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Using Morphology and Mitochondrial Cytochrome b Sequences in Determining Origins and Genetic Similarities among Populations of Rosyside Dace (*Clinostomus funduloides* Girard*)* in Western North Carolina

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Abstract

Clinostomus funduloides, commonly known as rosyside dace, are a Cyprinid minnow (*Cyprinidae*) species found in western North Carolina where they inhabit areas of narrow, rocky, headwater streams. Their range extends into Ohio, South Carolina, Tennessee, and Pennsylvania. Within western North Carolina, rosyside dace can be found in non-lacustrine bodies of water, and they have been recently described from in the Upper French Broad (UFB) river basin. Past work on the *cytb* gene of *C. funduloides* revealed a clade between the Catawba and French Broad samples dating back 2.4 million years. This led to a conclusion of bait-bucket release as the point of introduction of *C. funduloides* into the French Broad River basin, and this, along with relationships among populations of *C. funduloides*, was further examined in this study via phenotypic and genetic data collection. Morphological measurements of *C. funduloides* included measuring body depth and counting scales along the lateral line, but these data were inconclusive. Sequencing of the 1140 bp mitochondrial cytochrome-B gene (*cytb)* gene on fish from four *C. funduloides* populations (from the Broad, Catawba, and the French Broad river basins) showed that North Carolina fish are separate from Tennessee populations, and that there has been at least one bait bucket introduction.. *C. funduloides'* presence in the UFB is intriguing because of its separation from other populations by the Eastern Continental Divide. Further studies will add samples from a broader geographic range and will increase sample sizes.

1. Introduction

Clinostomus funduloides Girard, commonly known as rosyside dace, are a minnow species (Family Cyprinidae) found in western North Carolina where they inhabit areas of narrow, rocky, headwater streams. Native to the area, their range extends into Ohio, South Carolina, Tennessee, and a few parts of Pennsylvania. Within western North Carolina, rosyside dace can be found in almost every water basin and recently have been found in the Upper French Broad (UFB) river basin of North Carolina, where they were not historically located. As a bioindicator of healthy streams, intolerant of turbidity and silt, *C. funduloides* prefers deeper pools of headwater streams [1]. Although the International Union for the Conservation of Nature lists this fish as a species of least concern, and has a global ranking of G5 (secure), *C. funduloides* is considered an endangered species in Ohio [2]. These headwater streams are also important for reproduction of *C. funduloides. C. funduloides* is known to spawn in late April or early May, with eggs left in the nests of other minnows or suckers such as creek chub, striped dace, and trout.

The UFB is separated from other river basins in North Carolina containing *C. funduloides* by the Appalachian Eastern Continental Divide. The Appalachian Mountains started in the Ordovician period with the Iapetus ocean continental plate subducting beneath the North American Craton [2]. This subduction pushed the early Appalachian Mountains up, creating the Eastern Continental Divide and altering the flow of streams. Water west of the divide was forced into the Mississippi River and Gulf of Mexico, while water east of the divide could only flow east. Ancestral populations of *C. funduloides* within the Appalachian river basin were most likely separated and isolated by the formation of discrete lakes during the Miocene continental uplift and further disconnected during the Pleistocene glaciations [3]. Research on *C. funduloides* done by Simons (2017) suggested similarity among Eastern and Western (UFB) populations of this species by genetic analysis of the mitochondrial *cytochrome-B* (*cytb)* gene, supporting the bait-bucket hypothesis. *Cytb* is a highly conserved gene inherited maternally through the cytoplasm. Mitochondrial *cytb* gene is useful in determining origins of populations and can identify similarities across broad geographical ranges due to its relatively slow evolutionary rate among other mitochondrial regions [4,5,6].

We formulated two hypotheses as to how *C. funduloides* was introduced into the UFB. The first hypothesis suggests an introduction by means of bait-bucket release. Bait-bucket release refers to the act of releasing live bait fish into bodies of water that are most convenient for people who fish, whether or not the fish originated from that body of water. Bait-bucket release is a common method of introduction for new species [7]. According to the North Carolina Wildlife Resource Commission, white perch, zebra mussel, and large mouth bass have all been introduced into the states bodies of water via bait-bucket release. If our first hypothesis is true we expected to find *C. funduloides* more closely related to other rosyside dace of different drainages, who may or may not be closely related to one another. However, if we found *C. funduloides* to be distantly related to the neighboring fish and more closely related to fish from the same drainage, we would have support for our second hypothesis: *C. funduloides* is native to the Upper French Broad, but was not described from there until 2010.

2. Methods

2.1 Collection

Collection sites were chosen in streams where the United States Geological Survey [8] fish database had listed records of previously observed *C. funduloides*. An additional site was added for the Nolichucky, when investigators found a creek with deep pools and gravelly riffles (Table 1). Common fishes found in areas containing *C. funduloides* included central stoneroller (*Campostoma anomalum)*, bluehead chub (*Nocomis leptocephalus)*, sculpin (*Cottoidea*), tessellated darter (*Etheostoma olmstedi)*, blacknose dace (*Rhinichthys atratulus)*, and redbreast sunfish (*Lepomis aritis)*. A total of 23 rosyside dace were sampled. Collection was carried out using an electrofisher and net, and collections were done September 2017 until February 2018 with the help of volunteers. An overdose of Tricaine-S tricaine methanesulfonate (MS 222) solution from Western Chemical Inc. ™ was used to euthanize the sampled fish, which were then put on ice until morphological measurements.

Table 1. Locations from which *Clinostomus funduloides* (rosyside dace) was sampled.

Watershed	Location	New or Historical	Watershed	Side of Eastern Continental Divide
Nolichucky	Nolichucky River	Historical	Upper French Broad	West
Swannanoa	Swannanoa River	New	Upper French Broad	West
Little	Little River	New	Upper French Broad	West
Broad	Little Camp Creek	Historical	Broad	East
Catawba	Brevard Creek	Historical	Catawba	East

2.2 Morphology

Morphological measurements of the fish included: counting of scales along the lateral line, measuring body length and depth, as well as observing mouth parts. One distinguishing factor of *C. funduloides* is its large, oblique-mouth, with the lower jaw extending beyond the upper jaw [2].

2.3 Genetics

Twenty-three specimens of *C. funduloides* were thawed at room temperature or under hot water, along with nine specimens of *C. anomalum*. Extraction of epaxial and hypaxial muscle tissue followed the Qiagen DNeasy Blood & Tissue Kit™, using spin-column protocol. DNA concentration was measured by Nanodrop™ spectrophotometer, and *CYT-B* was amplified using previously published primers. PCR products were subjected to electrophoresis on ethidium bromide-stained 1.0% agarose gel at 120 volts, then visualized under ultraviolet light, using Carestream MI™ imaging software and apparatus.

Purified PCR product concentration were measured via Nanodrop™ spectrophotometry, and samples were prepared for both forward and reverse sequencing using techniques were adopted from the methods section of Simons. Final PCR products for both *C. funduloides* and *C. anomalum* were sent to Yale University for Sanger sequencing of forward and reverse primers. Sequences were then trimmed and subjected to *de novo* alignment of forward and reverse sequences in Geneious© v11.2.0.

Along with *C. elongates*, five specimens of *C. funduloides* were from GenBank, while thirteen *C. funduloides* sequences were generated from this study. A Tamura-Nei distance model was used to construct a neighbor joining tree which used bootstrap values to form a consensus phylogenetic tree out of 100,000 replicates. *Clinostomus elongatus*, redside dace, was used as an outgroup to root the tree.

3. Results

3.1 Morphology

Samples of fish from every river basin illustrated normal color patterns along with average length for *C. funduloides* [2,6]. Scale count along the lateral line ranged from 43-57, and fish had an average length of 6.7 cm[7]. The Nolichucky population had the greatest length and body depth, and the Swannanoa population had the smallest length and body depth.

3.2 Genetics

The consensus phylogenetic tree showed that rosyside dace from North Carolina are genetically separated from others in the region (Figure 1). In addition, the tree showed that not all fishes from a single basin are in single clades, and that genetic clustering does not always match geographic distributions (Figure 1).

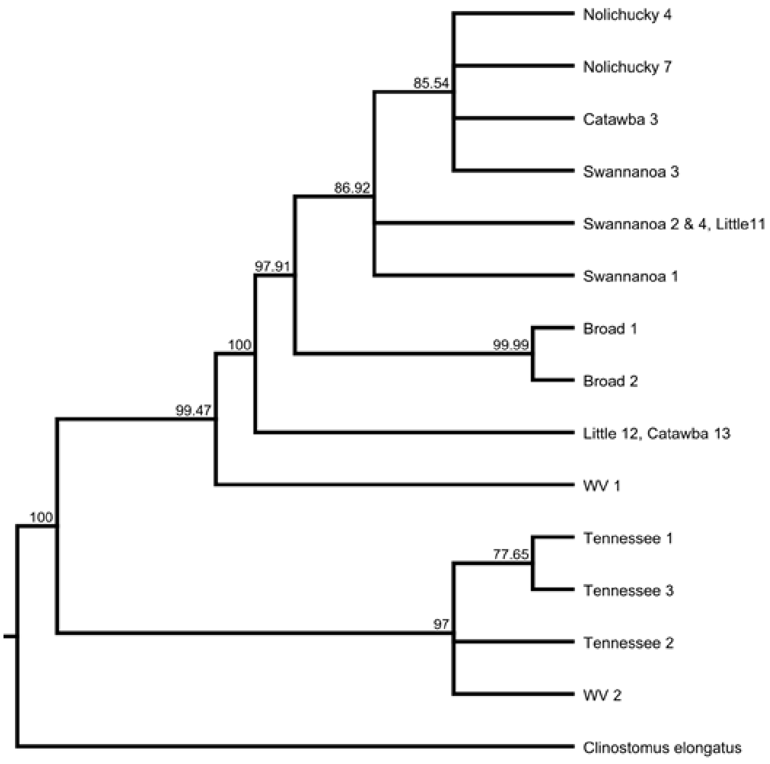

Figure 1. Consensus phylogenetic tree

Figure 1 Consensus phylogenetic tree with bootstrap support over nodes. Labels indicate river basins, with numbers showing the individual from that basin. Sequences for fish from Tennessee and West Virginia, as well as the outgroup (*Clinostomus elongatus*, redside dace), were downloaded from GenBank.

4. Discussion

The consensus phylogenetic tree partially supports the bait-bucket introduction hypothesis, since Catawba and Little River fish were both placed in multiple clades. What is not clear, however, is whether the Catawba fish were introduced to the Upper French Broad. This tree also shows us that, not only are there multiple clades in western North Carolina rivers, but fish from one river are in different clades, and newly described fish are in multiple clades. It is unexpected and unlikely for the Catawba fish to be in the same clade as the UFB fish. The Appalachian Eastern Continental Divide separates these rivers [9], making it impossible for the fish to co-occur naturally. The tree shows that the Tennessee and West Virginia clades are separate from the North Carolina clades, and there is strong support (99%) for the Broad River fish being their own clade. Morphology was not correlated with genetic variation, as demonstrated by the Nolichucky and Swannanoa fish belonging to the same clade.

Not much is known in terms of life history for *C. funduloides*. We do know, however, that headwater species are more likely to exhibit disjunct distributions, and are more heavily affected by periods of drying [10]. Between the late 1990's (when the USGS fish database) was last updated and present day, there seems to have been a fluctuation in *C. funduloides* population. Every site chosen for collection had population sizes well below previously reported numbers.

4.1 Conclusion

Although results of this study show support for bait-bucket introduction, there are still many questions to be answered. Because the mitochondrial *cyt-b* gene only codes for the maternal lineage of the fish, it would be beneficial to test nuclear genetic markers to examine patterns of biparental inheritance in *C. funduloides*. It would also be helpful to have a larger sample size from all river basins previously sampled, as well as add Tennessee and West Virginia to our list of collection sites. Complete morphological analysis of *C. funduloides* could also give us insight into relationships across sites. Furthermore, knowing more about the life history and behavior of *C. funduloides* and analyzing their demographic patterns in western North Carolina, could help us answer questions on what abiotic conditions are needed for rosyside dace, and whether or not the populations are stable.

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