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Phylogeny of the Orangethroat Darter (*Etheostoma spectabile*) species complex in the Ozark Highlands of Arkansas

McKenna George
University of Arkansas, Fayetteville

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**Phylogeny of the Orangethroat Darter (*Etheostoma spectabile*) species complex in the
Ozark Highlands of Arkansas**

McKenna George

University of Arkansas

Honors Mentor: Dr. Marlis Douglas

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Abstract

Darters are small, benthic fishes that live in freshwater rivers and streams and belong to the family Percidae. Pleistocene glaciations fragmented many darter species, resulting in speciation, but new species are often hard to detect if they are morphologically identical to pre-existing species. Intraspecific hybridization and resulting introgression, which occur frequently in glaciated areas, further complicate identification by introducing heterospecific genomes into mitochondrial DNA, making it difficult to accurately resolve phylogenetic relationships. The results of Bossu and Near's 2009 study highlight this issue, showing a large degree of incongruence between mitochondrial and nuclear gene trees.

This study analyzed samples from 50 collection sites along the White River Drainages in the Ozark Highlands region of Arkansas, an area that is high in both species richness and habitat diversity. SVDQuartets analysis generating bootstrap values for 1000 iterations recovered 12 species of *Etheostoma*, including 3 from the *E. spectabile* species complex, which was surprisingly non-monophyletic for the represented taxa. However, the relationships shown in the tree are consistent with previous studies which concluded that heterospecific DNA is being introgressed into the *E. spectabile* complex, although the sister-species relationships recovered differ from those found in Bossu and Near. The relationships displayed in this tree reveal the tendency for hybridization and introgression to occur between members of *E. spectabile* and other *Etheostoma*, however, sampling size and sampling area are both small, and further analysis is needed that includes more individuals and a broader sampling across a wide range of darter habitats to determine if these relationships are representative of the clade as a whole.

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Literature Review

Species complexes: A look into species concepts, molecular biology, and species conservation.

Introduction

In this review, I will be providing an overview of the concepts and research methods essential to the study of species complexes. First, I will discuss the development of several concepts that are used to delimitate species: the ecological species concept, biological species concept, and phylogenetic species concept. Afterwards I will explain several factors that studies show have substantial influence over the formation of species complexes and describe the various methods that are used to study them. Finally, I will emphasize the importance of studying and identifying species complexes in regard to how species are protected and conserved.

Species Concepts

In the 19th and early 20th century, many theorists still believed that all species arrived on Earth instantaneously. This idea, which Darwin referred to as “immutable production,” alleged that variations of those species could arise due to natural selection, but those variant individuals did not have the ability to become a distinct species (Darwin 1859). The major alternative to this theory followed the idea that new species develop through gradual modification of pre-existing species via natural selection (Rabosky 2009). Darwin, through studying separate populations of a single species that had become geographically isolated from each other, helped fully develop the idea of “descent with modification” that we now consider so fundamental to our understanding of species formation (Darwin 1859). He found that the adaptations developed by each population

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over time in response to their separate environments created morphological and ecological differences that resulted in a level of divergence beyond what one would observe within a single species (Darwin 1859).

Darwin's theories align with what is now known as the ecological species concept, which defines a species as all conspecific individuals occupying the same niche within an environment (Schluter 2001; de Queiroz 2007). It recognizes that species diverge when populations become environmentally isolated from one another, ultimately resulting in reproductive isolation as each population develops adaptations suited for their particular environment that may not be viable in the environment of other sub-populations (Brown and Wilson 1956; Schluter 2001; de Queiroz 2007; Moritz *et al.* 2017). There are two ways that environmental isolation can occur. Species that diverge in complete isolation are said to undergo allopatric speciation (Schluter 2001; Bickford *et al.* 2007; Weber and Strauss 2016; Moritz *et al.* 2017). Over time, these diverged populations may be reintroduced, especially in cases where community dispersal rates are high (Weber and Strauss 2016). For these species to successfully coexist they must often develop distinct ecological niches to minimize competition. Sympatric speciation will occur when these populations that have overlapping ranges reach reproductive isolation from one another. Developing changes in body size in order to take advantage of different resources as well as minimize shared predation is a common mechanism of selection that leads to divergent populations within the same region (Schluter 2001; Moritz 2017).

Dispersal rates dictate both how quickly these processes will occur and to what degree. High dispersal rates will initiate secondary contact after a shorter amount of time, and as a result gene flow and hybridization will occur more frequently than in populations that remain separated for a greater amount of time (Schluter 2001; Weber and Strauss 2016). Consequently, speciation

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will occur at a much slower rate and coexistence of recently diverged populations will suffer due to the persistent overlap in niche (Schluter 2001; Weber and Strauss 2016). However, when populations remain separated for a greater amount of time it allows for adaptations within sub-populations to accumulate, resulting in greater distinction between populations upon secondary contact (Brown and Wilson 1956; Vallin 2010; Weber and Strauss 2016). In this scenario, decreased gene flow due to increased prezygotic isolation causes speciation to occur more rapidly (Coyne and Orr 1988; Schluter 2001; Weber and Strauss 2016). Dobzhansky theorized that increased prezygotic isolation in cases of overlapping ranges was a result of selection acting against reduced hybrid fitness and hybrid inviability by reinforcing reproductive barriers (Dobzhansky 1937).

The biological species concept is arguably the most widely accepted concept and defines a species as a group of interbreeding populations that do not breed with members of other groups (de Quiroz 2007). Ernst Mayr and Theodosius Dobzhansky, who both produced landmark works in the world of evolutionary biology, developed and supported this concept throughout their careers. Mayr first proposed the concept in 1942, claiming that reproductive isolation was the most important factor that determined a species (Mayr 1942). Dobzhansky supported this idea, who in his first solo publication had cited mutation and genetic drift occurring through sexual selection as factors that could lead to the reproductive isolation of a population (Dobzhansky 1937). As our ability to understand the influence of genetics on speciation continues to increase, we are beginning to discover how changes in the genome of individuals can lead to the divergence of a population regardless of whether that change is favorable regarding selection (Orr *et al.* 2007; Schluter 2009). Research has shown that postzygotic isolation due to genetic mutations, specifically on sex chromosomes, can lead to the reproductive isolation of populations

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separated from one another by allopatric speciation (Schluter 2009). When the two populations develop sex-linked mutations independently of one another, it is possible that intraspecific mating between those two populations upon secondary contact results in either hybrid sterility or hybrid inviability (Orr *et al.* 2007). As a result, the two populations are then considered to be reproductively isolated from each other (Orr *et al.* 2007).

The phylogenetic species concept focuses on identifying populations that share a common ancestor and the traits derived from that ancestor (de Queiroz 2007). Brent Mishler and Michael Donoghue have developed this concept based on the idea of pluralism. They reject the notion that a single universal method for identifying species exists and instead encourage a more open-minded approach to species delimitation (Mishler and Donoghue 1982). They propose to distinguish species based on evidence of monophyly by identifying the smallest and least inclusive group of organisms based on fixed character-state differences between populations (Donoghue 1985; Mishler and Brandon 1987; Davis and Nixon 1992). Phylogenetic trees have become increasingly useful to ecologists and systematists because they can more accurately determine divergence time compared to other methods (Barracough and Nee 2001; Rabosky and McCune 2009; Weber and Strauss 2016). Furthermore, recent advances in molecular genetics provide a promising avenue for more quickly and accurately identifying members of a clade and calculating species richness (Donoghue 1985; Barracough and Nee 2001, Rabosky and McCune 2009).

Species Complexes

In addition to being familiar with the different concepts used to classify a species, there are several principles that one must understand to be able to study a species complex: character

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displacement, limiting similarity, and cryptic species. Character displacement describes the phenomenon where closely related species show divergence and displace one another where their ranges overlap but show convergence and are difficult to distinguish in places where only one or the other is found (Brown and Wilson 1956). When populations of a single species diverge through allopatric speciation but occupy similar environments it is likely that the populations will develop similar adaptations and converge over time (Brown and Wilson 1956). However, when two populations overlap and face competition they are more likely to diverge through sympatric speciation, each developing distinct characteristics in order to evolve in separate ecological niches (Brown and Wilson 1956).

It is believed that displacement occurs partly due to limiting similarity, or the maximum number of species that can successfully coexist (MacArthur and Levins 1976). MacArthur and Levins define limiting similarity as “the total number of species proportional to the total range of the environment divided by the niche breadth of the species (MacArthur and Levins 1976). Character displacement can make identifying all taxa within a species complex extremely difficult because it may be impossible to discern whether an allopatric population has diverged to the point of speciation or belongs to a species that has already been identified (Brown and Wilson 1956; Weber and Strauss 2016).

When two or more species are so morphologically identical that they cannot be defined by their phenotypic traits they are considered to be cryptic species (Bickford *et al.* 2007; Moritz 2017). Cryptic species are common among species complexes and present a challenge to researchers when trying to fill in gaps in evolutionary history (Bickford *et al.* 2007). Because all member of a species complex are closely related and morphologically similar, they are represented by a single, well defined species within the group (Moritz 2017).

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Methods for Studying Species Complexes

One of the most common methods for studying a species complex is to study and compare the morphological traits of species within the complex. When a new morphological structure develops it allows a species to invade new adaptive zones and gives the species a chance to diversify in its new environment (Simpson 1953). Identifying morphological “key innovations” can help researchers understand how diversification of species within a clade develops over time (Heard and Hauser 1995). However, many species complexes contain species that are morphologically identical and make identification based on character traits alone impossible, prompting the need for more precise methods (Weber and Strauss 2016; Moritz 2017).

Using molecular methods to study evolutionary relationships has resulted in major advancements in the world of evolutionary biology. Coyne and Orr produced a landmark paper in 1988 by measuring codon differences using electrophoretic magnetic distancing to calculate divergence time between species of *Drosophila* (Coyne and Orr 1988). Today, one of the most effective ways to study geographic distribution as well as evolutionary history is to analyze genetic markers found in mitochondrial DNA. Unlike nuclear DNA, mtDNA is haploid, does not recombine, and can accumulate mutations more rapidly, making it useful for clearing up discrepancies within nuclear gene trees (Wallis *et al.* 2017). However, analyzing mtDNA alone does not take into account cases of introgression, hybridization, and lineage sorting that can be found by analyzing multiple nuclear genes and identifying the differences between samples (Wallis *et al.* 2017). A recent study that analyzed 48 individual published works found that there was only a 43.9% concordance between nuclear and mitochondrial gene trees in freshwater fishes (Wallis *et al.* 2017). Still, mtDNA analysis can be rather useful when trying to uncover

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cryptic species (Moritz 2017). Molecular phylogenies can reveal a much greater level of diversity and species richness within a clade compared to morphological data alone (Rabosky and McCune 2009). When this molecular data is then combined with data collected on morphology and ecology of a species it can more accurately portray how environmental factors have influenced divergence rates of species clades (Rabosky and McCune 2009).

The methods for sequencing DNA are constantly evolving, with each procedure having its own strengths and weaknesses. The use of amplified fragment length polymorphisms (AFLPs) has recently replaced previously common approaches such as restriction fragment length polymorphisms (RFLPs) and randomly amplifies polymorphic DNAs (RAPDs) (Douglas and Douglas 2010). AFLPs can produce higher yields of data using a smaller amount of DNA than other methods (Savelkoul *et al.* 1999). They are useful for measuring variation at more immediate levels of divergence such as between individuals or populations, but become less effective when analyzing larger taxonomic groups (Savelkoul *et al.* 1999; Douglas and Douglas 2010). Another technique, and the one used for this study, provides a cheaper and more precise alternative to the RADseq method, which uses a restriction enzyme as well as random fragmentation to create segments of DNA (Peterson *et al.* 2012). Double digest RAD sequencing (ddRAD) instead uses two enzymes and precise selection of fragments resulting in more uniform sets of information and increased ability to compare across multiple individuals (Peterson *et al.* 2012). In addition to more advanced sequencing techniques, DNA barcoding, which identifies short genetic sequences unique to a particular species, has dramatically improved the process of species identification using genomic data (Herbert and Gregory 2005). Although it is not full proof, some studies have estimated that this system can accurately identify species up to 97% (Herbert and Gregory 2005).

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Conservation of Species Complexes

Studying species complexes and understanding how they interact with their environment is vital to species conservation. When cryptic species remain undiscovered, data that is important to creating and implementing a conservation plan such as species richness and number of invasive species can be inaccurate and lead to further mismanagement (Bickford *et al.* 2007). Recognizing that species identification cannot always keep up with the need for conservative action, biologists have tried to implement a protocol for identifying genetically important populations even if their taxonomic status is still up for debate (Ryder 1986; Taylor 1999; Fraser and Bernatchez 2001). The development of the evolutionarily significant unit (ESU) serves to determine which populations and sub-species are significant in terms of both genetic preservation and continued variation within a species (Ryder 1986). While the boundaries that determine an ESU are still being debated, this approach has the potential to be an effective guide for conservation efforts when discrepancies in phylogeny within species complex are evident (Fraser and Bernatchez 2001).

A species cannot be saved if its existence is unknown, and with recent climate change expected to cause widespread extinction events and changes in niche for many species, it has become imperative that our knowledge of speciation and how it is affected by the environment be as accurate as possible (Bickford *et al.* 2007, Vallin et al 2010). Cryptic species consistently complicate conservation efforts by distorting estimates of biodiversity (Angulo and Icochea 2010). What is thought to be one species may actually contain several cryptic species, each with varying levels of endangerment (Bickford *et al.* 2007, Angulo and Icochea 2010). Gaining support for management and protection of undescribed species is incredibly difficult because they often lack official assessment from entities such as the IUCN, who have a major influence

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over conservation efforts that are mandated by the Endangered Species Act (Frasier and Bernatchez 2001; Angulo and Icochea 2010).

Constant manipulation and degradation of the natural environment by humans over an extended amount of time has made habitat loss the number one threat to biodiversity (Bickford *et al.* 2007). Accurately defining a species' range has become an important focus of molecular genetics, as cryptic species are often revealed when sampling across the perceived range for a known species (Stuart *et al.* 2006; Bossu and Near 2009). Conservation efforts must be adapted when it is revealed that resource depletion is affecting a cryptic species with a restricted range rather than a sub-population of a species that was thought to have a wider range (Bickford *et al.* 2007).

Understanding how species have evolved up to this point could provide valuable insight as to how species will evolve in the future, which would help conservationists take preventative measures to insure the highest rates of species survival (Heard and Hauser 1995; Bickford *et al.* 2007; de Queiroz 2007). Molecular studies have shown success at resolving phylogenies across multiple levels of divergence (Mendelson and Wong 2010). These phylogenies, when cross-referenced with studies of morphology and ecology, could reveal how some species became extinct while others persisted.

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Introduction

Darters (Teleostei: Percidae: *Etheostoma*) are small, benthic fishes representing the most diverse group of freshwater fishes in North America, consisting of an estimated 250 endemic species. (Sloss *et al.* 2004; Lang and Mayden 2007; Ray *et al.* 2008; Bossu and Near 2009; Near *et al.* 2011). While the monophyly of darters, recognized as the sub-family Etheostomatinae, has been consistently demonstrated for some time, phylogenetic resolution of the species relationships within the darter clade has proven challenging (Bailey and Gosline 1955; Sloss *et al.* 2004; Lang and Mayden 2007; Near *et al.* 2011). Difficulty in reaching a consensus within genera and subgenera can be demonstrated by efforts to establish the subgenus *Oligocephalus* as a monophyletic group (Bailey and Etnier 1988; Lang and Mayden 2007). While the inability to identify a unifying characteristic possessed by all species belonging to the subgenus strongly suggests that the group is non-monophyletic, subsequent grouping into well-defined species clades is an ongoing debate (Lang and Mayden 2007; Bossu and Near 2009).

Tracing divergence through the evolutionary history of darters has been complicated by a series of recent events that resulted in rapid, large scale speciation in freshwater habitats (Bailey and Gosline 1955; Sloss *et al.* 2004; Near *et al.* 2011). Pleistocene glaciations caused fragmentation within many darter species and fragmented populations diverged in allopatry over time (Douglas and Douglas 2010). As a result, some extant species have a wide distribution, while others have been restricted to isolated drainages, making them vulnerable to extinction (Lang and Mayden 2007; Ray *et al.* 2008; Bossu and Near 2009). New species are difficult to detect if they display cryptic morphology, because species are primarily identified by phenotypic traits. Interspecific hybridization and resulting introgression further complicate the process of resolving species relationships by causing discordance between mitochondrial and nuclear gene

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phylogenies. Mitochondrial DNA is haploid and nonrecombining, allowing it to accumulate mutations more quickly than nuclear DNA, making it useful when trying to resolve phylogenetic relationships between closely related species (Bossu and Near 2009; Near *et al.* 2011; Wallis *et al.* 2017). The problem with using mtDNA alone to generate species trees is that these factors, along with its sex-biased gene flow, also cause introgression of heterospecific genomes to occur much more frequently in mtDNA than in nuclear DNA (Bossu and Near 2009; Irwin *et al.* 2009). Consequently, mtDNA phylogenies can present an inaccurate representation of evolutionary history, making it necessary to cross reference with nuclear gene phylogenies.

Darters are a species rich clade that occupy a wide range of habitats (Sloss *et al.* 2004; Near *et al.* 2011). Like many freshwater fishes, species within *Etheostoma* exhibit high levels of interspecific hybridization (Lang and Mayden 2007; Ray *et al.* 2008; Bossu and Near 2009; Near *et al.* 2011; Wallis *et al.* 2017). Freshwater habitats are rather diverse, ranging from small riffles, tributaries, and ponds to larger rivers and lakes, increasing the likeliness that allopatric speciation will occur after a population becomes isolated (Lang and Mayden 2007; Wallis *et al.* 2017). When climactic events or habitat modification results in the formation of new contact zones, the probability that sister and even non-sister species will hybridize is greater due to decreased prezygotic isolation (Ray *et al.* 2008; Wallis *et al.* 2017). Hybridization between *E. uniporum* and *E. caeruleum* and the resulting introgression of *E. caeruleum* haplotypes into the *Etheostoma spectabile* clade have been documented in multiple locations of sympatry across the Eastern United States (Distler 1968; Ray *et al.* 2008; Bossu and Near *et al.* 2009). In the river systems and drainages of the Ozark Highlands, where the two species' ranges largely overlap, studies have found that the mitochondrial genome of *E. uniporum* has been almost entirely replaced by the genome of *E. caeruleum* (Ray *et al.* 2008)

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The Orangethroat Darter (*Etheostoma spectabile*) complex has been recognized as a species clade that contains eight known species as well as several that have not yet been described (Bossu and Near 2009). This study will evaluate the distribution of the *E. spectabile* complex as well as how members of the clade interact with other darter species that have overlapping ranges. The objective of this study is to determine which darter species are represented in the drainage systems of the Ozark Highlands, and how the presence of those species might alter the phylogenetic relationships within the *E. spectabile* clade.

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Methods and Materials

Collection

Fish were collected by seine nets at 50 locations across the White River drainage, Arkansas, USA (Figure 1.) during the months of June and July 2017 after receiving IACUC approval and state permits. The specimens were anesthetized in MS-222 and preserved in 100% ethanol. Collections were then transported to the Conservation and Molecular Ecology Laboratory at the University of Arkansas where the species of each specimen was identified. The right pectoral fin and/or caudal fin and peduncle were taken from each individual as a tissue sample and stored in 100% ethanol at -20°C prior to extraction.

Sequencing

DNA was extracted from the tissue samples of 96 individuals using a Qiagen Fast Kit, following the manufacturer protocols (Bossu and Near 2009). The DNA was then prepared for ddRAD (double digest restriction associated DNA) sequencing by digesting the DNA with the restriction enzymes *MspI* and *PstI*. Each individual was ligated with a unique barcode and given 1 of 2 indices (48 individuals per index) so they could be pooled into a single lane for sequencing. Sequencing of the pooled DNA was conducted by the Genomics and Cell Characterization Core Facility at the University of Oregon.

Analysis

Sequence data was de-multiplexed, filtered, and aligned using Pyrad. Genomes were aligned at 8000 randomly selected loci with 100 base pairs each. Phylogenies were constructed using SVDQuartets analysis within the Paup program and nodal support was calculated by generating bootstrap values for 1000 iterations (Ray *et al.* 2008).

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Results

While previous nuclear gene phylogenies have resulted in the recovery of the *E. spectabile* species clade as a monophyletic group (Lang and Mayden 2007; Ray *et al.* 2008; Bossu and Near 2009), SVDQuartet analysis yielded a tree containing four major monophyletic, well supported clades that revealed non-monophyly between the *E. spectabile* species represented (Figure 2.). Clade 1 includes all sampled *Etheostoma* except for *E. Caeruleum* and *E. fragi*, who form monophyletic Clade 4 with strong bootstrap support at 83.5%.

Clade 2 contains members of several subgenera including *Etheostoma*, *Hololepis*, *Oligocephalus*, and *Ozarka* (Near *et al.* 2011). Bootstrap supports for the monophyletic relationships within this clade remain high until bifurcation at the final node results in the monophyly of *E. gracile* and *E. whipplei*, which is only 51.8% supported. Clade 3 contains the remaining two sampled members of *E. spectabile*, as well as the recovery of a sister-species relationship between *E. Euzonum* and *E. Uniporum* with 100% bootstrap support at the corresponding node. The resolution of Clade 3, which includes *E. spectabile* and *E. flabellare* as well, is also strongly supported at 99%.

Comparison with Bossu and Near's 2009 tree that was calculated using minimized deep coalescences reveals several distinct differences. First is the failure of the SVDQuartet tree to establish monophyly within the *E. spectabile* clade, whereas *E. uniporum*, *E. fragi*, and *E. spectabile* were recovered by Bossu and Near as a monophyletic group with very strong bootstrap support (Bossu and Near 2009). Another is the recovery in 2009 of *E. fragi* and *E. spectabile* as sister taxa. This contradicts the SVDQuartet tree (Figure 2.), which shows a more distant relationship within Clade 1. The Bossu and Near tree also shows that out of all species sampled for this study, *E. caeruleum* is most closely related to *E. whipplei*, not *E. fragi*.

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Discussion

SVDQuartet analysis of sampled DNA resulted in some interesting relationships between the species found in the Ozark Highlands of Arkansas, and raised some questions about the relationships that have been discovered in previous studies. The non-monophyly of the *E. spectabile* clade contradicts previous studies that have distinguished it as a monophyletic taxon using nuclear DNA (Ray *et al.* 2008; Bossu and Near 2009, Near *et al.* 2011). However, the resolution of sister species relationships between member of *E. spectabile* and other *Etheostoma*, though they may not be the same as those found in previous studies, is consistent with claims that large amounts of heterospecific DNA are being introgressed into the *E. spectabile* complex (Ray *et al.* 2008; Bossu and Near 2009, Near *et al.* 2011). It is also reveals how topography can shape species relationships and supports evidence that cases of hybridization and introgression are relatively high is relatively high in White River drainages, where habitat diversity has had a major influence on divergence patterns in the region (Distler 1968; Ray *et al.* 2008).

Despite the contrasts between the tree generated from this study and trees generated from previous studies like Bossu and Near, it is not meant to correct any previous findings, but rather to provide another perspective as to how closely related species might interact with one another in contact zones. This tree was generated by analyzing a total of only 96 samples, a much smaller data set than what was obtained by previous researchers (Ray *et al.* 2008; Bossu and Near 2009, Near *et al.* 2011). SVDQuartet analysis also uses randomly selected fragments of DNA, rather than targeting specific genes, and only nuclear DNA is analyzed, unlike other analyses like minimized deep coalescences, that compares the gene trees of several mitochondrial and nuclear genes to infer a single species tree (Bossu and Near 2009). It is also important to note that this study focuses on a single geographic region, while Bossu and Near gathered data from a much

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broader range, focusing more on the Eastern Highlands than the drainage systems found in Arkansas (Bossu and Near 2009, Near *et al.* 2011). Therefore, the results of this study should not be used to make more general predictions of species distribution and behavior in parts of their range that were not sampled.

To further evaluate the accuracy of the results generated by this study, more trials are needed that include larger sampling sizes, analysis of multiple genetic markers on both mitochondrial and nuclear DNA, and the generation and comparison of species trees using more than one method. The Ozark Highlands are an important example of how habitat diversity and species diversity are directly related, and studying how species interact in species rich environments is extremely important when trying to manage these areas and conserve biodiversity.

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Etheostoma flabellare Fantail Darter 2926 [Photograph found in Etheostoma, North American Native Fishes Association, Kankakee County, IL]. (n.d.). Retrieved April 15, 2018, from [http://gallery.nanfa.org/v/members/Uland/Family Percidae/Etheostoma/Etheostoma flabellare Fantail Darter 2926.JPG.html?g2_imageViewsIndex=2](http://gallery.nanfa.org/v/members/Uland/FamilyPercidae/Etheostoma/Etheostoma%20flabellare%20Fantail%20Darter%202926.JPG.html?g2_imageViewsIndex=2) (Originally photographed 2012, April 29)

Etheostoma fragi Strawberry Darter 215ws [Photograph found in Etheostoma, North American Native Fishes Association, Fulton County, AR]. (n.d.). Retrieved April 15, 2018, from [http://gallery.nanfa.org/v/members/Uland/Family Percidae/Etheostoma/Etheostoma fragi Strawberry Darter 215ws.JPG.html?g2_imageViewsIndex=2](http://gallery.nanfa.org/v/members/Uland/FamilyPercidae/Etheostoma/Etheostoma%20fragi%20Strawberry%20Darter%20215ws.JPG.html?g2_imageViewsIndex=2) (Originally photographed 2016, November 16)

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Etheostoma punctulatum Stippled Darter 79-1 [Photograph found in Etheostoma, North American Native Fishes Association, Bull Shoals Lake]. (n.d.). Retrieved April 15, 2018, from [http://gallery.nanfa.org/v/members/Uland/Family Percidae/Etheostoma/Etheostoma punctulatum Stippled Darter 79-1.JPG.html?g2_imageViewsIndex=2](http://gallery.nanfa.org/v/members/Uland/FamilyPercidae/Etheostoma/Etheostoma%20punctulatum%20Stippled%20Darter%2079-1.JPG.html?g2_imageViewsIndex=2) (Originally photographed 2012, April 12)

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Etheostoma whipplei Redfin Darter 3697w [Photograph found in Etheostoma, North American Native Fishes Association, White Co.]. (n.d.). Retrieved April 15, 2018, from http://gallery.nanfa.org/v/members/Uland/FamilyPercidae/Etheostoma/Etheostoma_whipplei_Redfin_Darter_3697ws.JPG.html?g2_imageViewsIndex=2 (Originally photographed 2015, November 05)

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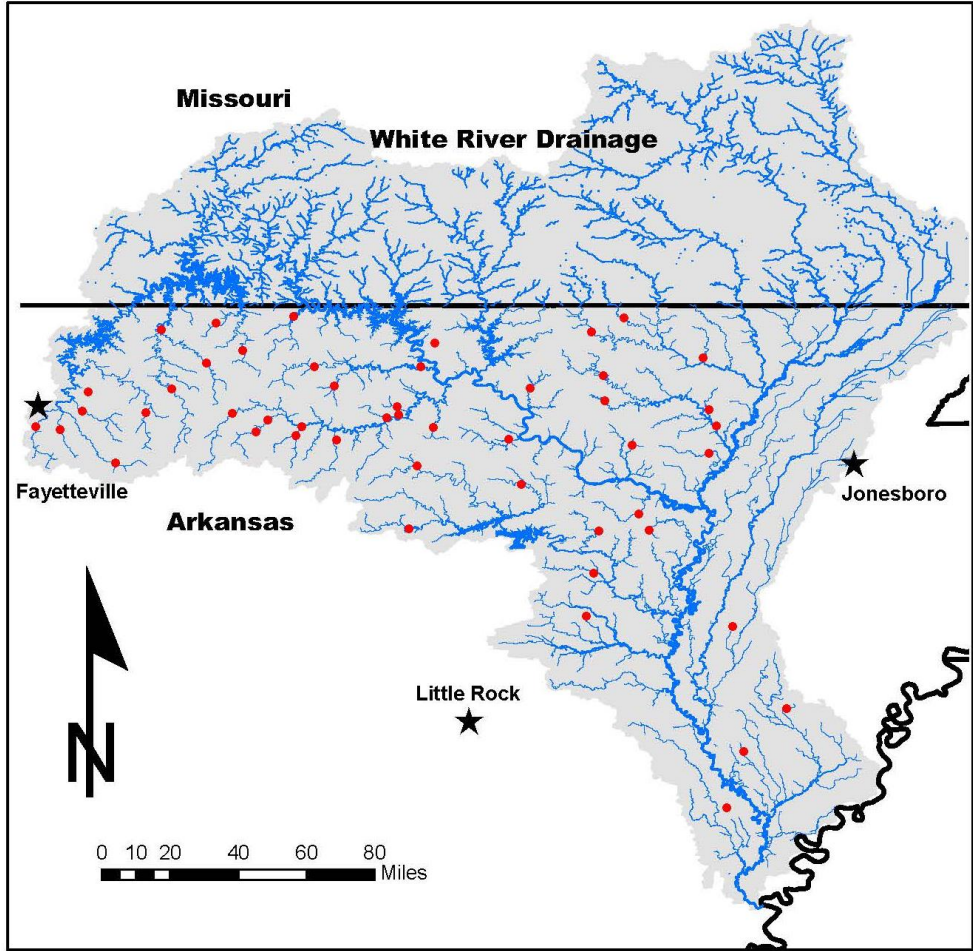
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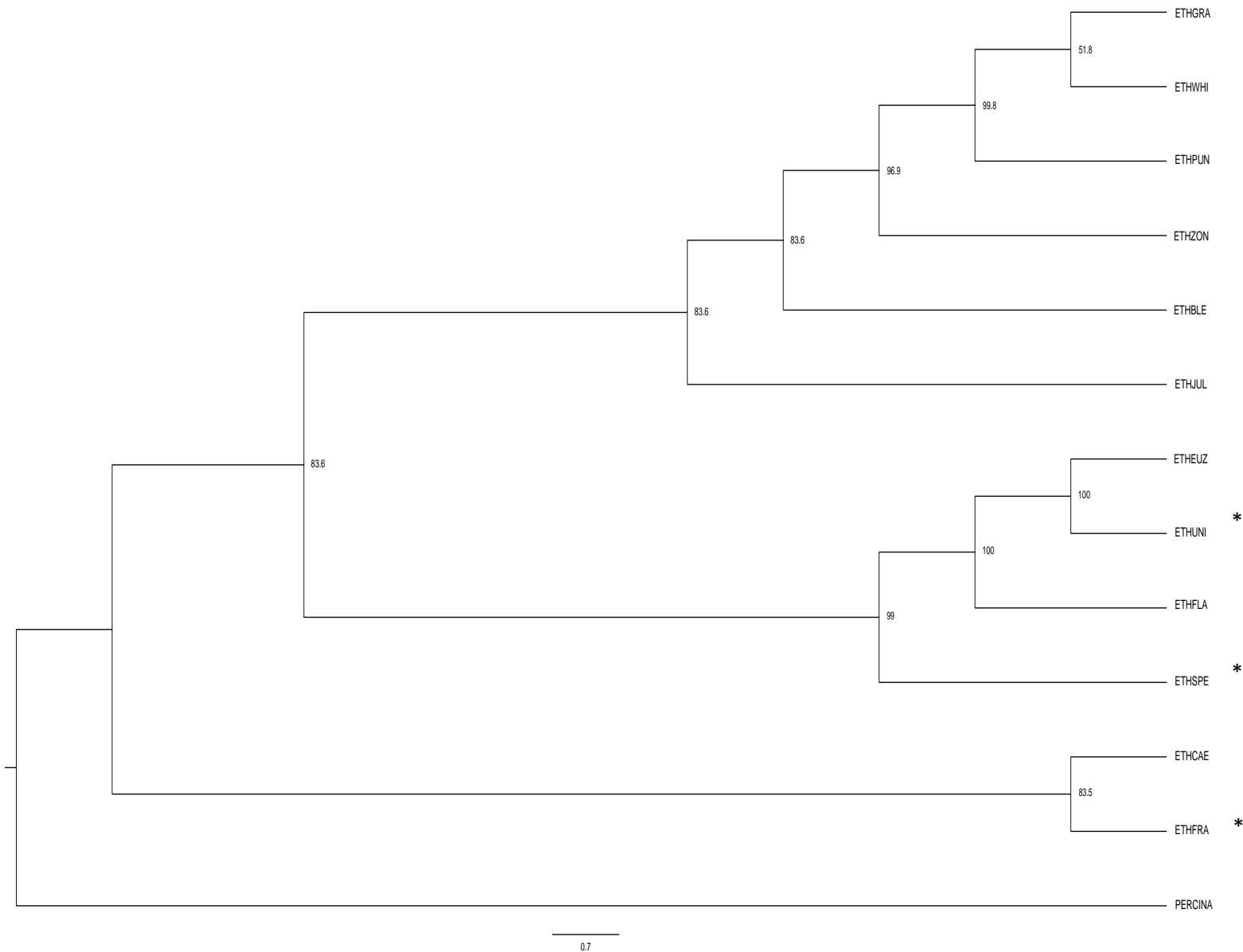
Figures

Figure 1. Map of the White River Drainage system in Missouri and Arkansas (grey). Red dots indicate the 50 locations where samples were collected in Arkansas.



PHYLOGENY OF THE ORANGETHROAT DARTER











Figure 2. Phylogeny generated from SVDQuartets analysis of 8000 nDNA loci with 100 base pairs per locus. Bootstrap values calculated for 1000 iterations are represented at the nodes. A total of 12 species of *Etheostoma* were recovered from 96 samples, including 3 from the *E. spectabile* complex, represented by an asterisks (*). Species codes are elaborated in Table 1.



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Tables

Table 1. Elaboration of species codes with corresponding scientific and common nomenclature. There are 12 species of *Etheostoma* represented as well as the outgroup *Percina*.

| SPECIES CODE | SPECIES | COMMON NAME | |
|--------------|-------------------------------|-------------------------|---|
| ETHGRA | <i>Etheostoma gracile</i> | Slough darter |  |
| ETHWHI | <i>Etheostoma whipplei</i> | Redfin darter |  |
| ETHPUN | <i>Etheostoma punctulatum</i> | Strippled darter |  |
| ETHZON | <i>Etheostoma zonale</i> | Banded darter |  |
| ETHBLE | <i>Etheostoma blenniodes</i> | Greenside darter |  |
| ETHJUL | <i>Etheostoma juliae</i> | Yoke darter |  |
| ETHEUZ | <i>Etheostoma euzonum</i> | Arkansas saddled darter |  |
| ETHUNI | <i>Etheostoma uniporum</i> | Current darter |  |
| ETHFLA | <i>Etheostoma flabellare</i> | Fantail darter |  |
| ETHSPE | <i>Etheostoma spectabile</i> | Orangethroat darter |  |

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| SPECIES CODE | SPECIES | COMMON NAME | |
|--------------|-----------------------------|-------------------|---|
| ETHCAE | <i>Etheostoma caeruleum</i> | Rainbow darter |  |
| ETHFRA | <i>Etheostoma fragi</i> | Strawberry darter |  |
| PERCINA | <i>Percina caprodes</i> | Logperch |  |

Table 1. (Continued)

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Office of Research Compliance

To: Marlis Douglas
Fr: Craig Coon
Date: May 4th, 2017
Subject: IACUC Approval
Expiration Date: May 3rd, 2020

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 17077: *Effective gene-flow between stream fish populations*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 3rd, 2020, you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Marlis Douglas and Zachery Zbinden. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem