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A Long-Recognized but Undescribed New Species of *Cyprinella* (Cypriniformes: Leuciscidae) from North Carolina and South Carolina, United States

Abstract

Cyprinella leptocheilus sp. nov., Siouan Thinlip Chub, is described as a new species that is endemic to Sand Hills and upper Coastal Plain streams in North Carolina and South Carolina. Recognized as an undescribed species since the early 1970s, this fish was known in the literature and in museum electronic databases as *Hybopsis* n. sp., *H.* sp. *cf. zanema*, *Cyprinella* n. sp., and *C.* sp. *cf. zanema*. Unofficially, it had gone by the common name Thinlip Chub. It was thought to be closely related to the two other barbeled *Cyprinella* species: Thicklip Chub, *C. labrosa*, and Santee Chub, *C. zanema*. *Cyprinella leptocheilus* is geographically, phenotypically, and genetically distinguishable from these two species. *Cyprinella leptocheilus* is confined to the Sand Hills and Coastal Plain regions of the Carolinas, whereas *C. labrosa* and *C. zanema* are more widely distributed in the Piedmont and Eastern Blue Ridge Foothills regions of the Santee River and Yadkin-Pee Dee River basins in the Carolinas and Virginia. Morphological differences detected were associated with the position of the dorsal-fin origin relative to the pelvic-fin origin; male tuberculation patterns and number of tubercles; upper lip thickness; mouth gape width; and the length of the maxillary barbel. Phylogenetic analyses of mitochondrial and mitochondrial-nuclear concatenated sequences consistently resolve each species as reciprocally monophyletic. *Cyprinella leptocheilus* is an evolutionary distinct sister to *C. zanema*; both are sister to *C. labrosa*. Within the *C. leptocheilus* clade, sequences from the Yadkin-Pee Dee River basin form a clade distinct from that of the Cape Fear River basin. These two clades are geographically isolated from one another. Given the divergence between the Cape Fear and Yadkin-Pee Dee River basins clades, it is recommended that these two *C. leptocheilus* clades receive protection as two evolutionarily significant units.

Keywords

Barbeled Cyprinella, Siouan Thinlip Chub, Leuciscidae

Cover Page Footnote

The authors are particularly grateful to Dr. Robert E. Jenkins (Professor Emeritus at Roanoke College, deceased) for graciously sharing with us the extensive and detailed data files that he and Dr. Ernest A. Lachner (National Museum of Natural History, Smithsonian Institution, deceased) had compiled in their earlier careers in the 1970s in their attempt to describe this new species of *Cyprinella*. This study could not have been completed without the encouragement from the North Carolina Museum of Natural Sciences staff: Gabriela M. Hogue—tissue preparation, curation of specimens, provided laboratory bench space, and use of laboratory photographic equipment and laptop computer by the senior author; Lily Hughes—photographic equipment and discussions regarding the resultant genetic data; and Bryan Stuart in the use of the electronic data imagery program. We would also like to express our gratitude to Janet Edgerton, Head Librarian of the Brimley Memorial Library at the North Carolina Museum of Natural Sciences, for her invaluable help in obtaining materials utilized for this project. Jesse L. Bissette (North Carolina Division of Marine Fisheries) photography; Scott A. Smith (North Carolina Division of Marine Fisheries) photography and imaging, assistance in the collection of fresh material and discussions of the project; Kyle Rachels and April Boggs (NCWRC) in the collection of fresh material and assistance in the field; and the North Carolina Chapter of the American Fisheries Society for two research grants to

purchase laboratory supplies for the genetic analyses. Lastly, the authors would like to thank the editors of the Southeastern Fishes Council Proceedings, Dr. Jonathan Armbruster (Auburn University), and an anonymous reviewer who collectively improved the quality of the manuscript. Lastly, this publication is dedicated to the memory of Dr. Robert E. Jenkins, Professor Emeritus of Roanoke College, Roanoke, VA – friend, colleague, and mentor

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INTRODUCTION

Twenty-nine described and at least eight undescribed or putative species exist in the genus *Cyprinella* (Schönhuth and Mayden 2010; Page et al. 2023), including an undescribed species endemic to the Sand Hills and upper Coastal Plain streams in North Carolina and South Carolina (Tracy et al. 2023a; Tracy et al. 2023b; Tracy et al. 2024; Figures 1 and 2). The earliest known vouchered specimen of this undescribed species was collected in June 1946 from the Lumber River, 17.7 km northeast of Laurinburg in Scotland County (CUMV 15278). Ichthyologists have recognized this form as a distinct undescribed species since the early 1970s (Jenkins 1972; Menhinick et al. 1974; Jenkins and Lachner 1980). This form has been referred to as *Hybopsis* n. sp., *H.* sp. *cf. zanema*, *Cyprinella* n. sp., and *C.* sp. *cf. zanema*. An informal common name, Thinlip Chub, was bestowed by Drs. Robert E. Jenkins and Ernest A. Lachner and appeared as such in published and gray literature and in electronic databases. Hereafter, until scientifically described later in this paper, we refer to this undescribed species as: *Cyprinella* sp. "Thinlip Chub".

Figure 1. *Cyprinella* sp. "Thinlip Chub", Lumber River, Columbus-Robeson counties, NC. Photograph by Fred C. (Fritz) Rohde.

Cyprinella sp. "Thinlip Chub" was believed to be closely related to two other *Cyprinella* species possessing a conspicuous maxillary barbel - Thicklip Chub, *C. labrosa* (Cope, 1870), and Santee Chub, *C. zanema* (Jordan and Brayton 1878; Figures 3 and 4). The latter two species had been previously placed in the genus *Hybopsis* (Jenkins and Lachner 1980), because both species possess maxillary barbels. Since 1991, *C. labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" have resided in the genus *Cyprinella* (Robins et al. 1991; Coburn and Cavender 1992; Jenkins and Burkhead 1994; Rohde et al. 2009; Page et al. 2023) even though they are the only species currently placed in the genus that possess a maxillary barbel. The body shapes of all three species are also more terete than other *Cyprinella* and these three taxa are more benthic in nature than the other *Cyprinella* who reside in upper- and mid-water habitats. All three taxa are found in North Carolina and South Carolina (Figures 2, 5, and 6).

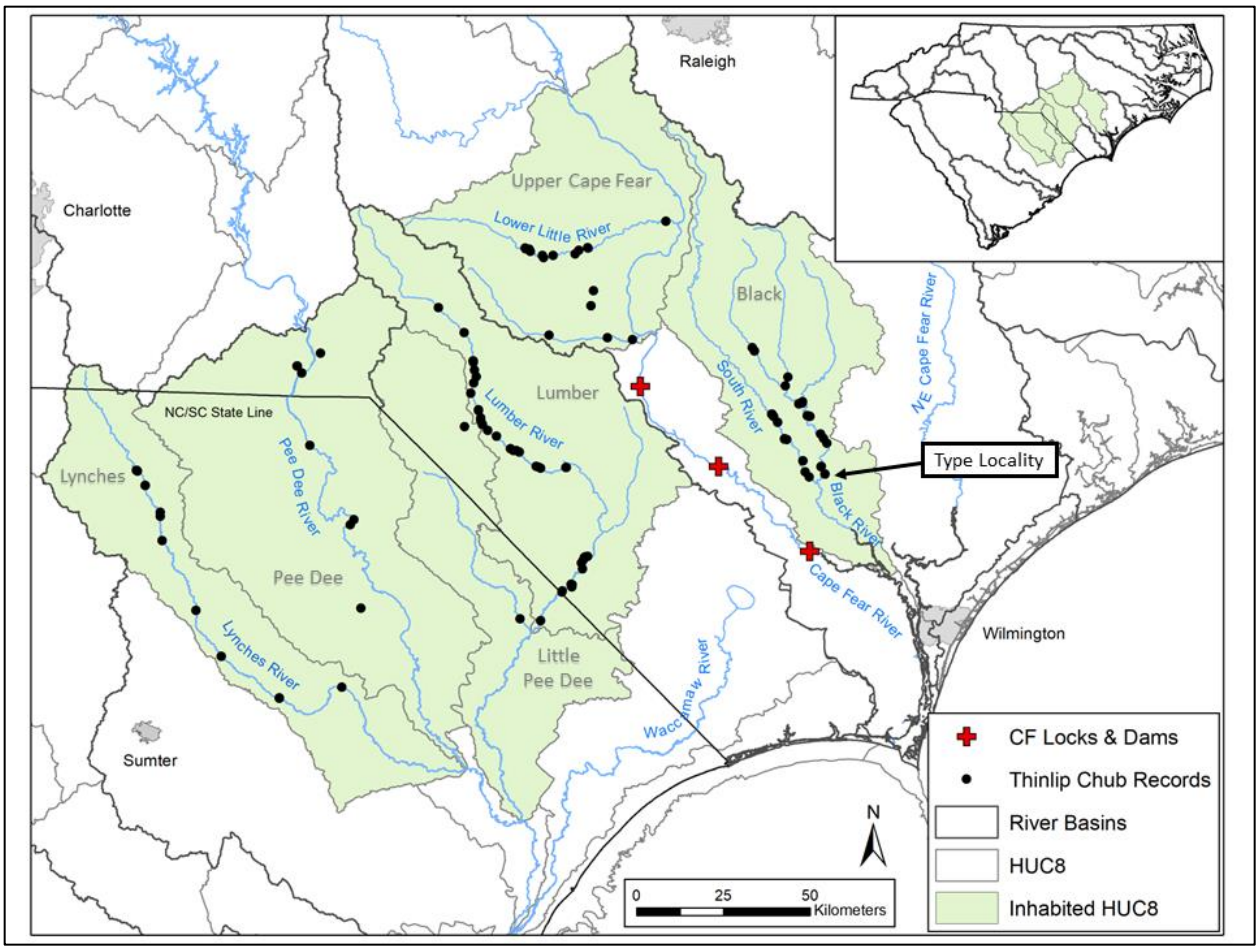

Figure 2. Known locales of *Cyprinella* sp. "Thinlip Chub" in North Carolina and South Carolina. Some locales may be represented by more than one collection. Gray text indicates subbasin/HUC8 name.

Figure 3. *Cyprinella labrosa*, Thicklip Chub, Henry Fork, Burke County, NC. Photograph by Jesse L. Bissette.

Figure 4. *Cyprinella zanema*, Santee Chub, Second Broad River, Rutherford County, NC. Photograph by Jesse L. Bissette.

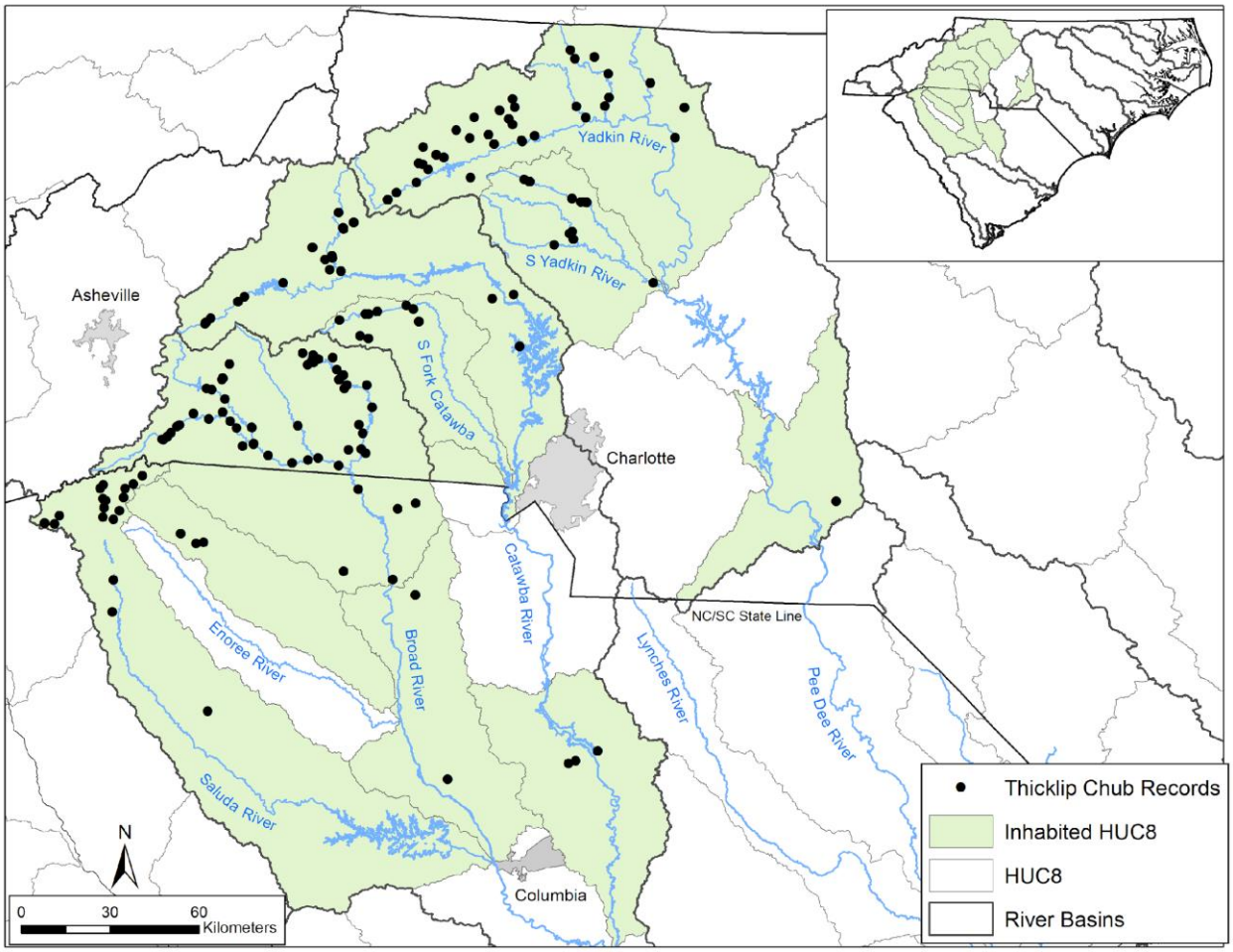

Figure 5. Known locales of *Cyprinella labrosa*, Thicklip Chub, in North Carolina and South Carolina. Previously known from one locality in the headwaters of the Ararat River in Virginia (USNM 105075), the species is now considered extirpated from Virginia (Jenkins and Burkhead 1994). Some locales may be represented by more than one collection. The single specimen in the Upper Pee Dee HUC 8 (NCSM 88758) was collected in 1967, and none have been collected from that HUC since then.

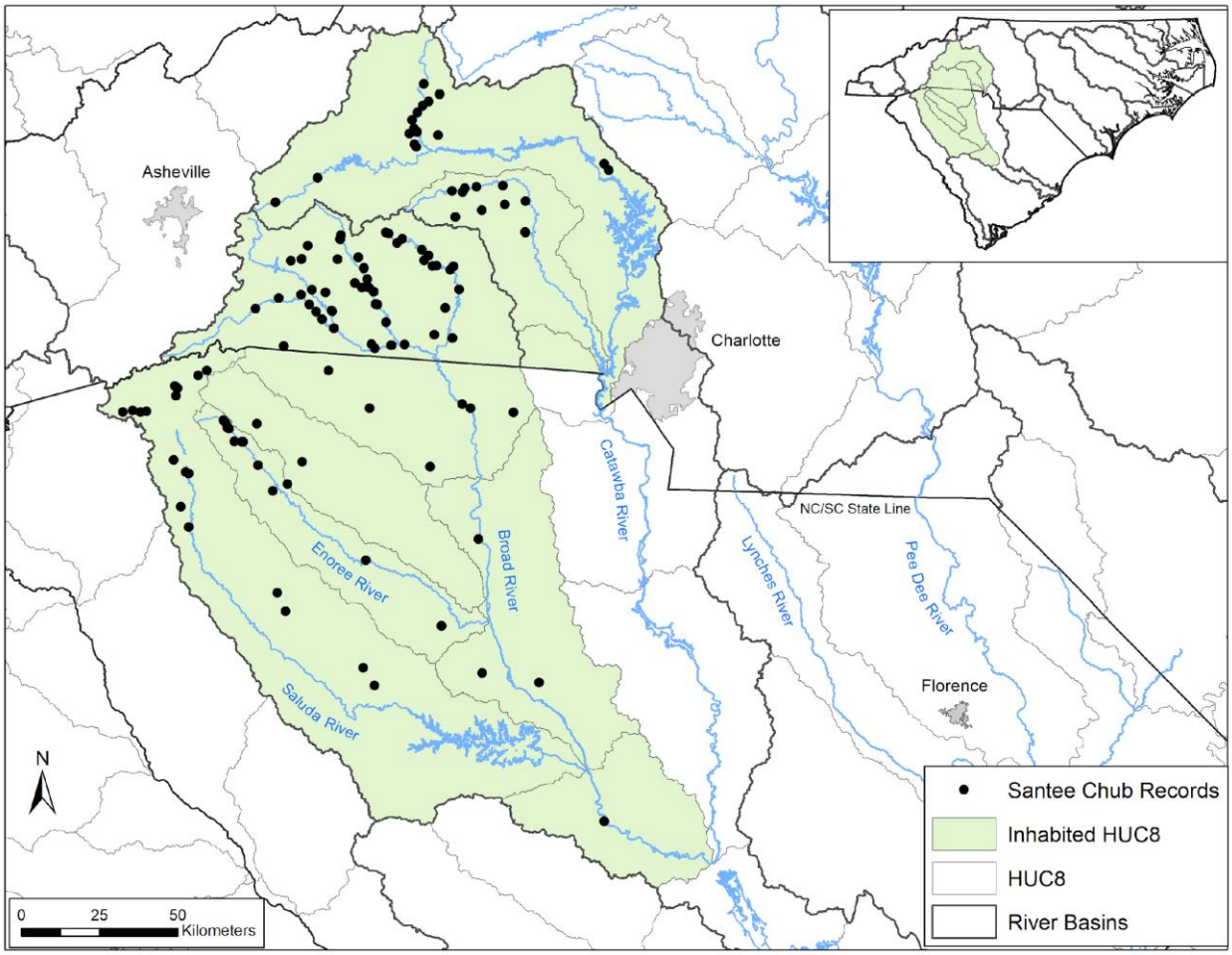

Figure 6. Known locales of *Cyprinella zanema*, Santee Chub, in North Carolina and South Carolina. Some locales may be represented by more than one collection.

Cyprinella sp. "Thinlip Chub" had tentatively been considered a subspecies of *C. zanema* based upon minor morphological differences (Jenkins and Lachner 1980; Jenkins and Burkhead 1994). However, DNA analyses using mitochondrial (NADH dehydrogenase subunit 2 [ND2] and cytochrome b genes) and nuclear (recombinant activation gene 1 [Rag1]) genes showed that *Cyprinella* sp. "Thinlip Chub" displayed reciprocal monophyly with respect to *C. zanema* and supported the suppositions that *Cyprinella* sp. "Thinlip Chub" was sister to *C. zanema* and was an evolutionarily distinct lineage. Both *Cyprinella* sp. "Thinlip Chub" and *C. zanema* were sister to *C. labrosa* (Anderson et al. 2001; Rohde et al. 2009; Schönhuth and Mayden 2010). Furthermore, the Cape Fear River basin population was found to be genetically distinct from the Pee Dee River basin (Lumber and Lynches River subbasins) populations (Anderson et al. 2001).

Broughton and Gold (2000) analyzed mitochondrial ND2 and NADH dehydrogenase subunit 4L (ND4L) nucleotide sequences and determined that *Cyprinella labrosa* was more closely related to many other species of *Cyprinella* than it was to its putative sister species, *C. zanema*, although they did not separate out *Cyprinella* sp. "Thinlip Chub" because at that time it was considered an undescribed species or subspecies of *C. zanema*. Later, Schönhuth and Mayden (2010) resolved *C. zanema* as the sister group to *Cyprinella* sp. "Thinlip Chub" with both species as a sister group to *C. labrosa*. These three barbeled species were always recovered as a well-

supported clade within the genus *Cyprinella*. Schönhuth and Mayden (2010) also found a 5.9% sequence divergence between the Cape Fear River and Yadkin-Pee Dee River basins suggesting that the *Cyprinella* sp. "Thinlip Chub" could be two species; however, they included only one specimen per basin in their analysis.

In this study we examined the systematics of *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" through an analysis of meristics, landmark-based linear morphometrics, and phylogenetic relationships inferred from mitochondrial and nuclear DNA. A description is provided herein for *Cyprinella* sp. "Thinlip Chub" and we propose uplisting the new species' state imperilment status.

METHODS

Materials Examined

Distributional data for North Carolina's vouchered specimens of *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" were obtained from Tracy et al. (2020) and from specimens collected since 2020 by the authors and colleagues. Distributional data for South Carolina's vouchered specimens of *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" were compiled from accessible material found at institutions accessed through the FishNet2 Portal (www.fishnet2.net, 2022). The North Carolina Museum of Natural Sciences (NCSM) is the largest repository of *Cyprinella* sp. "Thinlip Chub", specimens where 740 of the 893 known vouchered whole-bodied specimens are curated. The remaining 153 specimens are curated at 10 other institutions or resource agencies: CAS (6), CUMV (1), INHS (including SIUC) (18), KUI (3), North Carolina Wildlife Resources Commission (NCWRC, 2), TU (40), UF (41), UNCW (8), USNM (4), and UT (30) [Note: All museum codes follow Sabaj (2020)]. Additional distributional data for these three species were obtained for unvouchered specimens from Olmsted and Cloutman (1978), from the personal collection efforts by Fred C. Rohde, from the North Carolina Division of Water Resources (NCDWR, 1990-2016 data), from the North Carolina Wildlife Resources Commission (via the Portal Access to Wildlife Systems (http://www.ncpaws.org/), and from South Carolina Department of Natural Resources (SCDNR, Kevin Kubach, personal communication).

Meristic and landmark-based linear morphometric data for *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" were taken from 120 specimens curated at NCSM (Figures 4-6; Table 1 in Supplemental Meristic and Morphometric Files): 30 specimens of *C. labrosa*, 40 specimens of *C. zanema*, and 50 specimens of *Cyprinella* sp. "Thinlip Chub". To minimize the effects of ontogenetic change only the largest mature males and females in each lot were utilized for meristic and landmark-based linear morphometric data analyses. For *C. labrosa* the size range of the specimens was 62.2–78.9 mm Total Length (TL), 51.0–64.4 mm Standard Length (SL); for *C. zanema*—64.8–80.2 mm TL, 53.1–67.3 mm SL; and for *Cyprinella* sp. "Thinlip Chub"—60.2–91.5 mm TL, 48.6–74.0 mm SL.

Nine meristic characters and 27 landmark-based linear morphometrics (Tables 1 and 2; Figures 7-11) were evaluated following described methods (Hubbs and Lagler 1968; Pera and Armbruster 2006; Armbruster 2012; Gilbert et al. 2017). The meristic characters included dorsal-, pectoral-, pelvic-, anal-, and caudal-fin rays; lateral-line scales; circumferential scales;

predorsal scales; and caudal-peduncle scales (Table 2). In addition, also recorded for each specimen was the position of the dorsal-fin origin relative to the pelvic-fin origin (over or posterior to and how many scales posterior to); and whether or not the posterior corner of the upper lip, anterior to the maxillary barbel, extended vertically to the anteriormost edge of the orbit (referred to as the upper lip position; Figure 9). Characters were counted with the aid of an Accu Scope microscope with Excelis HD microscopy camera 0.35X equipped with CaptaVision+^{TM} PC software and ImageJ Software (https://imagej.net/ij/).

The landmark-based linear morphometric measurements were recorded to the nearest 0.1 mm using Fisher electronic digital calipers, traceable, Model 14-648-17, 11783217; an Accu Scope microscope with Excelis HD microscopy camera 0.35X equipped with CaptaVision+^{TM} PC software; a Canon EOS 5D$_{SR}$ camera equipped with a Canon Macro Lens EF 100 mm 1:2.8; and ImageJ Software (https://mirror.imagej.net/). Images of each specimen for these measurements were photographed against a black background. All images in Figures 7-11 were of a *Cyprinella* sp. "Thinlip Chub" specimen collected on September 13, 2022 from the Lumber River at the Fair Bluff boat access area, Columbus-Robeson counties, North Carolina, 34.31432/-79.03768, and photographed by Scott A. Smith.

Table 1. Nine meristic traits obtained from *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" specimens at NCSM.

Meristic Trait	**Description**
Lateral-Line Scales	The number of scales beginning with the first pored lateral scale on the shoulder (pectoral girdle) to the base of the caudal fin (hypural plate)
Circumferential Scales (Predorsal Circumferential Scale Rows)	The number of scales encircling the body anterior to the dorsal fin
Predorsal Scales	Scales from the nape to the dorsal fin
Circumcaudal-Peduncle Scales	The number of scales around the narrowest part of the caudal peduncle
Dorsal-Fin Rays	The number of principal rays whose tip reaches the distal margin of the fin; last two rays are counted as one because they share a common basal element
Pectoral-Fin Rays	The number of principal rays whose tip reaches the distal margin of the fin
Pelvic-Fin Rays	The number of principal rays whose tip reaches the distal margin of the fin
Anal-Fin Rays	The number of principal rays whose tip reaches the distal margin of the fin; last two rays are counted as one because they share a common basal element
Caudal-Fin Rays	The number of principal rays whose tip reaches the distal margin of the fin; procurrent rays are excluded

Table 2. Twenty-seven landmark-based linear morphometrics, dorsal-fin position, and upper lip position measured for *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" specimens at NCSM.

View/Position of the Body/Landmark-based Linear Morphometric
Lateral View/Whole Body
Total Length - distance from the anteriormost part of the head to the posteriormost part of the caudal fin with the caudal fin tips compressed together
Standard Length - distance from the anteriormost point on the head to the posterior end on the caudal-fin base (hypural plate)
Predorsal Length - distance from the tip of the snout to the structural base of the 1st dorsal-fin ray
Dorsal-Fin Base Length
Dorsal-Fin Origin to Pectoral-Fin Origin
Dorsal-Fin Origin to Pelvic-Fin Origin
Dorsal-Fin Origin to Anal-Fin Origin
Pectoral-Fin Origin to Pelvic-Fin Origin
Pelvic-Fin Origin to Anal-Fin Origin
Anal-Fin Base Length
Caudal Peduncle Depth
Dorsal-Fin Position (Origin) – dorsal-fin origin relative to pelvic-fin origin (over or the number of scales posterior to pelvic-fin origin)
Lateral View/Close-up of Head
Upper Jaw Length – distance from the anteriormost point of the premaxillary to the posteriormost edge of the maxillary barbel
Head Depth – distance from the top of the head, measured at the midline of the occiput, vertically downward to the ventral contour of the head
Head Length - distance from the most anterior point on the snout to the most distant point of the bony margin of the opercle
Snout Length - distance from the anteriormost point on the snout to the nearest anterior margin of the orbit
Snout to Top of Gill Slit Distance
Orbit Length - greatest distance between the free orbital rims
Maxillary Barbel Length – distance from the tip to the bottom of the upper lip
Upper Lip Width - measured at the midpoint of the length of the upper lip (from the junctures of the upper and lower lips) from the edge of the upper lip to the crease of the upper lip
Anterior of Upper Lip Thickness – thickness measured as a right angle from the top of the lip to the bottom of the lip
Upper Lip Position - whether or not the posterior corner of the upper lip, anterior to the maxillary barbel, extended vertically to the anteriormost edge of the orbit (Yes or No)
Dorsal View/Close-up of Head
Interorbital Width
Ventral View/Close-up of Head
Upper Lip Width - taken at the midline
Mouth Gape Width - greatest transverse distance across the opening of the mouth
Ventral View/Close-up of Pectoral and Pelvic Fins
Pectoral-Fin Base Length
Pectoral-Fin Length - distance from extreme base of the uppermost, outermost, or anteriormost ray to the farthest tip of the fin
Pelvic-Fin Base Length
Pelvic-Fin Length - distance from extreme base of the uppermost, outermost, or anteriormost ray to the farthest tip of the fin

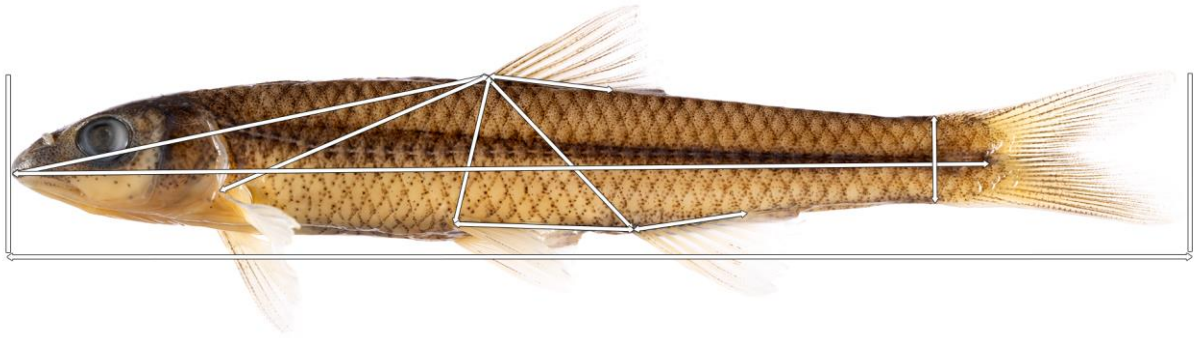

Figure 7. Lateral view, whole body - landmark-based linear morphometrics.

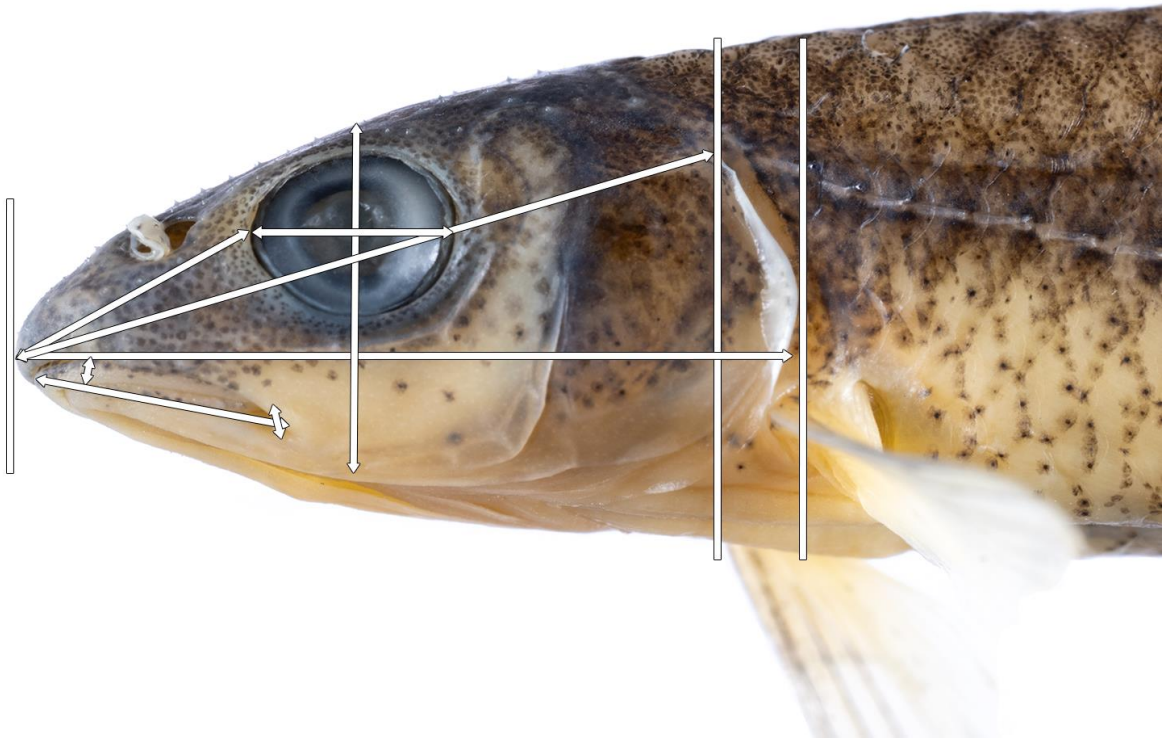

Figure 8. Lateral view, close-up of head - landmark-based linear morphometrics.

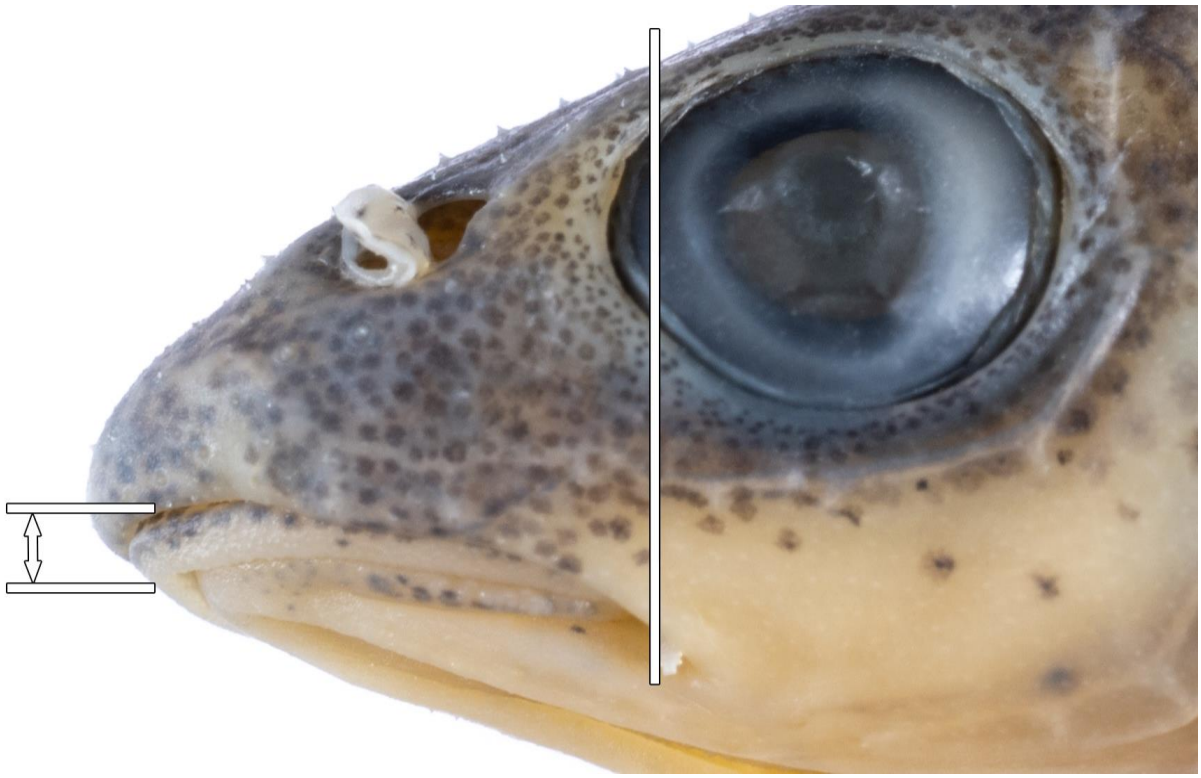

Figure 9. Lateral view, close-up of head, landmark-based linear morphometrics (anterior of upper lip thickness and upper lip position).

Figure 10. Left – ventral view, close-up of head (upper lip width at midline and mouth gape width); right – dorsal view, close-up of head (interorbital width), landmark-based linear morphometrics.

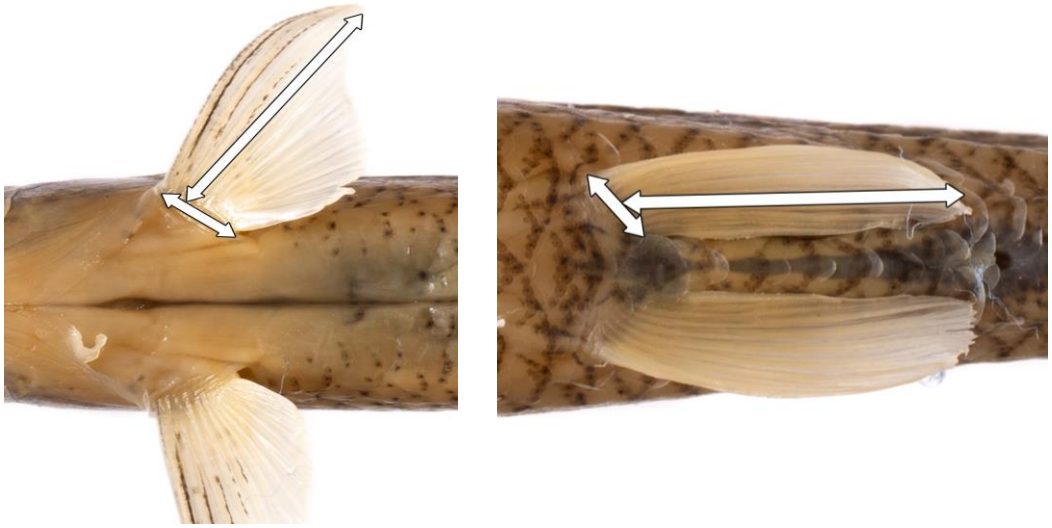

Figure 11. Ventral view, close-up of pectoral (left) and pelvic (right) fins, landmark-based linear morphometrics.

Determinations of color patterns of live or preserved specimens of *Cyprinella* sp. "Thinlip Chub" were made against either a white or black ground or from live specimens situated in a phototank. The descriptions of the coloration of live *Cyprinella* sp. "Thinlip Chub" specimens were obtained from R.E. Jenkins' correspondence files and recently from specimens photographed stream-side in a phototank from the Lynches, Black, and Lumber rivers (n=21, 6, and 3 images, respectively). Descriptions of the coloration of long-preserved adult specimens of *Cyprinella* sp. "Thinlip Chub" were obtained from the 50 specimens used in landmark-based linear morphometric analyses that had initially been preserved in formalin and then transferred into 70% ethanol or 50% isopropyl alcohol.

All lateral measurements and descriptions were taken from the left side unless noted, damaged, or otherwise anomalous. In several instances, the right pectoral or pelvic fin was measured because the left fins were damaged. The head depth, gape width, and upper lip width at

the ventral midline could not be measured in several instances due to the mouth gaping distortion of the preserved specimens or the upper lip at the ventral midline was closed too tightly for a measurement to be made. The descriptions of the nuptial tuberculation pattern follow that described in Jenkins and Burkhead (1994) and were based upon specimens photographed as part of the landmark-based linear morphometric analyses.

Statistical Methods

Simple, descriptive statistics (i.e., range, mean, mode, and standard deviation) were calculated for the meristics and landmark-based linear morphometrics data using Microsoft® Excel for Mac Version 16.80. Box and whisker plots that illustrated the maximum (uppermost line), minimum (lowermost line), mean (X symbol), median (horizontal line), the 1st and 3rd quartiles (bottom and top lines of the box), and outlier points were also produced in Excel. All analyses related to and including the random forest were conducted in R (Version 4.3.1, R Core Team 2023).

Random Forest Classification

Random Forest Modeling is a machine learning statistical classification technique that identifies characters useful for distinguishing different groups (Breiman 2001). In this case, we used Random Forest Modeling to determine which statistically significant landmark-based linear morphometric and meristic characters could be used to differentiate *Cyprinella* sp. "Thinlip Chub" from *C. labrosa* and *C. zanema* (Lee 2023).

For the three *Cyprinella* species, 38 variables (also known as features) (Tables 1 and 2) in addition to sex were collected from a total of 120 individuals. All features were continuous except upper lip position (yes/no) and dorsal-fin position (factor). The number of caudal-fin rays was not considered because the value was the same across all individuals. Upper lip position was removed from consideration because there were several missing values. For this variable, there were no satisfactory ways to substitute values because random forest models cannot handle missing values. Several of the continuous variables also had missing values. To fill in these missing values, the median value of the feature was computed from other observations within that same species and for the same basin in which it was observed. Total and standard lengths were not considered in the analysis because only the largest individuals were chosen for these measurements.

Before applying the random forest analysis, the features were evaluated for collinearity. Inclusion of variables with collinearity can result in irrelevant or redundant features, which can negatively impact model performance (Zuur et al. 2009). Therefore, we evaluated collinearity between variables by computing variance inflation factors (VIFs) for all features. The feature with the highest VIF over a value of 3 was removed. This process was repeated iteratively until all VIF values were less than 3. The next step was to split the data into training (n=84 individuals, 70%) and testing (n=36 individuals, 30%) data sets. The training data set is used to build the model, whereas the testing data set is used for model validation and prediction. Random forest works by combining multiple decision trees to make predictions. Each decision tree is built using a random subset of the available data and a random subset of the available features. The trees are constructed independently, and their predictions are combined by majority vote (for classification problems).

Important (or significant) features were selected using the Boruta algorithm (Kursa and Rudnicki 2010). Boruta is a powerful approach for the analysis of high-dimensional data sets. The Boruta algorithm works by first randomly shuffling the values of each feature. These permuted features are called shadow features. These shadow features are used to compare the importance of the original features, and any feature that has a higher importance than its corresponding shadow feature is considered important or confirmed (otherwise rejected). The algorithm stops either when all features get confirmed or rejected or it reaches a specified limit of random forest runs. The Boruta algorithm helps to reduce the risk of overfitting and ensures that the selected features are truly important and not just artifacts of the data set.

The features selected by the Boruta algorithm were used to fit the model to the training data using the default of 500 trees and 10-fold cross-validation. The final trained model was then applied to the test data set to make predictions. Model performance was evaluated by calculation of the percent accuracy based on confusion matrices. A confusion matrix is a method for expressing how many of the classifier's predictions were correct, and in the case of an incorrect call, the cause of the "confused" classification.

Genetic Sample Collection

Individuals encountered during field collections were either whole-body preserved in 95% ethanol or a fin clip was taken and preserved in 95% ethanol. For those specimens that were fin-clipped in the field, the whole body was preserved in 10% formalin. Once at NCSM, tissue samples were maintained in 95% ethanol in a -80°C ultracold freezer until analyzed.

A total of 121 samples were collected for analysis, including 47 *Cyprinella* sp. "Thinlip Chub" from the Lumber, Black/South, upper Cape Fear, Yadkin, and Lynches rivers. The remaining specimens, including sequences from GenBank, were from closely related species and outgroups (*C. analostana, C. caerulea, C. callisema, C. callistia, C. chloristia, C. galactura, C. gibbsi, C. labrosa, C. leedsi, C. lutrensis, C. nivea, C. pyrrhomelas, C. rutila, C. spiloptera, C. trichoristia, C. venusta, C. xaenura, C. zanema, Erimonax monachus, Erimystax insignis, Hybognathus regius, Hybopsis amblops, H. hypsinotus,* and *H. rubrifrons*) (Carlson et al. 2024). The number of samples successfully amplified varied by gene. Sample names that were included in each phylogeny, including GenBank sourced sequences, are outlined in Supplemental Genetics Files and are available on the National Center for Biotechnology Information (NCBI).

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

Extractions were processed following the Macherey-Nagel (Dueren, Germany) Nucleospin Tissue protocol and eluted in 100 µL of elution buffer. Two nuclear genes (Recombination Activating 1 (RAG1) and Homeobox C13 (Hoxc13; Paracchini et al. 2017) and two mitochondrial genes (Cytochrome oxidase one (COI; Folmer et al. 1994) and Cytochrome-b (Cyt-b)) were amplified and sequenced to build phylogenies (Table 3). All primers were tagged with M13 sequence for use in downstream sequencing.

Table 3. Primers, primer sequences, PCR protocols and cycling conditions for COI, Cytb, Rag1, and Homeobox13 genes.[1]

Gene	Forward Primer	Sequence	Reverse Primer	Sequence	Cycling Conditions	Reaction Protocol
COI	jgLCO1490	5'-GTA AAA CGA CGG CCAG TIT CIA CIA AYC AYA ARG AYA TTG- 3'	jgHCO2198	5'-CAG GAA ACA GCT ATG ACT AIA CYT CIG GRT GIC CRA ARA AYC A-3'	95°C 2 min 35 cycles (95°C 30 sec; 46°C 30 sec; 72°C 1 min) 72°C 10 min	0.5 µL F & R Primers 0.6 µL 25mM MgCl2 5 µL 1:10 polymerase
Cytb	tRNAGlu	5'-TGT AAA ACG ACG GCC AGT TGA CTT GAA GAA CCA CCG TT-3'	tRNAThr	5-'CAG GAA ACA GCT ATG ACA TCT TCG GAT TAC AAG ACC GA-3'	95°C 2 min 35 cycles (95°C 51 sec; 52°C 30 sec; 72°C 1 min) 72°C 10 min	0.5 µL F & R Primers 0.6 µL 25mM MgCl2 5 µL 1:10 polymerase
Rag1	Rag1-20F	5'- GTA AAA CGA CGG CCA GAT ATT CCA GCC CCT GCA C-3'	Rag1-1529R	5'- CAG GAA ACA GCT ATG ACA GGT GTA CAG CCA GTG GTG-3'	98°C 2 min 35 cycles (95°C 15 sec; 60°C 90 sec; 72°C 90 sec) 72°C 10 min	0.2 µL F & R Primers 4 µL HF-Buffer 0.2 µL 10 mM dNTP 0.2 µL Phusion Taq
	Rag1-141F	5'- GTA AAA CGA CGG CCA GAT GGC CTC TCT GGT TGG AC-3'	Rag1-1302R	5'- CAG GAA ACA GCT ATG ACA GGA GGT CAG CAA ATT GC- 3'		
	Rag1-161F	5'- GTA AAA CGA CGG CCA GAC CTT TGT TGA GGA TGT CCC-3'				
Homeobox13	10410-aad	5'-GTA AAA CGA CGG CCA GGG CGG ACA CCT TGA TGT ACG-3'	10410-aad	5'-CAG GAA ACA GCT ATG ACG CTG CAA GTT CAC GTT GTG C-3'	95°C 2 min 35 cycles (95°C 15 sec; 56°C 30 sec; 72°C 1 min) 72°C 10 min	0.1 µL F & R Primers 5 µL 1:10 polymerase

[1]All PCR primers were M13 tailed to facilitate downstream sequencing. 1:10 polymerase is a 1:10 mixture of Takara (San Jose, CA) PremixTaq DNA Polymerase and Promega (Madison, WI) GoTaq DNA Polymerase. Each reaction also included 1 µL template DNA and enough water for a total reaction volume of 10 µL.

Sanger Sequencing

PCR products were run on a 1.5-2% SYBR-safe dyed agarose gel to verify amplification and then cleaned using a 1:4 dilution of ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA). Samples were prepared for sequencing with 0.3 µL of corresponding PCR primer or M13, 0.25 µL BDX64 (Molecular Cloning Lab, San Francisco, CA), 0.125 µL BigDye (Thermo Fisher Scientific, Waltham, MA), 1.75 µL terminating buffer (Thermo Fisher Scientific, Waltham, MA), 6.38 µL H20, and 1.2 µL cleaned DNA. Cycling conditions included an initial 3-minute denaturing step at 96°C for 3 minutes followed by 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes and 30 seconds. Products were cleaned via ethanol precipitation, resuspended with 10 µL of formamide and then run on an ABI 3500XL genetic analyzer.

Sequence Analysis

Forward and reverse sequences were trimmed and aligned using Geneious software (Geneious Prime® 2024.0.3 https://www.geneious.com). For each gene, individual consensus sequences were aligned using the Geneious Clustal Omega plugin and trimmed to a uniform length. Severely truncated sequences (missing >25% of nucleotides) were not included in

subsequent phylogenies. The concatenated phylogeny was generated by stringing together the trimmed sequences for all samples that were successfully sequenced at all four genes. Phylogenetic trees were inferred in IQTree2 using the model selection tool (Akaike Information Criterion: TPM2u+F+R10; Corrected Akaike Information Criterion: TPM2u+F+R10; Bayesian Information Criterion (BIC): HKY+F+R10; Best-fit model: HKY+F+R10 chosen according to BIC) with ultrafast bootstrap replicates of 1000 (Kalyaanamoorthy et al. 2017; Hoang et al. 2018; Minh et al. 2020). Trees were visualized in FigTree (Rambaut 2018).

RESULTS

Meristics

Data for nine meristic variables, plus the dorsal-fin origin (position), are summarized in Table 4 and in Table 2 in Supplemental Meristic and Morphometric Files. The mode of the numbers of lateral-line scales, circumferential scales, and predorsal scales, were not different from one another for *Cyprinella zanema* and *Cyprinella* sp. "Thinlip Chub" but were slightly greater than those for *C. labrosa*. Additionally, the dorsal-fin origin (position) in *C. labrosa* was modally 1 scale (range: 0–2 scales) posterior to the pelvic-fin origin; in *C. zanema* it was modally 2.5 scales (range: 1.5–3 scales) posterior to the pelvic-fin origin; and in *Cyprinella* sp. "Thinlip Chub" it was modally 1.5 scales (range: 0–2.5 scales) posterior to the pelvic-fin origin (Table 4; Figure 12). The other six meristic variable characteristics were not different amongst the three species.

Table 4. Meristics and dorsal-fin origin (position) for *Cyprinella labrosa, C. zanema, and Cyprinella* sp. "Thinlip Chub". Gray shading denotes a difference.

Species	***Cyprinella labrosa***		***Cyprinella zanema***		***Cyprinella* sp. "Thinlip Chub"**	
Meristics	**Range**	**Mode**	**Range**	**Mode**	**Range**	**Mode**
Lateral-Line Scales	36-38	37	36-40	40	38-42	40
Circumferential Scales	24	24	24-26	26	24-26	26
Predorsal Scales	14-15	15	15-21	17	16-23	17
Circumcaudal-Peduncle Scales	12	12	12	12	12-14	12
Dorsal-Fin Rays	8- 9	8	8-9	8	8-9	8
Pectoral-Fin Rays	13-16	15	14-16	15	13-16	15
Pelvic-Fin Rays	8-9	8	8	8	7-9	8
Anal-Fin Rays	8	8	7 -8	8	7-9	8
Caudal-Fin Rays	19	19	19	19	19	19
Dorsal-Fin Origin (Position)	0.0-2.0	1.0	1.5-3.0	2.5	0.0-2.5	1.5

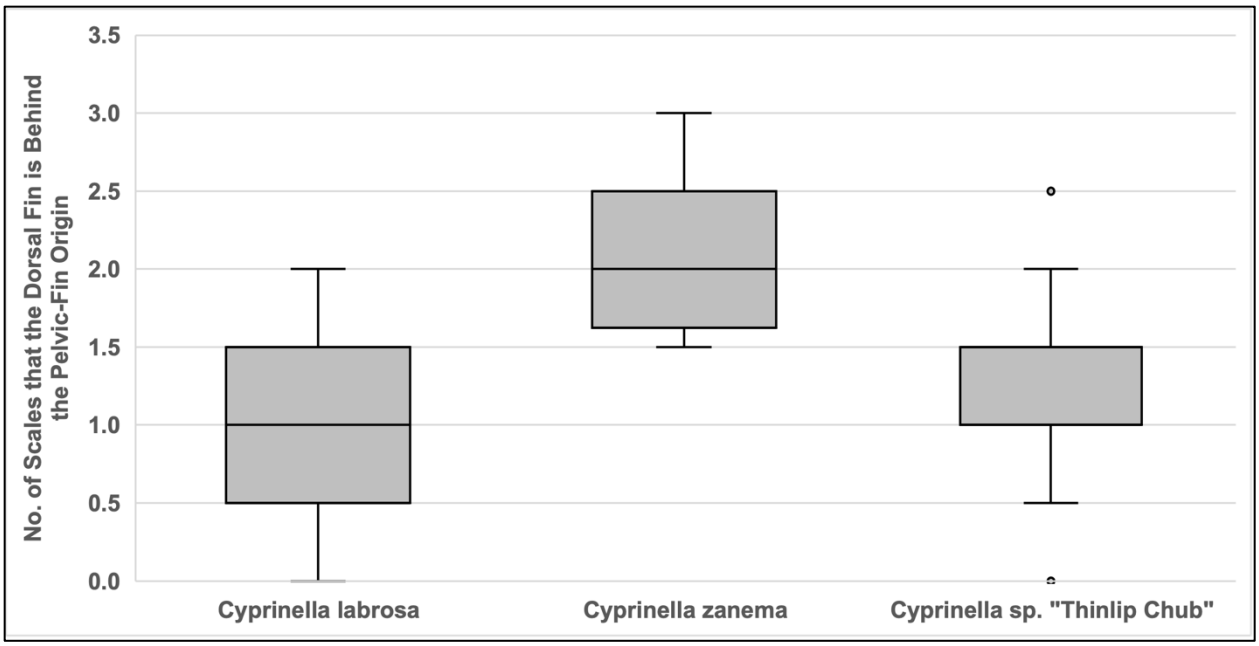

Figure 12. Box and whisker plots of the dorsal-fin origin relative to the pelvic-fin origin in *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub".

Also, in *Cyprinella labrosa*, the upper lip position, i.e., a line drawn from the posterior corner of the upper lip, immediately anterior to the maxillary barbel, did not extend vertically posterior to the anterior edge of the bony orbit in 27 of the 30 specimens (Figure 13). In *C. zanema* and *Cyprinella* sp. "Thinlip Chub", the upper lip position did extend vertically posterior to the anterior edge of the bony orbit in 26 of 40 specimens and in 47 of 50 specimens examined, respectively. The upper lip position could not be determined in three specimens each of the *C. labrosa* and *Cyprinella* sp. "Thinlip Chub" and 14 specimens of the *C. zanema* due to distortion of the specimens.

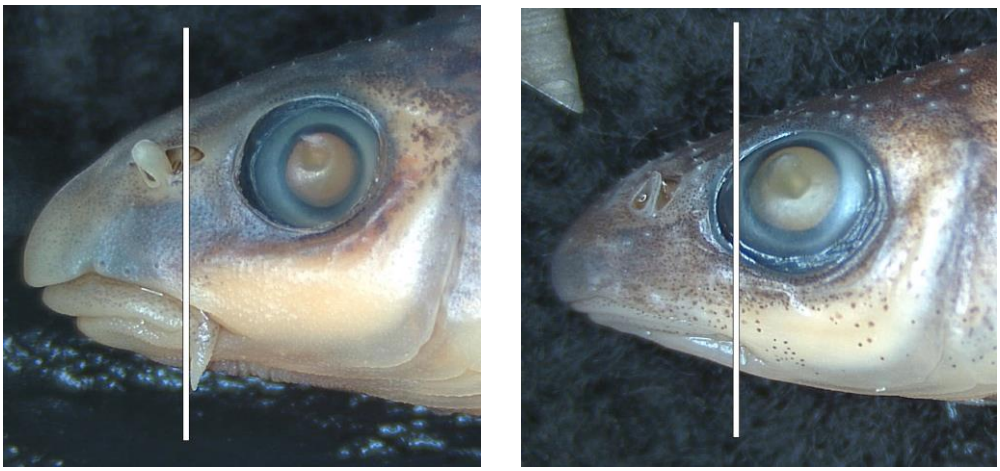

Figure 13. Lateral view, close-up of head, showing the upper lip position (i.e., a vertical line drawn from the posterior corner of the upper lip, immediately anterior to the maxillary barbel). Left: *Cyprinella labrosa*, Stony Fork, Wilkes County, Yadkin-Pee Dee River basin, NCSM 89947, Specimen No. 2. Right: *Cyprinella* sp. "Thinlip Chub", Little River, Hoke-Moore counties, Cape Fear River basin, NCSM 29309, Specimen No. 3. Photographs by Bryn H. Tracy.

Landmark-based Linear Morphometrics

Data for 25 landmark-based linear morphometric variables expressed as percentages of Standard Length are summarized in Table 5 and in Table 3 in Supplemental Meristic and Morphometric Files. Morphological characteristics pertaining to the upper lip (i.e., upper lip width at midpoint, upper lip width at midline, and anterior of upper lip thickness), and mouth gape width were all much less (i.e., thinner or narrower) for *Cyprinella* sp. "Thinlip Chub" than for *C. labrosa* and/or *C. zanema*. In addition, the maxillary barbel length was much shorter for *Cyprinella* sp. "Thinlip Chub" (mean 0.8% SL) than for *C. labrosa* and *C. zanema* (mean 2.1% and mean 1.9% SL, respectively). No differences were found in the remaining 20 landmark-based linear morphometric variables.

Table 5. Landmark-based linear morphometric data expressed as percentages of Standard Length for *Cyprinella labrosa, C. zanema, and Cyprinella* sp. "Thinlip Chub". Landmarks are defined in Table 2 and shown in Figures 7-11. Gray shading denotes a difference; SD=standard deviation.

Species	***Cyprinella labrosa***		***Cyprinella zanema***		***Cyprinella* sp. "Thinlip Chub"**	
Landmark-based Linear Morphometrics	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Head Depth	12.0-14.6	13.3 ± 0.6	10.8-12.7	11.8 ± 0.5	10.5-14.2	11.5 ± 0.7
Head Length	23.1-28.2	25.1 ± 1.3	21.8-26.3	24.3 ± 1.1	21.4-25.6	23.3 ± 1.1
Snout Length	7.3-10.0	8.5 ± 0.6	6.4-9.1	7.9 ± 0.6	5.6-8.6	7.0 ± 0.7
Snout to Top of Gill Slit	21.2-26.7	23.1 ± 1.1	219.6-23.8	22.0 ± 0.8	19.8-23.8	21.4 ± 1.0
Predorsal Length	50.3-55.6	52.8 ± 1.4	50.7-55.4	52.6 ± 1.1	48.4-54.8	51.7 ± 1.3
Orbit Length	5.4-6.7	6.1 ± 0.3	5.6-6.8	6.2 ± 0.2	5.3-7.1	6.2 ± 0.4
Dorsal-Fin Base Length	11.8-15.8	13.7 ± 0.9	10.6-14.7	12.9 ± 0.9	11.6-15.4	13.3 ± 0.9
Dorsal-Fin Origin to Pectoral-Fin Origin	28.5-35.5	31.9 ± 1.8	28.6-35.1	31.7 ± 1.4	28.5-34.7	31.3 ± 1.4
Dorsal-Fin Origin to Pelvic-Fin Origin	19.1-23.6	21.9 ± 1.1	17.2-22.3	19.4 ± 1.1	14.5-21.3	18.7 ± 1.7
Dorsal-Fin Origin to Anal-Fin Origin	25.6-29.1	27.4 ± 0.8	17.0-26.6	24.3 ± 1.6	21.4-26.9	24.5 ± 1.2
Pectoral-Fin Origin to Pelvic-Fin Origin	23.6-39.8	25.9 ± 1.4	21.6-26.9	24.0 ± 1.3	20.9-29.4	25.0 ± 1.5
Pelvic-Fin Origin to Anal-Fin Origin	16.7-21.8	18.8 ± 1.3	17.2-21.1	19.1 ± 0.7	14.7-20.0	18.4 ± 0.9
Anal-Fin Base Length	12.4-14.9	13.4 ± 0.6	11.6-15.0	13.1 ± 0.8	10.8-14.7	12.6 ± 0.9
Caudal Peduncle Depth	8.2-9.9	8.9 ± 0.5	7.8-9.9	8.9 ± 0.5	7.8-10.1	9.0 ± 0.5
Interorbital Width	8.1-10.0	8.9 ± 0.5	7.5-10.3	8.7 ± 0.6	7.3-9.7	8.5 ± 0.5
Pectoral-Fin Base Length	3.5-5.0	4.2± 0.4	2.9-4.6	3.8 ± 0.4	2.7-4.5	3.5 ± 0.4
Pectoral-Fin Length	15.3-20.0	17.9 ± 1.1	12.7-17.9	15.9 ± 1.1	13.2-18.2	15.7 ± 1.3
Pelvic-Fin Base Length	3.3-4.4	3.7 ± 0.3	2.6-4.6	3.5 ± 0.5	2.8-4.7	3.7 ± 0.4
Pelvic-Fin Length	12.2-16.3	14.4 ± 1.1	10.9-15.6	13.6 ± 1.1	10.6-15.1	12.9 ± 1.0
Upper Jaw Length	5.5-8.4	7.1 ± 0.6	5.2-8.7	7.6 ± 0.7	6.2-8.6	7.3 ± 0.6
Upper Lip Width at Midpoint	0.5-1.6	1.2 ± 0.3	0.5-1.3	0.9 ± 0.2	0.3-0.8	0.5 ± 0.1
Upper Lip Width at Midline	0.8-1.6	1.1 ± 0.2	0.7-1.3	1.0 ± 0.2	0.4-1.0	0.7 ± 0.1
Anterior of Upper Lip Thickness	0.5-1.3	0.9 ± 0.2	0.3-0.9	0.6 ± 0.1	0.3-0.7	0.5 ± 0.1
Mouth Gape Width	6.0-8.3	7.2 ± 0.5	5.9-9.4	7.4 ± 0.8	5.5-7.9	6.6 ± 0.6
Maxillary Barbel Length	1.3-3.9	2.1 ± 0.6	1.0-4.0	1.9 ± 0.5	0.3-1.5	0.8 ± 0.3

Data for nine landmark-based linear morphometric variables expressed as percentages of head length are summarized in Table 6. Similar to the variables expressed as percentages of Standard Length, morphological characteristics pertaining to the upper lip (i.e., upper lip width at midpoint, upper lip width at midline, and anterior of upper lip thickness; Table 5) were all much less (i.e., thinner) for *Cyprinella* sp. "Thinlip Chub" than for *C. labrosa* and/or *C. zanema* (Table 6; Figure 14). In addition, the maxillary barbel length was much shorter for *Cyprinella* sp. "Thinlip Chub" (mean 3.5% head length) than for *C. labrosa* and *C. zanema* (mean 8.5% and mean 8.0%

head length, respectively; Figure 14). No differences were found in the remaining five landmark-based linear morphometric variables.

Table 6. Landmark-based linear morphometric data expressed as percentages of head length for *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub". Landmarks are defined in Table 2 and shown in Figures 7-11. Gray shading denotes a difference; SD=standard deviation.

Species	*Cyprinella labrosa*		*Cyprinella zanema*		*Cyprinella* sp. "Thinlip Chub"	
Landmark-Based Variable	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Snout Length/Head Length (%)	30.7-37.4	33.7 ± 1.6	29.3-35.6	32.5 ± 1.8	25.5-34.2	30.1 ± 2.0
Snout to Top of Gill Slit/Head Length (%)	86.0-96.1	91.9 ± 2.3	84.0-95.5	90.7 ± 2.4	85.9-96.0	91.9 ± 2.0
Orbit Length/Head Length (%)	22.1-26.0	24.3 ± 1.2	22.0-28.2	25.4 ± 1.3	23.1-31.6	26.7 ± 1.5
Upper Jaw Length/Head Length (%)	23.2-31.4	28.1 ± 2.1	24.1-34.9	31.4 ± 3.0	25.0-34.3	31.2 ± 1.9
Upper Lip Width at Midpoint/Head Length (%)	2.2-6.5	4.6 ± 1.1	1.8-5.1	3.5 ± 0.7	1.3-3.2	2.1 ± 0.4
Upper Lip Width at Midline/Head Length (%)	3.2-6.3	4.3 ± 0.8	2.8-6.0	4.1 ± 0.7	1.7-4.4	2.9 ± 0.5
Anterior of Upper Lip Thickness/Head Length (%)	2.2-5.0	3.7 ± 0.6	1.4-3.5	2.5 ± 0.6	1.3-3.0	2.2 ± 0.5
Mouth Gape Width/Head Length (%)	24.1-33.8	28.5 ± 2.2	24.7-36.3	30.4 ± 2.5	23.6-33.3	28.4 ± 2.7
Maxillary Barbel Length/Head Length (%)	5.1-14.9	8.5 ± 2.2	4.0-15.3	8.0 ± 2.0	1.3-6.0	3.5 ± 1.3

When maxillary barbel length was plotted against the upper lip width at midline for each species, there was a distinct and almost complete separation and clustering of *Cyprinella* sp. "Thinlip Chub" from the other two species (Figure 15). A positive relationship exists between the two variables. A short barbel is correlated with a thin upper lip and both variables can be used to separate *Cyprinella* sp. "Thinlip Chub" from *C. labrosa* and *C. zanema*.

Random Forests Classification

There were a total of 120 observations (specimens across the three species) in the original data set (Lee 2023). All continuous features, except the number of caudal-fin rays, were evaluated for collinearity. This removed 10 features (head depth, head length, snout length, tip of snout to top of gill slit, dorsal-fin origin to pectoral-fin origin, dorsal-fin origin to pelvic-fin origin, dorsal-fin origin to anal-fin origin, pectoral fin length, maxillary barbel length, and upper lip width at midpoint) with 23 features remaining. Thirteen continuous features, plus the dorsal-fin position variable, were deemed important using the Boruta algorithm and are shown by green boxes in the Variable Importance Plot (Lee 2023; Figure 16). These 14 variables (also known as the confirmed features (i.e., upper lip width, number of predorsal scales, number of lateral-line scales, etc.)) are measurements and counts that were confirmed to inform the model, with the most informative variables, in order of increasing importance, towards the right on the horizontal axis (Figure 16). These 14 confirmed features are what differentiates *Cyprinella* sp. "Thinlip Chub" from *C. labrosa* and *C. zanema*. Upper lip width at midline was found to be the most important feature in the model. The 10 collinear features, previously confirmed as unimportant and hence removed from the model, are shown as red boxes; the three blue boxes represent dummy variables that did not inform the model (Figure 16).

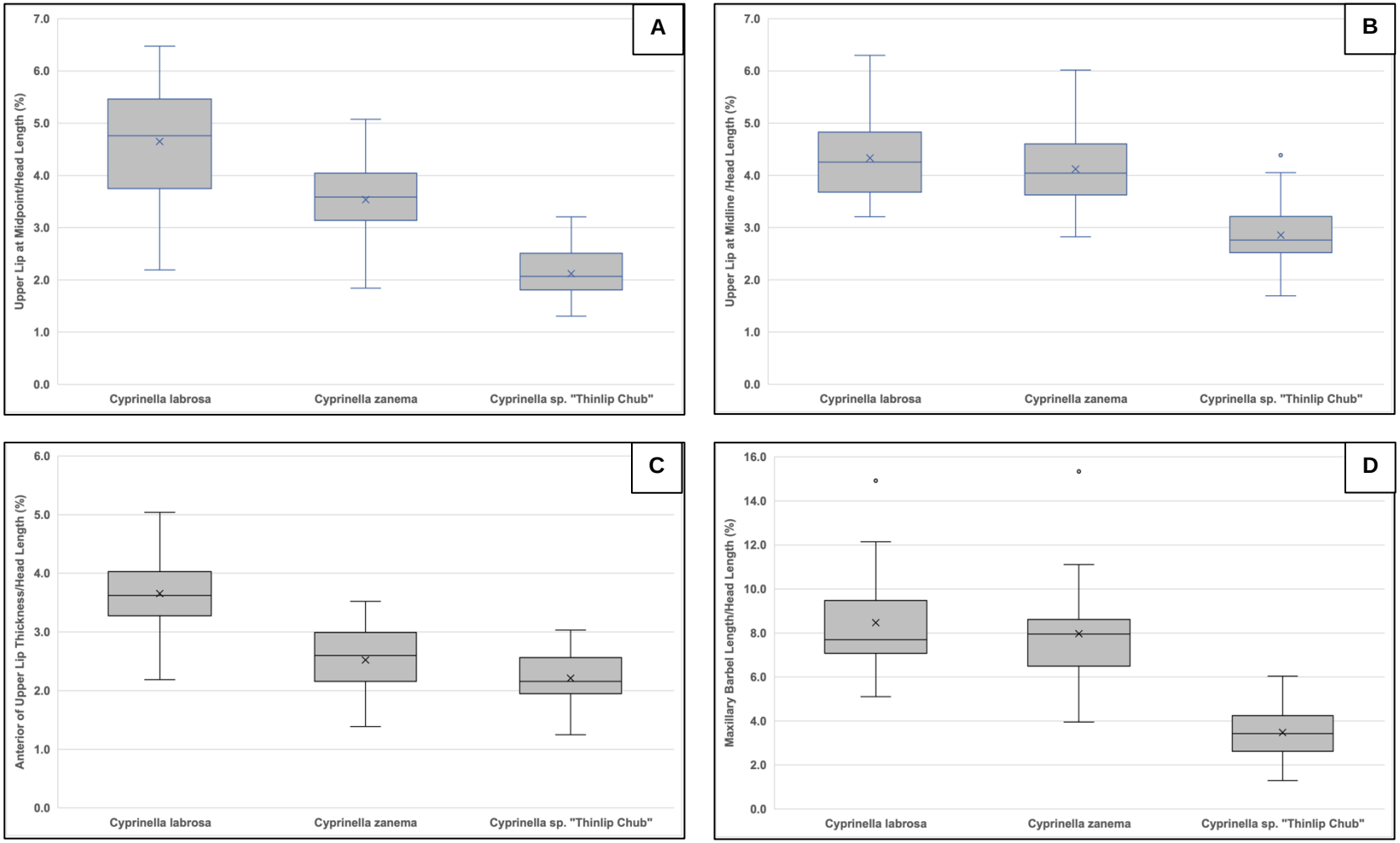

Figure 14. Figure 14. Box and whisker plots of the: A) upper lip width at midpoint/head length, B) upper lip width at midline/head length, C) anterior of upper lip thickness/head length, and D) maxillary barbel length/head length for *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. “Thinlip Chub”. The X symbol denotes the mean for each variable. The Y axis is the variable expressed as a percentage of head length.

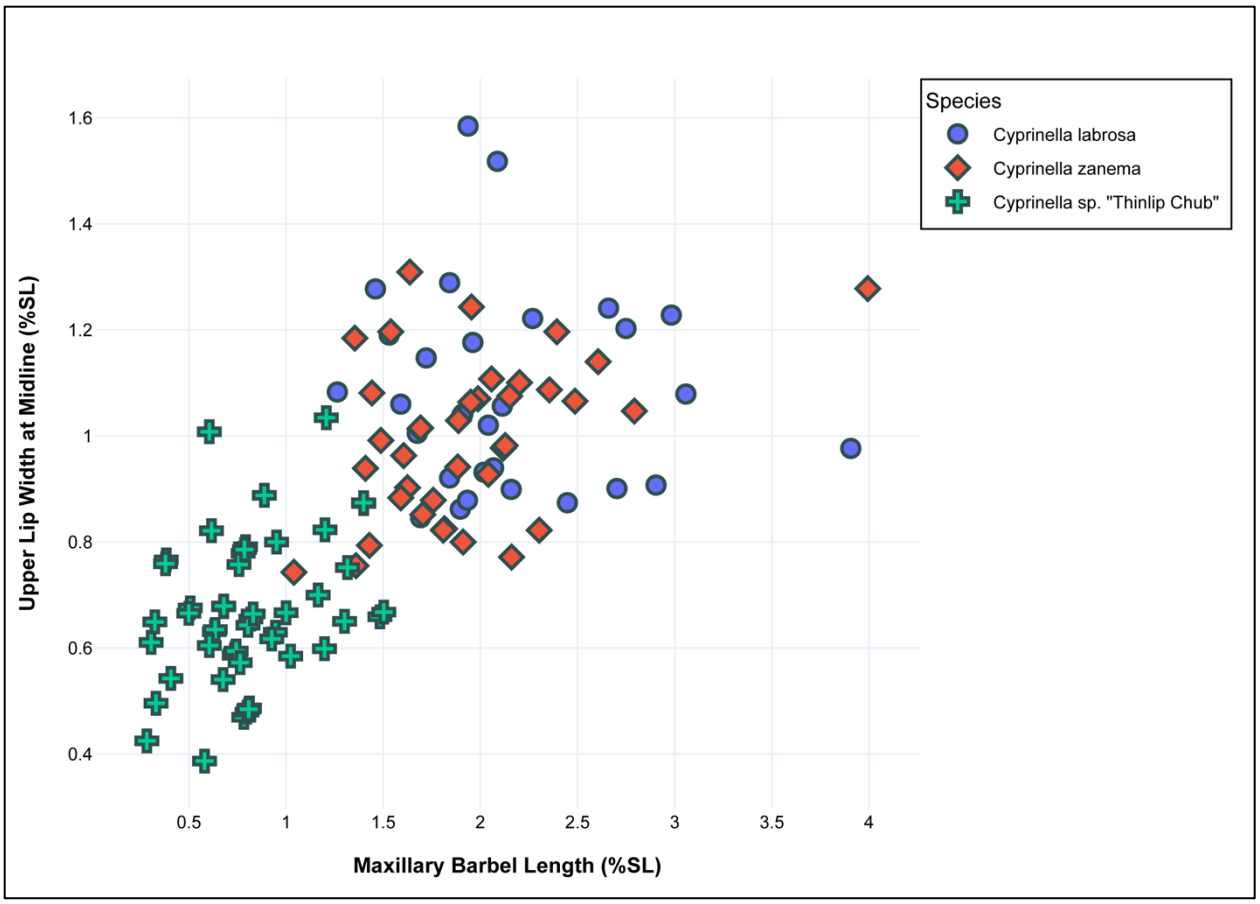

Figure 15. Bivariate plot of maxillary barbel length vs. upper lip width at midline expressed as percentages of Standard Length for *Cyprinella labrosa, C. zanema, and Cyprinella* sp. "Thinlip Chub".

The classification accuracy (random forest model performance) applied to the training and testing data sets and based upon the 14 features that were confirmed by the Boruta algorithm are summarized in confusion matrices (Table 7). The random forest model had 94% accuracy when applied to the training data; five of the individuals (6%) were incorrectly classified (i.e., three *Cyprinella zanema* were incorrectly classified as *Cyprinella* sp. "Thinlip Chub" and two *Cyprinella* sp. "Thinlip Chub" were incorrectly classified as *C. zanema*). For the testing data set, the random forest model had 100% accuracy for differentiating all three species (Table 7), which is evidence that the random forest model is a good classifier for these three species of *Cyprinella*.

Table 7. Confusion matrices of random forest classifier. The rows represent the observed species assignment and the columns represent the random forest model predictions. Cells along the diagonal represent the correct model predictions. Gray shading denotes 94% accuracy of the training data and 100% accuracy of the testing data.

	Species		
Observed Species Assignment	***Cyprinella labrosa***	***Cyprinella zanema***	***Cyprinella* sp. "Thinlip Chub"**
	Training Data (n=84)		
C. labrosa	21	0	0
C. zanema	0	25	3
***C.* sp. "Thinlip Chub"**	0	2	33
	Testing Data (n=36)		
C. labrosa	9	0	0
C. zanema	0	12	0
***C.* sp. "Thinlip Chub"**	0	0	15

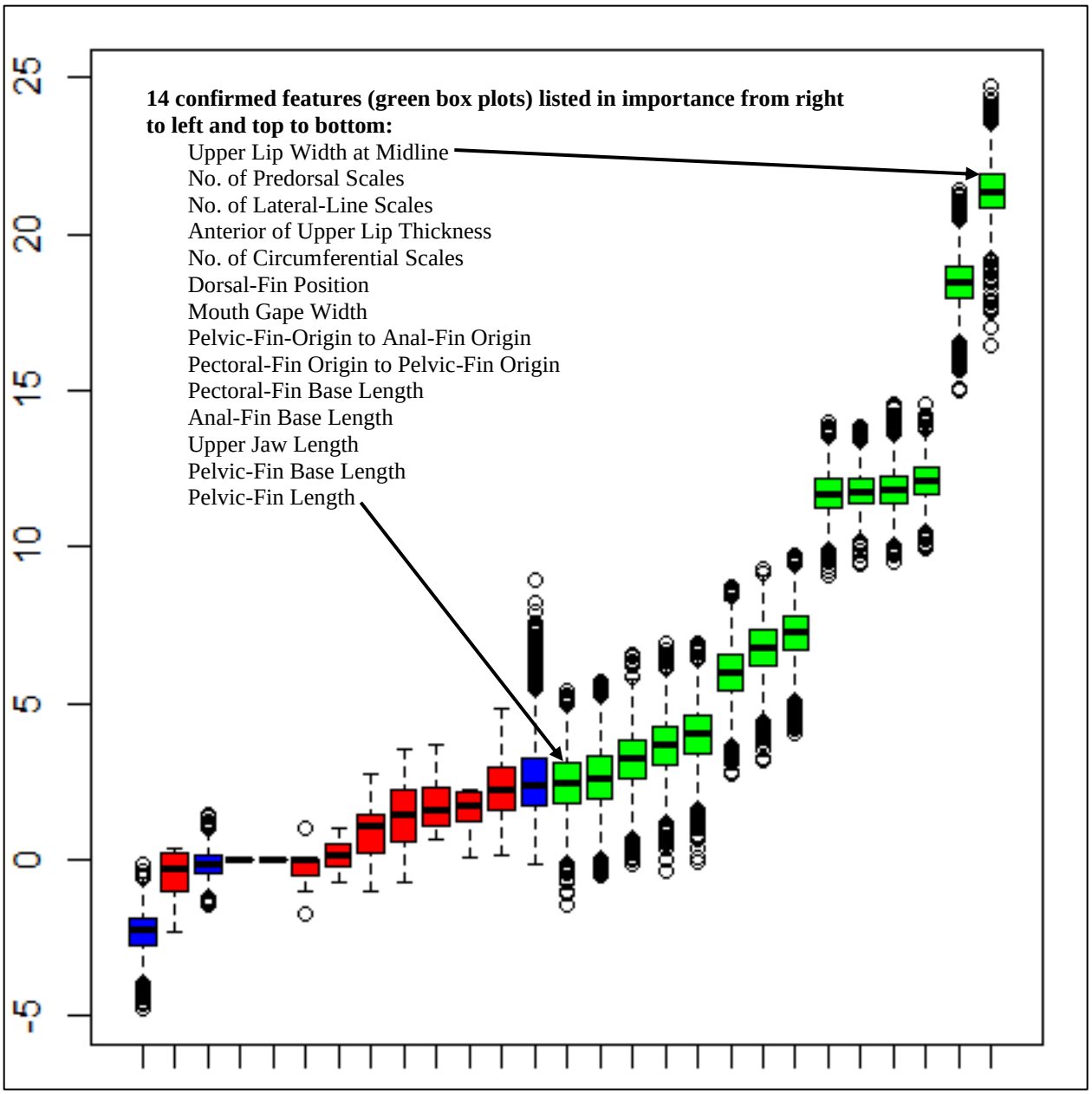

Figure 16. Variable Importance Plot of the landmark-based linear morphometric dataset for *Cyprinella* species classification using random forests. The horizontal axis represents the meristic and landmark-based linear morphometric variables; the vertical axis represents the relative importance of each variable.

Tuberculation Patterns

Male *Cyprinella* sp. "Thinlip Chub" and *C. zanema* have more internasal, interorbital, and occipital; lachrymal; sub-, post-, and supraorbital; preopercular, and opercular antrorse (directed upward and forward) tubercles than *C. labrosa*. (Table 6). Differences between *C. zanema* and *Cyprinella* sp. "Thinlip Chub" were less obvious, except that *Cyprinella* sp. "Thinlip Chub" appear to have fewer and smaller tubercles in the lachrymal region and on the sub-, post-, and supraorbital; preopercle, and opercle than did *C. zanema* (Table 8).

Table 8. Patterns of the antrorse tubercles on the dorsum (internasal, interorbital, and occipital (supraoccipital to posteromedial tip)) and lateral side (lachrymal; sub-, post-, and supraorbital; preopercle; and opercle) of the head in male *C. labrosa* (n=12, 52.3–58.8 mm SL), *C. zanema* (n=24, 53.1–67.3 mm SL), and *Cyprinella* sp. "Thinlip Chub" (n=17, 48.6–68.4 mm SL).

	Species		
Region	***Cyprinella labrosa***	***Cyprinella zanema***	***Cyprinella* sp. "Thinlip Chub"**
Internasal tubercles	Few (n=12), medium to very large size scattered tubercles	Numerous, large (n=19), or medium (n=2) size scattered tubercles	Variable in number; medium (n=11); large (n=4) or small (n=2) size scattered tubercles
Interorbital tubercles	Few (n=11), or very few (n=1), large to very large size scattered tubercles	Numerous, large size, scattered, (n=15); or numerous, large size, loosely arranged in two rows going from anterior to posterior (n=7); or numerous, medium size (n=1) scattered; or numerous, medium size (n=1), loosely arranged in two rows going from anterior to posterior tubercles	Variable in number; small to medium (n=8) size, scattered 8; or numerous, large size (n=5) scattered; or numerous, large to medium size (n=4), loosely arranged in two rows going from anterior to posterior tubercles
Occipital (Supraoccipital to Posteromedial Tip) tubercles	Few (n=11), or numerous (n=1), very large (n=11) or medium (n=1) size scattered tubercles	Numerous, large size (n=22); or small to medium size (n=2) scattered tubercles	Numerous, medium to large size (n=15), or small size (n=2) scattered tubercles
Lachrymal tubercles	Tubercles absent (n=7), or with very small to medium size, 0-2, tubercles near nares (n=5); tubercles absent (n=10), or small size (n=2) tubercles near upper lip	Numerous (n=21), or few (n=2) small to large size, scattered tubercles extending to upper lip	Few (n=11), or numerous (n=6), minute to medium size, scattered tubercles extending to upper lip
Sub-, Post-, and Supraorbital; Preopercle; and Opercle tubercles	None (n=12)	Several, small to large tubercles on the sub- and postorbital regions; small to medium tubercles along the lower edge, lower corner, or upper corner of preopercle; usually 1-4 tubercles at lower edge or lower corner of preopercle (n=17); or none (n=7)	None (n=12), or 1-3, very small size (n=5) tubercles on either the suborbital, supraorbital, or preopercular regions

Phylogenetic Analyses

The concatenated phylogeny largely reflects the patterns noted in other *Cyprinella* studies (Broughton and Gold 2000; Schönhuth and Mayden 2010) and supports the trends noted in the morphometric data (Figure 17; Supplemental Genetics Files). *Cyprinella labrosa*, *C. zanema*, and *Cyprinella* sp. "Thinlip Chub" form a larger monophyletic clade with distinct clades of each species. *Cyprinella* sp. "Thinlip Chub" is further split into the two clades of Cape Fear River and Yadkin-Pee Dee River basins. Divergence of species level clades is highly supported (Bootstrap Support Values (BSS)=100 for *C. labrosa* – *C. zanema* and *Cyprinella* sp. "Thinlip Chub" split; BSS=100 for *C. zanema* – *Cyprinella* sp. "Thinlip Chub" split); however, BSS is lower, though still supportive, for the two clades of *Cyprinella* sp. "Thinlip Chub" (BSS=87). While neither nuclear gene produced reciprocally monophyletic clades for the three species of barbeled *Cyprinella*, likely due to higher conservation and slower evolutionary rates of nuclear genes (Figures S3 and S4 in Genetic Figures S1-S4 File in Supplemental Genetics Files), both mitochondrial genes (Figures S1 and S2 in Genetic Figures S1-S4 File in Supplemental Genetics Files) and concatenated (Figure 16) analyses consistently resolved each of the three species as reciprocally monophyletic.

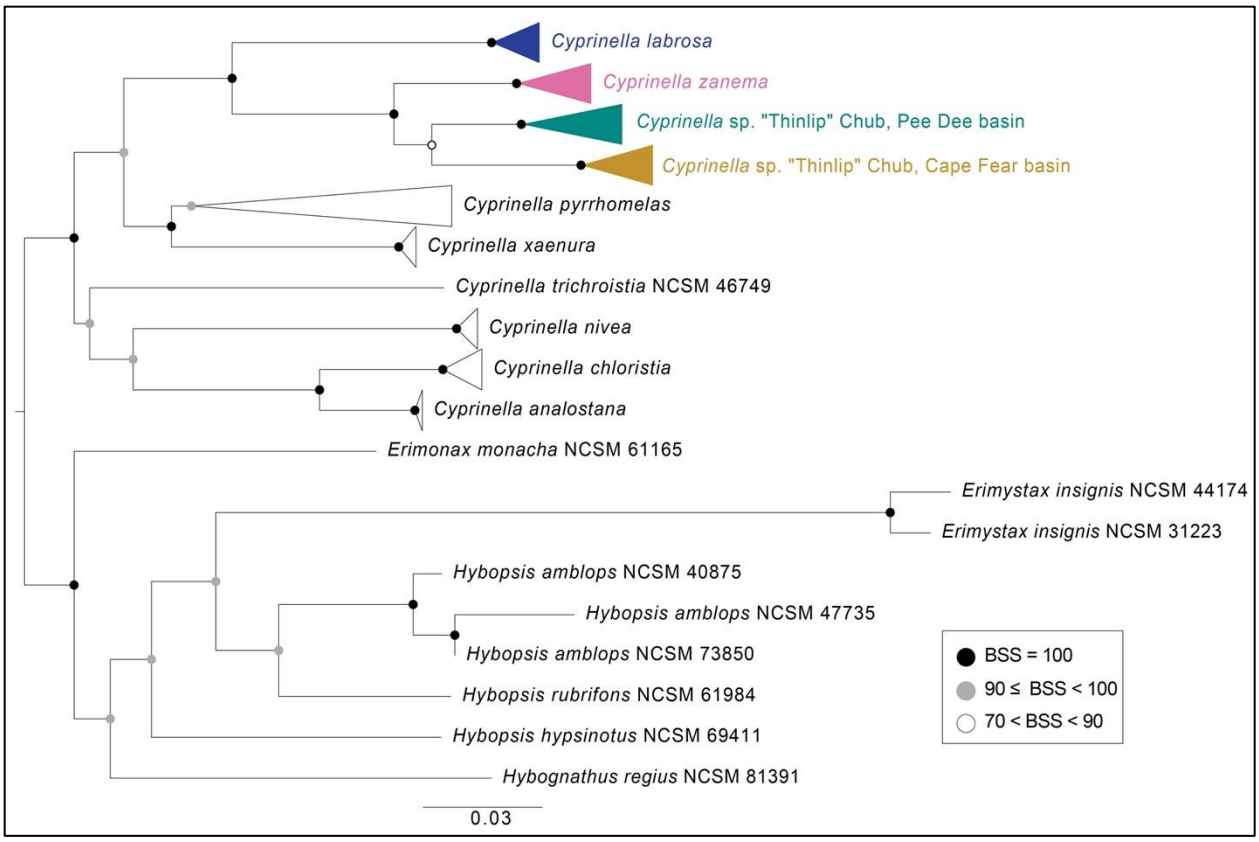

Figure 17. Concatenated phylogeny for *Cyprinella* and outgroups that includes sequences from RAG1, Homeobox13, COI, and Cyt-b. The species of interest, *C. labrosa*, *C. zanema*, and Yadkin-Pee Dee River basin and Cape Fear River basin *Cyprinella* sp. "Thinlip Chub", are denoted by blue, pink, teal, and yellow triangles, respectively. Bootstrap Support values are represented by black (BSS=100), gray (90 ≤ BSS < 100), and white circles (70 < BSS < 90).

We speculate that historically there was shared basin ancestry amongst the Santee (including the Catawba, Broad, and Saluda River subbasins), Yadkin-Pee Dee River (including the Yadkin, Lumber, Little Pee Dee, and Lynches River subbasins), and Cape Fear River (including the Upper Cape Fear River and South River/Black River subbasins) river basins. After dispersal of ancestral stock from the west to the east, the populations became isolated from one another leading to allopatric speciation resulting in the three species we see today.

***Cyprinella leptocheilus*, sp. nov., Tracy, Rohde, and Jenkins**
Siouan Thinlip Chub

urn:lsid:zoobank.org:act:F945BDDC-6E9E-4A50-A208-7631BC6213CF

Synonymies

Ceratichthys labrosus Cope (1870), in part, original species description.
Ceratichthys zanemus Jordan and Brayton (1878), in part, original species description.
Hybopsis labrosus (Cope 1870), in part, in Jordan and Evermann (1896), original species description.

Hybopsis labrosa (Cope 1870), in Louder (1962) and Ratledge et al. (1966) distribution; in Moore (1968) identification, distribution; in Eddy (1969) identification; in Jenkins and Lachner (1971) taxonomic characteristics.

Notropis volucellus (Cope 1865), in Louder (1963) misidentification, distribution.

Hybopsis n. sp., undescribed species in Menhinick et al. (1974) and Jenkins and Lachner (1980) distribution; in Menhinick (1987) distribution, imperilment status.

Hybopsis sp., Thinlip Chub undescribed species, in Olmstead and Cloutman (1978) distribution; in Anderson et al. (2001), phylogenetic relationships.

Hybopsis sp., in Mayden (1989) phylogenetic relationships.

Hybopsis zanema (Jordan and Brayton 1878), in Gilbert (1978) lectotype designation; in Page and Burr (1991) identification and distribution; in Menhinick (1991) Coastal Plain populations identification and distribution; in Rohde et al. (1994) identification, distribution, abundance, habitat, natural history

Hybopsis sp. *cf. zanema*, undescribed species, in Hocutt et al. (1986) distribution

Cyprinella zanema (Jordan and Brayton 1878) form, Thinlip Chub, undescribed species, in Menhinick (1997) description, range, habitat, and biology, significance, imperilment status, current protection, and recommendations; in Page and Burr (2011) identification and distribution.

Cyprinella zanema, in Mayden et al. (1992) known or presumed to be polytypic.

Cyprinella Thinlip Chub, undescribed species, in Rohde et al. (2009) identification, characteristics, meristics, distribution, habitat, biology, similar species, remarks, name; in Schönhuth and Mayden (2010) undescribed species, phylogenetic relationships.

Cyprinella sp. *cf. zanema*, undescribed species, in Tracy (2014), type specimen and type locality, description, range, habitat, life history and ecology, rationale for description, recommendations; in SCDNR (2015), description, population size and distribution, habitat or natural community requirements, challenges, conservation accomplishments, conservation recommendations, measures of success.

Cyprinella sp. “Thinlip” Chub, in Carlson et al. (2024) phylogenetic relationships; Tracy et al. (2020); Tracy et al. (2023a); Tracy et al. (2023b); Tracy et al. (2024) undescribed species distribution; undescribed species, identification, distribution.

Type Specimens

Holotype (Figures 18-20)

NCSM 113724, male, 70.9 mm TL, 58.3 mm SL, North Carolina, Sampson County, Black River, off SR 1100, about 700 m south center Ivanhoe, at North Carolina Wildlife Resources Commission’s boat access area, (34.60484, -78.24286), August 19, 2023, B.B. Kenney, F.C. Rohde, S.A. Smith, and B.H. Tracy.

Figure 18. Dorsal, lateral, and ventral views of the holotype, NCSM 113724, of *Cyprinella leptocheilus* sp. nov. Photographs by Jesse L. Bissette.

Figure 19. Close-up views of the ventral region anterior to the anal-fin origin and dorsum anterior to dorsal fin of the holotype, NCSM 113724, of *Cyprinella leptocheilus* sp. nov. Photographs by Jesse L. Bissette.

Figure 20. Close-up lateral view of the anterior region of the holotype, NCSM 113724, of *Cyprinella leptocheilus* sp. nov. Photograph by Jesse L. Bissette.

Paratopotypes

NCSM 115068, three females, 63-68 mm TL, 52-56 mm SL. Same locality and date as holotype.

USNM 477430, two females, 63 and 65 mm TL, 52 and 54 mm SL. Same locality and date as holotype.

ANSP 212136, two females, 53 and 58 mm TL, 45 and 47 mm SL. Same locality and date as holotype.

Paratypes

The following paratypes, comprising six lots and 20 specimens, were all collected from the Cape Fear River basin in North Carolina: 1) NCSM 28908, South River at NC 41, 20.0 km east of Elizabethtown, Bladen-Sampson counties, (34.69650, -78.36850), June 24, 1999, 2 specimens - 61.5 and 65.5 mm SL; 2) NCSM 89127, South River, below US 701, about 3.7 km south of Garland, Bladen-Sampson counties, (34.76106, -78.40754), October 8, 1977, 5 specimens – 61.9-67.7 mm SL; 3) NCSM 86397, South River, US 701, about 27.5 km south-southwest center of Clinton, Bladen-Sampson-counties, (34.76143, -78.40906), July 6, 1979, 2 specimens – 62.6 and 68.4 mm SL; 4) NCSM 115072, Rockfish Creek, at SR 1432, 10.6 km east-southeast of Raeford, Hoke County, (34.96810, -79.10960), October 26, 2000, 7 specimens – 59.0-68.2 mm SL; 5) NCSM 29309, Little River, at SR 2021, border Fort Liberty Military Reservation, 19.3 km east of Southern Pines, Hoke-Moore counties, (35.19380, -79.18540), August 28, 2000, 3 specimens – 60.0-64.2; and 6) NCSM 10615, Great Coharie Creek, at US 701 bridge, 4.0 km northeast of Garland, Sampson County, (34.817727, -78.362057), August 16, 1983, 74.0 mm SL. Complete

meristic, landmark-based linear morphometric, and locality data can be found in Tables 1-3 in Supplemental Meristic and Morphometric Files.

Other Specimens Examined

All lots of *Cyprinella leptocheilus* at NCSM were verified as to their correct identity. Additional lots of *C. leptocheilus* at USNM (216439, 216440, 216441), INHS (68642, 75236, and 88320), and UNCW (77-91-03 and U-645) were also verified. All specimens in all lots of *C. zanema* and *C. labrosa* at NCSM were verified of which a subset of each lot were used in this study (Table 1 in Supplemental Meristic and Morphometric Files).

Diagnosis

Cyprinella leptocheilus can be separated from all other species of *Cyprinella* except *C. labrosa* and *C. zanema* by possessing maxillary barbels. *Cyprinella leptocheilus* can be separated from *C. labrosa* and *C. zanema* by geographical distribution differences and by pigmentation, meristic, and morphometric characteristics. *Cyprinella leptocheilus* is restricted to the Cape Fear River and lower Yadkin-Pee Dee River basins, whereas *C. labrosa* and *C. zanema* are restricted to the Santee River and upper Yadkin-Pee Dee River basins. *Cyprinella leptocheilus* is distinguished from *C. labrosa* by having: 1) modally 40 lateral-line scales vs. 37 lateral-line scales; 2) no dark blotches or cross hatching on back and side vs. small, dark blotches and cross hatching on back and side; 3) no white spot at anterior base of dorsal fin vs. white spot present at anterior base of dorsal fin; 4) side of body well pigmented below lateral line vs. minimal pigment below lateral line; and 5) a line drawn from the posterior corner of the upper lip, immediately anterior to the barbel, extending vertically posterior to the anterior edge of the bony orbit vs. a line drawn from the posterior corner of the upper lip, immediately anterior to the barbel, extending vertically anterior to the anterior edge of the bony orbit. *Cyprinella leptocheilus* is distinguished from *C. zanema* by having: 1) dorsal-fin origin (position) modally 1.5 scales posterior to the pelvic-fin origin; vs. dorsal-fin origin modally 2.5 scales posterior to the pelvic-fin origin; 2) mean upper lip width at midpoint 2.1% of head length vs. mean upper lip width at midpoint 3.5% of head length; 3) mean upper lip width at midline 2.9% of head length vs. mean upper lip width at midline 4.1% of head length; and mean maxillary barbel length 3.5% of head length vs. mean maxillary barbel length 8.0% of head length.

Description

Adult *Cyprinella leptocheilus* range from about 45-92 mm TL; slender and elongate with large eyes and dorsoventrally compressed body deepest at nape and flattened ventrally (Figures 18–20). Frenum absent; horseshoe-shaped mouth inferior with snout projecting in front of upper jaw; single (very rarely double) maxillary barbel (of variable length) present at corner of mouth; and pharyngeals 0–1,4-4,0–1 (usually 1,4-4,1).

Dorsal-fin origin (0) 1 (2.5) scale behind pelvic fin. Dorsal-fin rays (8) 8 (9), (13) 15 (16) pectoral-fin rays, (7) 8 (9) pelvic-fin rays, (7) 8 (9) anal-fin rays, and 19 caudal-fin rays. Predorsal scales (16) 17 (23), (24) 26 (26) circumferential scales, 12 (14) circumcaudal-peduncle scales, and complete, decurved lateral line with (38) 40 (42) lateral-line scales. Lateral line, from origin near

shoulder, gradually dipping down into lower half of lateral stripe and then gradually rising to within middle of lateral stripe from beneath dorsal fin to caudal fin.

Meristic and landmark-based linear morphometric data for the holotype presented in Table 9. Additionally for the holotype, dorsal-fin origin approximately 1.75 scales posterior to pelvic-fin origin and line drawn from posterior corner of upper lip, immediately anterior to maxillary barbel, extends vertically posterior to anterior edge of bony orbit.

Table 9. Meristic and landmark-based linear morphometric data expressed as percentages of Standard Length for the holotype of *Cyprinella leptocheilus*, NCSM 113724.

Meristic Data		
Total Length (mm)	70.9	
Standard Length (mm)	58.3	
Predorsal Scales	17	
Lateral-Line Scales	40	
Circumferential Scales	26	
Circumcaudal-Peduncle Scales	14	
Dorsal-Fin Rays	8	
Pectoral-Fin Rays	13	
Pelvic-Fin Rays	8	
Anal-Fin Rays	8	
Caudal-Fin Rays	19	
Landmark-based Linear Morphometric Data (mm)		**Expressed as Percentages of Standard Length**
Head Depth	6.1	10.5
Head Length	13.5	23.2
Snout Length	3.8	6.5
Snout to Top of Gill Slit	12.1	20.8
Predorsal Length	28.9	49.6
Orbit Length	3.6	6.2
Dorsal-Fin Base Length	7.9	13.6
Dorsal-Fin Origin to Pectoral-Fin Origin	16.8	28.8
Dorsal-Fin Origin to Pelvic-Fin Origin	10.0	17.2
Dorsal-Fin Origin to Anal-Fin Origin	13.8	23.7
Pectoral-Fin Origin to Pelvic-Fin Origin	13.0	22.3
Pelvic-Fin Origin to Anal-Fin Origin	10.9	18.7
Anal-Fin Base Length	8.0	13.7
Caudal Peduncle Depth	5.5	9.4
Interorbital Width	5.1	8.7
Pectoral-Fin Base Length	2.2	3.8
Pectoral-Fin Length	8.6	14.8
Pelvic-Fin Base Length	1.3	2.6
Pelvic Fin Length	7.8	13.4
Upper Jaw Length	3.9	6.7
Upper Lip Width at Midpoint	0.2	0.3
Upper Lip Width at Midline	0.3	0.5
Anterior of Upper Lip Thickness	0.2	0.3
Mouth Gape Width	3.3	5.7
Maxillary Barbel Length	0.5	0.9

Tuberculation Patterns (Table 8)

In males, antrorse (directed upward and forward) tubercles large and more pronounced on dorsum of head from internasal to occipital region than on any other regions of head. Posterior to nares in middle of dorsum of head, tubercles in two ill-defined rows or loosely scattered from internasal to posterior region of interorbital area. Tubercles also present on anterior region of nape extending midway to dorsal fin, but smaller than on the head. Smaller tubercles also found on snout ventrally and lachrymal region laterally adjacent to anterior portion of upper lip. Smaller tubercles found below eye and posterior to lachrymal area; tubercles rarely found in subopercle

area and when present, small. Laterally, tubercles on scales one or two rows above and below lateral line but most pronounced above pelvic and anal fins to base of caudal fin and below caudal peduncle. Often 1-3 tubercles per scale on medial portion of exposed scale. Minute tubercles on pectoral fin (most pronounced on first six rays). In females, tubercles absent entirely or appearing on the head as minute prickles or buds and absent from predorsal area and pectoral and pelvic fins. Tubercles absent on dorsal, anal, and caudal fins in both sexes.

Color in Life

Body silvery with a silvery to dusky black lateral stripe, three scales in thickness from opercle to origin of dorsal fin, abruptly narrowing beneath dorsal fin, becoming diffuse and thinning to one scale in thickness as it extends to caudal-fin base. Gold stripe is sometimes visible dorsally to lateral stripe, extending from opercle to caudal fin. Black spot visible at anterior and posterior bases of dorsal fin. Dorsally, golden dashes scattered beneath dorsal fin and along dorsum and diffuse black stripe with scattered golden dashes extending from nape to caudal fin. Lateral-line pores outlined with a series of dashes from opercle to dorsal fin. Scales outlined in black with those dorsal to lateral line having interior of scales sometimes densely packed with melanophores from head to caudal fin; rarely without pigment. Edges of scales below lateral line outlined with black melanophores, scales' interior without pigment. Scales pigmented with melanophores along anal-fin base extending anterior to and surrounding vent. Preopercle, subopercle, and opercle silvery; preopercle and opercle often with scattered melanophores. Diffuse black stripe extending from snout to orbit; scattered golden flecks occasionally on head. Iris golden and pupil black. Laterally, upper lip with scattered melanophores, sometimes densely pigmented; upper lip may be without pigment. Lower lip with fewer melanophores than upper lip; maxillary barbel with or without melanophores. Dorsal-fin rays outlined with melanophores extending almost to fin tip. First pectoral-fin ray outlined with melanophores; pelvic- and anal-fin rays without pigment; all caudal-fin rays outlined with melanophores, extending to fin tip. Scattered gold flecks occasionally appearing on rays of all fins. Anterior rays and interradial membranes of dorsal fin tinged light yellow; interradial membranes faintly pigmented, but not densely pigmented as in some *Cyprinella* species. Pectoral-, pelvic-, and anal-fin interradial membranes without pigment; basal 2/3 of caudal-fin interradial membranes tinged light yellow-golden or orange.

Historical Descriptions of Color Patterns in Live Specimens

Specimens collected on August 1, 1976, from Black River at NC 411, Sampson County, North Carolina, Cape Fear River basin (TU 101097), were observed by F.C. Rohde (letters to R.E. Jenkins, August 10, 1976, and the week of August 20th, 1976). All dorsal-fin rays orange from base outward with all interradial membranes blackish, primarily posteriorly; edges clear. Anal-fin rays orange on posterior half with interradial membranes blackish. Caudal-fin rays orange with a light area at fin base with rays edged with black. Pectoral-fin rays clear, except for 1st ray edged with black; pelvic fins clear. Kodachrome© slide images (n=4, specimens ca. 60–65 mm TL) additionally showed lateral body as bright steely (silvery) blue; and dorsal rays outlined with melanophores.

Specimens collected on August 7, 1976, from the Lynches River at mouth of Swift Creek, Chesterfield-Kershaw counties, South Carolina, Yadkin-Pee Dee River basin, were observed by

D.G. Cloutman and L.L. Olmsted (letters from D.G. Cloutman to R.E. Jenkins, August 17 and 27, 1976). The specimens (NCSM 62135, n=3, specimens 52–70 mm TL) had dorsal and caudal fins light yellow and translucent. On May 7, 1977, male specimens from three sites on Lynches River, Chesterfield-Kershaw counties, observed to have nuptial tubercles; dorsal and caudal fins orange; and posterior margins of dorsal, caudal, anal, and pelvic fins milky white (letter from D.G. Cloutman to R.E. Jenkins, May 18, 1977).

Specimens collected on September 14, 1983, from Great Coharie at NC 903, Sampson County, North Carolina, Cape Fear River basin (UT 44.3086), were observed by J.R. Shute and P.W. Shute (letter from J.R. Shute to R.E. Jenkins, September 20, 1983). Specimens exhibited greenish-gold cheeks and opercles; sides faintly metallic blue-green; dorsal and caudal fins pale yellow; and tips of dorsal and anal fins whitish.

Color in Alcohol

Lateral stripe darkest from posterior base of dorsal fin to caudal peduncle, ca. 1 scale thick, extending to near base of caudal fin; diffuse and dusky, ca. 2–3 scales thick, from opercle to posterior base of dorsal fin. Black spot visible at anterior and posterior bases of dorsal fin. Dorsally, diffuse dorsal stripe extending from nape to caudal fin with diffuse pigment encircling dorsal fin. Dorsal stripe more distinct in specimens from Lumber River subbasin than Pee Dee, Lynches, and Upper Cape Fear River subbasins; and stripe absent in darkly pigmented specimens from Black River subbasin. Lateral-line pores outlined with a series of dashes from opercle to dorsal fin. Scales outlined with melanophores with outlines darker above than below lateral line; scales above lateral line and lateral stripe often densely filled with melanophores. Scales along anal-fin base pigmented with melanophores. Preopercle either unpigmented or sparsely scattered with melanophores; pigmentation of proximal region of opercle variable. Upper and lower lip pigmentation variable from unpigmented, to lightly-, to well-pigmented with melanophores. Ventrally, lower lip and chin pigmentation variable. Maxillary barbel pigmentation also variable: unpigmented (n=18 specimens), pigment concentrated at its base (n=13), pigment extending from base to tip of barbel (n=8), or maxillary barbel too small to discern any vestiges of pigment (n=11). Breast unpigmented in majority of specimens or very sparsely to sparsely pigmented with widely scattered melanophores. Pigmentation of belly scales variable from devoid of pigment to well-pigmented; melanophores appear to be near the edge but pigment actually closer to scale center rather than along its edge. Ventral caudal peduncle variously pigmented, but not concentrated as a distinct straight line: without pigment (n=6), sparsely pigmented (n=14), moderately pigmented (n=16), and well-pigmented (n=12).

Across the two river basins, outlines of dorsal-fin rays variable from faintly outlined to well-outlined with melanophores. Dorsal-fin interradial membrane pigmentation variable from being without pigment to being well-pigmented with melanophores. Outlines of pectoral-fin rays variable from rays devoid of pigment to all rays outlined with melanophores. In 44 of 50 specimens examined, pelvic-fin rays were without pigment, the remaining 6 specimens had most to all rays with scattered melanophores or only the 1st ray had scattered melanophores (n=3). Pectoral- and pelvic-fins' interradial membranes without pigment. Outlines of anal-fin rays in majority of specimens (n=36) were devoid of pigment; other 14 specimens with faintly to well-pigmented rays. Anal-fin interradial membranes without pigment (n=46) or faintly- to well-pigmented (n=14).

Caudal-fin rays faintly to well-outlined with melanophores. Pigmentation of caudal-fin interradial membranes also variable, ranging from an absence of pigment, to faintly pigmented, to well-pigmented; sometimes densely outlined with melanophores.

Distribution

Cyprinella leptocheilus is endemic to Sand Hills and upper Coastal Plain streams in North Carolina and South Carolina (Figure 2; Table 4 in Supplemental Meristic and Morphometric Files). In North Carolina, it is found in the upper Coastal Plain streams of the Cape Fear River basin (Beaver Creek in Cumberland County; Little River in Cumberland, Hoke, and Moore counties; Rockfish Creek in Hoke County; and the South and Black rivers and some of their tributaries in Bladen and Sampson counties); and in the Yadkin-Pee Dee River basin (Drowning Creek, Shoe Heel Creek, and Lumber River in Hoke, Columbus, Robeson, and Scotland counties, and Jones Creek in Anson County) (Tracy et al. 2020; Tracy et al. 2023a; Tracy et al. 2023b; Tracy et al. 2024; this publication)**.** There are no records of the species in the Cape Fear River downstream from Lock and Dam No. 3, located approximately 27 km south of Fayetteville (Figure 2).

Evermann (1916) reported *Hybopsis kentuckiensis* (historically often used as a synonym of Bluehead Chub, *Nocomis leptocephalus*) as common in the upper Lumber River subbasin, near Pinebluff, North Carolina. However, specimens from his trip could not be located or specimens were not vouchered to support his claim. During the NCWRC's 1962 Lumber River basinwide survey, no *N. leptocephalus* were encountered (Louder 1962; Starnes and Hogue 2011). Tracy et al. (2020) presumed *N. leptocephalus* populations in the Sand Hills region of this basin were the result of an introduction(s). It is possible that Evermann observed *C. leptocheilus*, because it has been commonly collected from the Lumber River downstream from Pinebluff.

Three NCWRC records from the 1960s whose specimens were not vouchered are now recognized as being valid collections of *Cyprinella leptocheilus* based upon recent collections from these or nearby streams:

1. Lumber River subbasin—two specimens were reported as *C. labrosa* from the Lumber River at Fair Bluff, NC 904, Columbus-Robeson counties, NC, June 29, 1961, Station 15J-2 (Louder 1962). This site was re-surveyed by NCWRC staff in 2014 and four specimens of *C. leptocheilus* were collected (1 lot of 2 specimens were vouchered, NCSM 76646; the other collection of 2 specimens was not).
2. Yadkin-Pee Dee River basin—one specimen was collected and identified as *Hybopsis* n. sp. from Hitchcock Creek at Cordova, Richmond County, NC, June 12, 1961, Station 18H-2 (Tatum et al. 1963). Since then, between 1985 and 1996, 14 specimens have been collected from nearby Jones Creek, which is downstream and on the west side of the Pee Dee River (NCSM 62143, NCSM 62145, and NCSM 62144).
3. Cape Fear River basin—one specimen was collected and identified as *Paranotropis volucellus* from Little Coharrie Creek, SR 1240, Sampson County, NC, June 27, 1962, Station 12H-4 (Louder 1963). Another specimen identified as *P. volucellus*, collected on June 12, 1962 from Rockfish Creek was re-identified as *C. leptocheilus* (NCSM 55864). This site on Little Coharrie Creek was re-surveyed in 2012 by NCWRC staff and they collected eight specimens of *C. leptocheilus*.

In South Carolina, *Cyprinella leptocheilus* is found exclusively in in the Yadkin-Pee Dee River basin (Little Pee Dee River in Marion County; Lumber River in Horry and Marion counties; Lynches River in Chesterfield, Darlington, Florence, Kershaw, Lee, and Sumter counties; Black Creek in Florence county; and Pee Dee River in Chesterfield, Darlington, and Marlboro counties (Rohde et al. 2009; Kevin Kubach and Mark Scott, SCDNR, personal communication).

Habitat

Cyprinella leptocheilus prefers shallow pool areas of medium-sized, from clear to turbid to tannin-stained to dark blackwater, warm water creeks and rivers with moderate velocity, over clean, sandy and gravelly substrates. It often schools near stumps or other cover but can also be found mid-channel. In the Lynches River, fish have been taken over gravel-bottom riffles and more commonly, in sand-bottom runs (Menhinick 1997; Rohde et al. 2009). In the Lumber and Black rivers, schools were observed moving along the edges of sandbars, also in sand-bottomed runs (B.K. Jones, personal communication). In the Lower Little River (Cape Fear River basin) in late July to mid-August 2021, *C. leptocheilus* were found in low specific conductance waters, 25–50 µS/cm (Kyle Rachels, NCWRC, personal communication).

D.G. Cloutman observed *Cyprinella leptocheilus* in the Lynches River in moderately swift current, mid-stream, over a sandy substrate at a depth of 1–3 ft; and associated with *C. nivea*, *C. pyrrhomelas*, and *Notropis scepticus* (letter from D.G. Cloutman to R.E. Jenkins, December 12, 1976). In other collections over the years from the Lynches River, *C. leptocheilus* has been collected alongside *Alburnops petersoni*, *C. analostana*, *Hybognathus regius*, *Miniellus procne*, *N. leptocephalus*, *Notemigonus crysoleucas*, *Notropis amoenus*, and *Pteronotropis cummingsae*. In the Black River it has been found alongside *A. petersoni*, C. *nivea*, *H. regius*, and *N. amoenus*.

Life History and Ecology

Little is known of the life history and ecology of *Cyprinella leptocheilus*. Based upon nuptial coloration, it probably spawns from mid- to late summer (Rohde et al. 2009). Stomach contents of fish from the Lynches River in May 1996 contained primarily dipteran larvae; mayfly and stonefly nymphs and caddisfly larvae were of minor importance. Maximum longevity is estimated to be four years (Rohde et al. 2009).

Conservation Status

In North Carolina and South Carolina, *Cyprinella leptocheilus*, Siouan Thinlip Chub, (listed as "Thinlip" chub, *Cyprinella* sp. cf. *zanema* and Thinlip chub, *Cyprinella sp*. 1, respectively) is ranked by NatureServe as S2-Imperiled, meaning the species is at high risk of extirpation in the jurisdiction due to restricted range, few populations or occurrences, steep declines, severe threats, or other factors (https://explorer.natureserve.org/AboutTheData/DataTypes/ConservationStatusCategories and https://explorer.natureserve.org/Taxon/ELEMENT_GLOBAL.2.100314/Cyprinella_sp_1, accessed 01/04/2023). In both states the species is considered a Species of Greatest Conservation Need (NCWRC 2015; SCDNR 2015; NCWRC 2021). In North Carolina the species is listed as

state Special Concern (NCWRC 2021; NCNHP 2022), but it is not listed in South Carolina (SCDNR 2015; https://schtportal.dnr.sc.gov/portal/apps/sites/#/natural-heritage-program, accessed 01/04/2023).

In North Carolina, a species must be officially described and given a scientific name before its imperilment status can be uplisted. *Cyprinella leptocheilus* historically, prior to 2010, occupied 28 HUC 12s that encompassed an area of 2281.2km^2 (Figure 21). Currently, from 2010 to present, it occupies 19 HUC 12s that encompass an area of 1491.4 km^2. Thus, *C. leptocheilus* only occupies 65% of its historical range in 19 HUC 12s, having been extirpated in 35% of its historical range. With a known range of < 5000 km^2, *C. leptocheilus* meets the designated criteria for Geographic Range under the North Carolina Species Assessment protocol (NCWRC 2020) to be uplisted to state Threatened. In South Carolina consideration should be given to list the species as imperiled.

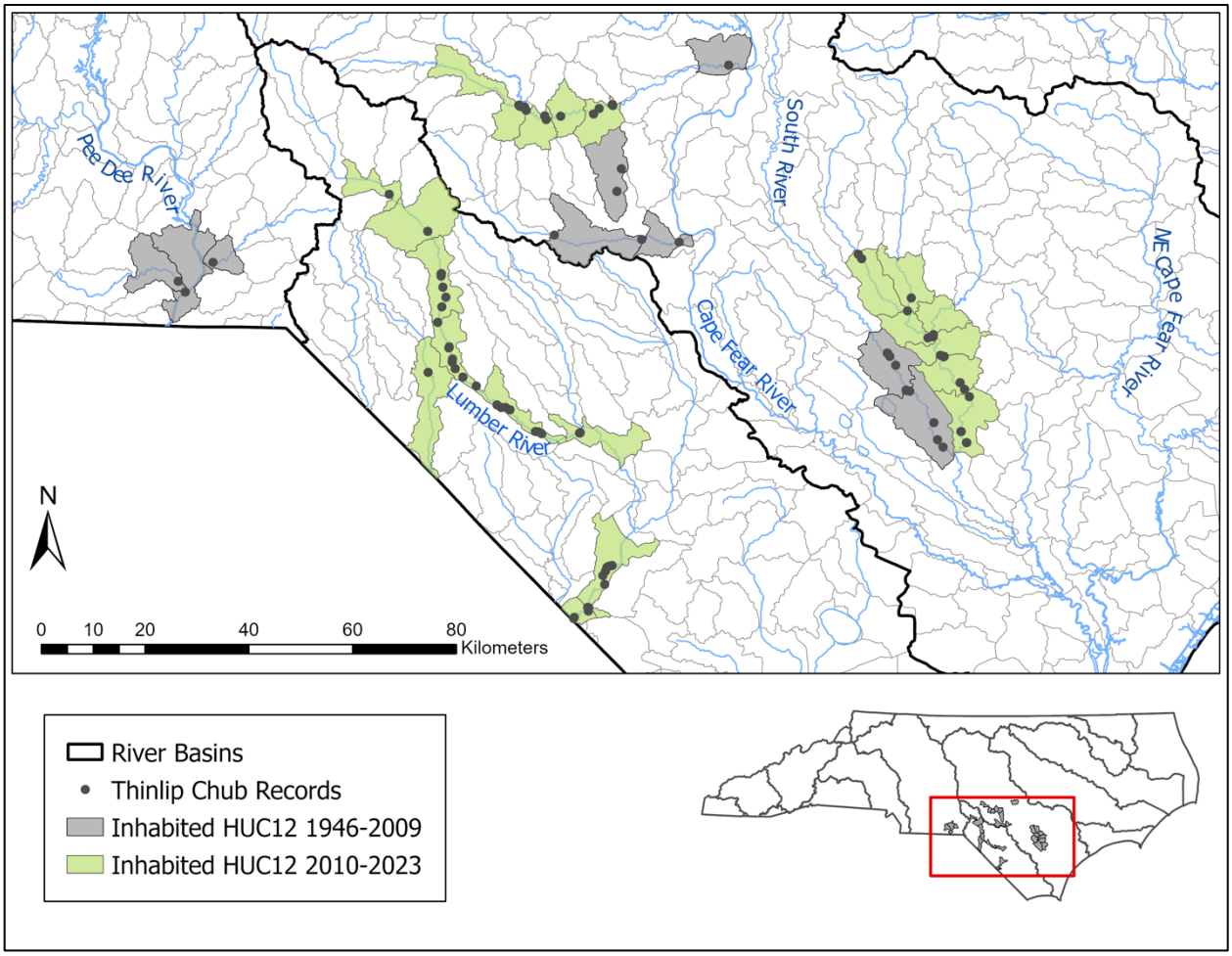

Figure 21. Occupancy of *Cyprinella leptocheilus* in North Carolina as denoted by inhabited HUC12s.

Threats to *C. leptocheilus* include habitat fragmentation and invasive species. The Black River/South River subbasin population has been geographically and genetically isolated from the Upper Cape Fear River subbasin population by a series of three Army Corps of Engineers locks and dams on the mainstem of the Cape Fear River that were built between 1915 and 1935 (https://www.saw.usace.army.mil/Locations/District-Lakes-and-Dams/Cape-Fear-Locks-and-

Dams/, accessed 01/10/2024; Figure 22). The truncated distributions, resulting in limiting genetic exchange between and intermingling of the two populations are also hampered by an abundance of three piscivorous, invasive catfish species: *Ictalurus punctatus*, *I. furcatus*, and *Pylodictis olivaris* that are found throughout these two river systems (Rachels and Fisk 2021a, 2021b; Rachels 2021).

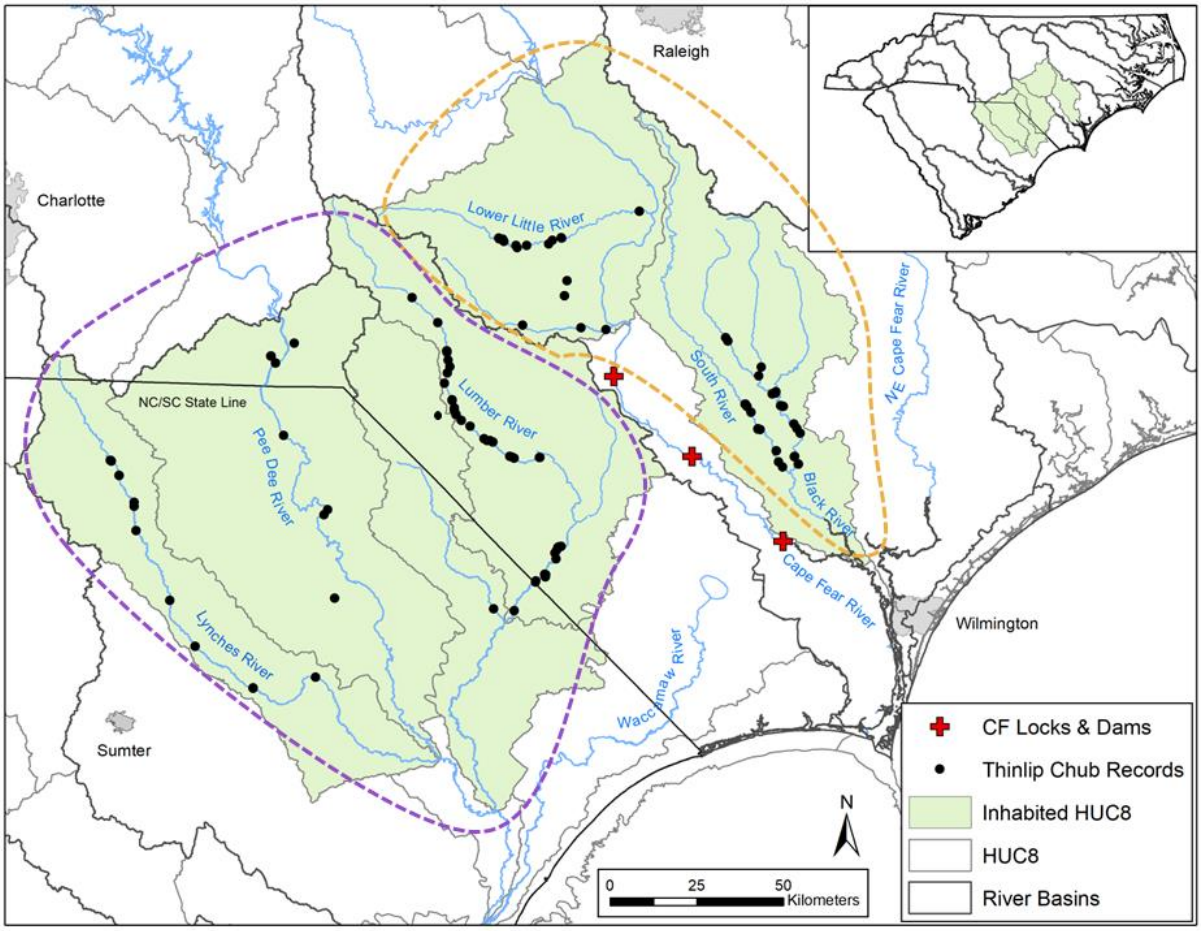

Figure 22. Population fragmentation and delineation of conservation units for two clades of *Cyprinella leptocheilus*, Siouan Thinlip Chub. The purple and yellow dashed lines delineate the Yadkin-Pee Dee River and Cape Fear River basin clades, respectively.

Furthermore, the Yadkin-Pee Dee River basin clade, including the Pee Dee River and Lynches River subbasins populations, and the Cape Fear River basin clade, including the Black River/South River and Upper Cape Fear River subbasin populations, are geographically isolated from one another (Figure 22). Given the divergence between the Cape Fear River and Yadkin-Pee Dee River basins populations, it is recommended that *C. leptocheilus* receive protection as two evolutionarily significant units as similarly proposed for two other endemic Atlantic slope southeastern species: *Etheostoma collis and E. mariae* (Krabbenhoft et al. 2008; Oswald et al. 2009).

Etymology

Cyprinella leptocheilus – *leptocheilus* — *leptós* (Greek λεπτός), fine or thin; *cheilus*, from *cheílos* (Greek χεῖλος), lip—a noun, thin lip. The species epithet was proposed by Drs. Robert E. Jenkins and Ernest A. Lachner in the early 1970s, and we concur with this name.

The common name, Siouan Thinlip Chub (pronounced Soo unn), honors the Carolina Siouan Language Territory of Cheraw, Chicora, Waccamaw, and Pee Dee First Peoples who originally inhabited the region in North Carolina and South Carolina where the species is found today. The adoption of this common name follows that of the Santee Chub, which was derived from the Santee Tribe, a historical tribe of Siouan language speakers from South Carolina (https://bit.ly/33LVLD5; Tracy 2022).

Intraspecific Variation within *Cyprinella leptocheilus*

Data for nine meristic variables for the two populations (clades) of *Cyprinella leptocheilus* are summarized in Table 10. There were no differences between the two basins regarding the nine meristic variables. Additionally, the position of the dorsal-fin origin relative to that of the pelvic-fin origin was not different between the two basins (in Cape Fear River basin mode 1.3 scales, range 0.5–2.5 scales; and in Yadkin-Pee Dee River basin mode 1.5 scales, range 0.0–2.5 scales; Table 10). Data for 25 landmark-based linear morphometric variables partitioned into the Cape Fear River and the Yadkin-Pee Dee River basins are summarized in Table 11. There were no differences between the two basins regarding the 25 landmark-based linear morphometric variables.

Table 10. Meristics for the Cape Fear River basin (n=20) and Yadkin-Pee Dee River basin (n=30) clades of *Cyprinella leptocheilus*.

Basin Clades	**Cape Fear River**		**Yadkin-Pee Dee River**	
Meristics	**Range**	**Mode**	**Range**	**Mode**
Lateral-Line Scales	38-41	40	38-42	40
Circumferential Scales	24-26	26	24-26	26
Predorsal Scales	16-23	17	16-20	17
Circumcaudal-Peduncle Scales	12	12	12-14	12
Dorsal-Fin Rays	8-9	8	8	8
Pectoral-Fin Rays	14-16	15	13-16	15
Pelvic-Fin Rays	7-9	8	7-8	8
Anal-Fin Rays	8	8	7-9	8
Caudal-Fin Rays	19	19	19	19
Dorsal-Fin Origin (Position)	0.5-2.5	1.5	0.0-2.5	1.5

There seemed to be no consistent differences in color patterns amongst the subbasins, although some specimens from the Lumber and Black River subbasins were more darkly pigmented than those from within their subbasins or from other subbasins. Recently preserved specimens from the Lumber River subbasin exhibited upper and lower lips and maxillary barbels that were well pigmented with melanophores, whereas those from the Black River subbasin had upper and lower lips that were devoid of pigment, or the lower lip had scattered melanophores and pigmentation of the maxillary barbels was confined to their bases.

Table 11. Landmark-based linear morphometric data expressed as percentage of Standard Length for the Cape Fear River basin (n=20) and Yadkin-Pee Dee basin (n=30) clades of *Cyprinella leptocheilus*; SD=standard deviation.

Basin Clades	**Cape Fear River**		**Yadkin-Pee Dee River**	
Landmark-based Linear Morphometrics	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Head Depth	10.5-12.5	11.3 ± 0.5	10.6-14.2	11.7 ± 0.8
Head Length	21.4-24.6	23.1 ± 0.9	21.8-25.6	23.5 ± 1.2
Snout Length	6.0-8.4	6.9 ± 0.6	5.6-8.6	7.1 ± 0.8
Snout to Top of Gill Slit	20.0-22.4	21.2 ± 0.7	19.8-23.8	21.6 ± 1.0
Predorsal Length	48.4-53.2	51.1 ± 1.1	49.3-54.8	52.2 ± 1.3
Orbit Length	5.8-6.9	6.3 ± 0.3	5.3-7.1	6.2 ± 0.4
Dorsal-Fin Base Length	11.6-15.3	13.3 ± 1.0	11.8-15.4	13.4 ± 0.9
Dorsal-Fin Origin to Pectoral-Fin Origin	28.5-33.3	30.7 ± 1.3	29.3-34.7	31.7 ± 1.4
Dorsal-Fin Origin to Pelvic-Fin Origin	14.5-20.6	17.5 ± 1.5	16.9-21.3	19.5 ± 1.2
Dorsal-Fin Origin to Anal-Fin Origin	22.1-26.5	24.2 ± 1.1	21.4-26.9	24.7 ± 1.2
Pectoral-Fin Origin to Pelvic-Fin Origin	23.2-27.2	25.1 ± 1.1	20.9-29.4	24.9 ± 1.7
Pelvic-Fin Origin to Anal-Fin Origin	17.3-20.0	18.7 ± 0.8	14.7-19.9	18.2 ± 0.9
Anal-Fin Base Length	10.8-14.7	12.8 ± 1.1	10.9-14.6	12.4 ± 0.8
Caudal Peduncle Depth	7.8-9.6	8.8 ± 0.4	8.3-10.1	9.2 ± 0.5
Interorbital Width	7.3-9.6	8.2 ± 0.5	7.6-9.7	8.7 ± 0.5
Pectoral-Fin Base Length	2.7-4.1	3.5 ± 0.4	2.8-4.5	3.6 ± 0.4
Pectoral-Fin Length	13.6-18.2	15.5 ± 1.1	13.2-18.2	15.8 ± 1.4
Pelvic-Fin Base Length	2.8-4.4	3.5 ± 0.5	3.1-4.7	3.8 ± 0.4
Pelvic-Fin Length	10.6-13.8	12.6 ± 0.9	10.7-15.1	13.1 ± 1.1
Upper Jaw Length	6.3-8.4	7.2 ± 0.5	6.2-8.6	7.3 ± 0.6
Upper Lip Width at Midpoint	0.3-0.7	0.5 ± 0.1	0.3-0.8	0.5 ± 0.1
Upper Lip Width at Midline	0.5-0.8	0.6 ± 0.1	0.4-1.0	0.7 ± 0.2
Anterior of Upper Lip Thickness	0.3-0.7	0.4 ± 0.1	0.4-0.7	0.5 ± 0.1
Mouth Gape Width	5.5-7.5	6.6 ± 0.6	5.5-7.9	6.6 ± 0.7
Maxillary Barbel Length	0.3-1.3	0.8 ± 0.2	0.3-1.5	0.8 ± 0.4

Our phylogenetic analyses supports the designation of *C. leptocheilus* as a distinct species (Figure 17; Supplemental Genetics Files). The genetic differences noted between the Yadkin-Pee Dee River and Cape Fear River basins clades may suggest the presence of additional species structure as was noted in other studies (Schönhuth and Mayden 2010). However, this goes beyond the scope of this species description. Given the lack of morphological variation between the two populations, more in-depth genetic analyses with broader genomic coverage are required to make those distinctions.

Key to the Barbeled Species of *Cyprinella*

As mentioned previously, *Cyprinella leptocheilus* is confined to the Sand Hills and Coastal Plain regions of North Carolina and South Carolina (Figure 2), whereas *C. zanema* is found in the Piedmont and Eastern Blue Ridge Foothills of the Santee River basin (including the Broad, Catawba, Enoree, and Saluda River subbasins) in North Carolina and South Carolina (Figure 6), and *C. labrosa* is found in the Piedmont and Eastern Blue Ridge Foothills in the upper Yadkin-Pee Dee River basin in North Carolina and Virginia and in the Santee River basin (including the Broad, Catawba, and Saluda River subbasins) in North Carolina and South Carolina (Figure 5; Jenkins and Burkhead 1994; Rohde et al. 2009; Tracy et al. 2020; Tracy et al. 2024).

Because there is no overlap of distributions (sympatry) between *Cyprinella leptocheilus* and the other two species, their identification is relatively straight-forward (Tracy et al. 2024). The three species may be identified and separated by the dichotomous key below, which is a slight modification from Tracy et al. (2024):

1a. Dorsal-fin origin modally 1 scale (range: 0–2) posterior to pelvic-fin origin. Line drawn from the posterior corner of the upper lip, immediately anterior to the maxillary barbel, does not extend vertically posterior to the anterior edge of the bony orbit. Lateral-line scales modally less than 40 (range: 36–38). Small, dark blotches and cross hatching on back and side. White spot at anterior base of dorsal fin; minimal pigment below lateral line..... .....Thicklip Chub, *Cyprinella labrosa*

1b. Dorsal-fin origin modally 1.5–2.5 scales (range: 0–3) posterior to pelvic-fin origin. Line drawn from the posterior corner of the upper lip, immediately anterior to the maxillary barbel, extends vertically posterior to the anterior edge of the bony orbit. Lateral-line scales modally greater than or equal to 40 (range: 36–42). No dark blotches or cross hatching on back and side. White spot absent at anterior base of dorsal fin; well pigmented below lateral line.....2

2a. Dorsal-fin origin modally 2.5 scales (range: 1.5–3) posterior to pelvic-fin origin. Mean of upper lip width at midpoint 3.5% head length (range 1.8-5.1%). Mean of upper lip width at midline 4.1% head length (range 2.8-6.0%). Mean of maxillary barbel length 8.0% head length (range 4.0-15.3%). Distribution restricted to the Santee River basin ....Santee Chub, *Cyprinella zanema*

2b. Dorsal-fin origin modally 1.5 scales (range: 0–2.5) posterior to pelvic-fin origin. Mean of upper lip width at midpoint 2.1% head length (range 1.3-3.2%). Mean of upper lip width at midline 2.9% head length (range 1.7-4.4%). Mean of maxillary barbel length 3.5% head length (range 1.3-6.0%). Distribution restricted to the Cape Fear River and Yadkin-Pee Dee River basins.....Siouan Thinlip Chub, *Cyprinella leptocheilus*

CONCLUSIONS

Cyprinella leptocheilus, Siouan Thinlip Chub, is geographically, phenotypically, and genetically distinguishable from the two other closely related barbeled *Cyprinella*: *C. labrosa* and *C. zanema*. *Cyprinella leptocheilus* is confined to the Sand Hills and Coastal Plain regions of the Carolinas, whereas *C. labrosa* and *C. zanema* are more widely distributed in the Piedmont and Eastern Blue Ridge Foothills regions of the Santee (including the Broad, Catawba, Enoree, and Saluda River subbasins) and the upper Yadkin-Pee Dee River basins in the Carolinas and Virginia (Jenkins and Burkhead 1994; Rohde et al. 2009; Tracy et al. 2020; Tracy et al. 2024).

The morphological differences detected were related to the position of the dorsal-fin origin relative to the pelvic-fin origin; male tuberculation patterns and number of tubercles; thickness of the upper lip width at midpoint, thickness of upper lip width at midline, anterior of upper lip thickness, mouth gape width, and the length of the maxillary barbel.

A concatenated phylogeny consisting of two nuclear genes (Rag1 and Homeobox13) and two mitochondrial genes (COI and Cyt-b) exhibited good species-scale resolution of nodes with little sequence variation between members of the same species. *Cyprinella labrosa, C. zanema,* and *C. leptocheilus* form a monophyly with each species clustering within their own distinct clades. The divergence of *C. labrosa* from *C. zanema* and the divergence of *C. leptocheilus* from *C. zanema* have high bootstrap support. Within the *C. leptocheilus* clade, sequences from the

Yadkin-Pee Dee River basin form a clade distinct from that of the Cape Fear River basin. These two clades are geographically isolated from one another. Given the divergence between the Cape Fear River and Pee Dee River basins clades, it is recommended that *C. leptocheilus* receive protection as two evolutionarily significant units and in North Carolina be uplisted from state Special Concern to state Threatened. In South Carolina consideration should be given to list the species as imperiled.

Supplemental Meristic and Morphometric, and Supplemental Genetics data files may be found at: https://doi.org/10.5281/zenodo.13327793.

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