Fish species that did not facilitate glochidia metamorphosis (number of trials, number of inoculated fish/number of surviving fish, range of days to rejection): longnose gar (*Lepisosteus osseus*) (2, 5/5, 3-12), American eel (*Anguilla rostrata*) (2, 6/5, 7-13), black bullhead (*Ameiurus melas*) (2, 11/11, 10), yellow bullhead (*Ameiurus natalis*) (1, 8/8, 8), tadpole madtom (*Noturus gyrinus*) (1, 3/3, 4), burbot (*Lota lota*) (1, 4/4, 9), common carp (*Cyprinus carpio*) (1, 1/1, 10), brassy minnow (*Hybognathus hankinsoni*) (1, 1/1, NR), northern redbelly dace (*Chrosomus eos*) (1, 12/12, NR), northern hogsucker (*Hypentilium nigricans*) (1, 2/2, 8), shorthead redhorse (*Moxostoma macrolepidotum*) (1, 5/5, 8), longear sunfish (*Lepomis megalotis*)* (1, 1/1, 24), logperch (*Percina caprodes*) (1, 10/8, 17), blackside darter (*Percina maculata*) (1, 4/4, 30), walleye (*Sander vitreus*) (2, 4/4, 8/13).

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The Hazards of DNA Barcoding, as Illustrated by the Pleurocerid Gastropods of East Tennessee

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Unusually high levels of intraspecific mtDNA sequence heterogeneity are not uncommonly reported in populations of pleurocerid gastropods sampled throughout the southeastern United States (Dillon & Frankis 2004, Dillon & Robinson 2009, Whelan & Strong 2016). The several hypotheses offered to account for this phenomenon (not mutually exclusive) include: great antiquity, mitochondrial introgression, pseudogenes, and cryptic speciation. Here we report a fresh example of striking mtDNA sequence heterogeneity in conspecific pleurocerid populations, controlled by a larger survey of allozyme variation at ten enzyme loci published by Dillon (2011).

Dillon (2011) sampled 30 to 50 individuals from each of 15 pleurocerid populations inhabiting East Tennessee and North Georgia: 9 populations of *Pleurocera clavaeformis*, 4 populations of *P. simplex*, and one population each of *P. carinifera* and *P. vestita*. To simulate a typical DNA barcoding approach, in the spring of 2011 we sampled single individuals from each of these 15 populations, cracking the shell and preserving the animal in absolute ethanol. Genomic DNA was isolated from an anterior portion of the foot muscle using a CTAB protocol. Then, an approximately 700 base-pair fragment of the mitochondrial COI gene was amplified via PCR using universal primers (Folmer et al. 1994) in a final volume of 25 μL. Reaction solutions and cycling protocols were slightly modified from those of Dillon & Robinson (2009) by

increasing $MgCl_2$ concentrations to 3 mM. PCR products were electrophoresed on 1% w/v agarose gels using GelRed, cleaned up with EXO/SAP, and cycle-sequenced in half-reactions of ABI Big Dye Terminator at the Georgia Genomics Facility on an ABI 3730 sequencer. Sequences were obtained in both directions multiple times, resulting in a total of four to five reads per individual.

Sequence alignments were performed using CodonCode Aligner. After trimming low-quality regions from the ends of the sequences, our alignment contained a total of 591 bp, with individual sequences ranging in length from 488 to 591 bp. Individual base pairs with sequence quality scores less than 20 were recoded as ambiguities before analysis. A matrix of raw pairwise percent sequence differences was calculated in R using the 'ape' package. We compared a total of 88 different models of molecular evolution using jModelTest ver. 0.1.1. We then used the best model from this analysis (HKY +I +G), with maximum likelihood parameter estimates and constructed a bootstrapped maximum-likelihood phylogeny (2000 replicates) in PAUP ver. 4.0b.

Figure 1 shows that only four of the 14 nodes in the maximum-likelihood phylogeny were supported by bootstrap values of 60% or greater. Setting a maximum sequence divergence within nominal species at 5%, only one (six-population) cluster of *P. clavaeformis* would appear conspecific, and setting the maximum divergence at 10% only added a seventh *clavaeformis* population to the cluster, leaving two additional *clavaeformis* populations united as a spurious second species. And even at 10% sequence divergence, none of our four control populations of *P. simplex* was depicted as conspecific with any other, their pairwise interpopulation divergences ranging from 16% (S6/S7) to 23% (S2/S8). Indeed, *simplex* sample S2 demonstrated greater sequence similarity with several *clavaeformis* samples (e.g. 85% with C6) than with any conspecific.

The use of DNA barcoding data to define species boundaries represents a return to the quality of 19th-century typological thinking long discredited by the modern synthesis. The striking contrast in resolving power between the single-character, single-individual results depicted in Figure 1 and the 10-gene, 450-individual analysis of Dillon (2011) should serve as a warning to those who imagine otherwise.

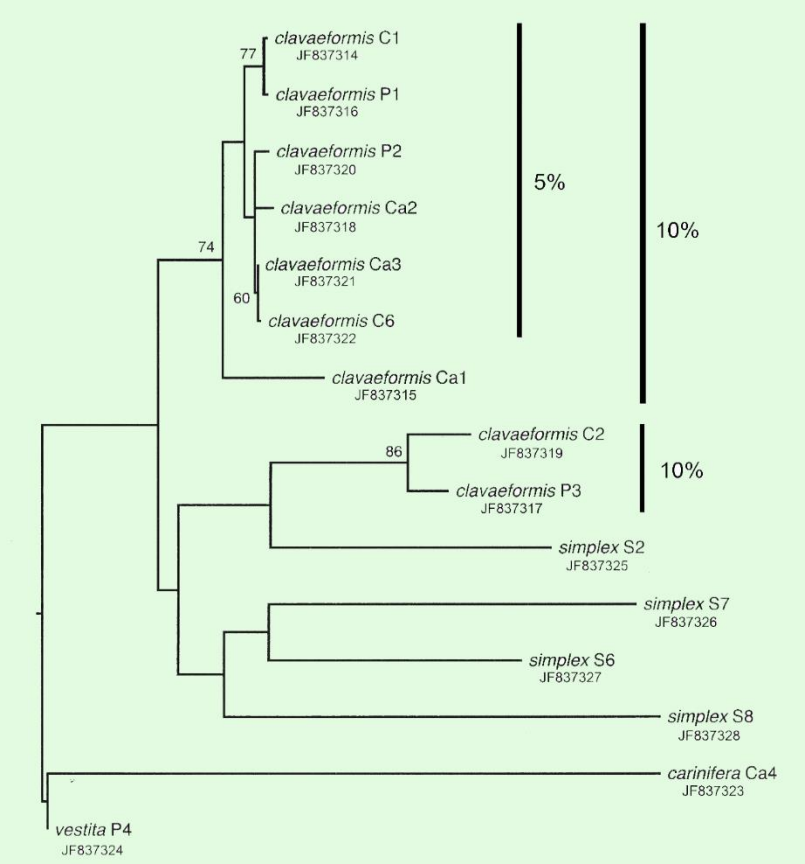

Figure 1. Maximum-likelihood phylogeny of mitochondrial COI genes sampled from the 15 pleurocerid populations of Dillon (2011), with GenBank accession numbers. Bootstrap support values are shown on nodes if at least 60% of the replicates supported the grouping.

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Ecophenotypes: When and Where They Do and Do Not Occur

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It is generally recognized among those of us working with unionids that some species develop distinctive ecophenotypes in different environments. One classic example occurs in Wabash Pigtoe (*Fusconaia flava*) that produces larger, heavier-shelled, inflated forms in larger rivers, but has smaller, thinner, more-compressed morphs in headwater streams (Figure 1). In other cases, sculpturing may be far more pronounced in large, flowing waterbodies and sometimes less so in small streams. Similarly, populations in natural lakes may be morphologically distinct from corresponding individuals in big-river environments. W.R. Haag (2012. North American Freshwater Mussels. Cambridge University Press, New York.) gave an excellent summary and discussion of these situations.

However, not all unionids produce distinctive ecophenotypes even in seemingly different environments. Queries recently crossed my desk from folks seeking images of specific Texas unionid forms from reservoirs, rivers, and small streams. But in point of fact, few such ecophenotypic differences between water-body types actually occur in Texas.

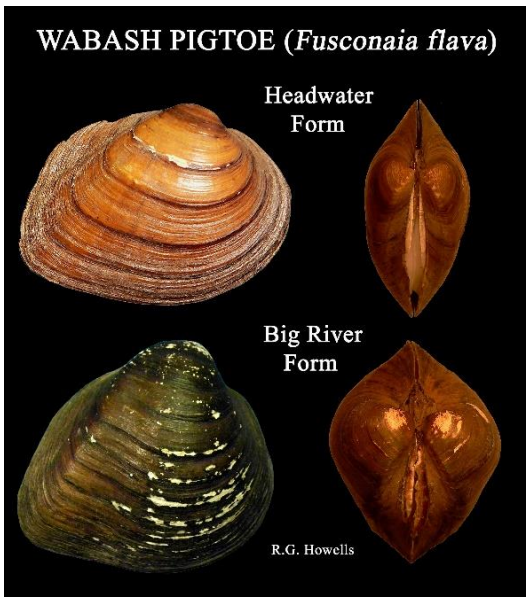

Figure 1. Wabash Pigtoe (*Fusconaia flava*) is an example of a unionid that has distinctive ecophenotypes from smaller headwater streams and larger rivers throughout much of its range. Although this species does occur in Texas, no major ecophenotypic distinctions have been recognized across the state.