Dorsal Fin length</i>	306-433	365.16	36.64	278-450	365.12	50.79	0.0028	0.4989
<i>Anal Fin Length</i>	169-256	212.8	27.47	127-278	216.875	46.03	-0.3781	0.3535
<i>CP Length</i>	176-269	229.76	24.69	165-274	228.71	23.43	0.1528	0.4396
<i>CP depth</i>	102-147	125.4	10.22	102-151	126.63	15.47	-0.3284	0.3720
<i>Head Length</i>	258-377	304.52	22.99	227-351	297.29	28.18	0.9857	0.1647
<i>Snout length</i>	38-55	45.84	4.72	40-64	51.42	5.68	-3.7435	0.0002
<i>Eye diameter</i>	68-90	78	5.97	63-85	72.25	6.72	3.1692	0.0013
<i>Upper jaw length</i>	61-96	75.8	7.8	59-84	70.92	7.25	2.2664	0.0140

Table 4. Proportional body measurement comparisons of *Elassoma zonatum* males from Cypress Creek (n=12) and Bayou de Chien (n=9). Proportions expressed as thousandths of standard length. A p value <0.05 is considered significant.

Measurement	Bayou de Chien			Cypress Creek			t-value	p
	Range	\bar{X}	SD	Range	\bar{X}	SD		
Standard Length, mm	26.97-33.89	29.15	2.32	22.93-29.62	25.96	1.84	1.7291	0.0011
Body Width	101-129	113.78	8.60	115-180	149.06	19.94	-4.9495	<0.0001
Body Depth	223-280	253.53	18.17	245-308	279.37	22.82	-2.7914	0.0058
Predorsal Length	432-578	518.13	45.06	438-571	483.47	43.28	1.7846	0.0452
Prepelvic length	362-401	378.70	15.37	312-407	375.88	24.17	0.3053	0.3817
Preanal length	558-640	594.49	27.93	571-741	608.48	45.01	-0.8191	0.2114
Dorsal Fin length	310-433	370.56	43.05	332-450	389.82	40.71	-1.0473	0.1541
Anal Fin Length	236-256	245.85	7.97	142-278	250.32	35.64	-10.3965	<0.0001
CP Length	176-261	218.97	31.60	200-274	229.44	20.76	-0.9171	0.1853
CP depth	128-147	135.12	5.74	119-151	139.48	9.02	-1.2645	0.1107
Head Length	292-377	319.14	26.99	257-351	309.84	27.06	0.7801	0.2225
Snout length	43-85	52.82	12.64	40-64	52.12	6.75	0.1633	0.4360
Eye diameter	59-90	77.65	9.74	64-81	72.72	5.80	1.4507	0.0816
Upper jaw length	67-96	79.84	8.38	65-82	74.13	6.01	1.8242	0.0419

Table 5. Proportional body measurement comparisons of *Elassoma zonatum* females from Cypress Creek (n=12) and Bayou de Chien (n=16). Proportions expressed as thousandths of standard length. A p value <0.05 is considered significant.

Measurement	Bayou de Chien			Cypress Creek			t-value	p
	Range	\bar{x}	SD	Range	\bar{x}	SD		
Standard Length, mm	28.2-35.51	30.82	1.79	23.92-31.96	27.32	2.71	4.1187	0.0002
Body Width	101-139	118.68	11.26	128-194	162.75	18.28	-7.8791	<0.0001
Body Depth	215-266	239.72	15.76	223-285	258.21	17.44	-2.9359	0.0034
Predorsal Length	443-567	497.57	33.58	460-586	517.27	36.00	-1.4901	0.0741
Prepelvic length	327-411	372.16	19.04	326-403	395.50	24.02	-2.8708	0.0040
Preanal length	577-678	622.65	31.20	615-727	653.67	32.73	-2.5497	0.0085
Dorsal Fin length	306-416	362.25	33.73	278-417	340.34	48.92	1.4041	0.0861
Anal Fin Length	169-214	194.28	12.51	127-223	183.47	26.93	1.4199	0.0838
CP Length	205-269	235.78	18.53	165-267	227.94	26.84	0.9156	0.1841
CP depth	102-136	119.94	7.73	102-123	113.89	7.81	2.0379	0.0259
Head Length	258-321	296.35	16.19	227-319	284.72	7.03	1.5178	0.0706
Snout length	38-53	43.92	4.11	40-58	50.83	4.64	-4.1540	0.0002
Eye diameter	68-87	76.82	5.37	63-85	71.70	7.85	-2.0492	0.0253
Upper jaw length	61-87	73.49	6.69	59-84	67.78	7.13	-2.1741	0.0191

Table 6. Summary classification for least discriminant analysis of *Elassoma zonatum* by drainage. Rows=given group, columns=predicted groups.

	<i>Bayou de Chien</i>	<i>Cypress Creek</i>	<i>Total</i>
<i>Bayou de Chien</i>	25	0	25
<i>Cypress Creek</i>	0	24	24
<i>Total</i>	25	24	49

Table 7. Summary classification for least discriminant analysis of male *Elassoma zonatum* by drainage. Rows=given group, columns=predicted groups.

	<i>Bayou de Chien</i>	<i>Cypress Creek</i>	<i>Total</i>
<i>Bayou de Chien</i>	9	0	9
<i>Cypress Creek</i>	0	12	12
<i>Total</i>	9	12	21

Table 8. Summary classification for least discriminant analysis of female *Elassoma zonatum* by drainage. Rows=given group, columns=predicted groups.

	<i>Bayou de Chien</i>	<i>Cypress Creek</i>	<i>Total</i>
<i>Bayou de Chien</i>	16	0	16
<i>Cypress Creek</i>	0	12	12
<i>Total</i>	16	12	28

APPENDIX B
FIGURES

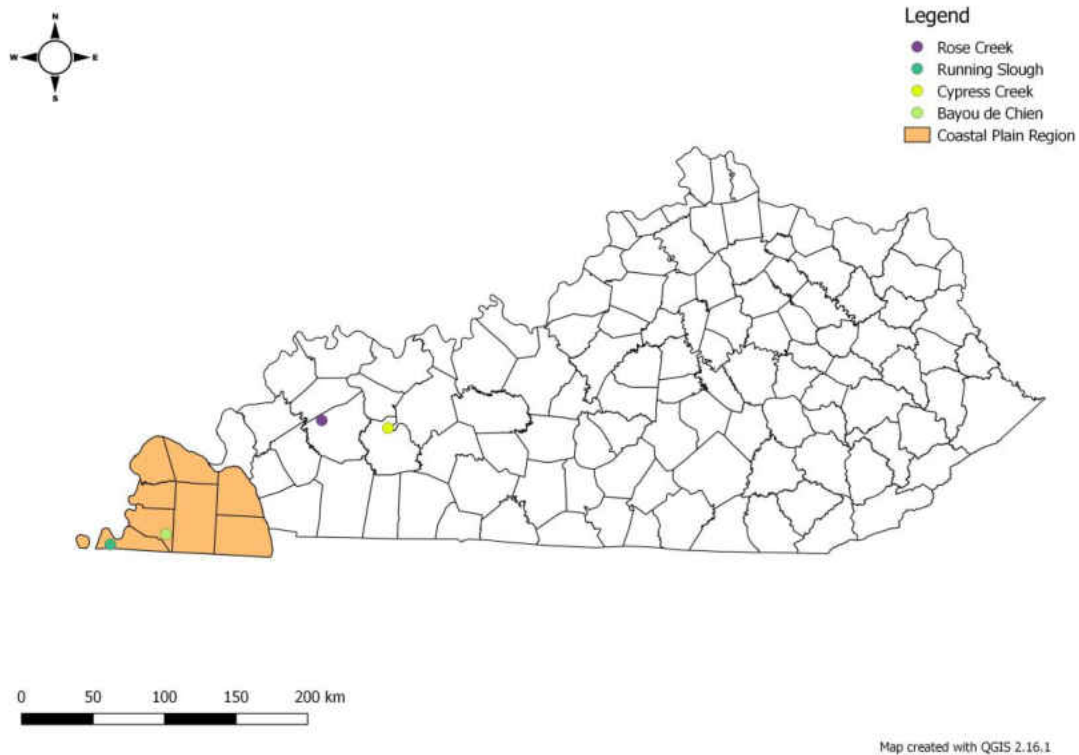
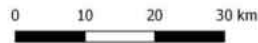
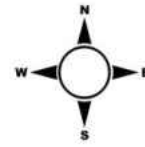


Figure 1. Locations of *Elassoma zonatum* sampling sites above and below the fall line in Kentucky. (Sources: shapefiles Kentucky Counties via kygeonet.ky.gov and Physiogeographic Regions via kygeonet.ky.gov).



Sampling site for *Elassoma zonatum* in Cypress Creek located in Muhlenberg County, Kentucky. Map created with QGIS 2.16.1

Figure 2. Cypress Creek *Elassoma zonatum* collection site located in Muhlenberg County, Kentucky. Part of the lower Green River drainage. (Sources: shapefiles NHD 24K Streams, Muhlenberg County via <http://www.uky.edu/KGS/gis/NHD24DOWN.htm> and Kentucky Counties via kygeonet.ky.gov).

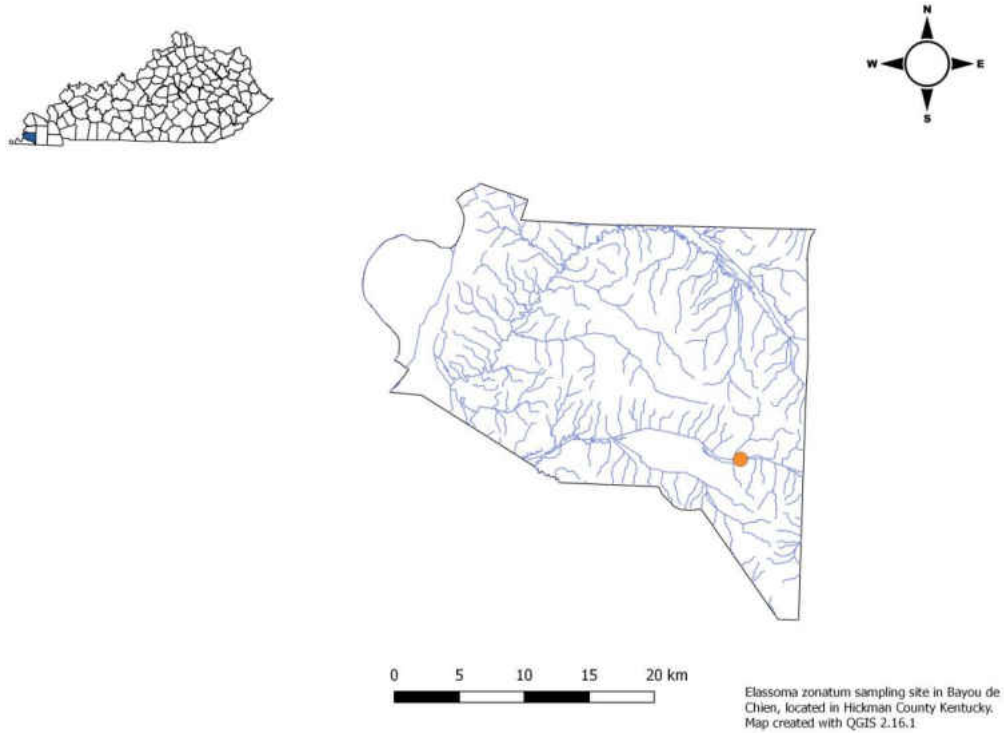


Figure 3. Bayou de Chien *Elassoma zonatum* collection site located in Hickman County, Kentucky. Part of the lower Mississippi River drainage. (Sources: shapefiles NHD 24K Streams, Fulton County via <http://www.uky.edu/KGS/gis/NHD24DOWN.htm> and Kentucky Counties via kygeonet.ky.gov).

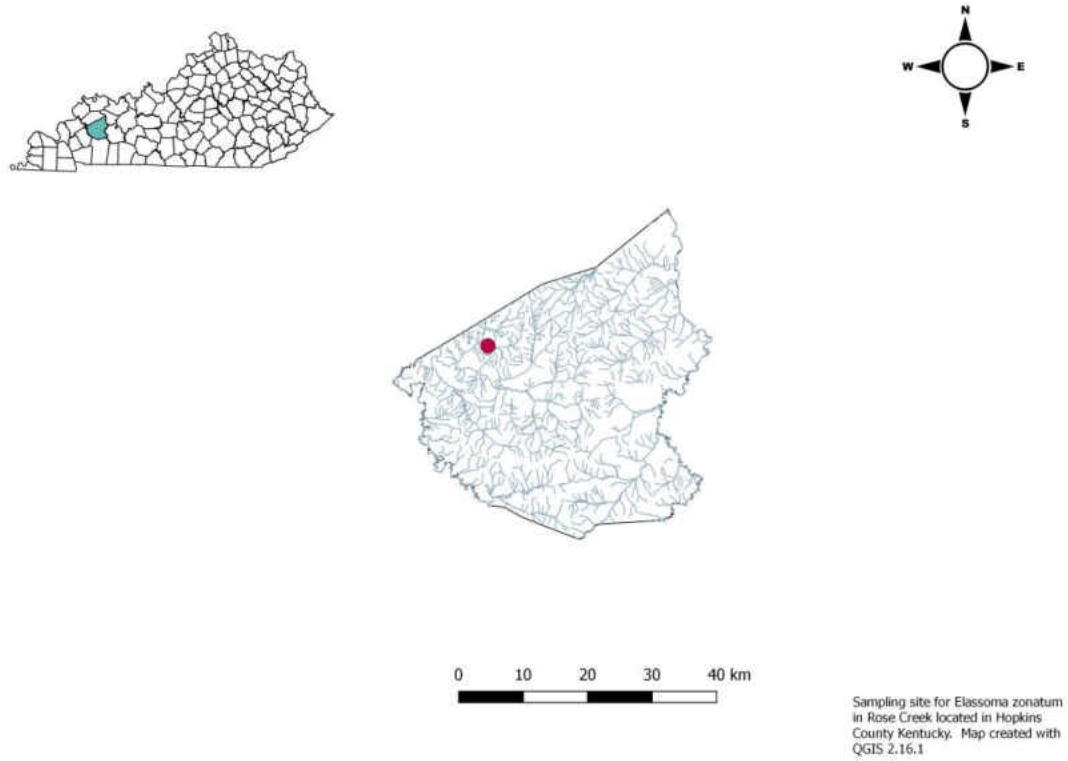
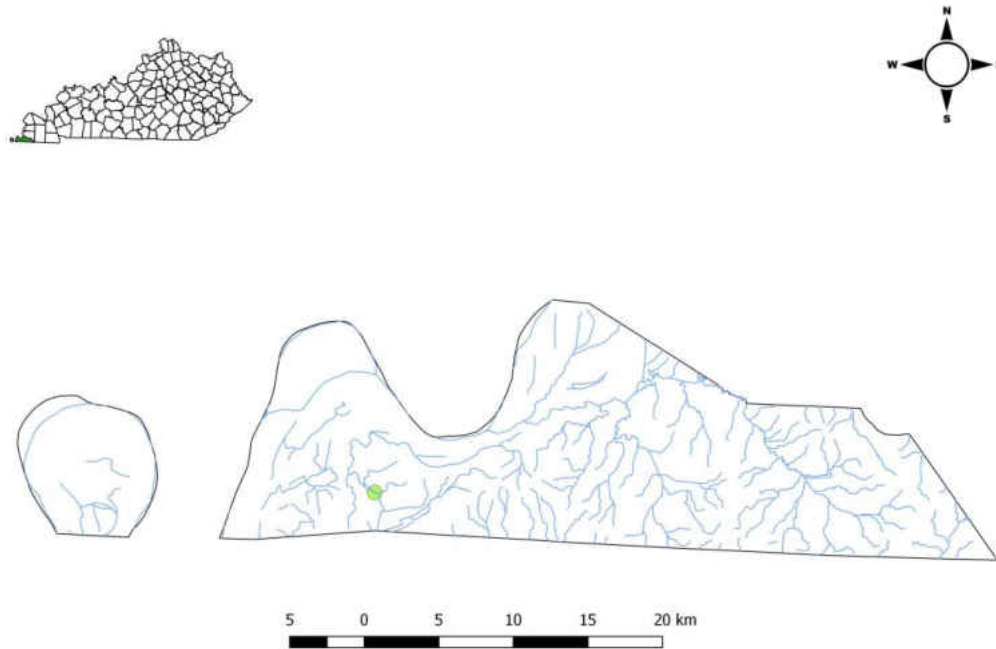


Figure 4. Rose Creek *Ellassoma zonatum* collection site located in Hopkins County, Kentucky. Part of the Tradewater River drainage. (Sources: shapefiles NHD 24K Streams, Hopkins County via <http://www.uky.edu/KGS/gis/NHD24DOWN.htm> and Kentucky Counties via kygeonet.ky.gov).



Sampling site for *Ellassoma zonatum* in Running Slough located in Fulton County, Kentucky. Map created with QGIS 2.16.1

Figure 5. Running Slough *Ellassoma zonatum* collection site located in Fulton County, Kentucky. Part of the lower Mississippi River drainage. (Sources: shapefiles NHD 24K Streams, Fulton County via <http://www.uky.edu/KGS/gis/NHD24DOWN.htm> and Kentucky Counties via kygeonet.ky.gov).



Figure 6. *Ellassoma zonatum* male illustrating the 12 landmarks used for geometric morphometric analysis.

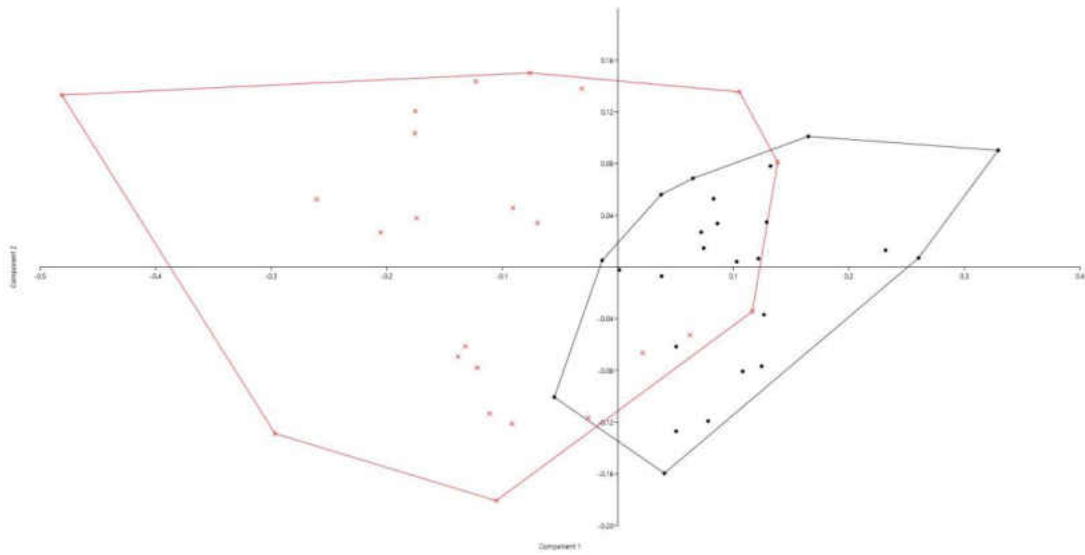


Figure 7. Principal Components Analysis (PCA) of \log_{10} transformed body measurements between *Elassoma zonatum* from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 (standard length) accounts for 48.938% of the variation while PC2 (Body depth) accounts for 16.7%.

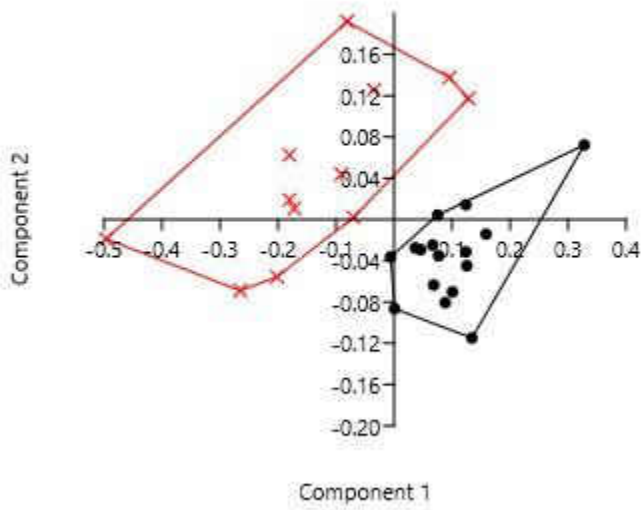


Figure 8. Principal Components Analysis (PCA) of \log_{10} transformed body measurements between *Elassoma zonatum* females from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 (standard length) accounts for 62% of the variation while PC2 (body depth) accounts for 12.55%.

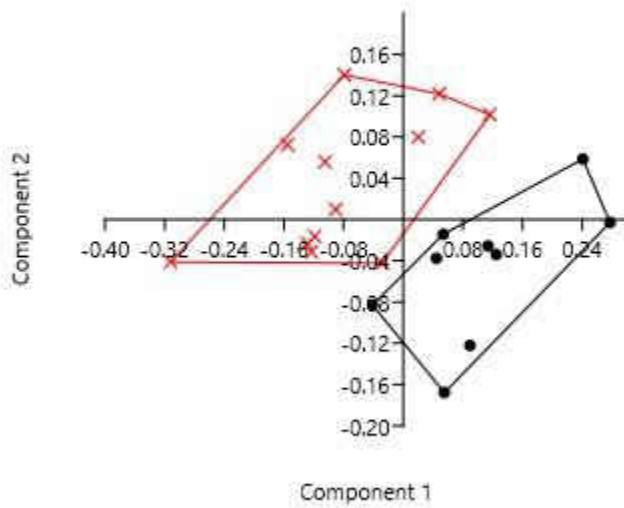


Figure 9. Principal Components Analysis (PCA) of \log_{10} transformed body measurements between *Elassoma zonatum* males from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 (standard length) accounts for 48.19% of the variation while PC2 (body depth) accounts for 15.02%.



Figure 10. Caudal peduncle scale of a female *Elassoma zonatam* from Bayou de Chien, illustrating lack of annulus observed in all specimens. All specimens were at or under 1 year of age when collected.

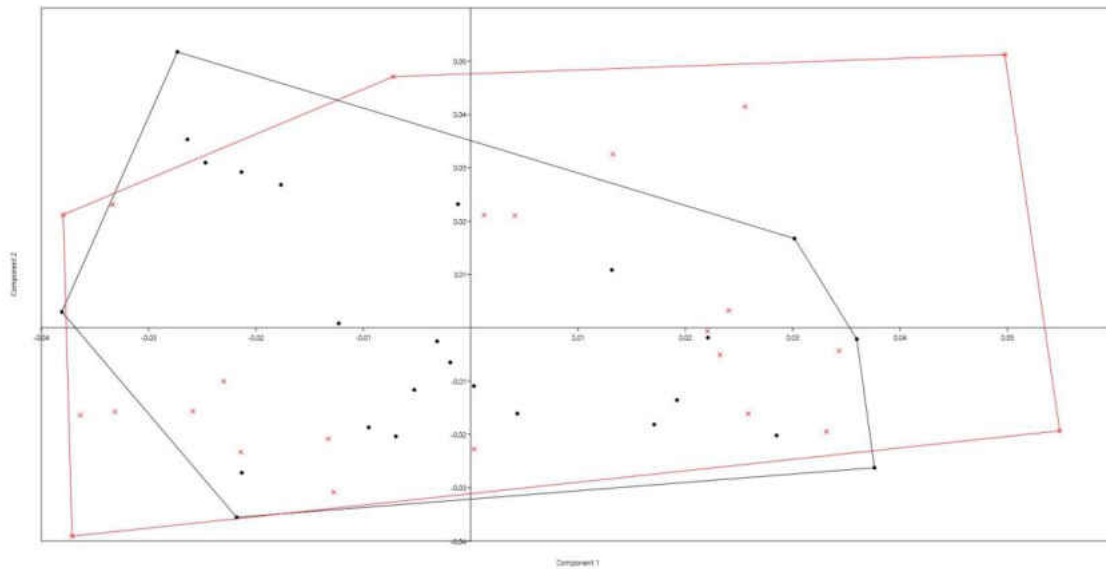


Figure 11. Principal Components Analysis (PCA) of Procrustes distances for the geometric morphometric analysis of *Ellassoma zonatum* from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 accounts for 28.02% of variation and PC2 accounts for 25.85%.

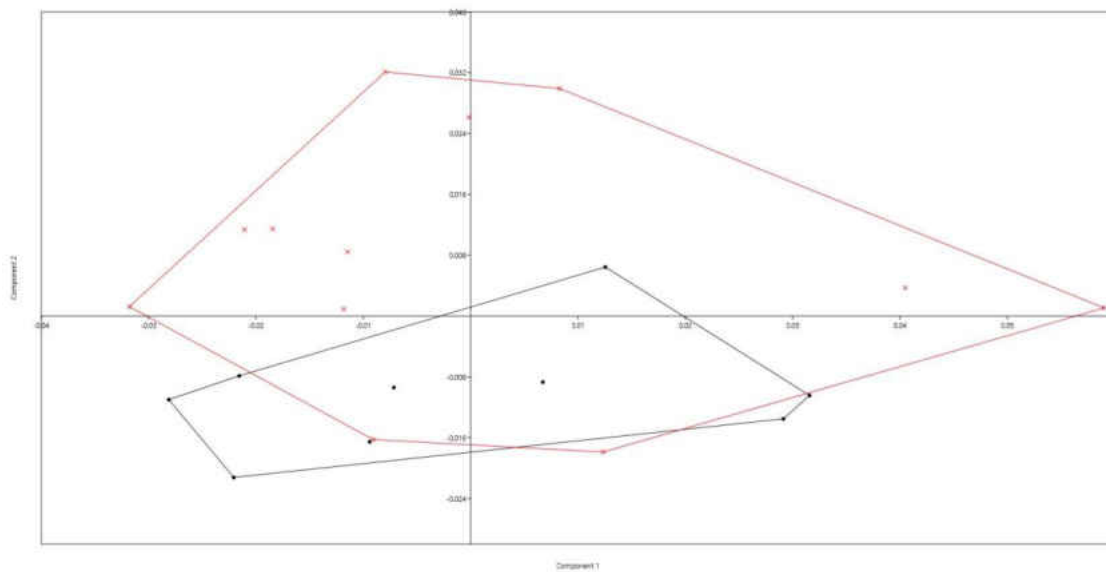


Figure 12. Principal Components Analysis (PCA) of Procrustes distances for the geometric morphometric analysis of *Ellassoma zonatum* males from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 accounts for 40.23% of variation and PC2 accounts for 17.32%.

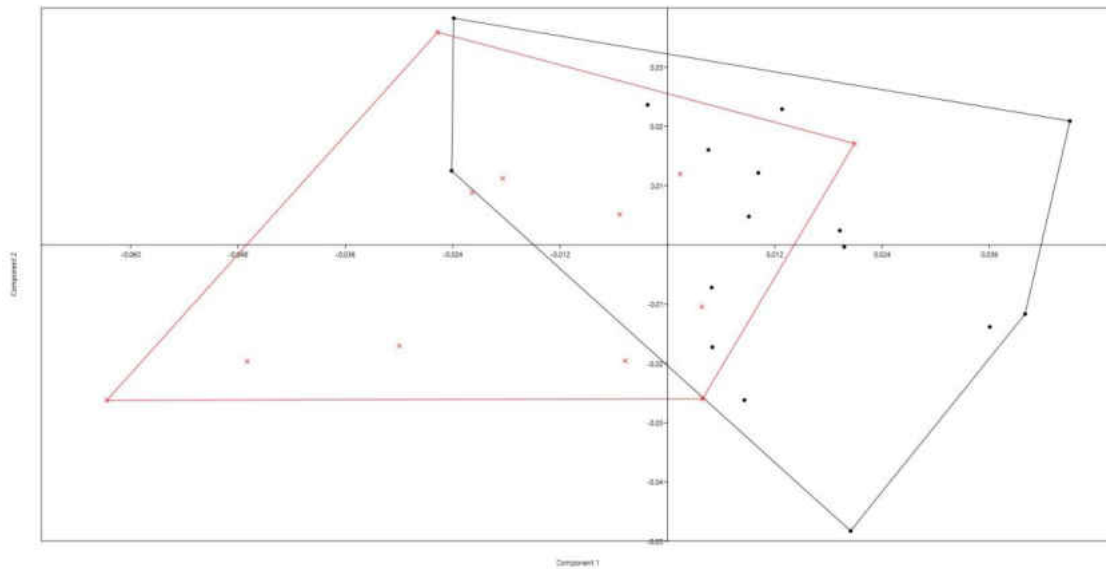


Figure 13. Principal Components Analysis (PCA) of Procrustes distances for the geometric morphometric analysis of *Ellassoma zonatum* females from Bayou de Chien (black dots) and Cypress Creek (red x). PC1 accounts for 35.31% of variation and PC2 accounts for 24.82%.

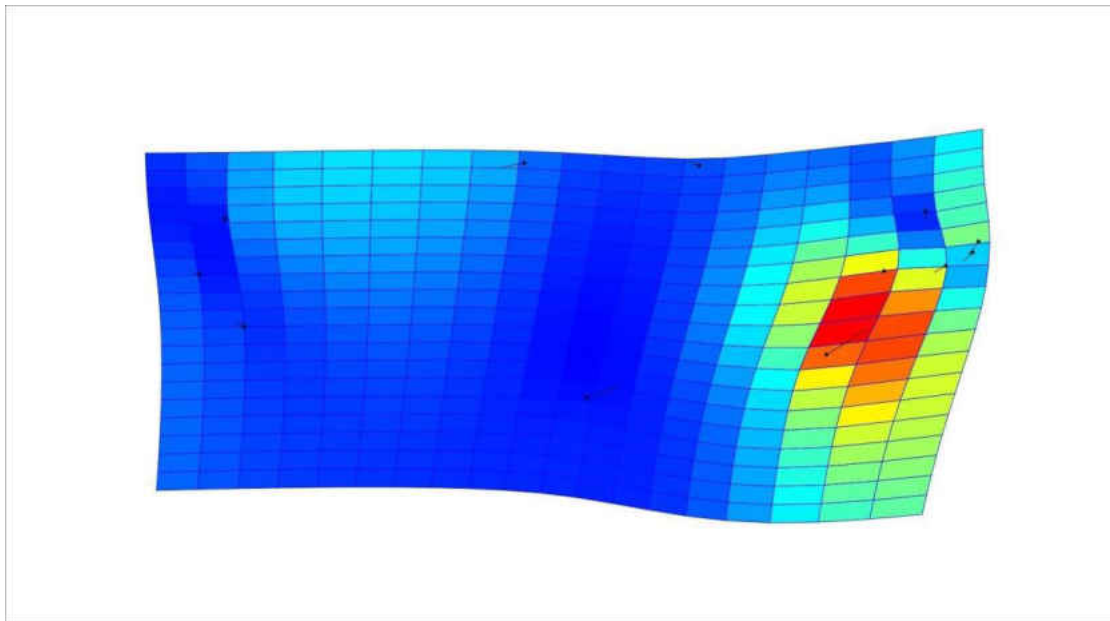


Figure 14. Thin-plate spline transformations using color coding between Cypress Creek and Bayou de Chien; head is to the right. Color coding shows green for expansion and purple for contraction. Vector arrows indicate directional stretching.

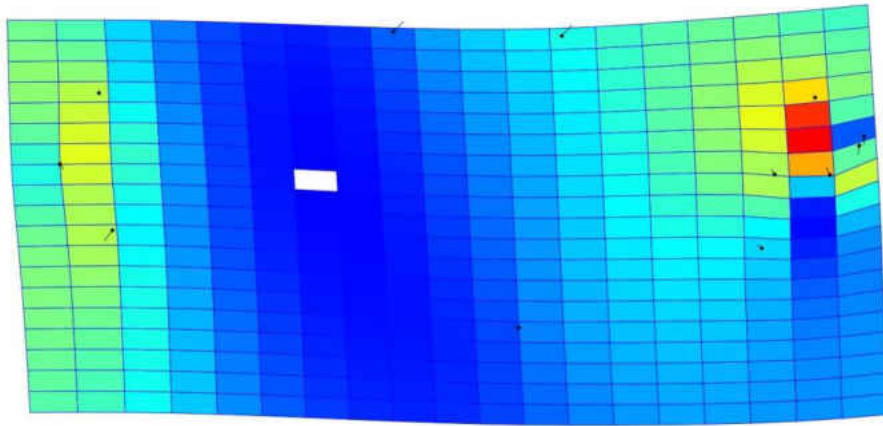


Figure 15. Thin-plate spline transformations using color coding between males at Cypress Creek and Bayou de Chien; head is to the right. Color coding shows green for expansion and purple for contraction. Vector arrows indicate directional stretching.

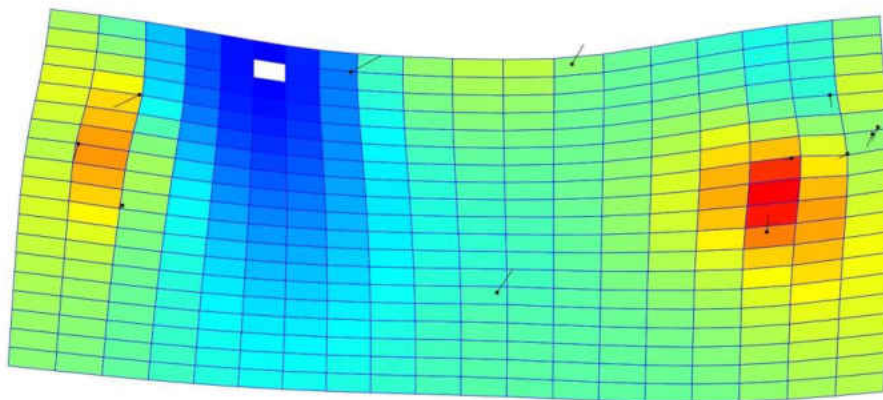


Figure 16. Thin-plate spline transformations using color coding between females at Cypress Creek and Bayou de Chien; head is to the right. Color coding shows green for expansion and purple for contraction. Vector arrows indicate directional stretching.

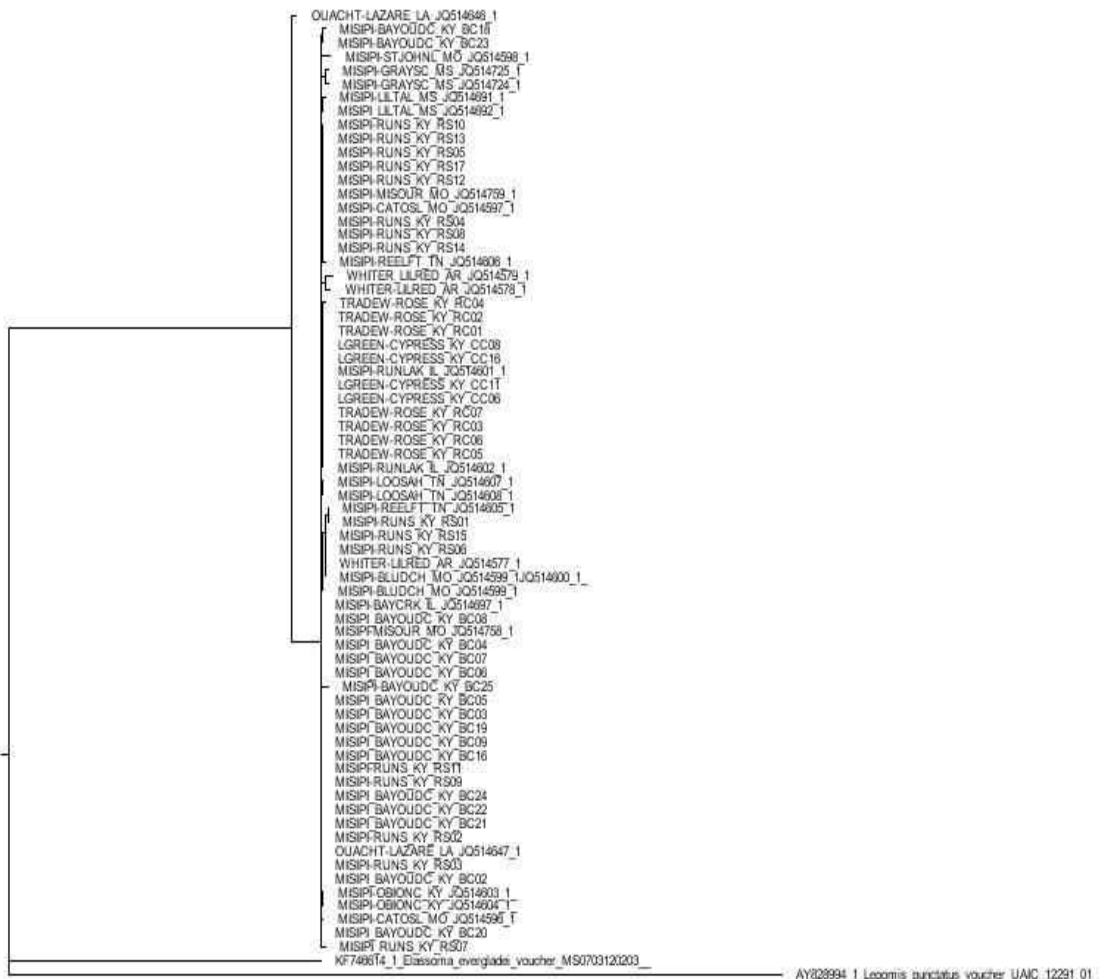


Figure 17. Phylogenetic tree constructed with RAxML using sequences from 43 sampled individuals in Kentucky and 25 sequences from other drainages located along the Mississippi and Lower Ohio river drainages accessed from Genbank.

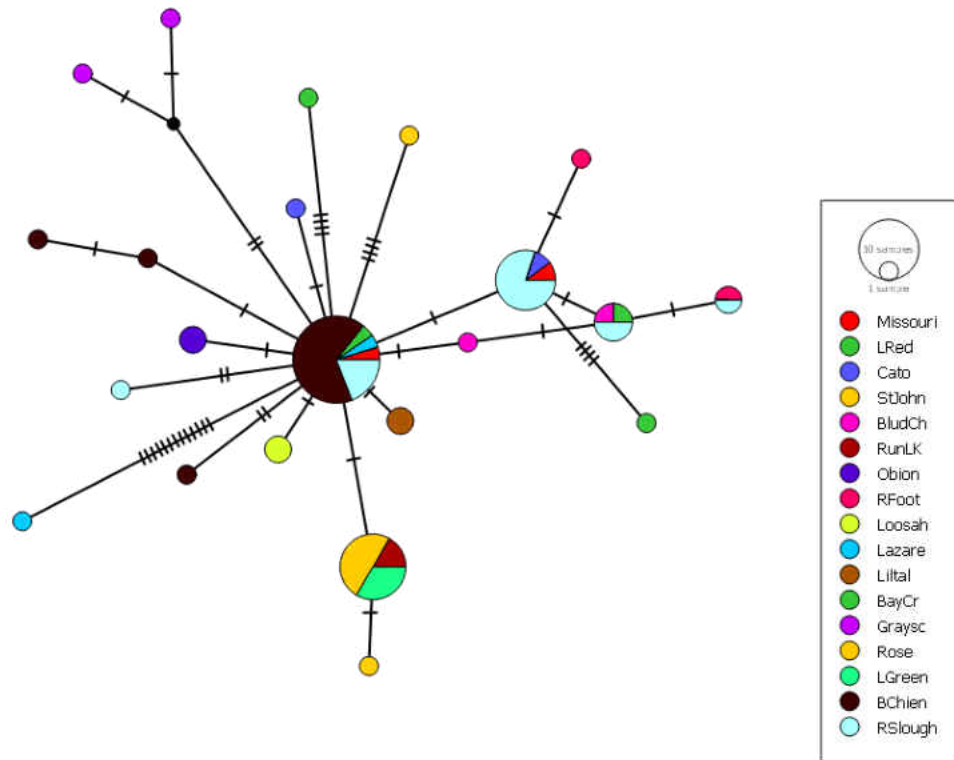


Figure 18. Haplotype network of 26 haplotypes of Cyt-b sequences of 68 *Elasmoma zonatum* specimens by sampling site using popart 1.7. Most significant variation is observed in the Lazare population located in the Ouachita River drainage in Louisiana.

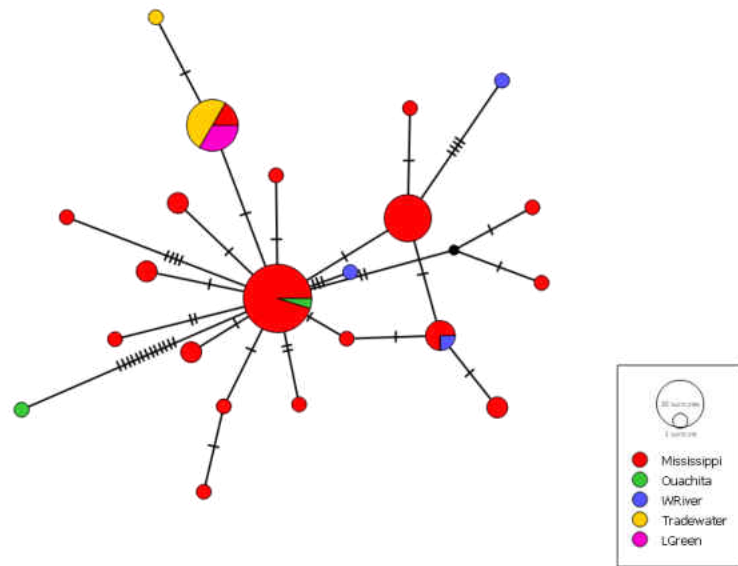


Figure 19. Haplotype network of 26 haplotypes of Cyt-b sequences of 68 *Ellassoma zonatum* specimens by major drainage using popart 1.7. Mississippi River made up bulk of haplotype sequences with only one population from Illinois sharing the same haplotype as individuals from the Lower Green and Tradewater drainages in Kentucky.

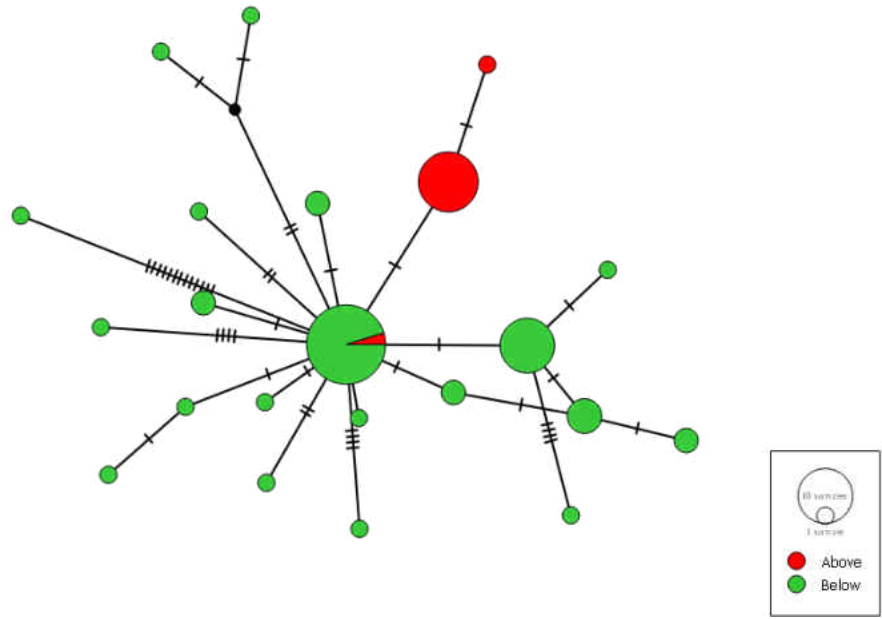


Figure 20. Haplotype network of 26 haplotypes of Cyt-b sequences of 68 *Ellassoma zonatum* specimens by location in relation to the fall line. Only individuals from Bay Creek, Illinois fell out with individuals below the fall line. Individuals from Tradewater, and Lower Green systems in Kentucky are shown above the fall line along with individuals from Running Lake, Illinois. Network created using popart 1.7.