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A DNA-barcoding approach to identifying juvenile freshwater mussels (Bivalvia:Unionidae) recovered from naturally infested fishes

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Abstract. We developed a multilocus deoxyribonucleic acid (DNA)-barcoding approach to identify newly transformed juvenile mussels collected from naturally infested fishes in a federally protected waterway that is home to a diverse mussel community, the St Croix River (Minnesota/Wisconsin, USA). We used new and publicly available data downloaded from GenBank to build reference databases for identified adult mussels. We assessed the efficacy of the mitochondrial loci cytochrome oxidase c subunit I (COI) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) for DNA barcoding. We concluded that the barcoding gap between average intra- and interspecific genetic distances is wider for ND1 than for COI, but both loci perform well for species identification in character-based phylogenetic analyses. Almost every species formed a monospecific clade with high bootstrap and posterior-probability support. We obtained newly transformed juvenile mussels by collecting individuals of 3 different fish species that were infested with unionid larvae. We held the fish in aquaria until the mussels emerged naturally. We then extracted DNA and sequenced our loci of interest. When sequences from the juveniles were included in phylogenetic analyses, they grouped with single species (or, in one case, a pair of closely related species) with high bootstrap and posterior-probability support. Identifying juveniles using morphology alone is difficult and, in some cases, impossible. Therefore, our approach will be useful to researchers interested in the relationship between unionid mussels and their fish hosts.

Key words: Unionidae, mussel, barcoding, mitochondrial DNA.

DNA barcoding is a method for species identification based on the nucleotide sequence of a short stretch of mitochondrial deoxyribonucleic acid (mtDNA). The method has been proposed as a universal approach applicable across all organisms and accessible to a wide range of researchers (Hebert et al. 2003). A biologist might be an expert on taxonomy and species identification within 1 or a few groups of organisms but might not be knowledgeable enough to identify species reliably across an entire community. The level of expertise of taxonomists making determinations of species identity as part of national stream surveys influenced the accuracy of the data obtained in those surveys (Stribling et al. 2008), and DNA barcoding could be a useful tool to address this issue (Kenney et al. 2009). The utility of the method has been established in some key groups of benthic invertebrates, such as nematodes (Bhadury et al. 2006), mayflies (Ball et al. 2005), and chironomid midges (Carew et al. 2007, Pfenninger et al. 2007). DNA barcoding has also been used to address questions about species-level taxonomy in mayflies (Alexander et al. 2009) and to distinguish among mussel species in the genus *Pleurobema*, whose shells lack significant sculpture or other distinguishing features (Campbell et al. 2008).

To date, much research has focused on the performance of the DNA-barcoding method and

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identification of appropriate loci for use in particular groups of organisms (e.g., Kress et al. 2005, Meyer and Paulay 2005, Linares et al. 2009); many fewer studies to date have used the barcoding technique to answer ecological or evolutionary questions (Golding et al. 2009). We describe a multilocus DNA-barcoding approach to the identification of recently transformed juvenile unionid mussels, which are, in some cases, impossible to identify to species based on morphology alone. Identification of these juveniles is crucial for conservation purposes and for the study of coevolutionary relationships between parasitic mussel larvae and the fishes they infest. We built reference-sequence data sets for 2 mitochondrial loci from adult mussels of known identity and successfully used our method to identify juveniles recovered from multiple species of naturally infested fishes in the St Croix River (Minnesota/Wisconsin, USA), a federally protected river that is home to a well-studied (Hornbach 2001) and diverse community of 40 mussel species.

Members of the mussel family Unionidae occur in aquatic environments worldwide and play key ecological roles. They influence water chemistry and clarity, serve as food for predators, such as otters and muskrat, and provide physical structure in the local habitat that is important to other organisms (Strayer 2008). Many of these species face severe population decline: ~70% are listed as endangered, threatened, or of special concern (Williams et al. 1993, Lydeard et al. 2004, Strayer 2008).

With few exceptions, unionid mussels have an unusual life history. The larvae (glochidia) are obligate parasites of fishes or amphibians and use their host for dispersal during development into the juvenile stage. Some mussel species rely on a single host, whereas others are generalists (Strayer 2008). Many mussel species have spectacular strategies for attracting hosts, such as the production of lure structures shaped like small fishes or blackfly larvae (Barnhart et al. 2008). Because unionid mussels depend on their vertebrate hosts, any conservation efforts aimed at a particular mussel species must take into account the host or hosts as well (McLain and Ross 2005, Allen et al. 2007).

Many studies have focused on identifying suitable hosts of mussels, and most were based on infestation trials done in the laboratory (e.g., Jones et al. 2004). However, these studies cannot determine the relative importance of fish hosts in the wild, which can differ substantially from laboratory preferences because of factors such as differential habitat use and behavioral characteristics of both host fish and mussel (Martel and Lauzon-Guay 2005). For example, investigators using laboratory trials have demonstrated that some endemic North American unionids have the ability to infest and metamorphose on goldfish (*Carassius auratus*), green swordtail (*Xiphophorus hellerii*), and guppies (*Poecilia reticulata*), none of which is native to North America (Watters and O'Dee 1998). Identification of juvenile mussels that have successfully metamorphosed on wild-infested fishes is essential for understanding the relationship between mussels and their fish hosts in natural settings. However, this task is complicated by the difficulty that may be involved in identifying recently released juveniles reliably to species based on morphology alone (Hoggarth 1992, Weiss and Layer 1995). At this stage of their life history, these animals are tiny (typically 200–300 μm in diameter) and display few diagnostic morphological characters.

A handful of studies have been done to create molecular identification keys based on restriction fragment length polymorphisms (RFLPs) for unionids (White et al. 1996, Gerke and Tiedemann 2001, Gustafson and Iwamoto 2005, Kneeland and Rhymer 2007, 2008). These studies have successfully produced useful identification keys, but this approach has several limitations. For example, creating keys for species-rich areas can be problematic because identifying a unique digestion pattern for every species present is difficult when many closely related species share the same geographic range. In addition, a different key must be created for each study area because species assemblages differ. This necessity creates a significant hurdle regional researchers must surmount before beginning studies in their location of interest.

Sequence-based DNA barcoding for the identification of adult bivalves has been undertaken. The early results indicate low levels of genetic variation within species and high levels of variation between species for key mitochondrial loci including cytochrome oxidase c subunit I (COI), the proposed standard barcoding locus for animals, and nicotinamide adenine dinucleotide dehydrogenase 1 (ND1) (Mikkelsen et al. 2007, Campbell et al. 2008). However, researchers have reported difficulty extracting DNA from juvenile mussels (Kneeland and Rhymer 2007, 2008), and perhaps for this reason, the efficacy of DNA barcoding for diagnosing host–parasite relationships between fish and mussels in the wild has not been explored. We present a DNA-barcoding method for identifying juvenile mussels with COI and ND1 sequences that has potential utility in development of conservation plans for endangered unionids and in studies of the coevolution of mussels and their hosts.

Methods

Evaluation of published sequences

We downloaded all previously published sequences for ND1 and COI for all unionid mussel species native to the St Croix River. Several published sequences were eliminated from our data sets because we had a priori reason to suspect that they did not represent the species with which they were associated. One previously published ND1 sequence for *Cyclonaias tuberculata* (voucher INHS 20590 from Serb et al. 2003) appeared as sister to 1 published and 1 new sequence from *Potamilus alatus* in initial phylogenetic analyses. The anomalous nature of this sequence was noted by Campbell et al. (2005). Three published sequences from *Obliquaria reflexa* (Roe and Lydeard 1998, Roe et al. 2001) were placed in a different part of the phylogenetic tree (within Quadrulini) than our new *O. reflexa* sequences (within Lampsilini). This unexpected result probably reflected a mislabeling of specimens associated with the published sequences (K. Roe, personal communication). The placement of our new sequences reflects current taxonomy proposed by Graf and Cummings (2007). In addition, we removed several sequences based on information provided by an anonymous referee of an earlier version of this paper: *Quadrula quadrula* ND1 and COI sequences from voucher UAUC 145 are actually from *Quadrula nobilis* (see also Krebs et al. 2003), a COI sequence from *Fusconaia flava* accession DQ298533 is from *Pleurobema cordatum*, a COI sequence from *F. flava* accession DQ298531-2 probably is from *Fusconaia hebetata*, and 2 anomalous COI sequences for *Lasmigona compressa* accession AF156503 and AF093846 probably are from *Lasmigona costata*.

Specimen collection: adult mussels

We obtained clips of mantle tissue from vouchers in the collections of the Bell Museum of Natural History and from live animals in the field, primarily in the St Croix River, but also in the upper Mississippi River, Minnesota; locality information is available from the authors. We obtained nonlethal samples from live animals with tools (nonserrated tweezers, dissecting scissors, flathead screwdriver) that had been sterilized in 95% ethanol. We propped the valves of the mussel open 1 cm by inserting and turning the screwdriver. We held the valves open and removed a 1 × 1.5-cm section of mantle by grasping a small section of the mantle tissue with tweezers, and then excising tissue with scissors. We preserved tissue clips in 95% ethanol. We returned the mussel to the river and placed it back into the sediment with the foot facing downward. Voucher photographs for adult mussels are available through MorphoBank (morphobank.org; project 376).

Specimen collection: fishes and juvenile mussels

We collected 3 species of fish from the St Croix River: walleye (*Sander vitreus*), freshwater drum (*Aplodinotus grunniens*), and spotfin shiner (*Cyprinella spiloptera*). We collected walleye and freshwater drum with a boom electrofisher. We examined individual walleye and drum for glochidial infestation on the gills and fins and retained fishes with >10 attached glochidia. Spotfin shiners were collected using a seine net.

We transported fish in aerated coolers to the University of Minnesota and placed them in aerated flow-through aquaria. We held spotfin shiners in groups of 30 to 40 individuals/40-L aquarium (90 individuals total). We held walleye (5 total) and drum (15 total) in groups of 2 to 3 individuals/100-L aquarium. We fed frozen brine shrimp and bloodworms to spotfin shiners and angle worms to walleye and drum. We waited for transformed juveniles to fall from the fish by natural means rather than attempting to excise them from the fish. We collected released juveniles by siphoning material from the floors of aquaria, and we poured siphonate through a 45-μm-mesh sieve. We collected sieved particulates in a Petri dish and examined them under a dissecting microscope. We identified as juveniles those mussels with a moving foot and valve growth beyond the margin of the glochidial shell or with 2 clearly apparent adductor muscles. We subsequently pipetted each juvenile into a 1.5-mL Eppendorf tube filled with 95% ethanol. Voucher photographs of juvenile mussels are available through MorphoBank (morphobank.org, project 379).

DNA extraction, polymerase chain reaction, and sequencing

We followed the manufacturer's protocol to extract DNA from adult mussel tissue with the DNEasy Kit from Qiagen (Valencia, California). We extracted DNA from juvenile mussels with the same kit, but we used a modified protocol (Kneeland and Rhymer 2007). For polymerase chain reaction (PCR) and sequencing, we used COI primers 5′-GTTCCACAAATCATAAGGATATTGG-3′ and 5′-TACACCTCAGGGTGACCAAAAAACCA-3′ (Campbell et al. 2005) and ND1 primers 5′-TGGCAGAAAAGTGCATCAGATTAAAGC-3′ and 5′-CCTGCTAAGGCAAGTGTACT-3′ (Serb et al. 2003). We prepared a mix

consisting of 13.25 μL of double-distilled H_2O, 5.0 μL of 5× PrimeSTAR® buffer, 0.25 μL of PrimeSTAR® HS DNA polymerase (TaKaRa Bio, Inc., Oksu, Japan), 2.0 μL of 2.5-mM deoxyribonucleotide triphosphate (dNTP), 2.5 μL of 2.5-μM primers, and 2 μL of template in a total reaction volume of 25 μL. Amplifications were done in an ABI 2720 Thermal Cycler (Applied Biosystems, Carlsbad, California). The PCR thermal profile for COI was: 95°C (2 min), 35× (95°C [30 s], 54°C [30 s], 72°C [30 s]), 72°C (2 min); hold at 4°C. The PCR thermal profile for ND1 was: 98°C (2 min), 30× (98°C [10 s], 57°C [5 s], 72°C [75 s]), 72°C (2 min); hold at 4°C. We purified PCR products using Qiagen's QIAquick PCR Purification kit (manufacturer's protocol) and quantified them on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, Delaware).

Sequencing reactions were done either at the University of Minnesota DNA Sequencing and Analysis Facility (Saint Paul, Minnesota) or at Macalester College. In-house sequencing was done in ½ volume but otherwise according to manufacturer's protocols using the Beckman Coulter GenomeLab™ DTCS Quick Start Kit (Beckman Coulter, Brea, California). Sequencing products were purified using Agencourt® CleanSEQ® magnetic beads according to the manufacturer's protocol (Beckman Coulter), and sequencing was done with a CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

Data analysis

We assembled consensus contig sequences from forward and reverse sequences using CodonCode Aligner (v.2.0.6; Codon Code Corporation, Dedham, Massachusetts). We constructed alignments in MacClade (Maddison and Maddison 2001). We submitted all new sequences to GenBank (Appendix; available online from: http://dx.doi.org/10.1899/10-004.1.s1). We calculated inter- and intraspecific distances for each data set with MEGA version 4.0 (Tamura et al. 2007) using the Kimura-2-parameter model with pairwise deletions when sequences were incomplete. Standard error was assessed using 500 bootstrap replicates.

We followed the recommendations of DeSalle et al. (2005) and used character-based phylogenetic methods to identify juvenile mussels. We considered only a subset of the available sequences in these phylogenetic analyses because of the very large volume of data available for certain species (e.g., 80 ND1 sequences for *Lampsilis cardium*). We used a maximum of 15 sequences per species from adult specimens (chosen to maximize the genetic diversity within each species) and all sequences from juvenile mussels for each locus. We constructed phylogenetic trees using both parsimony and Bayesian methods. We did parsimony analyses in PAUP* 4.0b10 (Swofford 2003) and assessed support from 100 bootstrap replicates. We did Bayesian analyses in MrBayes 3.1 (Huelsenbeck and Ronquist 2001). We used ModelTest 3.7 (Posada and Crandall 1998) to choose the model of sequence evolution under the Akaike Information Criterion as recommended by Posada and Buckley (2004). The model selected for analysis of the ND1 dataset was Tamura Nei + Invariant + Γ (TrN+I+G). We substituted General Time Reversible + Invariant + Γ (GTR+I+G) as the closest match supported in MrBayes (lset nst = 6, rates = invgamma). The model selected for analysis of COI was Transition Model + Γ (TIM+G), and we chose GTR+G as the closest match supported in MrBayes (lset nst = 6, rates = gamma). We ran both analyses for 3,000,000 generations with sampling every 1000th generation and discarded the first 300,000 generations as burn-in.

Results

We successfully sequenced a 591-basepair (bp) section of ND1 for 66 adult mussels and a 585-bp section of COI for 47 adult mussels (Appendix). We attempted to sequence an initial pool of 22 juvenile mussels for both loci. From that pool, we were able to sequence both loci for 8 juveniles, only ND1 for 13 juveniles, and only COI for 1 juvenile. After this initial screen, we focused our efforts on sequencing the ND1 locus from juveniles. Ultimately, ND1 was sequenced for 31 juveniles and COI was sequenced for 10 juveniles (Appendix).

For the COI locus, average intraspecific distances calculated using the Kimura-2-parameter model ranged from 0.001 to 0.020 (mean = 0.008; Table 1, Fig. 1). Interspecific distances ranged from 0.010 (*A. ligamentina* vs *L. higginsii*) to 0.237 (mean = 0.150; Fig. 1). For the ND1 locus, average intraspecific distances calculated using the Kimura-2-parameter model ranged from 0 to 0.023 (mean = 0.008; Table 1, Fig. 2). Interspecific distances ranged from 0.015 (*L. higginsii* vs *A. ligamentina*) to 0.356 (mean = 0.246; Fig. 2).

Phylogenetic analyses of the COI data set retrieved monophyly of every species except *L. higginsii* and *A. ligamentina*, which formed a clade within which the species were paraphyletic with respect to each other (Fig. 3). Every other species was retrieved as a monophyletic group with >95% posterior probability (pp) and >90% bootstrap support (bs) (bs values not shown in Fig. 3 to save space).

TABLE 1. Mean (SE) intraspecific distances (d) assessed using 500 bootstrap replicates based on the Kimura-2-parameter model calculated with pairwise deletion. *n* = number of individuals, COI = cytochrome oxidase c subunit I, ND1 = nicotinamide adenine dinucleotide dehydrogenase subunit 1.

	COI		ND1	
Species	*n*	d (SE)	*n*	d (SE)
Actinonaias ligamentina	5	0.007 (0.002)	5	0.007 (0.003)
Alasmidonta marginata	1	NA	2	0.003 (0.002)
Amblema plicata	43	0.005 (0.001)	3	0.009 (0.003)
Anodontoides ferussacianus	0	NA	4	0.002 (0.001)
Arcidens confragosus	0	NA	2	0.002 (0.002)
Cyclonaias tuberculata	2	0.020 (0.006)	4	0.009 (0.003)
Ellipsaria lineolata	3	0.005 (0.002)	4	0.006 (0.002)
Elliptio crassidens	5	0.010 (0.003)	5	0.011 (0.003)
Elliptio dilatata	4	0.012 (0.003)	3	0.006 (0.002)
Epioblasma triquetra	14	0.002 (0.001)	2	0 (0)
Fusconaia ebena	3	0.005 (0.003)	1	NA
Fusconaia flava	17	0.007 (0.002)	2	0.01 (0.004)
Lampsilis cardium	3	0.012 (0.004)	80	0.006 (0.002)
Lampsilis higginsii	8	0.009 (0.003)	2	0.019 (0.006)
Lampsilis siliquoidea	3	0.002 (0.002)	4	0.002 (0.002)
Lampsilis teres	3	0.011 (0.004)	1	NA
Lasmigona complanata	1	NA	2	0 (0)
Lasmigona compressa	1	NA	3	0.001 (0.001)
Lasmigona costata	2	0.008 (0.003)	4	0.011 (0.003)
Leptodea fragilis	4	0.014 (0.004)	3	0.002 (0.002)
Ligumia recta	4	0.017 (0.005)	2	0.009 (0.004)
Megalonaias nervosa	1	NA	3	0.007 (0.003)
Obliquaria reflexa	3	0.010 (0.004)	4	0.017 (0.004)
Obovaria olivaria	6	0.008 (0.002)	3	0.008 (0.003)
Pleurobema sintoxia	2	0.001 (0.001)	2	0.014 (0.005)
Potamilus alatus	5	0.003 (0.002)	2	0.002 (0.002)
Potamilus ohiensis	2	0.003 (0.002)	2	0.002 (0.002)
Pyganodon grandis	7	0.005 (0.002)	3	0.01 (0.003)
Quadrula metanevra	2	0.005 (0.003)	5	0.019 (0.003)
Quadrula nodulata	2	0.002 (0.002)	4	0.012 (0.003)
Quadrula pustulosa	1	NA	78	0.01 (0.002)
Quadrula quadrula	4	0.015 (0.004)	6	0.014 (0.004)
Strophitus undulatus	5	0.018 (0.004)	4	0.007 (0.002)
Toxolasma parvus	10	0.006 (0.001)	2	0.023 (0.006)
Tritogonia verrucosa	3	0.006 (0.002)	4	0.006 (0.002)
Truncilla donaciformis	1	NA	2	0.002 (0.002)
Truncilla truncata	2	0.003 (0.002)	2	0.005 (0.003)
Utterbackia imbecillis	0	NA	2	0.002 (0.002)
Average		**0.008**		**0.008**

Phylogenetic analyses of the ND1 data set retrieved monophyly of every species in both Bayesian and parsimony analyses with the exception of *L. higginsii* and *A. ligamentina*, which formed a clade within which the species were paraphyletic with respect to each other (Fig. 4). *Quadrula pustulosa* was retrieved as monophyletic, but support was low in both Bayesian analyses (65% pp) and parsimony analyses (<50% bs) (bs values not shown in Fig. 4 to save space). Every other species was retrieved as a monophyletic group with >95% pp and >90% bs, except *Truncilla truncata*, which received >95% pp but only 74% bs in parsimony analyses.

Thirty-one juveniles fell in monospecific clusters with >95% pp in analyses of the ND1 data set, and 10 juveniles retrieved from walleye formed a paraphyletic grade with *L. higginsii* and *A. ligamentina* (Fig. 4). All 10 sequenced juveniles were grouped in monospecific clades with ≥95% pp in the analysis of the COI dataset (Fig. 3).

Eight of the 9 juveniles retrieved from spotfin shiner minnows were identified as *F. flava* based on both COI sequences and ND1 sequences (100% pp and 98% bs for the clade with ND1; 100% pp and 99% bs with COI) (Figs 3, 4). The sequence for COI of the

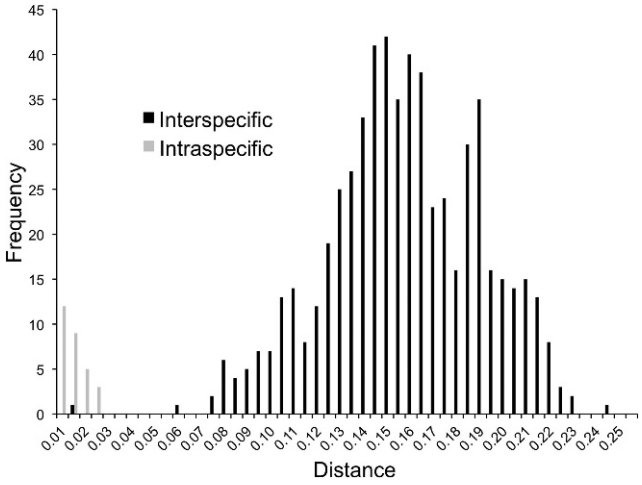

FIG. 1. Frequency distribution of Kimura-2-parameter distances for the cytochrome oxidase c subunit I (COI) locus. The small outlier interspecific distance is for a comparison of *Actinonaias ligamentina* and *Lampsilis higginsii*.

9th individual indicated identification as *Pleurobema sintoxia* (100% pp, 100% bs; Fig. 3).

ND1 was successfully sequenced for 12 juveniles retrieved from freshwater drum. Four juveniles were identified as *Ellipsaria lineolata* (100% pp and 100% bs), 2 were identified as *Leptodea fragilis* (100% pp, 100% bs), 2 as *Potamilus ohiensis* (100% pp, 100% bs), 4 as *T. truncata* (95% pp, 74% bs) (Fig. 4). COI was sequenced successfully for 1 additional juvenile from drum. The individual was identified as *T. truncata* (100% pp, 100% bs) (Fig. 3).

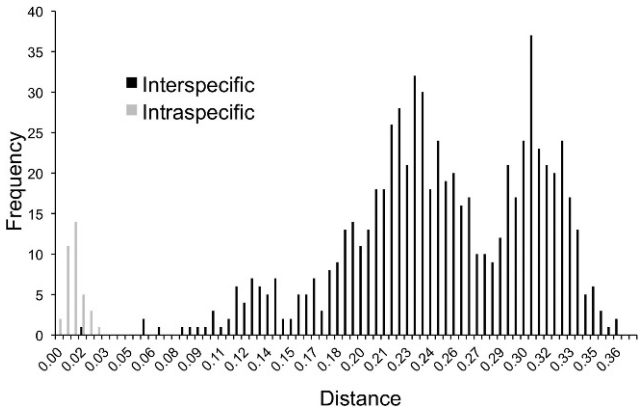

FIG. 2. Frequency of Kimura 2-parameter distances for the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) locus. The small outlier interspecific distance is for a comparison of *Actinonaias ligamentina* and *Lampsilis higginsii*.

ND1 was sequenced successfully for 11 juveniles retrieved from walleye. Ten were either *L. higginsii* or *A. ligamentina* (97% pp, 99% bs), and 1 was identified as *L. cardium* (100% pp, 100% bs) (Fig. 4).

Discussion

The primary goal of our project was to test the efficacy of DNA barcoding as a method for identifying newly transformed juvenile unionid mussels recovered from naturally infested fishes. We have demonstrated that the method performs well and that it will have utility for increasing our understanding of mussel life cycles. Even though the number of juveniles included in this project was small, our pilot study has identified previously unknown or unconfirmed natural hosts for several mussel species.

Efficacy of DNA barcoding for identifying unionids

Ideally, a barcoding locus should show clear separation between the distributions of intra- and interspecific distances, i.e., a barcoding gap (Meyer and Paulay 2005). We found minimal overlap between the ranges of intra- and interspecific distances in the ND1 and COI data sets for St Croix River unionids (Figs 1, 2) except for the very closely related species pair *A. ligamentina* and *L. higginsii*. This pattern could reflect very recent divergence of the 2 species or mitochondrial introgression caused by hybridization. The possibility of hybridization could be investigated using a nuclear locus, such as the internal transcribed spacer region (ITS), which has been used for species identification in previous studies (Campbell et al. 2008). *Lampsilis higginsii* is an endangered species, so the possibility of hybridization with the more-common *A. ligamentina* is of great interest. Hebert et al. (2004) proposed that a 10× difference between intra- and interspecific distances is desirable in a barcoding locus. Both of our data sets satisfied this criterion. With ND1, we found an average intraspecific distance of 0.008 and an average interspecific distance of 0.246, and with COI, we found an average intraspecific distance of 0.008 and an average interspecific distance of 0.150.

Based on our genetic-distance data, both ND1 and COI have excellent potential for species-level identification of unionids. However, ND1 presents a larger difference between average intra- and interspecific distances than COI, the proposed standard barcoding locus (Hebert et al. 2003). ND1 presented the lowest intraspecific and highest interspecific distances in a comparison of ND1, COI, and the nuclear gene ITS1 for use in DNA barcoding of freshwater mussels in the upper Coosa basin of the southeastern US

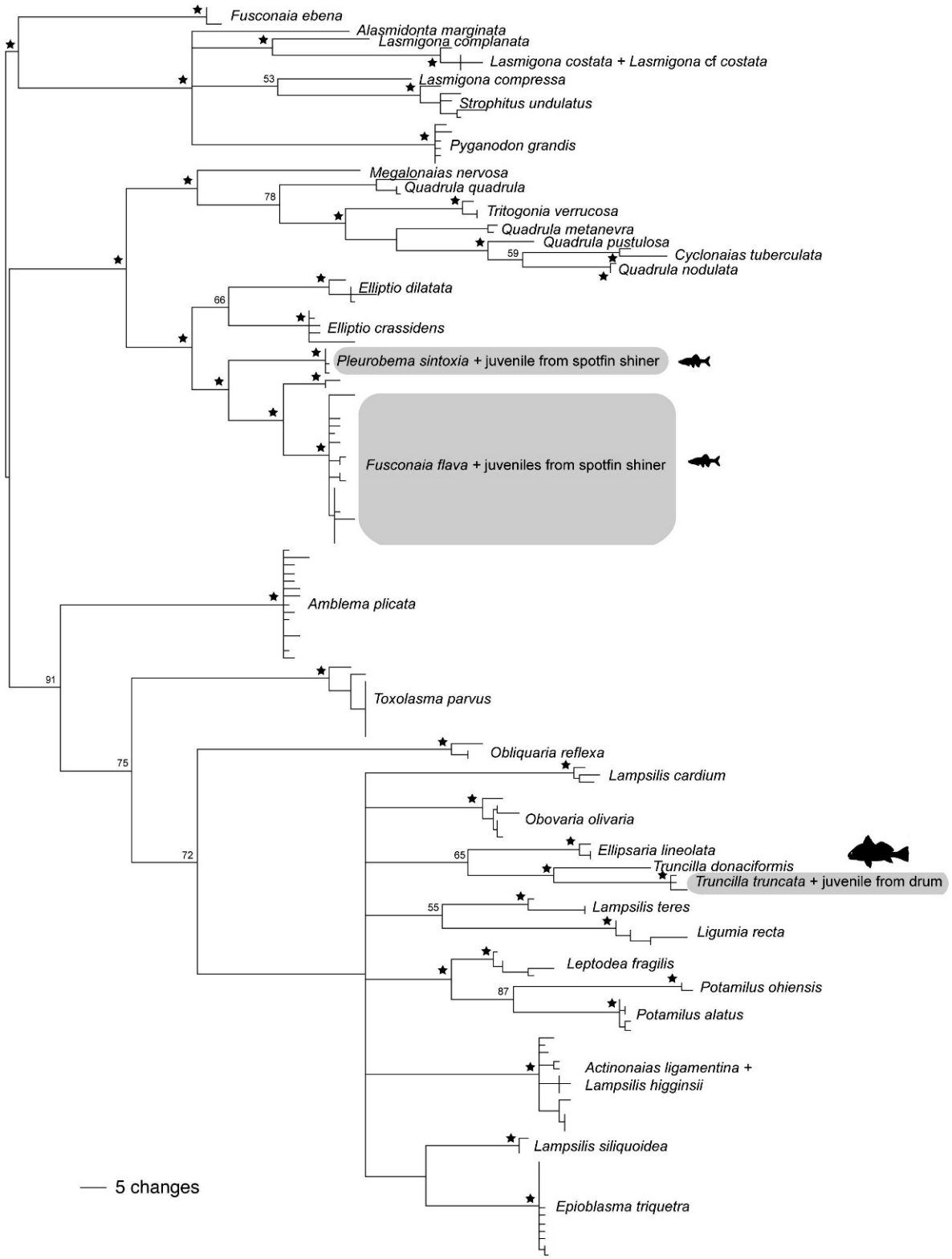

FIG. 3. Phylogeny for St Croix River unionids based on Bayesian analysis of cytochrome oxidase c subunit I (COI) data set. Stars indicate $\geq$95% posterior probability. Gray boxes indicate clades containing juvenile mussels retrieved from naturally infested fishes. Associated icons indicate fish species.

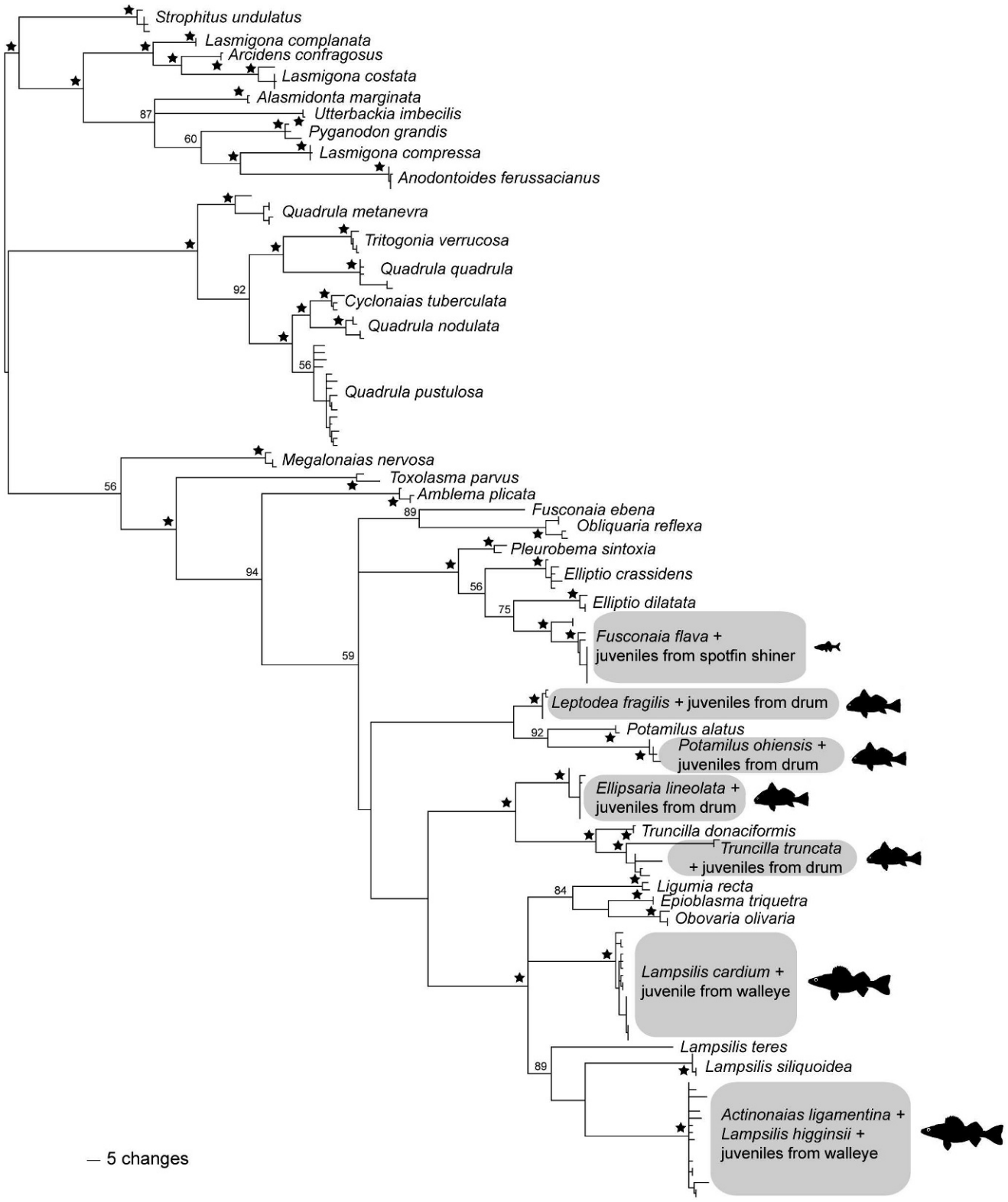

FIG. 4. Phylogeny for St Croix River unionids based on Bayesian analysis of nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) data set. Stars indicate ≥95% posterior probability. Gray boxes indicate clades containing juvenile mussels retrieved from naturally infested fishes. Associated icons indicate fish species.

(Campbell et al. 2008). Rach et al. (2008) demonstrated the utility of ND1 for barcoding Odonata, and this locus might prove useful across many different animal groups. We also found that ND1 sequence data generally were easier to obtain and of higher quality than COI sequence data. Our ND1 primers had a higher rate of successful amplification, and sequence data generally were cleaner.

The only cases of paraphyly in our phylogenetic analyses involved closely related *A. ligamentina* and *L. higginsii*, which were paraphyletic in analyses of both COI and ND1 data. We were unable to obtain tissue samples for 3 species that are rare in the St Croix River (*Anodonta suborbiculata*, *Quadrula fragosa*, and *Simpsonaias ambigua*). However, for every other unionid species in the St Croix River, we are confident that our reference data sets provide robust identification of juvenile mussels.

Mussel–host relationships

Sequence data obtained from juvenile mussels allowed us to identify 4 different species that successfully transformed on freshwater drum: *E. lineolata, L. fragilis, P. ohiensis*, and *T. truncata*. Drum are molluscivores that are thought to be a host for a variety of mussel species that lack conspicuous mantle lures (Barnhart et al. 2008). Drum are naturally infested with *E. lineolata*, *L. fragilis, P. ohiensis*, and *T. truncata* (Wilson 1916, Howard and Anson 1922), and laboratory host-suitability trials have demonstrated that drum facilitate metamorphosis of *E. lineolata* and *P. alatus* glochidia (Howard 1914, Brady et al. 2004, Rudh et al. 2007). Our study is the first to demonstrate that drum do in fact facilitate metamorphosis of *L. fragilis* glochidia.

Our study is the first to demonstrate that spotfin shiner is a natural host for *F. flava* (8 of 9 juvenile mussels retrieved from spotfin shiner were identified as *F. flava*). O'Dee and Watters (2000) demonstrated that creek chubs (*Semotilus atromaculatus*) and silver shiners (*Notropis photogenis*) facilitate *F. flava* glochidia metamorphosis in the laboratory, a result suggesting that this mussel might use a variety of cyprinid species under natural conditions. The 9th juvenile retrieved from spotfin shiner was identified as *Pleurobema sintoxia*. Laboratory trials have shown that the spotfin shiner can act as a host for this species (Hove et al. 1997).

One of the juveniles recovered from walleye was identified as *L. cardium*, a result that confirmed findings of laboratory infestation trials by Waller et al. (1985). Ten of the 11 juveniles recovered from walleye were probably *A. ligamentina*, but also could have been the federally endangered species *L. higginsii*. Previous studies of the host relationships of both mussels do not help specify the identification. Walleye is a potential host for *L. higginsii* (Sylvester et al. 1984, Waller and Holland-Bartels 1988), and many laboratory and natural-parasitism studies suggest that *A. ligamentina* is a generalist (Mussel/Host Database for the Molluscs Division of the Museum of Biological Diversity at the Ohio State University; http://128.146.250.235/MusselHost/). Both *A. ligamentina* and *L. higginsii* occur where the walleye used in our study were collected, but *A. ligamentina* is much more common (Hornbach 2001). In the future, data from a nuclear locus, such as the ITS region, or faster-evolving regions, such as microsatellites, might prove effective in distinguishing these 2 species.

Future directions: an integrated approach to identifying juvenile mussels

We have demonstrated the efficacy of DNA barcoding for identifying juvenile mussels, but we think that morphological methods should not be abandoned in favor of a purely genetic approach. Rather, the 2 approaches should be integrated in a synergistic manner. DeSalle et al. (2005, p. 1908) argued for a combined approach to species identification in general, stating that "[a] barcode should incorporate diagnostic characters from both the classical morphological approach and from the newer molecular approaches; one without the other misses the synergy that an integrated taxonomy is capable of attaining."

Juveniles from many unionid species cannot be distinguished with morphology alone, but morphology is adequate in some cases. For example, Miller (1999) used scanning electron microscopy (SEM) and found both qualitative and quantitative differences that enabled accurate differentiation and identification of the glochidia of 8 amblemine species from the St Croix River. Kennedy and Haag (2005) measured shell characteristics of glochidia from 21 species occurring in the Sipsey River, Alabama, and were able to correctly classify 72 to 79% of individuals to the species level with discriminant function analysis. Therefore, using molecular and morphological methods in tandem, the sort of holistic approach to mussel identification advocated by Jones et al. (2006), has great potential utility. Environmental SEM, which does not require coating specimens in metal, can be used to generate an SEM image of a juvenile mussel, and DNA can then be extracted and sequenced from that individual (SLB, NWJ, and MCH, personal experience). A hierarchical protocol for identifying

juvenile unionids that begins with morphology and relies on DNA barcoding when necessary may be the most efficient and effective method for identifying these unique and imperiled animals.

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