

Rediscovery and genetic confirmation of the Threeridge Mussel, *Amblema plicata* (Say, 1817) (Bivalvia, Unionidae), in the Choctawhatchee River, Florida, USA

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Abstract

Recent freshwater mussel research has resulted in rediscovery of several species presumed extinct. We report the rediscovery of *Amblema plicata* (Say, 1817) in 2019 from the Choctawhatchee River, Florida, USA. *Amblema plicata* has not been reported in the Choctawhatchee river basin since 1958, more than 61 years ago. This species was collected during the long-term monitoring of freshwater mussels in Florida streams. We provide genetic confirmation of our voucher identification using a DNA barcoding approach and discuss potential risks to *A. plicata* populations in the Choctawhatchee river basin.

Keywords

Biodiversity threats, distribution, DNA barcoding, freshwater, long-term monitoring, unionid

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Introduction

Freshwater mussels within the family Unionidae are one of the most imperiled faunal groups in the world, with over 65% of the 297 recognized North American species being of special concern, threatened, endangered, or extinct (Williams et al. 1993; Neves et al. 1997; Haag and Williams 2014). Historically, the southeastern United States had more freshwater mussel biodiversity than any other geographic region on the continent, but populations have experienced declines since the early 20th century (Neves et al. 1997). The highly imperiled status of unionid mussels reflects their sensitivity to changes in their environment but allows biologists to use them

as bioindicators of the overall health of freshwater ecosystems (Carlson et al. 2008; Grabarkiewicz and Davis 2008). Recent research and survey efforts have resulted in the rediscovery of several freshwater mussels that were presumed extinct (e.g., Campbell et al. 2008; Randleklev et al. 2012; Holcomb et al. 2015; Johnson et al. 2016; Pfeiffer et al. 2016) and the discovery of many previously unrecognized species (e.g., Johnson et al. 2018; Smith et al. 2018, 2019; Inoue et al. 2020).

The genus *Amblema* (Rafinesque, 1820) is endemic to North America and contains three currently recognized species: *A. elliottii* (Lea, 1856), *A. neislerii* (Lea,

1858), and *A. plicata* (Say, 1817) (Williams et al. 2017). The Threeeridge, *A. plicata*, was first described as *Unio plicata* Say, 1817. The type locality for *A. plicata* is in the Ohio River (Williams et al. 2014). There are at least 15 synonyms of *A. plicata* (Williams et al. 2008), the most notable being *A. perplicata* (Conrad, 1841), which has been assigned to populations of *Amblema* from the lower Mississippi river basin (Mulvey et al. 1997). However, Mulvey et al. (1997) reported *A. plicata* and *A. perplicata* to be conspecific based on genetic similarities recovered from the 16s rRNA gene, which was later confirmed by Elderkin et al. (2007). *Amblema elliottii* was considered a synonym of *A. plicata* prior to the genetic analysis by Mulvey et al. (1997), which demonstrated that *A. elliottii* is more closely related to *A. neislerii*. The Coosa Fiveridge, *A. elliottii*, is endemic to the Mobile river basin in Alabama and Georgia. The Fat Threeeridge, *A. neislerii*, is federally endangered and endemic to the Apalachicola and Flint river basins in Florida and Georgia.

Amblema plicata is currently recognized as stable throughout most of its range, but its conservation status in some states is undetermined (Williams et al. 2014). *Amblema plicata* has a widespread historical distribution in North America. In its northern distribution, *A. plicata* is present from the St. Lawrence River drainage in New York throughout the entire Mississippi river basin to Louisiana (Mulvey et al. 1997; Williams et al. 2008). On the Gulf Coast, it occurs from the San Antonio and Guadalupe rivers in Texas (Howells et al. 1996) east to the Choctawhatchee River in Florida (Blalock-Herod et al. 2005). The Choctawhatchee River continues into Alabama, but *A. plicata* is not known to occur in this reach (Williams et al. 2008, 2014). Within the state of Florida, *A. plicata* is known from the Escambia and Choctawhatchee River drainages. The population in the Escambia River is currently considered stable (Williams et al. 2014), while the population in the Choctawhatchee River was thought to be extirpated (Blalock-Herod et al. 2005) prior to locating a live individual in 2019 during one of our recent surveys. Here, we report the collection of *A. plicata* in the Choctawhatchee River in 2019, the first occurrence in the basin since 1958 (Butler 1989; Blalock-Herod et al. 2005), and provide the first genetic confirmation of the Choctawhatchee River population.

Methods

The Florida Fish and Wildlife Conservation Commission Freshwater Mussel Conservation Program (FMCP) routinely monitors freshwater mussel populations in Florida's freshwater streams and rivers to provide ecologically significant findings to conservation managers. Both quantitative and qualitative sampling procedures were performed during these long-term monitoring efforts. In brief, quantitative sampling procedures included excavation of five 0.5-m² quadrats along three 10-m transects placed perpendicular to flow. Qualitative sampling procedures included visual and tactile survey methods for

a minimum of 1-person hour. Sites were selected randomly using point layers generated in ArcMap v. 10.6 (Environmental Systems Research Institute, Inc., Redlands, CA, USA). Any vouchered specimens were placed on ice for transit and transferred to 95% non-denatured ethanol.

All surveys conducted by the FMCP in the Choctawhatchee river basin were compiled to develop a conservation status assessment map following the protocol created by the Georgia Department of Natural Resources (Georgia DNR 2020). The map was developed within ArcMap v. 10.6 (ESRI 2017). Hydrologic Unit Codes (HUC) 12 are shaded in relation to time, allowing the assessment of spatial and temporal changes. Surveys by FMCP were conducted from 18 June 2014 to 29 August 2019.

A DNA barcoding approach (Hebert et al. 2003) was conducted to verify our morphology-based identification. Genomic DNA was extracted from a small (≤ 5.0 mm²) piece of mantle tissue using the Qiagen PureGene DNA extraction kit following the standard extraction protocol (Qiagen, Hilden, Germany). The protein-coding mitochondrial DNA gene cytochrome c oxidase subunit I (COI) was amplified using previously published PCR primers (Campbell et al. 2005) and thermal cycling conditions (Johnson et al. 2018) in a 25 μ L mixture containing the following: MyTaqTM Red Mix (12.5 μ L; Meridian Bioscience, Memphis, TN, USA), primers (1.0 μ L each at 10 mM), DNA template (1.0 μ L at 100 ng μ L⁻¹), and 9.5 μ L molecular grade H₂O. PCR product was purified with Exonuclease I and Shrimp Alkaline Phosphatase (Promega Corp.) and bi-directionally sequenced on an ABI 3730 at Molecular Cloning Laboratories (McLAB, South San Francisco, CA, USA). Geneious v. 10.2.3 (Kearse et al. 2012) was used to assemble forward and reverse contigs before evaluating chromatograms and forming the consensus sequence, which was then translated into amino acid sequences to ensure the absence of gaps and stop codons using Mesquite v. 3.61 (Maddison and Maddison 2019). The consensus sequence was compared to published sequences on GenBank to determine species-level identification using the NCBI standard nucleotide BLASTN tool (Zhang et al. 2000) with default megablast and nt database options on 20 April 2021. A maximum likelihood analysis (ML) was performed to visualize the relationships of the newly generated sequence with the top 52 matches from the BLASTN search. The following nucleotide substitution models were determined for each codon using ModelFinder (Kalyaanamoorthy et al. 2017): codon 1 – TN+F+I; codon 2 – F81+F; and codon 3 – HKY+F. ML analyses were performed using IQ-TREE v. 2.0.6 (Nguyen et al. 2015) with 10 independent runs of initial tree search and 1000 ultrafast bootstrap replicates to assess nodal support (Hoang et al. 2018). The resulting phylogeny was presented as a 50% majority-rule consensus tree with *Villosa villosa* (B.H. Wright, 1898) (GenBank: MK044978.1) designated as the outgroup.

Repositories. UF: University of Florida Museum of Natural History, Invertebrate Zoology Collection. NCSM: North Carolina State Museum of Natural Sciences, Mollusks Collection.

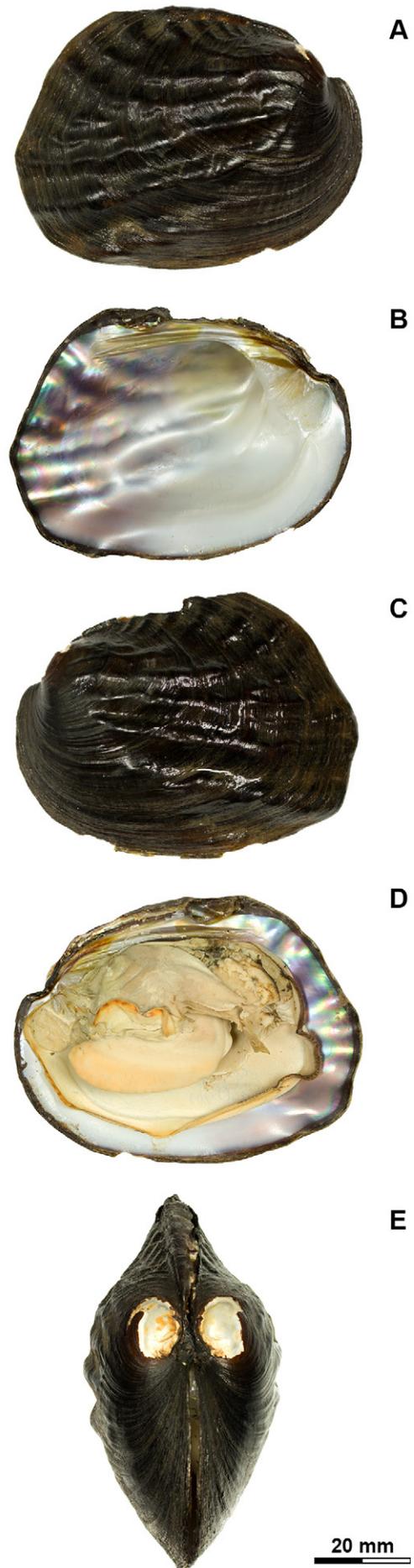
Results

New records. UNITED STATES OF AMERICA – Florida • Walton County, Choctawhatchee River; 30.4887, –85.8653; 5.VI.2019; L.N. Patterson, S.R. Geda, S.N. Casebolt leg.; 1 spec., 73 mm, 95% non-denatured ethanol, UF 439505.

From 2014 to 2019, the FMCP surveyed 110 sites in the Choctawhatchee river basin in Florida, totaling 117 person hours. A total of 16468 individual mussels representing 22 species were collected (Supplemental Table S1). Only one individual of *A. plicata* was found during these efforts (Fig. 1). The individual was collected on 5 June 2019 along the right descending bank of the mainstem Choctawhatchee River, just upstream from the mouth of an unnamed slough. The site was approximately 7.8 river kilometers (rkm) upstream of the Highway 20 bridge crossing and 1.7 rkm downstream from the confluence of Boynton Cutoff and the Choctawhatchee River mainstem. The location of this collection site is denoted as a white filled circle in Figure 2. The individual was found approximately 2.5 m from the bank at a depth of approximately 1 m, burrowed in a mixture of silt, mud, and sand, about 1.5 m downstream of a flow-obstructing log.

The study site had an average depth of 0.5 m and bottom substrate comprised mostly of sand, mud, and wood. Water temperature and dissolved oxygen were 28.3 °C and 89.7%, while specific conductivity, salinity, and pH were 182.7 $\mu\text{S cm}^{-1}$, 0.08 parts per thousand, and 7.61, respectively. Other unionid mussels collected at the same site, identified using morphological characteristics of known species in this basin, included *Elliptio mcMichaeli* (Clench & Turner, 1956), *E. pullata* (Lea, 1856), *E. purpurella* (Lea, 1857), *Glebula rotundata* (Lamarck, 1819), *Lampsilis floridensis* (Lea, 1852), *Pleurobema strodeanum* (B.H. Wright, 1898), *Uniomereus columbensis* (Lea, 1857), *Villosa vibex* (Conrad, 1834), and *V. lienosa* (Conrad, 1834).

Current and historical occurrences of *A. plicata* in the Choctawhatchee river basin were reported during five surveys distributed across 3 of 46 HUCs (Fig. 2). The 3 HUCs are represented by one with recent records (≤ 25 years or 1994–2019), one with no occurrences in the past 26–50 years, and one with no occurrences in over 75 years (Fig. 2). No other sightings of *A. plicata* are reported for the remaining 43 HUCs (Fig. 2). Surveys



► **Figure 1.** *Amblema plicata*. UF 439505, length 73 mm. **A.** Right valve, external view. **B.** Left valve, internal view. **C.** Left valve, external view. **D.** Right valve with soft morphology, internal view. **E.** Inflatedness, dorsal view. Specimen photo credit: Ava M. Laszlo.

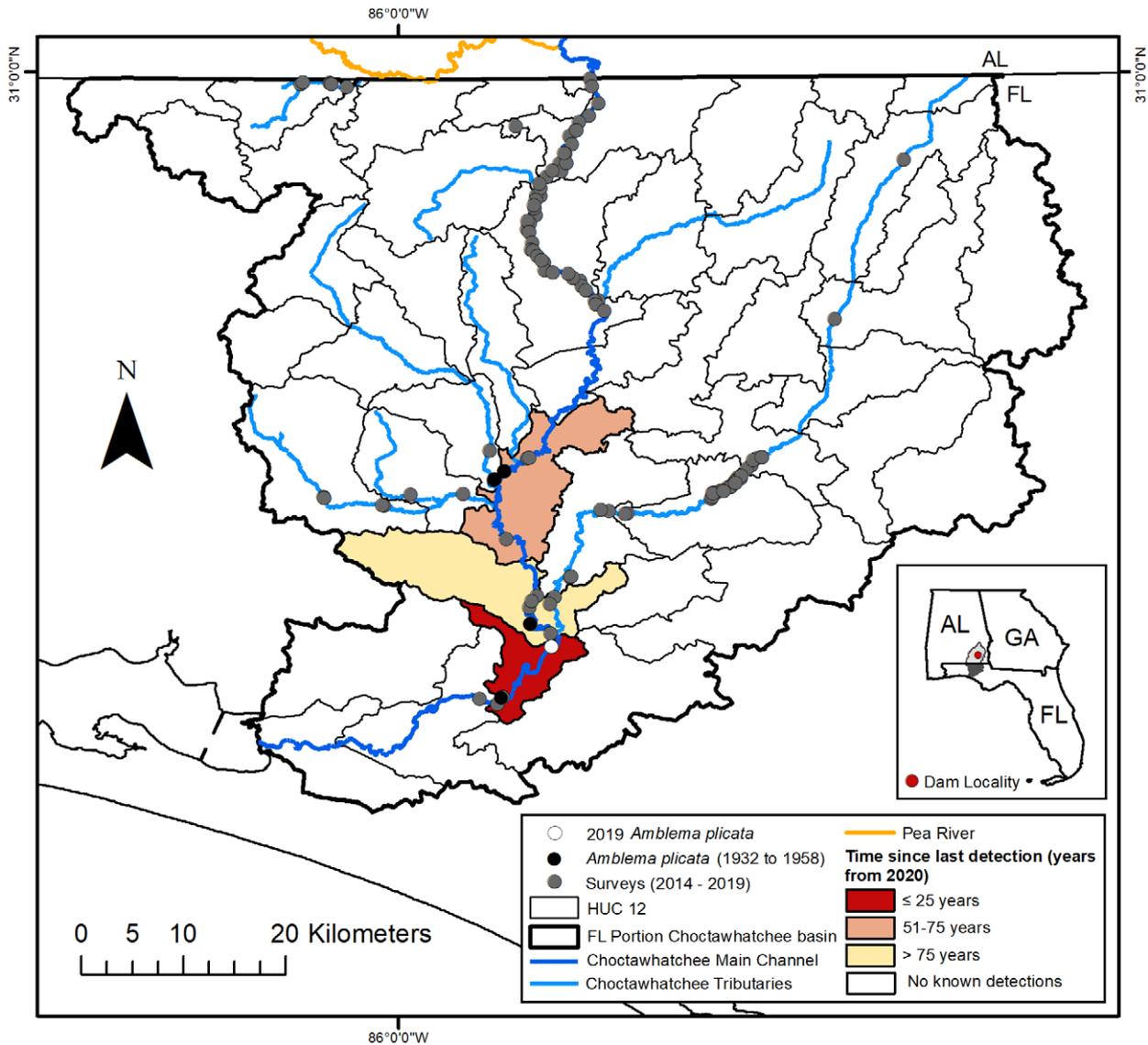


Figure 2. Conservation status map for *Amblema plicata*. Black or white circles denote presence and grey circles denote absence. Red dot on inset map indicates location of Elba Dam. Hydrological Unit Codes (HUC) 12-level are colored based upon when specimens were collected in that HUC. Map generated with ArcMap v. 10.6.

where an individual of *A. plicata* was not observed are denoted as grey filled circles in Figure 2.

Identification. The specimen was identified as *A. plicata* in the field using conchological characters (e.g., shape, width, external ornamentation) following Williams et al. (2014). The shell is quadrate with rounded posterior and anterior margins; has large, deep plications expressed as undulations along the posterior edge that obscure the posterior ridge; and has a broad umbo that is elevated slightly above the hinge line (Fig. 1). The shell also has corrugations that curve upward from the posterior ridge to the dorsal margin (Fig. 1A, C, E), not to be confused with the plications. The periostracum is transitioning to clothlike and dark brown, as typically seen in larger individuals, and the nacre is white to bluish white with a pink or purple tint towards the posterior (Fig. 1). The pseudo-cardinal teeth are large, thick, and triangular (Fig. 1B). There are also two long lateral teeth in the left valve (Fig.

1B) and one long lateral tooth in the right valve (Fig. 1D). *Amblema plicata* can resemble *Megaloniaias nervosa* (Rafinesque, 1820) and *A. neislerii*, but neither is known to occur in the Choctawhatchee river basin. In comparison, *M. nervosa* has more wrinkles, shallower plications, and a rougher umbo than *A. plicata*. *Amblema neislerii* is more inflated and has smaller plications than *A. plicata* (Williams et al. 2014).

Molecular analyses confirmed that the specimen collected from the Choctawhatchee River (UF 439505) is *A. plicata*. A 678-base-pair fragment of the COI gene was generated, submitted to GenBank (MT813471), and used for the BLASTN search summarized in Table 1. All of the top 50 BLASTN results represented a previously published sequence identified as *A. plicata* (Supplemental Table S2) except for one (GenBank: AY654991), which was listed as *A. elliottii* and represents the nearest-neighbor species with a pairwise sequence divergence of 1.83%. The ML phylogeny shows the query sequence

Table 1. Details for the top 10 BLASTN results producing significant alignments with the COI sequence generated using the specimen collected from the Choctawhatchee River. The BLASTN search was performed 20 April 2021.

Description	Drainage	Maximum score	Query coverage (%)	Sequence identity (%)
<i>Amblema plicata</i> (NC_050056.1)	Guadalupe River, Texas, USA	1225	100	99.26
<i>Amblema plicata</i> (MK044904.1)	Ohio River, Ohio, USA	1188	97	99.24
<i>Amblema plicata</i> (MK044903.1)	Ohio River, Ohio, USA	1182	97	99.09
<i>Amblema plicata</i> (EF033258.1)	Unavailable	1162	94	99.22
<i>Amblema plicata</i> (AF156512.1)	Grand River, Michigan, USA	1160	95	99.07
<i>Amblema plicata</i> (MH633633.1)	Colorado River, Texas, USA	1158	95	98.92
<i>Amblema plicata</i> (KT285618.1)	Sabine River, Texas, USA	1158	95	99.07
<i>Amblema plicata</i> (MH633632.1)	Colorado River, Texas, USA	1153	95	98.77
<i>Amblema plicata</i> (DQ648100.1)	Unavailable	1146	92	99.52
<i>Amblema plicata</i> (DQ648101.1)	Ohio River, Ohio, USA	1140	92	99.36

nested within a clade of *A. plicata* and sister to *A. elliottii* from the Mobile River (GenBank: AY654991.1) (Fig. 3).

Discussion

Amblema plicata was last reported in the Choctawhatchee river basin in 1958, more than 61 years prior to the collection reported here. Based on museum records, *A. plicata* was previously collected from four sites in the Choctawhatchee river basin, Florida: Oakey Bend in 1932 by D.B. Gillis (UF:3233), Yellow Bluff in 1933 by D.B. Gillis (UF:3236), Cowford Ferry in 1934 by L.M. Rushing (UF:3237), and a site surveyed by H.D. Athearn in 1958 (NCSM 113596; Butler 1989). The Oakey Bend, Yellow Bluff, and Athearn collections were approximately 6.3 rkm (2.9 linear km), 26.2 rkm (17.2 linear km), and 27.9 rkm (17.8 linear km) upstream of the FMCP collection, respectively. The Cowford Ferry collection was approximately 9.6 rkm (6.6 linear km) downstream of the FMCP collection. The locations of these museum records are denoted as black filled circles in Figure 2.

Prior attempts since 1959 to locate *A. plicata* at historical localities in the Choctawhatchee river basin have been unsuccessful. The United States Geological Survey surveyed the localities between 1998 and 2000 prior to reporting that the population may have been extirpated from the basin (Blalock-Herod et al. 2005). Other efforts to locate *A. plicata* in this reach of the Choctawhatchee River since 2000 were also unsuccessful. After collecting *A. plicata* from the 2019 locality, the FMCP used qualitative sampling procedures to thoroughly survey the historical localities, but no additional individuals of *A. plicata* were found. To date, *A. plicata* has been reported only in three adjacent sub-watersheds in the lower Choctawhatchee river basin, of which one has not had an *A. plicata* sighting in over 75 years (Fig. 2).

The BLASTN analysis of the COI gene sequence generated from the specimen we collected from the Choctawhatchee River confirmed our morphological identification of *A. plicata*. The top BLASTN result (GenBank: NC_050056.1) was *A. plicata* from the San Marcos River, Texas, a tributary of the Guadalupe River (Supplemental Table S2). Our ML phylogeny (Fig. 3) is congruent with BLASTN results and places

the Choctawhatchee specimen within a clade containing 49 other sequences identified as *A. plicata*. Our findings demonstrate sequence divergence at COI is sufficient to differentiate between *A. elliottii*, *A. neislerii*, and *A. plicata*. The phylogenetic relationships between the three *Amblema* species, however, is incongruent with the previous work by Mulvey et al. (1997), which showed *A. elliottii* and *A. neislerii* to be sister species based on the 16S rRNA gene. Additional taxon and genetic sampling are needed to further resolve the relationships among current recognized *Amblema* species.

A total of 25 unionid species have been reported from the Choctawhatchee river basin, of which six are federally listed and one is presumed extinct. Only 348 individual mussels representing four federally listed species have been sampled from 39 of the 110 sites surveyed in the Choctawhatchee river basin by the FMCP (Supplemental Table S1). Several factors can contribute to the decline in freshwater mussel diversity, but the most notable are habitat destruction, deterioration of water quality, organic pollution, toxic spills, and the introduction of invasive species (Williams et al. 1993; Brim Box and Mossa 1999; Haag 2012).

Favorable habitat conditions for *A. plicata* in the Choctawhatchee river basin are unknown (Williams et al. 2014), but here we present similarities in the substrate composition of each collection locality as it was surveyed in 2019. Substrates consisting primarily of sand, mud, and silt were recorded among the historical collection localities. The Oakey Bend and Athearn collection localities also had a prominent (20–40%) presence of large woody debris and detritus. For a comparison of favorable habitat conditions within Florida, *A. plicata* in the Escambia river basin is typically found in deep water with substrates containing a mixture of sand, clay, and fine gravel (Williams et al. 2014). Mussel populations within a drainage typically have clumped distributions because of restriction to microhabitats with stable substrate, low shear stress, and low sediment mobilization (Strayer 2008). These habitat characteristics enable the survival and persistence of these sensitive bivalves, which recover slowly from disturbance (Strayer 2008).

Freshwater mussel distribution is also directly impacted by changes in the distribution of native fishes.

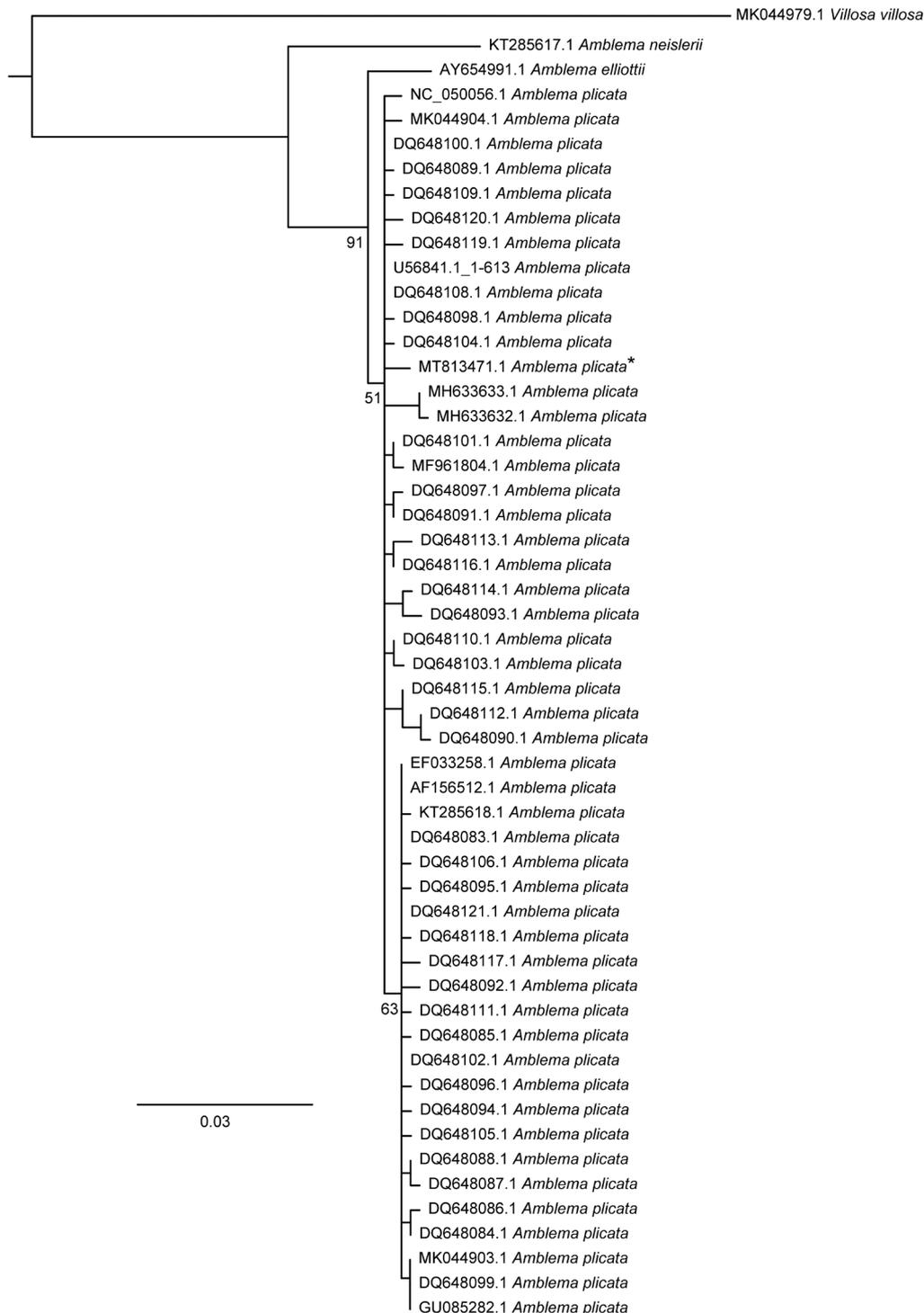


Figure 3. Maximum likelihood phylogeny showing the relationships of the newly generated sequence with the top 52 matches from the NCBI BLASTN search results performed on 20 April 2021. Bootstrap support values are provided for major clades and the query sequence (MT813471) is denoted with an asterisk (*).

Freshwater mussels exhibit complex life-history strategies involving a larval stage of development, known as a glochidium, which is an obligate ectoparasite of aquatic vertebrates, primarily fishes (Barnhart et al. 2008). In brief, glochidia encyst on the gills and/or fins of the host where they transform into juveniles over the period of several weeks before dropping off into the substrate (Haag 2012). A single mussel species may have an array of suitable host fishes or may be specialized to only

complete metamorphosis on a single host species (Johnson et al. 2016; McLeod et al. 2017). *Amblyma plicata* is considered a host generalist, known to parasitize at least 25 fishes from 10 families for the purpose of glochidial metamorphosis (Williams et al. 2014). Once transformed, juveniles of *A. plicata* continue to grow into adults, which can reach a length of 178 mm and live an average of 31 years (Haag 2012; Williams et al. 2014). The *A. plicata* collected in 2019 was only 73 mm in length, suggesting

that younger size and age classes might be present and recruitment has occurred recently (i.e., within the last 30 years) in the Choctawhatchee river basin. Parasitizing native fishes has enabled sedentary mussels to effectively disperse upstream or into other rivers and lakes (Haag 2012), but the dispersal of the fish could be impeded by impoundments. The Pea River, which occurs predominantly in the state of Alabama (Fig. 2) and is the largest tributary of the Choctawhatchee River, has the only impoundment in the Choctawhatchee river basin (Williams et al. 2008). The Elba Dam was built in 1903 and acts as a barrier for upstream fish and mussel migration (Williams et al. 2008). The location of the dam (31.3638, -86.0937) is denoted as a red dot in the inset map of Figure 2. The lack of upstream host fish migration may explain why *A. plicata* is not known from the reaches of the Choctawhatchee River in Alabama.

Status assessments of mussel communities, including abundance, distribution, and habitat usage, will provide a comparative baseline for future studies. Environmental monitoring, in terms of water quality and substrate composition, will aid in understanding changes in the mussel communities over time. Conservation focused land management and public awareness can help prevent habitat destruction, poor water quality, and pollution, all of which are notable causes for declines in mussel populations (Haag 2012). Given that biodiverse mussel assemblages are integral functional components of freshwater ecosystems (Howard and Cuffey 2006; Strayer 2008; Vaughn and Hoellein 2018), population and environmental monitoring, conservation focused land management practices, and public awareness represent a few of the necessities to ensure the persistence of freshwater mussel populations.

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Authors' Contributions

All authors played a role in the species identification. LNP and SRG conducted the survey, made the field identification, and took measurements. SRG generated the

map. NAJ conducted the genetic identification and analyses. All authors contributed to and approved the final manuscript.

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Supplemental Data

Supplemental Table S1. Data repository that includes surveys conducted by the Freshwater Mussel Conservation Program (FMCP) in the Choctawhatchee river basin.

Supplemental Table S2. The top 100 BLASTN results of previously published COI sequences from NCBI GenBank as of 20 April 2021.