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# Diagnosis and Distribution of Florida Sand Darter, Ammocrypta bifascia (Teleostei; Percidae), in the Flint River, Georgia

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# Diagnosis and Distribution of Florida Sand Darter, Ammocrypta bifascia (Teleostei; Percidae), in the Flint River, Georgia


## Abstract

*Abstract:* In 2013, we observed the presence of an undocumented *Ammocrypta* species in the lower Flint River, Georgia. The occurrence represents the first record of the genus in Georgia. Subsequent surveys at 24 sites, using seining or snorkeling, documented additional specimens from mainstem sites (n = 3) between Albany and Bainbridge and from Ichawaynochaway Creek (n = 5 sites), a large tributary to the Flint River. We used morphological and genetic data to identify specimens to species. Morphological examination included 23 morphometric and 8 meristic characters from fifteen specimens that were compared to specimens from Williams (1975). For genetic analyses, we targeted the mtDNA cytochrome c oxidase and nuclear ribosomal internal transcribed spacer 2 (ITS-2) genes and compared them to specimens from throughout the geographic range of *Ammocrypta*. Morphological analyses showed broad overlap in measurements and counts with *A. bifascia* (Florida Sand Darter). The mtDNA data also grouped our specimens with *A. bifascia* from the Choctawhatchee River, which is the type locality of *A. bifascia.* While nuclear data was monomorphic when compared to other *A. bifascia*, the mtDNA of Flint River *A. bifascia* shared a unique haplotype that was one or more substitutions apart from other *A. bifascia* haplotypes. Georgia specimens appear to be native, having a haplotype that is different from other *A. bifascia* and a distribution pattern that corresponds closely to other fishes. We observed *A. bifascia* from only a handful of scattered localities in the mainstem of the river and the lower portion of its largest tributary. Past surveys may have failed to detect the species due its limited distribution and the difficulty of collecting small benthic fishes in large river habitats.



## Keywords

cryptic biodiversity, Apalachicola drainage, Ichawaynochaway Creek, seining, snorkeling





## Cover Page Footnote

Initial records of A. bifascia in the Flint River were made during Paddle Georgia (June 2013) and Fall Float on the Flint (October 2014), events of the Georgia River Network, then organized by April Ingle, Joe Cook, Dana Skelton, and Gwyneth Moody. Field assistance was provided by Garrett Hopper, Nathan Herron, and the Aquatic Biology lab (The Jones Center at Ichauway), Deborah Weiler and Peter Dimmick (Georgia DNR), Kenneth Swift and Paddle Georgia participants. Matthew Rowe and Zachariah Abouhamdan (Georgia DNR) assisted with photo editing and map production, respectively. Stephen Golladay, Krista Capps and Mary Freeman are thanked for their advisement and Mary and Bud Freeman for their wisdom regarding Ichawaynochaway Creek fishes. We thank Sandra Bohn, Ashantye Williams, Shannon Julian and Meredith Bartron (USFWS) for sequencing and data curation along with the University Kansas Biodiversity Institute and the Florida Museum of Natural History for tissue samples, and the Georgia Museum of Natural History for specimen archival. Support for genetic analyses was provided by Mansfield University's Faculty Professional Development Committee Grant (GRM) and by The Environmental Resource Network, Inc. (T.E.R.N.), the official "Friends Group" of the Wildlife Conservation Section of the Wildlife Resources Division of Georgia DNR.

## INTRODUCTION

The percid genus *Ammocrypta* comprises six species endemic to central and eastern North America. Members of the genus often inhabit sandy runs of small to medium rivers (Page and Burr, 1991), hence their common name, sand darters (Etnier and Starnes 1993). The Florida Sand Darter, *A. bifascia*, which is the focus of our study, was first described by Williams (1975) from specimens collected in the Choctawhatchee River, Florida. Williams (1975) diagnosed *A. bifascia* as distinct from *A. beanii* (the Naked Sand Darter) based on geographic distribution, tuberculation, and medial fin banding pattern (*bifascia* refers to two bands). The geographic distribution of *A. bifascia* encompasses Gulf of Mexico drainages of the Perdido, Escambia, Blackwater, Yellow, and Choctawhatchee rivers (Boschung and Mayden 2004).

Starnes and Starnes (1979) collected specimens from the Apalachicola River, Florida, which is east of the Choctawhatchee River and outside the known distribution of *A. bifascia*. The Apalachicola specimens were collected a few hundred meters below Jim Woodruff Dam, which impounds Lake Seminole, and is the confluence of the Chattahoochee and Flint rivers near the Florida, Alabama, and Georgia border (Fig. 1). Only two specimens were collected on two occasions, but Starnes and Starnes (1979) considered the Apalachicola specimens native, likely missed by earlier sampling efforts not extensive to the main river. In contrast, others (Stauffer et al. 1980; Swift et al. 1986; Fuller et al. 1999; Boschung and Mayden 2004) considered the scarcity of records in the Apalachicola as evidence of occasional bait-bucket introductions by anglers given the proximity to the tail water fishery below Jim Woodruff Dam. Mettee et al. (1996) did not mention the Apalachicola specimens in their account of *A. bifascia*, while Page (1983), Page and Burr (1991; 2011), and Robins et al. (2018) accepted the Apalachicola specimens as native without comment. Robins et al. (2018) also included additional localities in the Apalachicola and Chipola rivers (Apalachicola drainage) on their distribution map for *A. bifascia.*

We collected *Ammocrypta* specimens in the lower Flint River upstream of Jim Woodruff Dam in 2013, representing the first record of the genus in Georgia. Given their significance, our primary objective was to accurately identify these specimens using both morphological and genetic data. While *A. bifascia* was likely, given their known occurrence further downstream in the drainage, we also considered the possibility that our specimens represented an undescribed and potentially endemic species. Boschung and Mayden (2004) recognized 9 fishes endemic to the Apalachicola drainage. The recently described Halloween Darter

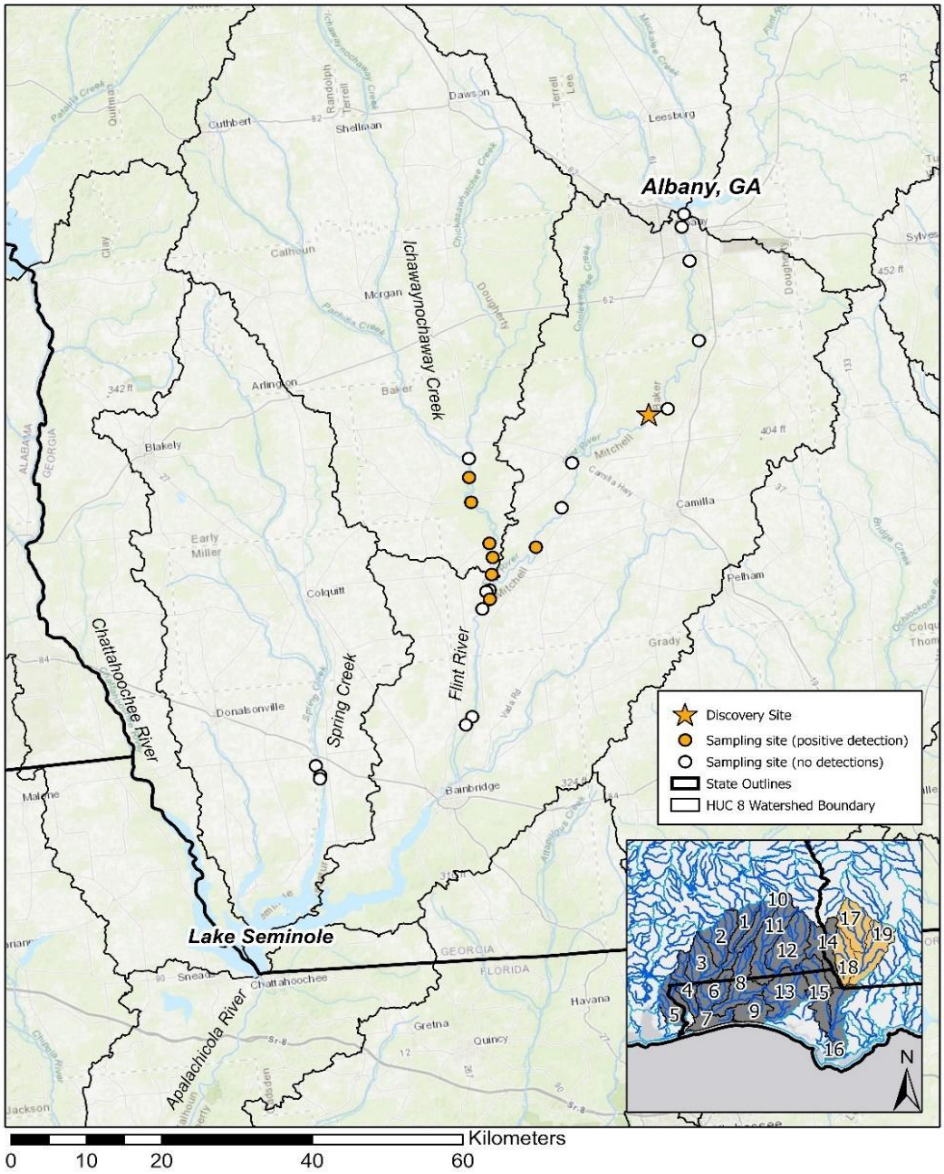


**Figure 1**. Sites where targeted searches for *Ammocrypta* were completed as part of this study. Lower inset shows watersheds included within the known range of *A. bifascia*, with watersheds sampled in Georgia highlighted. 1 = Patsaliga, 2 = Sepulga, 3 = lower Conecuh, 4 = Escambia, 5 = Perdido, 6 = Blackwater, 7 = Pensacola Bay, 8 = Yellow, 9 = Choctawhatchee Bay, 10 = upper Conecuh, 11 = Pea, 12 = upper Choctawhatchee, 13 = lower Choctawhatchee, 14 = lower Chattahoochee, 15 = Chipola, 16 = Apalachicola, 17 = Ichawaynochaway, 18 = Spring, 19 = lower Flint.

(*Percina crypta*; Freeman et al. 2008) is one of the recognized endemics, but warrants additional taxonomic study (Hayes and Piller 2018).

Our secondary objective was to better define the distribution of *Ammocrypta* in Georgia through additional surveys. While Flint River sport fish populations have been the focus of extensive research and monitoring (e.g., Sammons et al. 2019), sampling for smaller fishes has largely focused on Flint River tributary streams (e.g., Albanese et al. 2007; McCargo and Peterson 2010). This bias in sampling reflects the inability to effectively sample small fishes with boat electrofishing and the difficulty of accessing shallow habitats that can be sampled by other gear types.

## METHODS AND MATERIALS

### Study Area

The lower Flint River basin (LFRB) is located within the Dougherty Plain district of the Coastal Plain physiographic province of southwestern Georgia (Rugel et al. 2012; Gore and Witherspoon 2013) and receives significant groundwater inputs from the upper Floridan aquifer (Hicks et al. 1987; Rugel et al. 2016). Coastal Plain rivers of the southeast are often characterized by low-gradient meandering channels, broad floodplains, high sediment deposition, and point bar formations (Hupp 2000). However, river channels in the LFRB are deeply incised into the Ocala Limestone formation, and erosional features exist as springs, fractures, and stream bedrock outcrops (Rugel et al. 2012; Rugel et al. 2016). Therefore, since hydrology in the Dougherty Plain is largely controlled by mantled karst geology (Hicks et al. 1981) and confined channel morphology (Atkinson et al. 2009), reaches in this region of the LFRB remain distinct from other alluvial reaches characteristic of the Coastal Plain. Notably, due to the influence of the limestone outcrops on river morphology, sandbars are rare and often submerged within our study reach.

### Sampling

After our initial discovery of *Ammocrypta* (n = 5 fish) in the Flint River near Newton GA during June 2013, we targeted sandbars and sand dominated runs from 24 additional sites in the lower Flint Basin. Sites were distributed in the mainstem Flint River (Albany to Lake Seminole, n = 15 sites), Spring Creek (between Colquitt and the confluence of Spring Creek with Lake Seminole, n = 3 sites), Ichawaynochaway Creek (n = 5 sites), and Chickasawhatchee Creek (n = 1 site). Collections were made with small seines 3.1 to 5 m long, 1.2-1.8 m deep with 3-6

mm mesh. All specimens were either fixed in 10% formalin or preserved with 95% ethanol for genetic analyses under authority of the Georgia Department of Natural Resources. For Ichawaynochaway Creek, we also used snorkel crews (2-4 individuals) to search suitable sand bar habitat for a minimum of 60 minutes or until *Ammocrypta* were found. The objective of the snorkeling surveys was to detect *Ammocrypta* in multiple distinct locales within the lower reaches of the Ichawaynochaway Creek. Therefore, no fishes were collected during snorkeling surveys and search efforts focused on behavior and habitat observations once *Ammocrypta* were successfully detected.

## Species Identification

### Morphological

Morphometric (n = 23) and meristic (n = 8) characters were recorded under a dissecting microscope with dial calipers to the nearest one tenth of a millimeter and compared to the same characters found in Williams (1975). Counts and measurements followed Hubbs and Lagler (2004) with a few modifications to account for scale features unique to *Ammocrypta* (Williams 1975) and were made on 14 females and one male that were initially preserved in formalin and later transferred to 70% ethanol. The focus on female specimens was not deliberate but reflects what was captured during our surveys. Counts and measurements were compared to the five females measured by Williams (1975) from the Choctawhatchee/Yellow systems. Counts and measurements were not completed on the genetic specimens described below due to effects of 95% ethanol preservation on morphology. Similarly, genetic analyses were not carried out on the 15 formalin-fixed specimens.

### Genetic

We targeted the mtDNA cytochrome c oxidase (COI) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) genes. We sequenced 20 individuals for COI (n = 9 Flint River, n = 9 Choctawhatchee River, n = 2 Apalachicola River); Choctawhatchee and Apalachicola tissue samples were obtained from the University of Kansas Biodiversity Institute and the Florida Museum of Natural History, respectively. We sequenced 32 individuals for ITS-2 (n = 20 Flint River, n = 12 Escambia River; see Appendix). We extracted DNA from tissue using the DNeasy Blood and Tissue kit (QIAGEN, Inc.). The DNA was inspected visually for molecular weight via agarose electrophoresis (2% agarose in 1x TAE).

Polymerase chain reaction (PCR) amplification used primers VF2_t1 and FR1d_t1 (Ivanova et al. 2007) for COI, and ITS-2-F (5'-CTACGCCTGTCTGAGTGTC) and ITS-2-R (5'-ATATGCTTAAATTCAGCGGG) for ITS-2 (Phillips et al. 1995). PCR amplifications (25 μL reaction volume) included 30-80 ng/uL DNA, 1 × *Taq* reaction buffer (GoTaq Flexi, Promega, Madison, WI), 3.125 mM $MgCl_2$, 0.375 mM of each dNTP, 0.50 uM of each primer, and 0.05 U *Taq* DNA polymerase (GoTaq Flexi, Promega). Thermal cycle conditions were an initial 94 ˚C (2 min) denaturation followed by 35 cycles of 95 ˚C (30s), 58˚C (30s) 72 ˚C (30s) and a final 72 ˚C (7 min) extension.

PCR products were cleaned using a QIAquick Purification Kit (QIAGEN, Inc.) and cycle sequencing (both forward and reverse strands) followed the BigDye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) using primers above. Products were purified using standard ethanol/EDTA precipitation (BigDye Terminator v3.1 Cycle Sequencing Kit User Guide). We ran the purified products on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Inc.). Sequences were checked against original chromatograms using Bioedit v. 7.0.1 (Hall 1999) and contiguous sequences assembled using the computer program Geneious v. 8.1.6 (https://www.geneious.com).

For mtDNA phylogenetic reconstruction, we downloaded 53 *Ammocrypta* sequences from the Barcode of Life Database (BOLD) repository comprising all six species of *Ammocrypta* (Appendix). We compared ITS-2 sequences between Escambia River *A. bifascia* and Flint River specimens to assess nuclear genetic variation. Sequences of each gene were aligned by eye.

Our resulting alignments were used to estimate a nucleotide substitution model and perform maximum likelihood (ML) phylogenetic reconstruction using MEGA (v10.0.5; Kumar et al. 2018). Resolution was assessed via bootstrap re-sampling with 500 pseudo-replicates. We used *Etheostoma cinereum* (BNAFA574-08), *Crystallaria asprella* (BNAFA008-08) and *Percina maculata* (BCF308-07) as outgroups. We constructed haplotype networks for *A. bifascia* (n = 27) and *A. beanii* (n = 9) mtDNA sequences using the R v3.6.2 (R Core Team 2019) library pegas (Paradis 2010).

## RESULTS

### Distribution and Habitat

We documented *Ammocrypta* in the mainstem Flint River (n = 3 sites) and in Ichawaynochaway Creek (n = 5 sites; Fig. 1; Table 1). Specimens were collected and observed along sandbars in flowing water habitats over a range of depths that could be accessed while wading and/or snorkeling (Fig. 2; Table 1). We only made relatively large collections at the discovery site, which is one of a few large sandbars we encountered on the Flint River between Albany and Bainbridge. Benthic taxa collected with *Ammocrypta* included *Notropis longirostris* (Longnose Shiner), *Notropis amplamala* (Longjaw Minnow) and *Percina westfalli* (Eastern Blackbanded Darter). All collections were made during June-October during relatively low-flows.

**Figure 2**. Initial discovery site on the Flint River where we made our largest collections of *Ammocrypta bifascia*.

### Morphological Identification

Live specimens had medial and distal bands on the dorsal, caudal, and anal fins (Figs. 3 and 4). All individuals were similar in appearance to those described by Boschung and Mayden (2004) - being translucent with yellow and orange

**Figure 3**. *Ammocrypta bifascia* adult male captured from the Flint River, GA in July 2015.

**Figure 4**. *Ammocrypta bifascia* adult male observed while snorkeling in Ichawaynochaway Creek, GA in August 2019.

reflections and having shades of iridescent greens and yellows along the operculum and lateral line.

We documented overlap in almost all morphometric characters (Table 2) between Flint River and Choctawhatchee/Yellow drainage specimens measured by Williams (1975). The average value of each measurement tended to be less than measured by Williams (1975), which may be attributed to the larger size of his specimens. Exceptions to this pattern are longer anal spine length, longer and non-overlapping pelvic fin length, and a longer hiatus between dorsal fins in Flint River specimens. We also note that average snout length was shorter in Flint River specimens and did not overlap with data from Williams (1975). All meristic characters overlapped, but average counts and ranges of counts were lower for Flint River specimens.

**Table 1.** Observation data for sites where *Ammocrypta* were documented in Georgia. The number (No.) indicates animals observed or collected during one or more survey events. SL = standard length, rm = river miles.

| Waterbody | Locality | County | Latitude | Longitude | No. | SL (mm) | Habitat |
|---|---|---|---|---|---|---|---|
| Flint River | 7.5 rm upstream of GA route 37 (discovery site) | Baker/Mitchell | 31.35476 | -84.2257 | 5-25 | 35-51 | Long sandbar that slopes steeply to main channel |
| Flint River | 9.5 rm downstream of GA route 37 | Baker/Mitchell | 31.20536 | -84.4066 | 1 | "Adult" | Gravel sand-run next to sandbar. |
| Flint River | 2.0 rm downstream of confluence with Ichawaynochaway Creek. | Baker/Mitchell | 31.14719 | -84.4797 | 6 | 37-48 | Sandbar below limestone outcrop. |
| Ichawaynochaway Creek | 0.7 rm upstream confluence with Flint River. | Baker | 31.17762 | -84.4729 | 1 | 50 | Sandbar on island with cypress trees |
| Ichawaynochaway Creek | 1.25 rm downstream of GA route 91. | Baker | 31.19891 | -84.4680 | 1 | 50 | Mid-channel sandbar |
| Ichawaynochaway Creek | 0.33 rm upstream of GA route 91. | Baker | 31.21668 | -84.4704 | 1-4 | 30-35 | Sandbar on island with cypress trees |
| Ichawaynochaway Creek | 0.15 rm downstream of GA route 200 | Baker | 31.27083 | -84.4884 | 5 | 40-65 | Sandbar in side channel |
| Ichawaynochaway Creek | 1.5 rm downstream confluence with Chickasawhatchee Creek | Baker | 31.30211 | -84.4868 | 1 | 35 | Mid-channel sandbar |

**Table 2.** Comparison of morphological data between *Ammocrypta* collected from the Flint River (this study) and Choctawhatchee/Yellow rivers from Williams (1975). Data are based on 14 females and one male (SL = 37.4-47.5 mm) from the Flint River and five females (SL = 50-54 mm) from Williams (1975). * Indicates characters not measured in both studies. Dia = diameter, bet = between, jct = junction. Measurements are to the nearest tenth of a millimeter.

| | Flint River | | Choctawhatchee/Yellow | |
|---|---|---|---|---|
| Measurements | Range | Average (SE) | Range | Average |
| Snout tip- 1st dorsal origin | 355-389 | 370 (2.7) | 383-399 | 391 |
| Snout tip-2nd dorsal origin | 607-652 | 636 (3.1) | 639-657 | 646 |
| Snout tip-anal origin | 588-657 | 627 (4.5) | 633-653 | 641 |
| Snout tip-pelvic insertion | 261-285 | 274 (2.4) | 282-295 | 280 |
| Snout tip-jct gill membranes | 123-167 | 149 (2.9) | 144-163 | 154 |
| Caudal Peduncle length | 182-251 | 209 (5.2) | 213-220 | 216 |
| Caudal Peduncle depth | 43-76 | 66 (2.2) | 65-70 | 67 |
| Body depth, 1st dorsal origin | 95-146 | 118 (3.7) | 117-137 | 129 |
| Body width | 95-114 | 102 (1.5) | 109-118 | 114 |
| Longest dorsal spine | 96-130 | 113 (2.8) | 106-111 | 109 |
| Longest dorsal ray | 95-132 | 111 (2.7) | 112-120 | 116 |
| Caudal fin length | 133-182 | 161 (3.4) | 161-172 | 166 |
| Anal spine length | 49-76 | 63 (2.0) | 42-51 | 48 |
| Longest anal ray | 92-140 | 123 (3.3) | 123-135 | 128 |
| Left pectoral fin length | 172-239 | 202 (4.2) | 206-217 | 210 |
| Left pelvic fin length | 171-198 | 177 (2.9) | 147-157 | 153 |
| Head length | 219-265 | 242 (2.7) | 252-262 | 257 |
| Horizontal dia. fleshy orbit | 51-61 | 55 (0.8) | 56-67 | 63 |
| Snout length | 56-78 | 66 (1.8) | 80-83 | 81 |
| Upper Jaw length | 59-78 | 72 (1.3) | 77-86 | 82 |
| Bony interorbital* | -- | -- | 13-18 | 15 |
| Fleshy interorbital* | 17-51 | 31 (2.4) | -- | -- |
| Dorsal hiatus bet dorsal fins | 41-81 | 64 (2.6) | 29-64 | 49 |
| Dorsal spine count | 8-11 | 9.3 (0.2) | 8-12 | 9.6 |
| Dorsal ray count | 8-11 | 9.9 (0.2) | 10-12 | 11.0 |
| Anal ray count | 7-9 | 8.2 (0.2) | 8-11 | 9.3 |
| Left pectoral ray count | 11-13 | 12.1 (0.2) | 12-13 | 12.9 |
| Lateral line scale count | 63-73 | 68.2 (0.9) | 63-78 | 70.28 |
| Scale count above lateral line | 1 | 1 (0.0) | 0-2 | 1 |
| Scale count below lateral line | 0-2 | 1 (0.1) | 0-3 | 1.29 |
| Transverse scale row count | 3-5 | 3.3 (0.2) | 3-7 | 4.55 |

## Genetic Identification

The 76 (73 *Ammocrypta* + 3 outgroups) aligned COI sequences were 608 nucleotides (nt) of which 176 positions were variable and 153 parsimony informative. The mean base frequencies were as follows: A=0.23 (SE = 0.07), C=0.29 (0.08), G=0.20 (0.05) and T=0.28 (0.08). The 32 aligned ITS sequences were 405 nt and were monomorphic across the geographic range of *A. bifascia*. Due to the lack of variation in ITS, no subsequent analyses were performed on this gene. The Kimura 2-parameter model with rate variation and invariable sites (K2+G+I) was selected (Bayesian information criterion = 7178.98) to best describe the substitution pattern of the mtDNA COI gene. The resulting ML phylogeny (Fig. 5) produced two well-resolved *A. bifascia* clades. The first placed Flint River *A. bifascia* with Choctawhatchee and Apalachicola river samples (herein called the eastern clade). The other was rendered paraphyletic by the presence of *A. beanii* with members of *A. bifascia* from west of the Choctawhatchee River (herein called the western clade).

The 36 sequenced *A. bifascia* and *A. beanii* comprised 20 unique mtDNA COI haplotypes (Table 3). Supporting the ML phylogeny, there were 40 nucleotide substitutions between *Ammocrypta* haplotypes from the western clade vs. the eastern clade (Fig. 6). Haplotype II was common to both *A. beanii* and western *A. bifascia* (Table 3). Flint River specimens had identical sequences (haplotype VIII; Table 3) and shared no haplotypes with Choctawhatchee or Apalachicola samples (Table 3, Fig. 6). The number of substitutions between Flint and Choctawhatchee samples ranged from one (haplotype VIII vs XII) to six (haplotypes VIII vs IX or XV). One Apalachicola specimen shared haplotype XVI with Choctawhatchee samples (Table 3,) while the other (XVIII) had one substitutional difference when compared to Flint River samples (Fig. 6).

The average genetic distance, inferred from a Kimura 2-parameter model of nucleotide substitution, between *A. bifascia* eastern and western clades was 8% and approximated that among species (average = 9%, Table 4). The average genetic distance between the western clade of *A. bifascia* and *A. beanii* was 0.08%. Average genetic distances among comparisons within the eastern clade ranged from (0.03-0.24%).

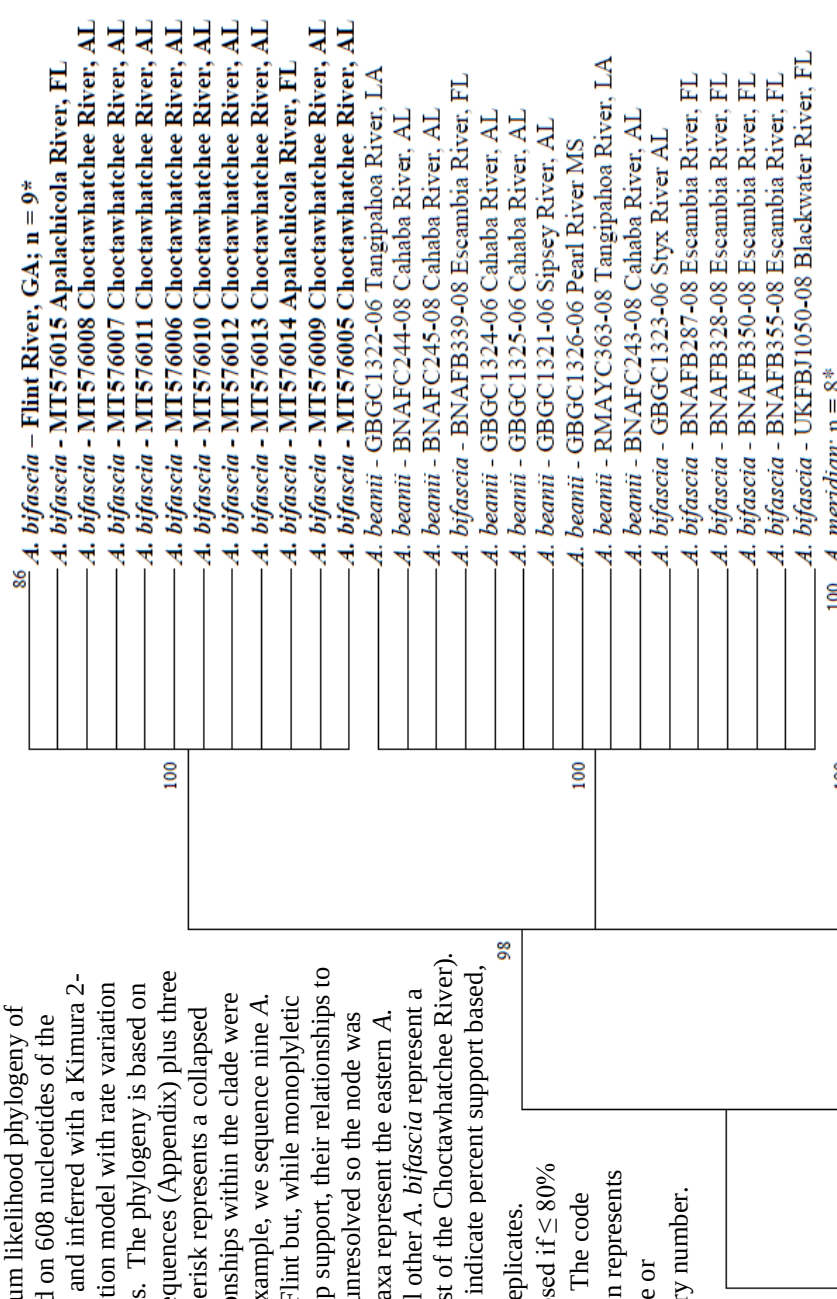


**Figure 5.** Maximum likelihood phylogeny of *Ammocrypta* based on 608 nucleotides of the mtDNA COI gene and inferred with a Kimura 2-parameter substitution model with rate variation and invariable sites. The phylogeny is based on 73 *Ammocrypta* sequences (Appendix) plus three outgroups. An asterisk represents a collapsed node where relationships within the clade were unresolved. For example, we sequence nine *A. bifascia* from the Flint but, while monoplyletic with 86% bootstrap support, their relationships to one another were unresolved so the node was collapsed. Bold taxa represent the eastern *A. bifascia* clade. All other *A. bifascia* represent a western clade (west of the Choctawhatchee River). Numbers at nodes indicate percent support based, on 500 bootstrap replicates. Nodes were collapsed if ≤ 80% bootstrap support. The code after each specimen represents the Barcode of Life or GenBank repository number.

**Table 3.** Mitochondrial COI haplotypes of *Ammocrypta bifascia* and *A. beanii* per drainage.

| | *A. bifascia* | | | | | | *A. beanii* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | Apalachicola | Flint | Choctawhatchee | Blackwater | Escambia | Styx (Perdido) | Cahaba | Sipsey | Tangipahoa | Pearl |
| I | 0 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 |
| II | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 1 | 0 | 0 |
| III | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| IV | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| V | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| VI | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| VII | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| VIII | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IX | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| X | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XI | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XII | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XIII | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XIV | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XV | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XVI | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XVII | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XVIII | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| XIX | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| XX | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |

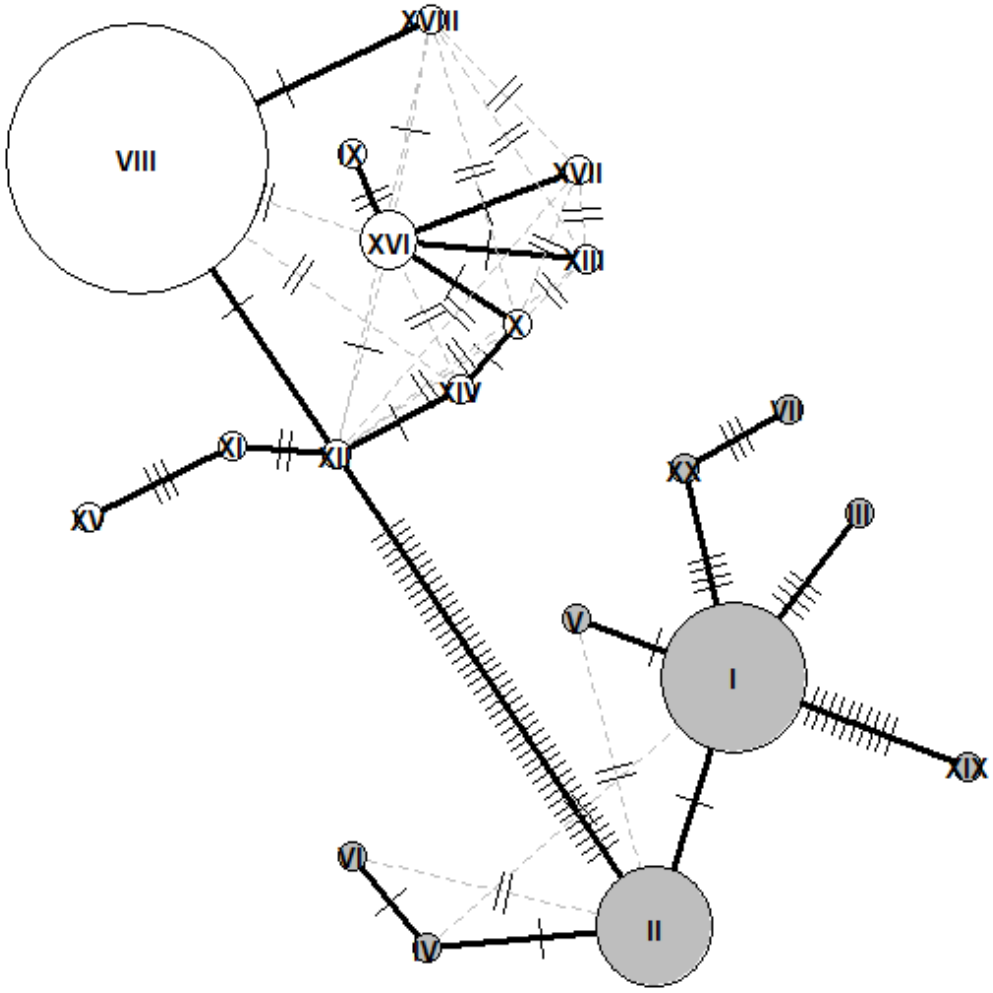


**Figure 6**. Mitochondrial DNA COI haplotype network for *Ammocrypta bifascia* and *A. beanii*. Light circles represent haplotypes from eastern *A. bifascia* (Apalachicola, Flint, Choctawhatchee rivers). Dark circles are haplotypes from the western *A. bifascia* clade (Blackwater, Escambia, Perdido rivers) and *A. beanii* haplotypes from the Cahaba, Sipsey, Tangipahoa, and Pearl rivers. Roman numerals in circles correspond to haplotypes found in Table 3. Note that haplotype II is shared between *A. bifascia* and *A. beanii*. Solid dark edges represent the minimum spanning tree and the alternative relationships are inferred from light dashed edges. Smaller perpendicular edges represent nucleotide substitutions between haplotypes.

**Table 4**. Genetic distances among *Ammocrypta* species inferred from a Kimura 2-parameter model of nucleotide substitution. Comparisons between the eastern and western clade of *A. bifasica* are reported in text.

| | *A. pellucida* | *A. vivax* | *A. meridiana* | *A. clara* | *A. beanii* |
|---|---|---|---|---|---|
| *A. pellucida* | | | | | |
| *A. vivax* | 0.1178 | | | | |
| *A. meridiana* | 0.1105 | 0.0585 | | | |
| *A. clara* | 0.0659 | 0.0930 | 0.0893 | | |
| *A. beanii* | 0.1362 | 0.0930 | 0.0880 | 0.1054 | |
| *A. bifascia* | 0.1014 | 0.0939 | 0.0781 | 0.0865 | 0.0415 |

## DISCUSSION

### Specimen Identification

We documented Georgia's first record of *Ammocrypta,* finding it in both the lower Flint River and Ichawaynochaway Creek. Morphometric and meristic data along with pigmentation patterns of Flint River specimens are congruent with *A. bifascia* described by Williams (1975). Morphological differences that we documented in Flint River specimens, such as longer pelvic fin length and shorter snout length may reflect true population-level variation or differences in sample size between the two studies. The mtDNA phylogeny grouped Flint River *Ammocrypta* with those from the Choctawhatchee River, which is the type locality of *A. bifascia*. While Flint River basin and Choctawhatchee River specimens did not share a common haplotype, most were only one or two substitutional differences apart and were more closely related to each other than to the western *A. bifascia* + *A. beanii* clade, further supporting that Flint River specimens are *A. bifascia*. The lack of shared haplotypes may indicate minimal exchange of maternal genetic material or could be due to limited sample sizes. The Choctawhatchee specimens also exhibited greater genetic (mtDNA) diversity than in the Flint River, which may have implications for conservation (Moyer et al. 2019).

Interestingly, both the mtDNA phylogeny and haplotype network showed two well-supported geographic clades of *A. bifascia*. An eastern clade comprising individuals sampled from the Choctawhatchee and east (including specimens from the Flint River), and a western clade from drainages west of the Choctawhatchee River. The genetic east/west pattern recovered by our study has also been observed in the Longnose Shiner (*Notropis longirostris*), which has a similar distribution (Stout 2017). The western clade appears paraphyletic with respects to the *A. beanii*

individuals downloaded from BOLD. There are a variety of hypotheses to explain this pattern including, hybridization and/or the retention of ancestral polymorphisms. Unfortunately, our study was not designed to discern competing hypotheses. The paraphyly of this group warrants further taxonomic study.

**Natural History and Distribution**

Occupied sites for *A. bifascia* in the Flint River and Ichawaynochaway Creek exhibited habitat conditions typical of the species (Williams 1975; Boschung and Mayden 2004) and included reaches with strong to moderate current over a shifting sand bottom. We collected *A. bifascia* typically on unconsolidated barren sand bars, along sandy edges of cypress island formations, or on patches of clean sand surrounded by large rocks, woody debris, and aquatic vegetation. We observed fish on mid-channel sand bars in clear water at depths ranging from 0.15-1.5 m with most observations being between 0.5-1.0 m – a finding similar to Williams (1975). For cypress island formations, we observed fish at both upstream and downstream ends of island locations, which typically had swift and moderate flows, respectively.

Our snorkeling observations in Ichawaynochaway Creek showed that *Ammocrypta* were often solitary. However, we also observed groups of 2-5 ($\bar{x}$ = 2.33; SE = 0.95) individuals dispersed throughout sand bars and often associated with larger groups ($n \geq 25$) of mature and juvenile Eastern Blackbanded Darter (*Percina westfalli*) and juvenile leuciscids (*sensu* Schonhuth et al. 2018). We also observed the sand burying behavior first described by Williams (1975) for an adult female and a juvenile at two sites surveyed in September. The burying behavior has been proposed as a mechanism to conserve energy in moderate currents and to potentially avoid predation (Williams 1975).

We observed *Ammocrypta* at all Ichawaynochaway Creek sites sampled within a 12.6 mile reach, suggesting that they are widely distributed between the confluence of the Flint River and Chickasawhatchee Creek. In contrast, sites where we detected *Ammocrypta* in the Flint River were more isolated spatially. Our observation could reflect a true distribution pattern reflecting the more sparse distribution of sandbar habitats or the lower probability of detecting the species in a large river. We did not detect the species in Chickasawhatchee (site sampled 0.5 mi upstream of Ichawaynochaway Creek confluence; Fig. 1) or Spring Creek, which were targeted because they are smaller but still comparable in size to Ichawaynochaway Creek. Smaller tributary streams throughout the lower Flint River system have been extensively sampled using seining and electrofishing but

have never reported *Ammocrypta* (Albanese et al. 2007; McCargo and Peterson 2010; Davis et al. 2020). Lower Ichawaynochaway Creek has received relatively extensive fish sampling due to the presence of an ecological research center (The Jones Center at Ichauway). Smith et al. (2006) reported 61 fishes taken by electrofishing, trapping, seining, angling, and rotenone at the Jones Center, but never reported *Ammocrypta*. Collectively, these results suggest that *A. bifascia* is distributed in larger tributaries and rivers in the lower Flint River system where they are inherently more difficult to detect. Their ability to bury in sand may exacerbate their low detection probability. We recommend additional surveys targeting sandbar habitats in larger tributary streams using both seining and snorkeling to better document the distribution of *A. bifascia* in Georgia. Additional surveys coupled with robust methods to estimate population presence and abundance of *A. bifascia* would contribute to a better understanding of this potentially rare taxon in Georgia.

**Status as a Native Taxon**

Our unexpected finding of *A. bifascia* in the Flint River system and the rarity of collections in the Apalachicola River raises the possibility that this species was introduced to the Apalachicola drainage. However, both purposeful (Moyer et al. 2014) and accidental (Moyer et al. 2005) introductions seem unlikely since *Ammocrypta* are not sought after as aquarium fishes or desirable baitfishes. It is also unlikely that *Ammocrypta* would be collected with native schooling baitfishes via a variety of typical baitfish capture methods (e.g., cast net, minnow trap, hook and line) due to their small size and benthic orientation.

Genetic data support our hypothesis that *A. bifascia* (Flint River) is native to the system because they share a unique haplotype not found in other *A. bifascia*; however, our sample size was limited, and shared haplotypes with *A. bifasicia* from other drainages could still exist. Further support for this hypothesis is from numerous other native fishes that share a distribution pattern similar to *A. bifascia*, such as *Lythrurus atrapiculus* (Blacktip Shiner), *N. longirostris*, *N. amplamala*, and *Pteronotropis harperi* (Redeye Chub) (Boschung and Mayden 2004).

The mtDNA data remain inconclusive regarding the presence of *A. bifascia* in the Apalachicola River. If genetic analyses showed that the Apalachicola specimens had a common haplotype with Flint River samples, then it would be indicative of being native to the system. One Apalachicola specimen shared a haplotype with Choctawhatchee individuals indicating that it either retained an ancestral polymorphism with the Choctawhatchee or was introduced into the

system. The other Apalachicola specimen shared haplotype affinities with Flint River specimens, but then again, so do Choctawhatchee samples. Teasing apart these competing scenarios for the Apalachicola specimens would require additional nuclear markers and larger samples sizes from the Apalachicola system (including the Chipola River) and other drainages.

Starnes and Starnes (1979) were first to contend that *A. bifascia* was native to the Apalachicola River. The distribution pattern we documented in the mainstem river and lower Ichawaynochaway Creek, where they may be difficult to detect using traditional sampling methods, may explain why this species has eluded detection in the Flint River system. Our recent discovery of a new native species in Georgia underscores the importance of exploratory sampling in under-sampled habitats for documenting and conserving aquatic biodiversity.

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**Appendix.** General locality and GenBank/Barcode of Life Database (BOLD) numbers for specimens used in this study. Asterisk signifies a specimen that was sequenced for this study.

| Species | General Locality | COI GenBank/BOLD # | ITS GenBank # |
|---|---|---|---|
| *A. bifascia** | Flint River, GA | | 14719 |
| *A. bifascia** | Flint River, GA | | 14720 |
| *A. bifascia** | Flint River, GA | | 14721 |
| *A. bifascia** | Flint River, GA | | 14723 |
| *A. bifascia** | Flint River, GA | | 14724 |
| *A. bifascia** | Flint River, GA | | 14725 |
| *A. bifascia** | Flint River, GA | | 14726 |
| *A. bifascia** | Flint River, GA | MT575996 | |
| *A. bifascia** | Flint River, GA | MT575997 | 14706 |
| *A. bifascia** | Flint River, GA | MT575998 | 14707 |
| *A. bifascia** | Flint River, GA | MT575999 | 14708 |
| *A. bifascia** | Flint River, GA | MT576000 | 14709 |
| *A. bifascia** | Flint River, GA | MT576001 | 14710 |
| *A. bifascia** | Flint River, GA | MT576002 | 14711 |
| *A. bifascia** | Flint River, GA | | 14712 |
| *A. bifascia** | Flint River, GA | | 14713 |
| *A. bifascia** | Flint River, GA | | 14714 |
| *A. bifascia** | Flint River, GA | | 14715 |
| *A. bifascia** | Flint River, GA | | 14716 |
| *A. bifascia** | Flint River, GA | MT576004 | 14717 |
| *A. bifascia** | Flint River, GA | MT576003 | 14718 |
| *A. bifascia** | Choctawhatchee River, AL | MT576005 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576006 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576007 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576008 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576009 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576010 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576011 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576012 | |
| *A. bifascia** | Choctawhatchee River, AL | MT576013 | |
| *A. bifascia** | Apalachicola River, FL | MT576014 | |
| *A. bifascia** | Apalachicola River, FL | MT576015 | |
| *A. bifascia** | Escambia River, FL | | 14775 |
| *A. bifascia** | Escambia River, FL | | 14776 |

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| *A. bifascia** | Escambia River, FL | | 14777 |
| *A. bifascia** | Escambia River, FL | | 14778 |
| *A. bifascia** | Escambia River, FL | | 14779 |
| *A. bifascia** | Escambia River, FL | | 14780 |
| *A. bifascia** | Escambia River, FL | | 14781 |
| *A. bifascia** | Escambia River, FL | | 14782 |
| *A. bifascia** | Escambia River, FL | | 14783 |
| *A. bifascia** | Escambia River, FL | | 14784 |
| *A. bifascia** | Escambia River, FL | | 14785 |
| *A. bifascia** | Escambia River, FL | | 14786 |
| *A. bifascia* | Escambia River, FL | BNAFB287-08 | |
| *A. bifascia* | Escambia River, FL | BNAFB328-08 | |
| *A. bifascia* | Escambia River, FL | BNAFB339-08 | |
| *A. bifascia* | Escambia River, FL | BNAFB350-08 | |
| *A. bifascia* | Escambia River, FL | BNAFB355-08 | |
| *A. bifascia* | Styx River, AL | GBGC1323-06 | |
| *A. bifascia* | Blackwater River, FL | UKFBJ1050-08 | |
| *A. beanii* | Cahaba River, AL | BNAFC243-08 | |
| *A. beanii* | Cahaba River, AL | BNAFC244-08 | |
| *A. beanii* | Cahaba River, AL | BNAFC245-08 | |
| *A. beanii* | Sipsey River, | GBGC1321-06 | |
| *A. beanii* | Tangipahoa River, LA | GBGC1322-06 | |
| *A. beanii* | Cahaba River, AL | GBGC1324-06 | |
| *A. beanii* | Cahaba River, AL | GBGC1325-06 | |
| *A. beanii* | Pearl River, MS | GBGC1326-06 | |
| *A. beanii* | Tangipahoa River, LA | RMAYC363-08 | |
| *A. clara* | White River, AR | BNAFC233-08 | |
| *A. clara* | White River, AR | BNAFC234-08 | |
| *A. clara* | White River, AR | BNAFC235-08 | |
| *A. clara* | White River, AR | BNAFC236-08 | |
| *A. clara* | White River, AR | BNAFC237-08 | |
| *A. clara* | Clinch River, VA | BNAFC238-08 | |
| *A. clara* | Clinch River, VA | BNAFC239-08 | |
| *A. clara* | Clinch River, VA | BNAFC240-08 | |
| *A. clara* | Clinch River, VA | BNAFC241-08 | |
| *A. clara* | Clinch River, VA | BNAFC242-08 | |
| *A. clara* | Clinch River, VA | UKFBJ155-08 | |
| *A. meridiana* | Cahaba River, AL | BNAFC226-08 | |

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|---|---|---|
| *A. meridiana* | Cahaba River, AL | BNAFC227-08 |
| *A. meridiana* | Cahaba River, AL | BNAFC228-08 |
| *A. meridiana* | Cahaba River, AL | BNAFC229-08 |
| *A. meridiana* | Cahaba River, AL | BNAFC230-08 |
| *A. meridiana* | Cahaba River, AL | BNAFC231-08 |
| *A. meridiana* | Cahaba River, AL | BNAFC232-08 |
| *A. meridiana* | Noxubee River. MS | UKFBJ144-08 |
| *A. pellucida* | St. Lawrence River, QC | BCF225-07 |
| *A. pellucida* | Grand River, ON | BCF226-07 |
| *A. pellucida* | Grand River, ON | BCF227-07 |
| *A. pellucida* | Grand River, ON | BCF229-07 |
| *A. pellucida* | Grand River, ON | BCF230-07 |
| *A. pellucida* | Grand River, ON | BCF231-07 |
| *A. pellucida* | Licking River, KY | BNAFA004-08 |
| *A. pellucida* | Licking River, KY | BNAFA005-08 |
| *A. vivax* | Saline River, AR | BNAFA001-08 |
| *A. vivax* | Saline River, AR | BNAFA002-08 |
| *A. vivax* | Saline River, AR | BNAFA003-08 |
| *A. vivax* | Saline River, AR | BNAFB034-08 |
| *A. vivax* | Saline River, AR | BNAFB057-08 |
| *A. vivax* | Saline River, AR | BNAFB069-08 |
| *A. vivax* | Saline River, AR | BNAFB081-08 |
| *A. vivax* | Saline River, AR | BNAFB093-08 |
| *A. vivax* | Saline River, AR | RMAYC454-08 |
| *A. vivax* | no locality data | UKFBJ161-08 |