# Taxonomic identification of aquatic species for Loxahatchee National Wildlife Refuge via DNA barcoding

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Traditional species identification is reliant on hierarchical taxonomic identification keys base on phenotypic characters; in constrast, DNA barcoding aims to provide an alternative method for species-level identifications using a species specific molecular tag derived from the 5′ region of the mitochondrial cytochrome c oxidase I (*COI*) gene (Hubert et al. 2008). Despite the great promise of DNA barcoding, it has been controversial because the efficiency of the method hinges on the degree of sequence divergence among species. Species-level identifications are relatively straightforward when the average genetic distance among individuals within a species does not exceed the average genetic distance between sister species. As a result, DNA barcoding can become arduous for species that have diverged rather recently (i.e., average genetic distance among individuals within a species tends to exceed the average genetic distance between sister species) or for organisms that have undergone a recent hyrdization event. Despite these potential pitfalls, studies have illustrated some straightforward benefits from the use of a standardized molecular approach for species identification (Hebert et al. 2003; Hebert and Gregory 2005). First, intraspecific phenotypic variation often overlaps that of sister taxa in nature, which can lead to incorrect identifications if based on phenotype only (Pfenninger et al. 2006; Moyer and Diaz-Ferguson 2012). Second, DNA barcodes are effective whatever the life stage under scrutiny (Caterino and Tishechkin 2006). Third, cryptic variation and often spectacular levels of undetected taxonomic diversity have been frequently reported (Hebert et al. 2004; Witt et al. 2006; Smith et al. 2007) Finally, DNA barcode libraries are fully available as they are deposited in a major sequence database, and attached to a voucher specimen whose origin and current location are recorded (Hebert and Gregory 2005; Hebert et al 2004).

The Fish Barcode of Life Initiative (FISH-BOL; www.fishbol.org) is a DNA barcode reference library for all fish species derived from voucher specimens with authoritative taxonomic identifications (Ward et al. 2009). FISH-BOL allows for a fast, accurate, and cost-effective system for molecular identification of the world's ichthyofauna. The benefits of this work include facilitating species

identification, flagging potentially previously unrecognized species, and enabling identifications where traditional methods are not applicable, such as for immature stages or body fragments. Herein, we provide DNA barcode confirmation of species identification for specimens collected from Loxahatchee National Wildlife Refuge.

***Methods***

Tissue samples were obtained by United States Fish and Wildlife biologists via boat electrofishing on waters in the Loxahatchee National Wildlife Refuge and placed in individually labeled vials containing 1 mL of 95% non denatured ethanol. All tissue samples were archived at the USFWS Conservation Genetics Laboratory in Warm Springs, GA. DNA was extracted from each tissue sample using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia, California). Final DNA templates were eluted in 200 uL of AE buffer (QIAGEN, Inc) and DNA concentration (50-400 ng/uL) estimated using a Nanodrop spectrophotometer (Thermo Scientific, Inc).

We targeted a partial coding segment of the mtDNA *COI* gene approximating 655 nucleotides (nt) in length. The segment of *COI* was amplified via the polymerase chain reaction (PCR) using universal FishF2 and FishR2 primers (Ward et al. 2005) in a 20 µL reaction volume containing approximately 100 ng/uL DNA, 0.5 × *Taq* reaction buffer (GoTaq Flexi, Promega, Madison, WI), 3.0 mM $MgCl_2$, 0.25 mM of each dNTP, 0.50 uM of each primer, and 0.05 U Taq DNA polymerase (GoTaq, Promega, Madison, WI). Optimized thermal cycle conditions for *COI* were an initial 94 ˚C (2 min) denaturation followed by 35 cycles of 95 ˚C (1 min.), 55˚C (1 min.), and 72 ˚C (1 min.) and a final 72 ˚C (4 min.) extension.

PCR products were cleaned using the QIAquick Purification Kit (QIAGEN, Inc). Cycle sequencing was conducted following the Big Dye Terminator v3.1 protocol (Applied Biosystems, Inc., Foster City, CA) using the FishF2 primer under the following thermal cycle parameters: 25 cycles of 96˚C for 10 s, 50˚C for 5 s and 60˚C for 4 min. Cycle sequencing PCR products were purified using the BigDye XTerminator Purification kit (Applied Biosystems, Inc.), visualized on an ABI PRISM 3130 genetic analyzer (Applied

Biosystems, Inc.), and analyzed with Sequence Analysis software v5.2 (Applied Biosystems, Inc.). Each sequence was then submitted to The Barcode of Life Data System (BOLD; http://www.barcodinglife.org) following the methods outlined by Ratnasingham and Hebert (2007). BOLD categorizes barcode records in its reference (search) library as either verified or unvalidated. Verified barcodes are defined as species with a minimum of three representatives and a maximum conspecific divergence of two percent. Unvalidated barcodes do not meet these criteria. Thus, for each sequence that we submitted to BOLD, we reported the percent sequence similarity given by BOLD and categorized the sequence as either verified or unvalidated. Often BOLD reported unvalidated sequences as having more than one species as a possible candidate. In these cases we reported all potential species return under the BOLD search criteria.

**RESULTS/DISCUSSION**

A total of 108 tissue samples were analyzed. One sample (USFWS 194) failed to PCR amplify even after repeated attempts and DNA re extraction. Read lengths were all approximately 500 nt long, No insertions, deletions or stop codons were observed in any sequence. The lack of stop codons is consistent with all amplified sequences being functional mitochondrial *COI* sequences. There were nine samples for which the species identification based on morphology did not match the *COI* barcode results (Table 1). Even though the DNA from the nine samples was re extracted and all PCR and sequencing chemicals replaced (in case of contamination), our subsequent results were the same as previous analyses. This finding suggests that the discrepancy is an artifact of misplacement of tissues (tissue placed in wrong tube) or record keeping (incorrect scientific name associated with the tissue vial). Of the remaining sequences, 31 of 98 (32%) were verified by BOLD (Table 1). While BOLD categorized the remaining 67 sequences as unvalidated, they had a high (often >95%) sequence similarity to the morphological species identification (Table 1).

**CONCLUSIONS**

Freshwater fish species can be efficiently identified or verified through the use of DNA barcoding; however, some species may be problematic due to high sequence similarity or the lack of validated sequence data in BOLD.

**LITERATURE CITED**

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Table 1. Species identification and confirmation using mtDNA *COI* barcoding. Percent similarity is the percent sequence similarity between the specimen of interest and that found in the Barcode of Life database. Verified barcodes are defined as species with a minimum of three representatives and a maximum conspecific divergence of two percent. Unvalidated barcodes do not meet these criteria. An asterisk represents individuals where morphological and mtDNA barcoding results do not match.

| USFWS# | Morphological identification | Bold results (Genus) | Bold results (species) | % Similarity | BOLD categorization |
|---|---|---|---|---|---|
| 81 | Brown Bullhead | *Ameiurus* | *nebulosus* | 99.81 | verified |
| 82 | Inland Silverside | *Labidesthes* | *sicculus* | 98.43 | unvalidated |
| 85 | Inland Silverside | *Labidesthes* | *sicculus* | 97.00 | unvalidated |
| 86 | Inland Silverside | *Labidesthes* | *sicculus* | 98.87 | verified |
| 87 | Inland Silverside | *Labidesthes* | *sicculus* | 97.74 | unvalidated |
| 88 | Chain Pickerel | *Esox* | *americanus; niger* | 99.61 | unvalidated |
| 89 | Chain Pickerel | *Esox* | *americanus; niger* | 97.52 | unvalidated |
| 90 | Chain Pickerel | *Esox* | *americanus; niger* | 94.07 | unvalidated |
| 92 | Dollar sunfish | *Lepomis* | *marginatus* | 99.62 | verified |
| 93* | Dollar sunfish | *Enneacanthus* | *obesus; gloriosus* | 97.61 | unvalidated |
| 94 | Dollar sunfish | *Lepomis* | *marginatus* | 98.89 | verified |
| 95 | Bluespotted sunfish | *Enneacanthus* | *obesus; gloriosus* | 99.25 | unvalidated |
| 181 | Bluespotted sunfish | *Enneacanthus* | *obesus; gloriosus* | 99.04 | unvalidated |
| 182 | Bluespotted sunfish | *Enneacanthus* | *obesus; gloriosus* | 99.43 | unvalidated |
| 183 | Bluespotted sunfish | *Enneacanthus* | *obesus; gloriosus* | 99.23 | unvalidated |
| 184 | Inland Silverside | *Labidesthes* | *sicculus* | 96.30 | unvalidated |
| 185 | Bluefin Killifish | *Lucania* | *goodei* | 100.00 | verified |
| 186 | Yellow bullhead | *Ameiurus* | *natalis* | 96.02 | unvalidated |
| 187 | Mosquitofish | *Gambusia* | *holbrooki; affinis* | 99.80 | unvalidated |
| 188 | Mosquitofish | *Gambusia* | *holbrooki; affinis* | 99.39 | unvalidated |
| 189 | Mosquitofish | *Gambusia* | *holbrooki; affinis* | 99.80 | unvalidated |
| 190 | Black Crappie | *Pomoxis* | *nigromaculatus* | 96.46 | unvalidated |
| 191 | Brown Bullhead | *Ameiurus* | *nebulosus* | 95.08 | unvalidated |
| 192 | Tilapia spp (?Nile) | *Oreochromis* | *sp.; aureus* | 98.55 | verified |
| 193 | Tilapia spp (?Nile) | *Oreochromis* | *sp.; aureus; niloticus* | 99.80 | unvalidated |

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|---|---|---|---|---|---|
| 194 | Chain Pickerel | unable to PCR | | | |
| 195 | Chain Pickerel | *Esox* | *americanus; niger* | 99.61 | unvalidated |
| 196 | Spotted Sunfish | *Lepomis* | *punctatus* | 99.29 | verified |
| 197* | Lake chubsucker | *Pomoxis* | *nigromaculatus* | 95.96 | |
| 198 | Lake chubsucker | *Erimyzon* | *sucetta* | 65.34 | unvalidated |
| 199 | Lake chubsucker | *Erimyzon* | *sucetta* | 90.40 | unvalidated |
| 200 | Spotted Sunfish | *Lepomis* | *punctatus* | 97.30 | unvalidated |
| 201 | Golden shiner | *Notemigonus* | *crysoleucas* | 98.28 | unvalidated |
| 202 | Brown bullhead | *Lepomis* | *punctatus* | 93.52 | |
| 203 | Brown Bullhead | *Ameiurus* | *nebulosus* | 99.25 | verified |
| 204 | Brown Bullhead | *Ameiurus* | *nebulosus* | 98.98 | verified |
| 205 | Mosquito fish | *Gambusia* | *holbrooki; affinis* | 99.79 | unvalidated |
| 206 | Mosquitofish | *Gambusia* | *holbrooki; affinis* | 99.80 | unvalidated |
| 207 | Gizzard Shad | *Dorosoma* | *cepedianum; anale* | 99.79 | unvalidated |
| 208 | Sailfin catfish | *Hypostomus; Pterygoplichthys* | *plecostomus; pardali; disjunctivuss; joselimaianus* | 99.47 | unvalidated |
| 209 | Sailfin catfish | *Hypostomus; Pterygoplichthys* | *plecostomus; pardali; disjunctivuss; joselimaianus* | 95.38 | unvalidated |
| 210 | Lake chubsucker | *Erimyzon* | *sucetta* | 80.00 | |
| 211 | Channel catfish | *Ictalurus* | *punctatus* | 99.79 | verified |
| 212 | Bluegill | *Lepomis* | *macrochirus* | 99.48 | verified |
| 213 | Bluespotted sunfish | *Enneacanthus* | *obesus; gloriosus* | 99.41 | unvalidated |
| 214 | Inland Silverside | *Labidesthes* | *sicculus* | 97.98 | unvalidated |
| 215 | Black Crappie | *Pomoxis* | *nigromaculatus* | 96.00 | unvalidated |
| 216 | Black Crappie | *Pomoxis* | *nigromaculatus* | 97.00 | unvalidated |
| 217 | Warmouth | *Lepomis* | *gulosus* | 98.56 | verified |
| 218 | Golden shiner | *Notemigonus* | *crysoleucas* | 96.96 | unvalidated |
| 219 | Golden shiner | *Notemigonus* | *crysoleucas* | 98.45 | unvalidated |
| 220 | Dollar sunfish | *Lepomis* | *marginatus* | 99.40 | verified |
| 221 | Black crappie | *Pomoxis* | *nigromaculatus* | 96.00 | unvalidated |
| 826 | Black Crappie | *Pomoxis* | *nigromaculatus* | 97.00 | unvalidated |
| 827* | Lake Chubsucker | *Pomoxis* | *nigromaculatus* | 93.74 | |
| 828 | Yellow bullhead | *Ameiurus* | *natalis* | 89.48 | unvalidated |

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|---|---|---|---|---|---|
| 829 | Gizzard Shad | *Dorosoma* | *cepedianum* | 99.02 | unvalidated |
| 830 | Gizzard Shad | *Dorosoma* | *cepedianum; anale* | 99.82 | unvalidated |
| 831 | Bowfin | *Amia* | *calva* | 98.75 | verified |
| 832 | Bowfin | *Amia* | *calva* | 98.77 | verified |
| 833 | Not given | *Micropterus* | *salmoides; floridanus* | 99.40 | unvalidated |
| 834 | Bowfin | *Amia* | *calva* | 99.05 | verified |
| 835 | Largemouth Bass | *Micropterus* | *salmoides; floridanus* | 99.65 | unvalidated |
| 16349 | Not given | *Lepomis* | *gulosus* | 93.13 | unvalidated |
| 16401 | Largemouth Bass | *Micropterus* | *salmoides; floridanus* | 98.42 | unvalidated |
| 16402 | Dollar sunfish | *Lepomis* | *marginatus* | 99.26 | verified |
| 16403 | Redear | *Lepomis* | *microlophus* | 99.82 | verified |
| 16509* | Sailfin catfish | *Oreochromis* | *sp.* | 99.38 | |
| 16510* | Sailfin catfish | *Oreochromis* | *sp.* | 100.00 | |
| 16511 | Florida Gar | *Lepisosteus* | *oculatus; platyrhincus* | 99.09 | unvalidated |
| 16512 | Florida Gar | *Lepisosteus* | *oculatus; platyrhincus* | 99.26 | unvalidated |
| 16513 | Florida Gar | *Lepisosteus* | *oculatus; platyrhincus* | 98.89 | unvalidated |
| 16514 | Florida Gar | *Lepisosteus* | *oculatus; platyrhincus* | 98.90 | unvalidated |
| 16515 | Florida Gar | *Lepisosteus* | *oculatus; platyrhincus; osseus* | 99.45 | unvalidated |
| 16516 | Bowfin | *Amia* | *calva* | 98.49 | unvalidated |
| 16517 | Bowfin | *Amia* | *calva* | 92.03 | unvalidated |
| 16518 | Redear | *Lepomis* | *microlophus* | 99.82 | verified |
| 16519 | Redear | *Lepomis* | *microlophus* | 97.83 | unvalidated |
| 16520 | Gizzard Shad | *Dorosoma* | *cepedianum; anale* | 99.81 | unvalidated |
| 16521 | Gizzard Shad | *Dorosoma* | *cepedianum; anale* | 95.67 | unvalidated |
| 16522 | Warmouth | *Lepomis* | *gulosus* | 93.57 | unvalidated |
| 16523 | Warmouth | *Lepomis* | *gulosus* | 99.08 | verified |
| 16524 | Warmouth | *Lepomis* | *gulosus* | 97.42 | unvalidated |
| 16525 | Warmouth | *Lepomis* | *gulosus* | 97.78 | unvalidated |
| 16526 | Yellow bullhead | *Oreochromis* | *natalis* | 87.83 | unvalidated |
| 16527 | Tilapia sp (?Nile) | *Oreochromis* | *sp.; aureus; niloticus* | 99.81 | unvalidated |
| 16528* | Golden Shiner | *Oreochromis* | *sp.* | 93.19 | |

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|---|---|---|---|---|---|
| 16529* | Golden Shiner | *Lepomis* | *microlophus* | 97.25 | |
| 16530 | Bluegill | *Lepomis* | *macrochirus* | 100.00 | verified |
| 16531 | Bluegill | *Lepomis* | *macrochirus* | 98.73 | verified |
| 16532 | Bluegill | *Lepomis* | *macrochirus* | 99.27 | verified |
| 16533 | Bluegill | *Lepomis* | *macrochirus* | 98.96 | verified |
| 16534 | Bluegill | *Lepomis* | *macrochirus* | 99.13 | verified |
| 16535 | Redear | *Lepomis* | *microlophus* | 97.79 | unvalidated |
| 16536 | Redear | *Lepomis* | *microlophus* | 95.66 | unvalidated |
| 16537 | Redear | *Lepomis* | *microlophus* | 99.43 | verified |
| 18746 | Yellow bullhead | *Ameiurus* | *natalis* | 95.83 | unvalidated |
| 18747 | Mayan Cichlid (Whole specimen in Jar) | *Cichlasoma* | *urophthalmus* | 99.65 | verified |
| 18748 | Not given | *Oreochromis* | *aureus* | 100.00 | unvalidated |
| 18749 | Tilapia spp (?Nile) | *Oreochromis* | *aureus* | 100.00 | verified |
| 18750 | Mayan Cichlid (Whole specimen in Jar) | *Cichlasoma* | *urophthalmus* | 100.00 | verified |
| 18751 | Yellow bullhead | *Ameiurus* | *natalis* | 92.32 | unvalidated |
| 18752 | Longnose Gar | *Lepisosteus* | *osseus* | 98.11 | unvalidated |
| 18753 | Not given | *Fundulus* | *catenatus* | 89.41 | unvalidated |
| 18754 | Spotted Sunfish | *Lepomis* | *punctatus* | 99.63 | verified |
| 18755* | Seminole Killifish | *Lepomis* | *punctatus* | 99.43 | |
| 18756 | Spotted Sunfish | *Lepomis* | *punctatus* | 100.00 | verified |
| 18757 | Striped Mullet | *Mugil* | *cepahlus* | 100.00 | unvalidated |