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RESEARCH NOTE

# A molecular identification key for freshwater mussel glochidia encysted on naturally parasitized fish hosts in Maine, USA

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The freshwater mussel (Bivalvia: Unionidae) fauna of North America is the richest of any continent, yet over 70% of these molluscs are listed as 'endangered', 'threatened' or of 'special concern' (Williams *et al*., 1993). Declines in richness and abundance at local and regional scales have continued largely due to anthropogenic activities (Nedeau, McCollough & Swartz, 2000). With few exceptions, the larvae of freshwater mussels, called glochidia, are obligate parasites of specific fish species, and mussel dispersal occurs primarily while glochidia are attached to the gills, fins or scales of a host fish. Their dependence on suitable host fish to metamorphose into juveniles and complete their life cycle (Watters, 1992) increases their susceptibility to environmental perturbation, as declines in host fish abundance, introduction of exotic fishes and alterations of fish communities can have drastic consequences on the ability of mussels to reproduce successfully.

Host identification has been the focus of considerable research but, for many mussel species, the list of suitable host fish is incomplete or restricted to species in a limited portion of the mussel's range. Identification of host fish is generally done under laboratory conditions; however, comparatively little is known about host use in the wild, and the relative importance of some host species to recruitment and dispersal of freshwater mussels (Martel & Lauzon-Guay, 2005) cannot be determined in the laboratory. Laboratory designation of host fish may not take into consideration the behavioural characteristics of the host fish, such as habitat use (Martel & Lauzon-Guay, 2005) and attraction to mantle lures or conglutinates, and host fish deemed suitable in the laboratory may serve a limited role in natural populations. On a larger scale, relationships between mussels and their host fish are further complicated as fish communities and host abundance differ among localities and watersheds (Vaughn & Taylor, 2000; Martel & Lauzon-Guay, 2005), and the ability of mussels to adapt to local host species (Rogers, Watson & Neves, 2001) demonstrates the need for a more complete understanding of host use in the wild. This information is critical for conservation and effective planning for natural recovery of many declining mussel species.

The study of natural parasitism is greatly inhibited by the difficulty in obtaining reliable identification of encysted glochidia (Hoggarth, 1992). Identification to species using morphological characters is not always possible, and in some mussel communities it is not possible to identify glochidia below the level of subfamily (Weiss & Layzer, 1995). In an effort to provide a more reliable method for identification of glochidia, White, McPheron & Stauffer (1996) developed a dichotomous molecular identification key for the unionids of French Creek, PA, USA, that utilized restriction-fragment length polymorphisms (RFLPs) visualized on agarose gels for species identification. Using a similar approach that added a nested polymerase chain reaction (PCR), a dichotomous RFLP-based molecular key was developed for the unionids of Europe (Gerke & Tiedemann, 2001). However, these keys have not been rigorously applied to the study of natural parasitism in unionids, and there has been difficulty in effectively extracting DNA from a single glochidium using the published methods.

The purpose of creating a molecular identification key to the ten species of freshwater mussels that occur in Maine was to determine host fish use in the wild for two species that are state-listed as 'threatened', the yellow lampmussel [*Lampsilis cariosa* (Say, 1817)] and tidewater mucket [*Leptodea ochracea* (Say, 1817)]. The goals were to examine host use in multiple localities, to determine if host fish identified in the laboratory are used as hosts in the wild and to determine if additional species are potential hosts by capturing naturally parasitized fish and identifying glochidia with the key. In addition, three other mussel species are currently state-listed as 'special concern', so identification of fish hosts for wild populations of mussels in Maine is essential information for regional conservation planning.

To develop the key, all ten species were sampled at or within close proximity to localities in the three river drainages where *Lampsilis cariosa* and *Leptodea ochracea* occur (Fig. 1). Approximately 15 mg of mantle tissue was sampled from morphologically identified adult mussels using methods that are minimally invasive (Berg *et al*., 1995), and DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions for the Tissue Protocol. The NADH dehydrogenase subunit 1 (ND1) gene of the mitochondrial (mtDNA) genome was amplified from each species using the unionid-specific primers Leu-uurF and LoGlyR (Serb, Buhay & Lydeard, 2003). Two specimens of each species from the widest possible distribution within the study area were selected for sequencing to examine intraspecific variation within the ND1 gene. PCR reactions consisted of ~200 ng genomic DNA, 1×PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward and reverse primers, 1.25 U *Taq* Polymerase (Invitrogen, Carlsbad, CA, USA), and sterile water to a total reaction volume of 50 μl. Reactions were amplified with a PTC programmable thermocycler with a heated lid (MJC Research, Inc., Watertown, MA, USA). Reaction conditions for double-stranded amplification consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 54°C for 60 s, and 72°C for 60 s; and a final extension of 72°C for 5 min. PCR products were purified with Nanosep 30 k Centrifugal Devices (Pall Corp., Ann Arbor, MI, USA) following the manufacturer's instructions. Products were sequenced in both directions using Big Dye (Perkin Elmer) terminator cycle sequencing (v. 3.1) and read with an ABI 3730 Capillary Sequencer.

Glochidia were obtained from gravid female mussels by flushing the marsupium with water using a syringe. On naturally parasitized fish, individual glochidia were removed from ethanol-preserved gills with dissecting probes under 10× magnification using a dissecting microscope, and effort was made to minimize the amount of gill tissue attached to each glochidium. DNA was extracted from a single glochidium using a QIAamp DNA Mini Kit with a slightly modified Tissue Protocol. Detailed instructions of the Tissue Protocol included in the QIAamp DNA

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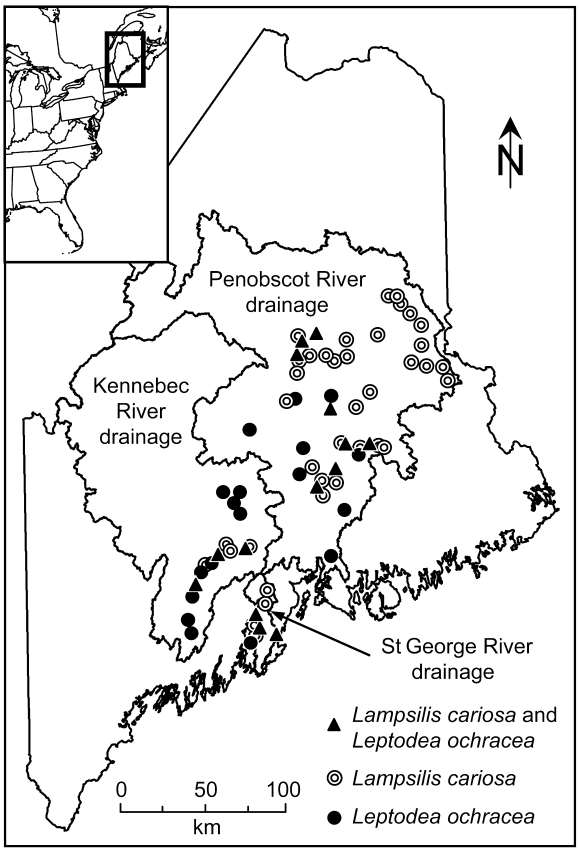


**Figure 1.** Distribution of *Lampsilis cariosa* and *Leptodea ochracea* in the Penobscot, Kennebec, and St George River drainages in Maine. (Insert: rectangle indicates the location of Maine in eastern North America).

**Table 1.** Molecular identification key to the freshwater mussels of Maine, USA.

| | | |
|---|---|---|
| 1. PCR products digested with *Nae*I | | |
| a. Cut into two fragments | | |
| 717 and 302 bp | Lampsilinae | → 2 |
| b. Uncut | | |
| 1019 bp | Ambleminae, Anodontinae or Margaritiferidae | → 2 |
| 2. PCR products digested with *Nla*IV | | |
| a. Cut into three fragments | | |
| 518, 224 and 196 bp | *Leptodea ochracea* | |
| 456, 258 and 181 bp | *Lampsilis radiata radiata* (Gmelin, 1781) | |
| 456, 339 and 224 bp | *Elliptio complanata* | |
| 649, 189 and 181 bp | *Anodonta implicata* Say, 1829 | |
| 456, 305 and 258 bp | *Margaritifera margaritifera* (Linnaeus, 1758) | |
| b. Cut into two fragments | | |
| 714 and 224 bp | *Lampsilis cariosa* | |
| 563 and 456 bp | | → 3 |
| c. Uncut | | |
| 1019 bp | | → 4 |
| 3. PCR products digested with *Apo*I | | |
| a. Cut into three fragments | | |
| 465, 396 and 158 bp | *Alasmidonta undulata* (Say, 1817) | |
| b. Cut into two fragments | | |
| 861 and 158 bp | *Strophitus undulatus* (Say, 1817) | |
| 4. PCR products digested with *Apo*I | | |
| a. Cut into three fragments | | |
| 465, 396 and 158 bp | *Alasmidonta varicosa* (Lamarck, 1819) | |
| 587, 274 and 158 bp | *Pyganodon cataracta* (Say, 1817) | |

Fragment sizes estimated from cut positions on the ND1 gene. Fragments less than 100 bp were not visible on agarose gels and are not included in the key.

Mini Kit were used with the following exceptions: amounts of buffers, proteinase K and ethanol used in each step were reduced by one-half, DNA was resuspended in 75 μl of sterile water and the final elution step was not repeated due to low expected yield. Otherwise, the procedure remained unchanged. The amount of DNA recovered varied among glochidia, and 4–7.5 μl of undiluted DNA (~5–100 ng) provided the template for PCR amplification using the conditions previously described.

Sequences were aligned with the Clustal W algorithm in Sequence Navigator™ v. 1.01 (Applied Biosystems Inc., 1994) and inspected for errors. RestrictionMapper (available online at http://www.restrictionmapper.org/) was used to search a regularly updated database of all commercially available restriction enzymes and find the base pair positions where each restriction enzyme cut the ND1 sequences for each mussel species. Sequences were examined for intraspecific differences in restriction sites to identify any variable sites in the gene that could result in species identification errors. Interspecific differences in restriction sites were examined to find the most efficient suite of diagnostic restriction enzymes to construct the key to identify each mussel species. Only restriction enzymes that produced less than five cut positions in any species were considered for use, since digests resulting in more complex banding patterns with many small DNA fragments would complicate species identification.

Alignment of the coding region of the ND1 gene yielded 903 bp of usable sequence for inter- and intraspecific comparisons (GenBank accession numbers: EF446096–EF446105). The number of variable sites was low for most species, ranging from 0 to 5, with the exception of 19 variable sites in *Elliptio complanata* (Lightfoot, 1786). In cases where intraspecific variation in the ND1 sequences occurred within restriction enzyme recognition sites (33 of the 157 commercially available restriction enzymes), those enzymes were eliminated from consideration. A total of 64 restriction enzymes were informative for species recognition and met our criteria, so a wide variety of enzymes were available to construct the most efficient identification key.

Selection of restriction enzymes for the molecular identification key (Table 1) was biased towards the most effective means of identifying *Lampsilis cariosa* and *Leptodea ochracea* because of their 'threatened' status. The primary restriction enzymes selected were *NaeI* (5'GCC/GGC3') (New England BioLabs Inc., Ipswich, MA, USA) and *Nla*IV (5'GGN/NCC3') (New England BioLabs Inc., Ipswich, MA, USA), which represented steps 1 and 2 of the key, respectively. *Nae*I yielded restriction patterns that could be used to separate the three Lampsiline species from the remaining species (Fig. 2A), and *Nla*IV further identified six of the ten species in Maine (Fig. 2B). Although *Nae*I is not necessary for identification purposes, it was included to ensure accuracy of the key should

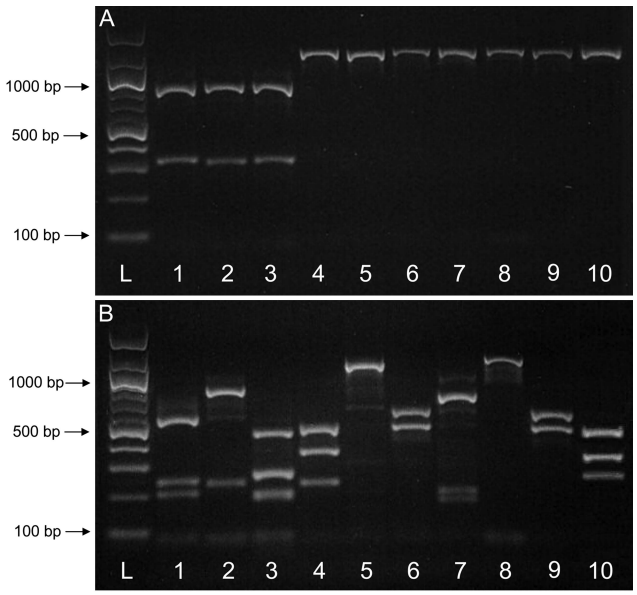


**Figure 2.** RFLP patterns of the ND1 gene when digested with *Nae* I (**A**) and *Nla* IV (**B**) L: 100 bp DNA size standard, 1: *Leptodea ochracea*, 2: *Lampsilis cariosa*, 3: *Lampsilis radiata*, 4: *Elliptio complanata*, 5: *Alasmidonta varicosa*, 6: *Alasmidonta undulata*, 7: *Anodonta implicata*, 8: *Pyganodon cataracta*, 9: *Strophitus undulatus*, 10: *Margaritifera margaritifera*.

unexpected restriction fragment patterns arise due to mutation. For the remaining four mussel species, *Apo* I (5'Pu/AATTPy3') (New England BioLabs Inc., Ipswich, MA, USA) was used in step 3 (Fig. 3A) and step 4 (Fig. 3B) of the key, which are separated for identification purposes according to fragment patterns of *Nla* IV.

To confirm accuracy of the key, morphologically identified specimens of each mussel species were selected from study sites throughout the region for testing. One specimen of each species from each sample location was randomly chosen, and DNA was extracted and amplified as previously described. Restriction enzyme digests consisted of 8 μl of PCR product, 0.3 U of restriction enzyme, 0.1 ug/ml BSA and the manufacturer's supplied buffer at a concentration of 1×. Complete digestion took 3 h of incubation in a shaking water bath set to the manufacturer's recommended temperature. Digested samples were assayed on a 1.5% agarose gel and run for 2 h at 55 V in TAE buffer with a 100 bp DNA Ladder (New England BioLabs Inc., Ipswich, MA, USA) size standard to determine fragment sizes and identify species.

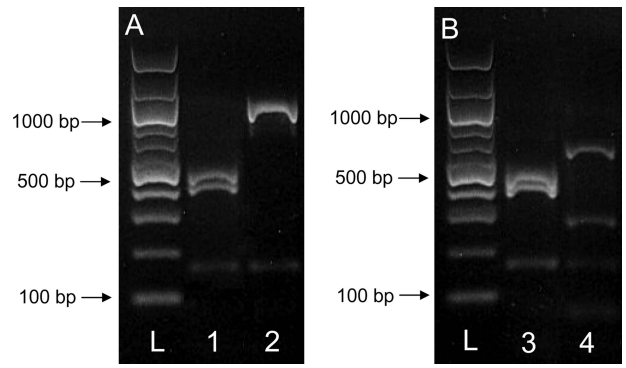


**Figure 3.** RFLP patterns of the ND1 gene when digested with *Apo* I in step 3 (**A**) and step 4 (**B**) of the molecular identification key. L: 100 bp DNA size standard, 1: *Alasmidonta undulata*, 2: *Strophitus undulatus*, 3: *Alasmidonta varicosa*, 4: *Pyganodon cataracta*.

All ten mussel species in the Maine region were identified with 100% accuracy using the three restriction enzymes in the key. Species could easily be matched to the correct restriction fragment pattern(s), and novel patterns were not present in any specimen. The key was also tested on glochidia taken from *Lampsilis cariosa* and *Leptodea ochracea* using the modified QIAamp DNA extraction Tissue Protocol. DNA from individual glochidia was successfully extracted and amplified with a success rate of 100% ($n = 8$). Each glochidium had the correct species-specific restriction fragment pattern when PCR products were digested with *Nae* I and *Nla* IV, confirming that the DNA key worked well for identifying a single glochidium to species.

The value of molecular identification keys lies in their ability to effectively identify glochidia from naturally parasitized fish, and this key was used extensively to identify individual glochidia on 18 species of fish from 13 localities in the study area (Kneeland, 2006). A total of 848 glochidia encysted in the gills of 230 fish were processed for identification. DNA from 687 (81%) was successfully amplified and identified to species, and no ambiguous restriction fragment patterns were observed. PCR failure was likely due to an insufficient quantity of DNA from most of these glochidia, which could not be identified as a result. Glochidia from six of the ten mussel species that occur in Maine were identified from ethanol-preserved gills of naturally parasitized fish. Potential new host fish were identified for five of six mussel species, including *Lampsilis cariosa*, *Leptodea ochracea* and a species of 'special concern', *Alasmidonta undulata*. Some fish species that had been previously identified as effective hosts in laboratory trials were not used as extensively as expected in natural mussel populations.

As a conservation tool, molecular identification keys provide efficient and accurate information on host use by the entire mussel community. With an amplification rate of 81% for glochidia from naturally parasitized fish, the effectiveness of the key and extraction methods developed for mussels in our region is extremely useful for the study of natural parasitism. When compared to the identification key for European mussels (Gerke & Tiedemann, 2001), the methods used here resulted in a simpler identification process that involved fewer steps to identify more species. Accuracy of the key was confirmed on known adult mussels and none of the glochidia identified in our study had ambiguous restriction fragment patterns. Consequently, the use of RFLPs proved to be a faster, more cost effective alternative to DNA sequencing of each glochidium. With a maximum distance of 486 river kilometres between fish sampling localities in separate river drainages, the key effectively identified glochidia over a relatively large scale compared to other studies of natural parasitism (Weiss & Layzer, 1995; White *et al*., 1996; Martel & Lauzon-Guay, 2005). Valuable information was provided for several mussel species, including the 'threatened' *Lampsitis cariosa* and *Leptodea ochracea*, for which little was known about their host fish (Wick, 2006). When combined with laboratory host identification trials, molecular identification keys can provide information on host use in the wild, which is essential for recovery of natural populations.

Molecular identification keys would have to be tested for use outside their intended range, and inclusion of additional mussel species would likely alter the key's construction. Keys could be created for mussel communities in any region, regardless of species richness, and the methods used here for selecting restriction enzymes can be applied to create a key that requires relatively few steps. The methods developed for extracting DNA from a single glochidium can also be applied for efficient use of the key. Selecting a DNA region for amplification, such as ND1, that is appropriate for species-level identification (Campbell *et al*., 2005) is the most important component. Targeting regions such as the ribosomal

DNA internal transcribed spacer 1 (ITS-1) can be problematic because of intra-individual variation in some mussel species (King *et al*., 1999). Use of universal primers should be avoided due to the potential for cross-species amplification of host fish DNA. Unionid-specific primers, such as the primer pair used in our study, are more appropriate as they amplify DNA from a wide variety of mussel species without the presence of ambiguous RFLP patterns. If other DNA regions are used in developing an identification key, the complete mitochondrial genome map of *Lampsilis ornata* (Conrad, 1835) (Serb & Lydeard, 2003) can aid in the design of unionid-specific primers.

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## REFERENCES

BERG, D.J., HAAG, W.R., GUTTMAN, S.I. & SICKEL, J.B. 1995. Mantle biopsy: a technique for nondestructive tissue-sampling of freshwater mussels. *Journal of the North American Benthological Society*, **14**: 577–581.

CAMPBELL, D.C., SERB, J.M., BUHAY, J.E., ROE, K.J., MINTON, R.L. & LYDEARD, C. 2005. Phylogeny of North American amblemines (Bivalvia, Unionoida): prodigious polyphyly proves pervasive across genera. *Invertebrate Biology*, **124**: 131–164.

GERKE, N. & TIEDEMANN, R. 2001. A PCR-based molecular identification key to the glochidia of European freshwater mussels (Unionidae). *Conservation Genetics*, **2**: 285–287.

HOGGARTH, M.A. 1992. An examination of the glochidia-host relationships reported in the literature for North American species of Unionacea (Mollusca: Bivalvia). *Malacology Data Net*, **3**: 1–30.

KING, T.L., EACKLES, M.S., GJETVAJ, B. & HOEH, W.R. 1999. Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Union-idae): conservation implications of range discontinuity. *Molecular Ecology*, **8**: S65–S78.

KNEELAND, S.C. 2006. *Identification of fish hosts for wild populations of rare freshwater mussels* (Lampsilis cariosa *and* Leptodea ochracea) *using a molecular DNA key*. MSc thesis, University of Maine, Orono, Maine, USA.

MARTEL, A.L. & LAUZON-GUAY, J.S. 2005. Distribution and density of glochidia of the freshwater mussel *Anodonta kennerlyi* on fish hosts in lakes of the temperate rain forest of Vancouver Island. *Canadian Journal of Zoology*, **83**: 419–431.

NEDEAU, E.J., McCOLLOUGH, M.A. & SWARTZ, B.I. 2000. *The freshwater mussels of Maine*. Maine Department of Inland Fisheries and Wildlife, Augusta, ME.

ROGERS, S.O., WATSON, B.T., & NEVES, R.J. 2001. Life history and population biology of the endangered tan riffleshell (*Epioblasma florentina walkeri*) (Bivalvia: Unionidae). *Journal of the North American Benthological Society*, **20**: 582–594.

SERB, J.M., BUHAY, J.E. & LYDEARD, C. 2003. Molecular systematics of the North American freshwater bivalve genus *Quadrula* (Unionidae: Ambleminae) based on mitochondrial ND1 sequences. *Molecular Phylogenetics and Evolution*, **28**: 1–11.

SERB, J.M. & LYDEARD, C. 2003. Complete mtDNA sequence of the North American freshwater mussel, *Lampsilis ornata* (Unionidae): an examination of the evolution and phylogenetic utility of mitochondrial genome organization in Bivalvia (Mollusca). *Molecular Biology and Evolution*, **20**: 1854–1866.

VAUGHN, C.C. & TAYLOR, C.M. 2000. Macroecology of a host-parasite relationship. *Ecography*, **23**: 11–20.

WATTERS, G.T. 1992. Unionids, fishes, and the species-area curve. *Journal of Biogeography*, **19**: 481–490.

WEISS, J.L., & LAYZER, J.B. 1995. Infestations of glochidia on fishes in the Barren River, Kentucky. *American Malacological Bulletin*, **11**: 153–159.

WHITE, L.R., McPHERON, B.A. & STAUFFER, J.R. Jr. 1996. Molecular genetic identification tools for the unionids of French Creek, Pennsylvania. *Malacologia*, **38**: 181–202.

WICK, P. 2006. *Fish hosts and demographics of* Lampsilis cariosa *and* Leptodea ochracea, *two threatened freshwater mussels in Maine*. MSc thesis, University of Maine, Orono, Maine, USA.

WILLIAMS, J.D., WARREN, M.L., CUMMINGS, K.S., HARRIS, J.L. & NEVES, R.J. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries*, **18**: 6–22.

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