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Development of species-specific primers with potential for amplifying eDNA from imperilled freshwater unionid mussels.

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RUNNING HEAD: UNIONID ENVIRONMENTAL DNA

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Abstract

Environmental DNA (eDNA) is emerging as a potentially powerful tool for inferring species' presence, and hence occupancy, from DNA that is shed into environmental samples such as water. Although eDNA screening has been used to detect DNA from a variety of taxonomic groups, it has not yet been used to identify DNA from species with numerous potentially sympatric confamilial species, a situation that may preclude the development of species-specific markers. There are 41 native freshwater mussel species (Unionidae) in Ontario, Canada. Many of these are potentially sympatric, and 14 species have been formally assessed as endangered, threatened, or special concern. We investigated whether there was sufficient variation within the cytochrome oxidase region (COI) to develop species-specific eDNA markers for at-risk unionids. We developed 32 COI markers for eight unionid species, and tested each of these on the target species plus 29 potentially sympatric unionid taxa. Six of these markers amplified DNA only from the intended target species. We then extracted and amplified mussel eDNA from rearing-tank water samples. We conclude that despite high species diversity, it should be possible to develop eDNA COI markers and screen water samples for habitat occupancy by unionid mussels.

Keywords: eDNA; cytochrome oxidase I; mtDNA; unionidae; freshwater mussels

Characterization of environmental DNA (eDNA) is emerging as a potentially powerful tool for inferring species' presence, and hence occupancy, from DNA that is shed into

environmental samples such as water. eDNA screening has been used to detect DNA from a variety of taxonomic groups, including invasive species (Smart et al. 2015; Amberg et al. 2015) and species-at-risk (Davy et al. 2015; Janosik and Johnston 2015). However, this method has not yet been tested on species with numerous potentially sympatric confamilial species, and we therefore do not know the feasibility of developing species-specific markers in such groups. Fourteen out of 41 native freshwater mussels (Bivalvia: Unionidae) in the province of Ontario, Canada, are listed as either Special Concern, Threatened, or Endangered (COSEWIC 2014). Identifying unionid populations is a major conservation challenge, made more difficult by their parasitic larval stage and the burrowing behaviour of juveniles (Boyer et al. 2011; Metcalfe-Smith et al. 2007). In addition, physical site searches are costly, time consuming, and limited by various seasonal and environmental factors (Dejean et al. 2012). A potentially more rapid and cost-effective survey method involves screening environmental DNA (eDNA) from water samples using species-specific markers (Ficetola et al. 2008; Mahon et al. 2013; Wilcox et al. 2013). Unionids in Ontario, which include several sets of congeneric species, collectively exhibit a combination of close and more distant evolutionary relationships (Lydeard et al. 2000; Manendo et al. 2008). We tested the feasibility of designing species-specific markers on eight at-risk unionid species, and then determined whether we could extract and amplify unionid eDNA from water samples.

Methods

Cytochrome oxidase I (COI) mitochondrial DNA (mtDNA) sequences from 38 unionid species native to Ontario were obtained from GenBank (<http://ncbi.nlm.nih.gov/genbank>) (Table 1) and aligned using the ClustalW multiple alignment function in BioEdit Ver 7.2.5 (Hall 1999). A total of 32 primer pairs were designed *in silico* for eight species of unionid mussels using

Primer-BLAST (Ye et al. 2012) based on a global search in GenBank against available COI sequence data (Table 1) for native Ontario unionids (Campbell et al. 2008; Boyer et al. 2011; Bronnenhuber and Wilson 2013). We assumed that mussel species in the database had been accurately identified if the following conditions were met: 1) the associated published study provided an explanation of how mussel identifications were made, and 2) a BLAST search (Altschul et al. 1990) identified the closest matches for each sequence as conspecific sequences. Post-hoc taxonomic verifications should not be considered infallible, but our validation of primers using DNA samples extracted from mussels that had been identified by one or more experienced mussel taxonomists further increased our confidence that the available sequence data were reliable. The number of available sequences for each species varied greatly: we obtained only one sequence for *Amphinaias pustulosa*, but more than one hundred sequences for *Villosa iris* (Table 1). Primers were designed to incorporate the maximum number of nucleotide mismatches with other unionids, and to minimizing annealing regions that included intraspecific nucleotide polymorphisms. Four primer pairs annealed to regions that had either one or two nucleotide mismatches within species, but only one of these primer-pairs was later identified as species-specific (see below). Each of the resulting 32 primer pairs had a minimum of 2 species-specific nucleotides (mismatches with all other taxa) within each of the forward and reverse primer binding sites. Primer pairs were designed to amplify regions that were 150-400 bp in length.

To test primers for species-specificity, we collected tissue samples of 30 unionid species from the Ausable and Sydenham rivers in southern Ontario (Table 1) by nonlethal swab sampling (Reid et al. 2012). Mussel shell valves were gently separated and held with a steel rod 4-5 mm in diameter, and the mussel foot and viscera were swabbed using a sterile brush. The

majority were swabbed with a 6 mm diameter Qiagen buccal swab brush, although smaller unionids such as *Villosa fabalis* and *Strophitus undulatus* were swabbed with a 2 mm diameter Microbrush® International in order to minimize the stress to the animals. Swabbed brush tips were cut off and stored in 2 mL screw-cap cryovials with 1 mL 1X TNES-urea buffer at room temperature (Karlsson et al. 2013). DNA was extracted from 200 µL of each swab solution that contained 6 mm brushes using a DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's *Supplementary Protocol DY15*. Qiagen extractions from swab solutions that contained the 2 mm diameter brushes yielded no detectable DNA, and were therefore extracted by alkaline lysis and ethanol precipitation (0.1X volume NaOH and 2X volume 100% EtOH, followed by centrifugation, 70% EtOH wash, centrifugation, and resuspension in 50 µL TE buffer).

Gradient PCRs were performed in Mastercycler epGradient thermal cyclers (Eppendorf). Each reaction included 1U *DreamTaq* (ThermoScientific™), 0.2 mM dNTPs, 1X *DreamTaq* Buffer with 2mM MgCl₂ (ThermoScientific™), 0.2 µM of each primer, and 2 µL DNA in a final volume of 25 µL. Reactions included 2 min at 94°C followed by 30 cycles of 15 s at 94°C, 15 s at the annealing temperature gradient, and 30 s at 72°C, with a final step of 72°C for 1 min. Optimal annealing temperatures for subsequent PCRs were identified from the gradient PCRs (Table 2). We tested each primer pair on all 30 of the species for which we had DNA samples (Table 1).

To test whether we could extract and amplify unionid eDNA, six replicate water samples were each collected in 1 L Mason jars from a tank rearing several hundred juvenile (each approximately 10.6 mm long) Wavyrayed Lampmussel (*Lampsilis fasciola*) at the Ontario Ministry of Natural Resources and Forestry (OMNRF) White Lake Hatchery (Sharbot Lake, ON).

Before sample collection, the Mason jars and transportation cooler were soaked in a 10% bleach solution for five minutes and then triple rinsed with deionized water. One 1-litre jar was filled with deionized water and then returned to the cooler as a negative control. Samples were collected from a 270L tank for which water is recirculated through a sand filter, UV sterilization, and a 1 micron-mesh bag filter before returning to the tank. Once a week, one-third of the water (90L) is replaced with water from a 1800L reservoir which has been through a parallel filtration (combined sand, UV, and 1µm filtration). A small amount of unfiltered water (<2%) is supplied directly from the local waterbody (White Lake) to make up for evaporative and splash loss. Each 1 L sample was filtered onto WhatmanTM GF/C glass microfiber filters (1 µm pore size) with an EZ SteamTM pump (PALL Corporation) upon return to Trent University (within approximately two hours). Prior to filtering, lab equipment was soaked in a 10% bleach solution for five minutes and then triple rinsed in deionized water. Filtering funnels were soaked in a 10% bleach solution for at least two minutes and then triple rinsed with deionized water between each sample. Negative funnel controls were made by filtering deionized water through funnels before and after samples were filtered. The filters were stored in 1.5 mL microtubes at -20°C until DNA was extracted using a CTAB/magnetic bead protocol (Dempster et al. 1999; Coyne et al. 2006).

Results and Discussion

Based on visualization of conventional PCR amplicons on an agarose gel, we identified one primer pair for each of six species that amplified DNA only from the target species (Table 2). The remaining primer pairs either generated no amplicon; generated amplicons from multiple species; or were tested only on the target species (because a species-specific marker had already been found) (Table 2). One primer pair (*PfaCOI2*) amplified DNA from 28 out of 30 unionid species and may have potential as a pseudo-universal unionid marker. We were able to amplify

mussel eDNA using the generalist primer pair referred to above (*Pfa*COI2), and also using two additional primer pairs (*GLfa*COI3 and *Lfa*COIc) that had previously amplified *L. fasciola* swab DNA extractions, but were not species-specific (i.e. each of these amplified DNA from the target plus two additional species).

We were unable to design putatively species-specific markers for multiple species, and future research on these species should investigate additional gene regions or the potential increased specificity of primer-probe combinations that can be utilized in quantitative PCR. However, the specificity of six of our markers and the successful extraction and amplification of unionid mussel eDNA are important demonstrations when investigating the feasibility of using eDNA as a supplementary tool for monitoring mussel populations and identifying their habitats (Stoeckle et al. in press). Although we had neither DNA samples nor haplotype accessions from three Ontario Unionid species, one of these (*Elliptio crassidens*) has never been collected alive from Ontario, and the remaining two are rare headwater species (*Alasmidonta viridis* and *Anodontoides ferussacianus*) that have very minimal spatial overlap with any of the species at risk. However, sympatric species will vary between regions and researchers should sequence initial positive PCRs to confirm target species amplification (Scriver et al. 2015). This caveat may be particularly pertinent for primer pair *Vfa*COI3F, the species-specific primer pair that we designed for *Villosa fabalis*, because there was a single intraspecific nucleotide polymorphism within its primer binding site (Table 2).

We have shown that monitoring freshwater mussel populations from eDNA may provide a feasible alternative to more traditional search methods, which are time-consuming and may be susceptible to bias associated with animal size (Hornbach and Deneka, 1996) or position (burrowed vs. unburrowed) (Amyot and Downing, 1991). Furthermore, genetic approaches to

species detection can reduce errors in identification when morphological characters are unclear (Cyr et al 2007; Doucet-Beaupre et al 2012), and future applications of these markers could include identification of cryptic species and life history stages (Zieritz et al. 2012). More generally, we have demonstrated that mitochondrial COI sequences can be sufficiently variable for designing species-specific markers even within an extensive group of confamilial species, and conclude that eDNA surveys have the potential to be a valuable tool in unionid mussel conservation.

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Table 1: Accession numbers, sample sites and DNA extraction method for the Ontario unionid sequences that were used for primer design and/or for which swab samples were collected. Sample locations: AR = Ausable River; SR = Sydenham River; Y = Yes; "-" = none available.

Scientific Name	Common Name	Swab Sample?	Sample Sites	Extraction Method	Available GenBank COI Sequences Accession #
<i>Actinonaias ligamentina</i>	Mucket	Y	SR	DNeasy Blood & Tissue kit	EF033300.1, EF033263.1, GU085281.1, GU085280.1, AY785392.1, AF406796.1, AF231730.1
<i>Alasmidonta marginata</i>	Elktoe	Y	AR, SR	DNeasy Blood & Tissue kit	HM849059.1, AF156502.1
<i>Alasmidonta undulatus</i>	Triangle Floater	-	-		HM849062.1, HM849061.1, HM849060.1
<i>Ambelma plicata</i>	Threeridge	Y	AR, SR	DNeasy Blood & Tissue kit	EF033258.1, EF033295.1, DQ648118.1, DQ648117.1, DQ648113.1, DQ648107.1, DQ648106.1, DQ648101.1, DQ648100.1, DQ648099.1, DQ648097.1, DQ648095.1, DQ648093.1, DQ648092.1, DQ648089.1, DQ648088.1, DQ648087.1, DQ648086.1, DQ648083.1, DQ648120.1, DQ648119.1, DQ648109.1, DQ648116.1, DQ648110.1, DQ648108.1, DQ648105.1, DQ648102.1, DQ648098.1, DQ648096.1, DQ648091.1, DQ648115.1, DQ648114.1, DQ648112.1, DQ648111.1, DQ648104.1, DQ648103.1, DQ648094.1, DQ648090.1, DQ648085.1, DQ648084.1, U56841.1, GU085282.1, GU550953.1, GU550951.1, AF156512.1
<i>Amphinaias pustulosa</i>	Pimpleback	Y	SR	DNeasy Blood & Tissue kit	GU085318.1
<i>Cyclonaias tuerkulata</i>	Purple Wartback	Y	AR, SR	DNeasy Blood & Tissue kit	HM849069.1, HM230410.1, HM849070.1, GU085284.1, GU085283.1
<i>Elliptio complanata</i>	Eastern Elliptio	-	-		HQ153535.1, KC708473.1, KC708472.1, KC708471.1, KC708470.1, KC708469.1, KC708468.1, KC708467.1, KC708466.1, KC708465.1, KC708464.1, HQ153534.1, EU448185.1, EU448184.1, EU448183.1, EU448182.1, EU448181.1, EU448180.1, EU448179.1, EU448178.1, EU448177.1, EU448176.1, EU448175.1, EU448174.1, EU448173.1, EU448172.1
<i>Elliptio dilatata</i>	Spike	Y	AR, SR	DNeasy Blood & Tissue kit	KF035280.1, GQ921082.1, EU448188.1, AF156507.1, AF156506.1, AF231751.1
<i>Epioblasma torulosa rangiana</i>	Northern Riffleshell	Y	AR, SR	DNeasy Blood & Tissue kit	DQ479949.1, DQ479948.1, DQ479947.1, DQ479946.1, DQ479945.1, DQ479944.1, DQ479943.1, DQ479942.1, DQ479941.1, DQ479940.1, DQ479939.1, DQ479938.1, JF326432.1, DQ220724.1

<i>Epioblasma triquetra</i>	Snuffbox	Y	AR, SR	DNeasy Blood & Tissue kit	EF507816.1, EF507815.1, EF507814.1, EF507813.1, EF507812.1, EF507811.1, EF507810.1, EF507809.1, EF507808.1, EF507807.1, EF507806.1, EF507805.1, EF507804.1, DQ208590.1, AF156528.1
<i>Fusconaia flava</i>	Wabash Pigtoe	Y	AR, SR	DNeasy Blood & Tissue kit	AF232822.1, DQ298536.1, DQ298535.1, DQ298534.1, DQ298533.1, DQ298532.1, DQ298531.1, DQ298530.1, DQ298529.1, DQ298528.1, DQ298527.1, DQ298526.1, DQ298525.1, DQ298524.1, HM230370.1, HM230366.1, HM230367.1, HM849073.1, AF406799.1, EF033307.1, EF033261.1, AF156510.1, AF231733.1
<i>Lampsilis cardium</i>	Plain Pocketbook	Y	AR, SR	DNeasy Blood & Tissue kit	AF120653.1, GQ921084.1, GQ921083.1, AF156519.1, AF156518.1
<i>Lampsilis fasciola</i>	Wavy-rayed Lampmussel	Y	AR, GR	DNeasy Blood & Tissue kit	GQ921098.1, GQ921143.1, AF156520.1
<i>Lampsilis radiata</i>	Eastern Lampmussel	-	-		KC408801.1, KC408800.1, KC408799.1, KC408798.1, KC408797.1, KC408796.1, KC408771.1, KC408770.1, KC408769.1, HQ153608.1, HQ153607.1, HQ153606.1, HQ153605.1
<i>Lampsilis siliquoidea</i>	Fatmucket	Y	AR, SR	DNeasy Blood & Tissue kit	KC408792.1, KC408795.1, KC408794.1, KC408793.1, KC408791.1, KC408790.1, KC408789.1, KC408788.1, KC408787.1, KC408786.1, KC408785.1, KC408784.1, KC408783.1, KC408782.1, KC408781.1, KC408780.1, KC408779.1, KC408778.1, KC408777.1, KC408776.1, KC408775.1, KC408774.1, KC408773.1, KC408772.1, KC408768.1, KC408767.1, KC408766.1, KC408765.1, KC408764.1, KC408763.1, KC408762.1, KC408761.1, KC408760.1, KC408759.1, KC408758.1, KC408757.1, KC408756.1, KC408755.1, KC408754.1, KC408753.1, KC408752.1, KC408751.1, KC408750.1, KC408749.1, KC408748.1, KC408747.1, KC408746.1, KC408745.1, KC408744.1, HM849076.1, DQ494752.1, AF156522.1, AF156521.1
<i>Lasmigona complanata</i>	White Heelsplitter	Y	AR, SR	DNeasy Blood & Tissue kit	HM849078.1, HM849077.1, AF093845.1
<i>Lasmigona compressa</i>	Creek Heelsplitter	-	-		HM849084.1, HM849085.1, HM849081.1, HM849080.1, HM849079.1, HM849083.1, HM849082.1, AF093847.1, AF093846.1, AF156503.1
<i>Lasmigona costata</i>	Flutedshell	Y	AR, SR	DNeasy Blood & Tissue kit	GU085296.1, HM849089.1, HM849088.1, HM849087.1, HM849086.1, GU085295.1, AF093848.1

<i>Leptodea fragilis</i>	Fragile Papershell	Y	SR	DNeasy Blood & Tissue kit	GU085298.1, GQ921186.1, GU085297.1, AF049519.1, AF049518.1
<i>Ligumia nasuta</i>	Eastern Pondmussel	-	-	-	AF156515.1
<i>Ligumia recta</i>	Black Sandshell	Y	AR, SR	DNeasy Blood & Tissue kit	KC291717.1, KC291716.1, GU085299.1, AF406795.1, AF385110.1, AF156516.1, AF231748.1
<i>Obliquaria reflexa</i>	Threehorn Wartyback	Y	SR, GR	DNeasy Blood & Tissue kit	GU085300.1, AY655008.1, GU085301.1, EF033292.1, EF033254.1, AF049521.1, AF385114.1
<i>Obovaria olivaria</i>	Hickorynut	-	-		KF035243.1, KF035244.1, KF035242.1, KF035241.1, KF035238.1, KF035237.1, KF035235.1, KF035234.1, KF035232.1, KF035229.1, KF035240.1, KF035239.1, KF035233.1, KF035236.1, KF035231.1, KF035230.1, HM230408.1, GU085304.1, GU085302.1, GU085303.1, EF033306.1, EF033267.1, AF232812.1
<i>Obovaria subrotunda</i>	Round Hickorynut	Y	SR	DNeasy Blood & Tissue kit	KF035263.1, KF035268.1, KF035257.1, KF035267.1, KF035266.1, KF035262.1, KF035261.1, KF035260.1, KF035259.1, KF035258.1, KF035256.1, KF035255.1, KF035253.1, KF035252.1, KF035251.1, KF035250.1, KF035249.1, KF035248.1, KF035247.1, KF035265.1, KF035254.1, KF035246.1, KF035245.1, KF035264.1, AY655010.1
<i>Pleurobema sintoxia</i>	Round Pigtoe	Y	SR	DNeasy Blood & Tissue kit	EF033253.1, AY655019.1, GU085309.1, GU085308.1, EF033291.1
<i>Potamilus alatus</i>	Pink Heelsplitter	Y	AR, SR	DNeasy Blood & Tissue kit	GU085310.1, GU085311.1, AF049511.1, AF049510.1, AF231752.1
<i>Ptychobranchus fasciolaris</i>	Kidneyshell	Y	AR, SR	DNeasy Blood & Tissue kit	EF033265.1, EF033301.1, AF156514.1
<i>Pyganodon cataracta</i>	Eastern Floater	-	-		JX101481.1, JX101491.1, JX101490.1, JX101489.1, JX101488.1, JX101487.1, JX101486.1, JX101485.1, JX101484.1, JX101483.1, JX101482.1, JX101480.1, JX101479.1, JX101478.1, EF488186.1, EF488185.1, EF418023.1, EF418016.1
<i>Pyganodon grandis</i>	Giant Floater	Y	AR, SR	DNeasy Blood & Tissue kit	KM262551.1, HM849107.1, HM849106.1, HM849105.1, HM849104.1, HM849103.1, HM849102.1, HM849101.1, HM849100.1, GU085313.1, GU085312.1, AF406801.1, EF418024.1, EF418022.1, EF418019.1, AF156504.1, AF231734.1
<i>Quadrula quadrula</i>	Mapleleaf	Y	AR, SR	DNeasy Blood & Tissue kit	AF156511.1, EF033308.1, EF033268.1, AF231757.1, AF232823.1, FJ809751.1 (haplotype male), FJ809750.1 (haplotype female)

<i>Simpsonaias ambigua</i>	Salamander Mussel	Y	SR	DNeasy Blood & Tissue kit	N/A
<i>Strophitus undulatus</i>	Creepers	Y	AR, SR	EtOH Precipitation	HM849121.1, GQ981409.1, HM849122.1, HM849120.1, HM849119.1, HM849118.1, HM849117.1, HM849116.1, HM849115.1, GU085320.1, GU085319.1, AF093839.1, AF156505.1, AF231740.1
<i>Toxolasma parvus</i>	Lilliput	Y	GR	DNeasy Blood & Tissue kit	HM849151.1, DQ206791.1, DQ206790.1, DQ206789.1, DQ206788.1, DQ206787.1, DQ206786.1, DQ206785.1, DQ206784.1, AY655022.1, HM849154.1, HM849153.1, HM849152.1, HM849150.1, HM849149.1, HM849148.1, HM849147.1, HM849146.1, HM849145.1, HM849144.1, HM849143.1, HM849142.1, HM849141.1, HM849140.1, HM849139.1, HM849138.1, HM849137.1, HM849136.1, HM849135.1, HM849134.1, Q612178.1, GU085321.1
<i>Truncilla donaciformis</i>	Fawnsfoot	Y	SR	EtOH Precipitation	GU085323.1
<i>Truncilla truncata</i>	Deertoe	Y	AR, SR	DNeasy Blood & Tissue kit	HM852947.1, HM852948.1, GU085324.1, AF156513.1
<i>Utterbackia imbecillis</i>	Paper Pondshell	-	-		HM849172.1, HM849173.1, HM849172.1, HM849171.1, HM849170.1, HM849169.1, HM849168.1, HM849167.1, HM849166.1
<i>Villosa fabalis</i>	Rayed Bean	Y	SR	EtOH Precipitation	GQ921212.1, GQ921211.1, GQ921137.1, DQ220726.1
<i>Villosa iris</i>	Rainbow	Y	AR	DNeasy Blood & Tissue kit	GQ920976.1, GQ920980.1, GQ920979.1, GQ920978.1, GQ920977.1, GQ920975.1, GQ920974.1, GQ920973.1 (plus >100 not reported here)

Table 2. 32 COI primer pairs designed for eight native Ontario unionids. The six primers that amplified only target DNA templates are shown in bold. Intraspecific variation refers to intraspecific variation within the primer binding regions. T_A = annealing temperature; N/A = cross-species PCR not performed; NT = nucleotide.

Species	Primer ID	Sequence 5'-3'	Length of Amplified Fragment (bp)	T _A (°C)	Non-target species amplifications	Intraspecific Variation
<i>Epioblasma torulosa rangiana</i> (Northern Riffleshell)	EtoCOI4F	TGGGACAGCCAGGTAGGTTA	252	69.4	<u>Species-specific</u>	None
	EtoCOI4R	CAGTCCCAACACCACTCTCC				
	EtoCOI5F	GCCAGGTAGGTTATTGGGGG	246	63.7	<i>Potamilus alatus</i>	None
	EtoCOI5R	CCAGTCCCAACACCACTCTC				
<i>Epioblasma triquetra</i> (Snuffbox)	EtrCOI1F	TAATTCGGGCTGAGTTGGGG	268	66.1	12 species	None
	EtrCOI1R	CCAGTCCCAACACCACTCTC				
	EtrCOIaF	TTGATTATTAGTACCGGCTTTGT	245	63.1	<i>Lampsilis fasciola</i>	None
	EtrCOIaR	ACAAAGGAATCCGCTCAGC				
	EtrCOIbF	CCCACCATTATCTGGTAATGTGG	164	64.5	Unreliable amplification	None
	EtrCOIbR	ACAAAGGAATCCGCTCAGC				
	GLfaCOI3F	TGGCGTTGTGGTCTGGTTTA	505	68	<i>Elliptio dilatata</i> , <i>Lampsilis siliquoidea</i>	None
	GLfaCOI3R	ACGGTAACAGCCCACACAAA				
<i>Lampsilis fasciola</i> (Wavy-rayed Lampmussel)	LfaCOIaF	ACTGGGGCAACCTGGTAG	287	62.1	<i>Epioblasma torulosa rangiana</i> , <i>Actinonaias ligamentina</i>	None
	LfaCOIaR	CATTTCCAGACAATGGGGGG				
	LfaCOIbF	ACTGGGGCAACCTGGTAGGT	413	Failed	N/A	None
	LfaCOIbR	TGTGTGGGCTGTTACCGTGA				
	LfaCOIcF	CCCCCATTGTCTGGAAATG	183	64.1	11 species	None
	LfaCOIcR	ACGGTAACAGCCCACACA				

Table 1: Accession numbers, sample sites and DNA extraction method for the Ontario unionid sequences that were used for primer design and/or for which swab samples were collected. Sample locations: AR = Ausable River; SR = Sydenham River; Y = Yes; "-" = none available.

Scientific Name	Common Name	Swab Sample?	Sample Sites	Extraction Method	Available GenBank COI Sequences Accession #
<i>Actinonaias ligamentina</i>	Mucket	Y	SR	DNeasy Blood & Tissue kit	EF033300.1, EF033263.1, GU085281.1, GU085280.1, AY785392.1, AF406796.1, AF231730.1
<i>Alasmidonta marginata</i>	Elktoe	Y	AR, SR	DNeasy Blood & Tissue kit	HM849059.1, AF156502.1
<i>Alasmidonta undulatus</i>	Triangle Floater	-	-		HM849062.1, HM849061.1, HM849060.1
<i>Ambelma plicata</i>	Threeridge	Y	AR, SR	DNeasy Blood & Tissue kit	EF033258.1, EF033295.1, DQ648118.1, DQ648117.1, DQ648113.1, DQ648107.1, DQ648106.1, DQ648101.1, DQ648100.1, DQ648099.1, DQ648097.1, DQ648095.1, DQ648093.1, DQ648092.1, DQ648089.1, DQ648088.1, DQ648087.1, DQ648086.1, DQ648083.1, DQ648120.1, DQ648119.1, DQ648109.1, DQ648116.1, DQ648110.1, DQ648108.1, DQ648105.1, DQ648102.1, DQ648098.1, DQ648096.1, DQ648091.1, DQ648115.1, DQ648114.1, DQ648112.1, DQ648111.1, DQ648104.1, DQ648103.1, DQ648094.1, DQ648090.1, DQ648085.1, DQ648084.1, U56841.1, GU085282.1, GU550953.1, GU550951.1, AF156512.1
<i>Amphinaias pustulosa</i>	Pimpleback	Y	SR	DNeasy Blood & Tissue kit	GU085318.1
<i>Cyclonaias tuerkulata</i>	Purple Wartback	Y	AR, SR	DNeasy Blood & Tissue kit	HM849069.1, HM230410.1, HM849070.1, GU085284.1, GU085283.1
<i>Elliptio complanata</i>	Eastern Elliptio	-	-		HQ153535.1, KC708473.1, KC708472.1, KC708471.1, KC708470.1, KC708469.1, KC708468.1, KC708467.1, KC708466.1, KC708465.1, KC708464.1, HQ153534.1, EU448185.1, EU448184.1, EU448183.1, EU448182.1, EU448181.1, EU448180.1, EU448179.1, EU448178.1, EU448177.1, EU448176.1, EU448175.1, EU448174.1, EU448173.1, EU448172.1
<i>Elliptio dilatata</i>	Spike	Y	AR, SR	DNeasy Blood & Tissue kit	KF035280.1, GQ921082.1, EU448188.1, AF156507.1, AF156506.1, AF231751.1
<i>Epioblasma torulosa rangiana</i>	Northern Riffleshell	Y	AR, SR	DNeasy Blood & Tissue kit	DQ479949.1, DQ479948.1, DQ479947.1, DQ479946.1, DQ479945.1, DQ479944.1, DQ479943.1, DQ479942.1, DQ479941.1, DQ479940.1, DQ479939.1, DQ479938.1, JF326432.1, DQ220724.1

<i>Epioblasma triquetra</i>	Snuffbox	Y	AR, SR	DNeasy Blood & Tissue kit	EF507816.1, EF507815.1, EF507814.1, EF507813.1, EF507812.1, EF507811.1, EF507810.1, EF507809.1, EF507808.1, EF507807.1, EF507806.1, EF507805.1, EF507804.1, DQ208590.1, AF156528.1
<i>Fusconaia flava</i>	Wabash Pigtoe	Y	AR, SR	DNeasy Blood & Tissue kit	AF232822.1, DQ298536.1, DQ298535.1, DQ298534.1, DQ298533.1, DQ298532.1, DQ298531.1, DQ298530.1, DQ298529.1, DQ298528.1, DQ298527.1, DQ298526.1, DQ298525.1, DQ298524.1, HM230370.1, HM230366.1, HM230367.1, HM849073.1, AF406799.1, EF033307.1, EF033261.1, AF156510.1, AF231733.1
<i>Lampsilis cardium</i>	Plain Pocketbook	Y	AR, SR	DNeasy Blood & Tissue kit	AF120653.1, GQ921084.1, GQ921083.1, AF156519.1, AF156518.1
<i>Lampsilis fasciola</i>	Wavy-rayed Lampmussel	Y	AR, GR	DNeasy Blood & Tissue kit	GQ921098.1, GQ921143.1, AF156520.1
<i>Lampsilis radiata</i>	Eastern Lampmussel	-	-		KC408801.1, KC408800.1, KC408799.1, KC408798.1, KC408797.1, KC408796.1, KC408771.1, KC408770.1, KC408769.1, HQ153608.1, HQ153607.1, HQ153606.1, HQ153605.1
<i>Lampsilis siliquoidea</i>	Fatmucket	Y	AR, SR	DNeasy Blood & Tissue kit	KC408792.1, KC408795.1, KC408794.1, KC408793.1, KC408791.1, KC408790.1, KC408789.1, KC408788.1, KC408787.1, KC408786.1, KC408785.1, KC408784.1, KC408783.1, KC408782.1, KC408781.1, KC408780.1, KC408779.1, KC408778.1, KC408777.1, KC408776.1, KC408775.1, KC408774.1, KC408773.1, KC408772.1, KC408768.1, KC408767.1, KC408766.1, KC408765.1, KC408764.1, KC408763.1, KC408762.1, KC408761.1, KC408760.1, KC408759.1, KC408758.1, KC408757.1, KC408756.1, KC408755.1, KC408754.1, KC408753.1, KC408752.1, KC408751.1, KC408750.1, KC408749.1, KC408748.1, KC408747.1, KC408746.1, KC408745.1, KC408744.1, HM849076.1, DQ494752.1, AF156522.1, AF156521.1
<i>Lasmigona complanata</i>	White Heelsplitter	Y	AR, SR	DNeasy Blood & Tissue kit	HM849078.1, HM849077.1, AF093845.1
<i>Lasmigona compressa</i>	Creek Heelsplitter	-	-		HM849084.1, HM849085.1, HM849081.1, HM849080.1, HM849079.1, HM849083.1, HM849082.1, AF093847.1, AF093846.1, AF156503.1
<i>Lasmigona costata</i>	Flutedshell	Y	AR, SR	DNeasy Blood & Tissue kit	GU085296.1, HM849089.1, HM849088.1, HM849087.1, HM849086.1, GU085295.1, AF093848.1

<i>Leptodea fragilis</i>	Fragile Papershell	Y	SR	DNeasy Blood & Tissue kit	GU085298.1, GQ921186.1, GU085297.1, AF049519.1, AF049518.1
<i>Ligumia nasuta</i>	Eastern Pondmussel	-	-	-	AF156515.1
<i>Ligumia recta</i>	Black Sandshell	Y	AR, SR	DNeasy Blood & Tissue kit	KC291717.1, KC291716.1, GU085299.1, AF406795.1, AF385110.1, AF156516.1, AF231748.1
<i>Obliquaria reflexa</i>	Threehorn Wartyback	Y	SR, GR	DNeasy Blood & Tissue kit	GU085300.1, AY655008.1, GU085301.1, EF033292.1, EF033254.1, AF049521.1, AF385114.1
<i>Obovaria olivaria</i>	Hickorynut	-	-		KF035243.1, KF035244.1, KF035242.1, KF035241.1, KF035238.1, KF035237.1, KF035235.1, KF035234.1, KF035232.1, KF035229.1, KF035240.1, KF035239.1, KF035233.1, KF035236.1, KF035231.1, KF035230.1, HM230408.1, GU085304.1, GU085302.1, GU085303.1, EF033306.1, EF033267.1, AF232812.1
<i>Obovaria subrotunda</i>	Round Hickorynut	Y	SR	DNeasy Blood & Tissue kit	KF035263.1, KF035268.1, KF035257.1, KF035267.1, KF035266.1, KF035262.1, KF035261.1, KF035260.1, KF035259.1, KF035258.1, KF035256.1, KF035255.1, KF035253.1, KF035252.1, KF035251.1, KF035250.1, KF035249.1, KF035248.1, KF035247.1, KF035265.1, KF035254.1, KF035246.1, KF035245.1, KF035264.1, AY655010.1
<i>Pleurobema sintoxia</i>	Round Pigtoe	Y	SR	DNeasy Blood & Tissue kit	EF033253.1, AY655019.1, GU085309.1, GU085308.1, EF033291.1
<i>Potamilus alatus</i>	Pink Heelsplitter	Y	AR, SR	DNeasy Blood & Tissue kit	GU085310.1, GU085311.1, AF049511.1, AF049510.1, AF231752.1
<i>Ptychobranchius fasciolaris</i>	Kidneyshell	Y	AR, SR	DNeasy Blood & Tissue kit	EF033265.1, EF033301.1, AF156514.1
<i>Pyganodon cataracta</i>	Eastern Floater	-	-		JX101481.1, JX101491.1, JX101490.1, JX101489.1, JX101488.1, JX101487.1, JX101486.1, JX101485.1, JX101484.1, JX101483.1, JX101482.1, JX101480.1, JX101479.1, JX101478.1, EF488186.1, EF488185.1, EF418023.1, EF418016.1
<i>Pyganodon grandis</i>	Giant Floater	Y	AR, SR	DNeasy Blood & Tissue kit	KM262551.1, HM849107.1, HM849106.1, HM849105.1, HM849104.1, HM849103.1, HM849102.1, HM849101.1, HM849100.1, GU085313.1, GU085312.1, AF406801.1, EF418024.1, EF418022.1, EF418019.1, AF156504.1, AF231734.1
<i>Quadrula quadrula</i>	Mapleleaf	Y	AR, SR	DNeasy Blood & Tissue kit	AF156511.1, EF033308.1, EF033268.1, AF231757.1, AF232823.1, FJ809751.1 (haplotype male), FJ809750.1 (haplotype female)

<i>Simpsonaias ambigua</i>	Salamander Mussel	Y	SR	DNeasy Blood & Tissue kit	N/A
<i>Strophitus undulatus</i>	Creepers	Y	AR, SR	EtOH Precipitation	HM849121.1, GQ981409.1, HM849122.1, HM849120.1, HM849119.1, HM849118.1, HM849117.1, HM849116.1, HM849115.1, GU085320.1, GU085319.1, AF093839.1, AF156505.1, AF231740.1
<i>Toxolasma parvus</i>	Lilliput	Y	GR	DNeasy Blood & Tissue kit	HM849151.1, DQ206791.1, DQ206790.1, DQ206789.1, DQ206788.1, DQ206787.1, DQ206786.1, DQ206785.1, DQ206784.1, AY655022.1, HM849154.1, HM849153.1, HM849152.1, HM849150.1, HM849149.1, HM849148.1, HM849147.1, HM849146.1, HM849145.1, HM849144.1, HM849143.1, HM849142.1, HM849141.1, HM849140.1, HM849139.1, HM849138.1, HM849137.1, HM849136.1, HM849135.1, HM849134.1, Q612178.1, GU085321.1
<i>Truncilla donaciformis</i>	Fawnsfoot	Y	SR	EtOH Precipitation	GU085323.1
<i>Truncilla truncata</i>	Deertoe	Y	AR, SR	DNeasy Blood & Tissue kit	HM852947.1, HM852948.1, GU085324.1, AF156513.1
<i>Utterbackia imbecillis</i>	Paper Pondshell	-	-		HM849172.1, HM849173.1, HM849172.1, HM849171.1, HM849170.1, HM849169.1, HM849168.1, HM849167.1, HM849166.1
<i>Villosa fabalis</i>	Rayed Bean	Y	SR	EtOH Precipitation	GQ921212.1, GQ921211.1, GQ921137.1, DQ220726.1
<i>Villosa iris</i>	Rainbow	Y	AR	DNeasy Blood & Tissue kit	GQ920976.1, GQ920980.1, GQ920979.1, GQ920978.1, GQ920977.1, GQ920975.1, GQ920974.1, GQ920973.1 (plus >100 not reported here)

Draft

Table 2. 32 COI primer pairs designed for eight native Ontario unionids. The six primers that amplified only target DNA templates are shown in bold. Intraspecific variation refers to intraspecific variation within the primer binding regions. T_A = annealing temperature; N/A = cross-species PCR not performed; NT = nucleotide.

Species	Primer ID	Sequence 5'-3'	Length of Amplified Fragment (bp)	T _A (°C)	Non-target species amplifications	Intraspecific Variation
<i>Epioblasma torulosa rangiana</i> (Northern Riffleshell)	EtoCOI4F	TGGGACAGCCAGGTAGGTTA	252	69.4	<u>Species-specific</u>	None
	EtoCOI4R	CAGTCCCAACACCACTCTCC				
	EtoCOI5F	GCCAGGTAGGTTATTGGGGG	246	63.7	<i>Potamilus alatus</i>	None
	EtoCOI5R	CCAGTCCCAACACCACTCTC				
<i>Epioblasma triquetra</i> (Snuffbox)	EtrCOI1F	TAATTCGGGCTGAGTTGGGG	268	66.1	12 species	None
	EtrCOI1R	CCAGTCCCAACACCACTCTC				
	EtrCOIaF	TTGATTATTAGTACCGGCTTTGT	245	63.1	<i>Lampsilis fasciola</i>	None
	EtrCOIaR	ACAAAGGAATCCGCTCAGC				
	EtrCOIbF	CCCACCATTATCTGGTAATGTGG	164	64.5	Unreliable amplification	None
	EtrCOIbR	ACAAAGGAATCCGCTCAGC				
<i>Lampsilis fasciola</i> (Wavy-rayed Lampmussel)	GLfaCOI3F	TGGCGTTGTGGTCTGGTTTA	505	68	<i>Elliptio dilatata</i> , <i>Lampsilis siliquoidea</i>	None
	GLfaCOI3R	ACGGTAACAGCCCACACAAA				
	LfaCOIaF	ACTGGGGCAACCTGGTAG	287	62.1	<i>Epioblasma torulosa rangiana</i> , <i>Actinonaias ligamentina</i>	None
	LfaCOIaR	CATTTCCAGACAATGGGGGG				
	LfaCOIbF	ACTGGGGCAACCTGGTAGGT	413	Failed	N/A	None
	LfaCOIbR	TGTGTGGGCTGTTACCGTGA				
	LfaCOIcF	CCCCCATTGTCTGGAAATG	183	64.1	11 species	None
	LfaCOIcR	ACGGTAACAGCCCACACA				

<i>Pleurobema sintoxia</i> (Round Pigtoe)	<i>APsi</i> COI1F	CTTTGGTGGAGAGGGGTGTC	189	68	11 species	None
	<i>APsi</i> COI1R	CAGCAACCAACCCAGGAGAT				
	<i>GPsi</i> COI1F	TGGTTGGGTTGGCTTTGAGT	453	66.1	<i>Potamilus alatus</i>	None
	<i>GPsi</i> COI1R	CAGCAACCAACCCAGGAGAT				
	<i>Psi</i>COI2F	GATTGTTACGGCGCATGCTT	199	63.7	<u>Species-specific</u>	None
	<i>Psi</i>COI2R	GACACCCCTCTCCACCAAAG				
<i>Ptychobranthus fasciolaris</i> (Kidneyshell)	<i>Pfa</i> COI1F	GAGAGTGGTGTGGGACTGG	250	67	<i>Epioblasma torulosa rangiana, Ligumia recta</i>	None
	<i>Pfa</i> COI1R	AAACAGGCAACGATGCAACC				
	<i>Pfa</i> COI2F	AGGCTTTTGATTCTGGGCTGA	275	50.4	28 species	None
	<i>Pfa</i> COI2R	CCAGTCCCAACACCACTCTC				
	<i>Pfa</i> COI3F	GGTTGTTAGTGCCGGCTTTG	243	64.5	<i>Potamilus alatus, Quadrula quadrula</i>	None
	<i>Pfa</i> COI3R	ACAAGGGAATTCGCTCAGCA				
	<i>Pfa</i> COIaF	CAATGTGATTGTAACAGCGCA	419	66.1	N/A	1 NT
	<i>Pfa</i> COIaR	AAACCGCCGTCACAGTAACT				
	<i>Pfa</i>COIbF	CTTTTGGTTGTTAGTGCCGG	265	63.1	<u>Species-specific</u>	None
	<i>Pfa</i>COIbR	AGTAACTGCTCACACAAACAAG				
	<i>Pfa</i> COIcF	CAATGTGATTGTAACAGCGC	406	63.1	N/A	None
	<i>Pfa</i> COIcR	AGTAACTGCTCACACAAACAAG				
	<i>Pfa</i> COIdF	CTTTTGGTTGTTAGTGCCGG	278	64.1	N/A	None
	<i>Pfa</i> COIdR	AAACCGCCGTCACAGTAACT				
	<i>Pfa</i> COIeF	CTTTTGGTTGTTAGTGCCG	356	62.1	N/A	None
	<i>Pfa</i> COIeR	AGAAAGATGTGTTAATGTTCCG				
<i>Quadrula quadrula</i> (Mapleleaf)	<i>EQqu</i> COI3F	GATTGGGGCTCCGGATATGG	170	61	8 species	2 NT
	<i>EQqu</i> COI3R	CTGAAGCCCCAGAATGAGCA				
	<i>AQqu</i>COI2F	GCTCATTCTGGGGCTTCAGT	197	69.4	<u>Species-specific</u>	1 NT
	<i>AQqu</i>COI2R	AAAACAGGTAACGCCGCAAC				

Villosa fabalis (Rayed Bean)	VfaCOI2F	GCTTCGGTGGACTTGGCTAT	105	66.1	Unreliable amplification	None
	VfaCOI2R	CCCAGGAGACCGCATATTCC				
	VfaCOI3F	GTGACTGCGCACGCTTTTAT	298	63.7	Species-specific	1 NT
	VfaCOI3R	AAGAAGCACCAGCAAGGTGT				
	VfaCOI5F	GTGGAGAGTGGTGTGGGAC	218	70	Epioblasma torulosa rangiana, Lampsilis fasciola, Lampsilis cardium, Lasmigona complanata	None
Villosa iris (Rainbow)	VfiCOI5R	ACCGTAACAGCCACACAAA				
	ViCOI2F	TGTGGCTCATCTCTGGTGCTT	160	70	Obovaria subrotunda	None
	ViCOI2R	AACAGCCACACAAACAACG				
	GVirCOI1F	GAGAGTGGTGTGGGACTGG	253	68	Epioblasma torulosa rangiana, Lampsilis fasciola, Potamilus alatus	None
	GVirCOI1R	CCAAAACAGGCAATGACGCA				
	GVirCOI2F	GGAGAGTGGTGTGGGACTG	251	70	Lampsilis fasciola	None
	GVirCOI2R	AAACAGGCAATGACGCAACC				
	ViriCOIaF	CTGAGTTCTTCTTTGGTGGAG	274	63.6	Species-specific	None
	ViriCOIaR	CAGCCAAAACAGGCAATGA				
	ViriCOIbF	TTGGCTGGTGCTTCTTCTAT	157	63.6	N/A	None
	ViriCOIbR	CAGCCAAAACAGGCAATGA				
	ViriCOIcF	GCGATTTTCTTTACATTTGGC	175	65.1	N/A	None
	ViriCOIcR	CAGCCAAAACAGGCAATGA				
	ViriCOIdF	CTGAGTTCTTCTTTGGTGGA	139	62.1	N/A	None

*ViriCOIdR*AAATAGAAGAAGCACCAGCC

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