Proceedings of the Nova Scotian Institute of Science (2025)
Volume 54, Part 1, pp. 111-134

MULTIPLEX PCR FOR SPECIES-LEVEL DISCRIMINATION OF YELLOW LAMPMUSSEL (*LAMPSILIS CARIOSA* (SAY, 1817)) AND TIDEWATER MUCKET (*ATLANTICONCHA OCHRACEA* (SAY, 1817))

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ABSTRACT

Effective management of imperiled freshwater mussel populations (Order Unionida) is dependent on accurate field identifications. Standard methods of identifying living mussels utilizing external shell characteristics, however, can be unreliable for some species given high levels of phenotypic plasticity and morphological overlap with other taxa. In Canada, Yellow Lampmussel (*Lampsilis cariosa* (Say, 1817)), a species of Special Concern, is limited in distribution to isolated populations within Nova Scotia and New Brunswick. Efforts to monitor this species can be complicated by difficulties in distinguishing Yellow Lampmussel from the Tidewater Mucket (*Atlanticoncha ochracea* (Say, 1817)). Both species are known from the Saint John River and its tributaries in New Brunswick and are also sympatric within the Sydney River watershed in Nova Scotia. In our survey of biology students and faculty at Cape Breton University, participants correctly identified these two species based on photographs of external shells only 61.7% of the time, with even the most experienced individuals achieving a success rate of just 68.8%. To facilitate species-level identification, here we have developed a simple genetic-based tool to differentiate between live Yellow Lampmussel and Tidewater Mucket. Using custom-designed primers and a single multiplex PCR reaction, the identity of these two species can be determined based on amplification product size. This tool should be of enormous value to freshwater mussel ecologists working to monitor Yellow Lampmussel populations and to explore other aspects of their biology and ecology.

Keywords: Freshwater mussels, Unionida, Yellow Lampmussel, Tidewater Mucket, Multiplex PCR

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INTRODUCTION

Freshwater mussels are among the most threatened taxonomic groups worldwide, with populations declining due to habitat loss, introduction of non-native species, and pollution (Lopes-Lima *et al.* 2018). More than half of all freshwater mussel species are considered Near Threatened, Vulnerable, or Endangered globally, and 7.7% are listed as Extinct (IUCN 2023). Given the important role of freshwater mussels within aquatic habitats and associated ecosystem benefits (Vaughn 2018), these species are in need of protection through targeted conservation and management plans (Haag and Williams 2014).

In the Canadian Maritimes, Yellow Lampmussel (*Lampsilis cariosa* (Say 1817)) is one of ten freshwater mussel species present and has been listed as a species of Special Concern federally under Canada's Species at Risk Act (SARA) since 2005, with additional designations of Special Concern in New Brunswick and Threatened in Nova Scotia (Fisheries and Oceans Canada 2010, Government of Canada 2024, Nova Scotia Department of Natural Resources and Renewables 2022). Management and recovery plans have been implemented at both the provincial and federal levels to support remaining populations. In Canada, this species is limited to the Saint John River and its tributaries in New Brunswick, and the Sydney River watershed, including Blacketts Lake, Gillis Lake, Forresters Lake, and Pottle Lake, in

Fig 1 **Two Yellow Lampmussel from Blacketts Lake, Cape Breton, in the summer of 2023, showing intraspecific variation in colour, size, and shape. Upper mussel: female; lower mussel: male. Scale bar: 1.5cm.**

Nova Scotia (Martel *et al.* 2010, COSEWIC 2013, NS Department of Natural Resources and Renewables 2022) (Fig 1). Across its broader global range, spanning the Northeast Atlantic slope of North America from eastern Canada to 15 eastern U.S. states, Yellow Lampmussel is considered extirpated or possibly extirpated in several jurisdictions (NatureServe 2024).

Accurate species identification is essential for estimating freshwater mussel population sizes and distributions, which directly inform conservation status assessments and management plans (Fisheries and Oceans Canada 2010, Shea *et al.* 2011). For Yellow Lampmussel, field identification is especially challenging due to its close resemblance to Tidewater Mucket (*Atlanticoncha ochracea* (Say 1817)) and their overlapping distributions in the Saint John and Sydney River watersheds (Johnson 1947, Nedeau *et al.* 2000, Sabine *et al.* 2004, Martel *et al.* 2010, Fig 2). Both species exhibit considerable morphological variation in shell shape and colour, influenced by age, sex, and environmental factors such as water flow and substrate type (Mehlhop and Cifelli 1997, Haag 2012, Howells *et al.* 2017, Keogh 2023). This intraspecific variability, particularly between males and females and among individuals from different environments, results in substantial overlap in external shell traits and has long contributed to species-level confusion (Johnson 1947, Nedeau *et al.* 2000).

The presence of fine green rays on the shell is often cited as a distinguishing feature of Tidewater Mucket (e.g. Massachusetts Division of Fisheries and Wildlife, n.d.), but ray expression can vary and is absent in some populations, notably those in the Sydney River watershed of Nova Scotia, limiting its usefulness where the two species co-occur (Nova Scotia Department of Natural Resources and Renewables 2022). Similarly, a bronze tinge to the external shell is listed as a diagnostic feature in Maine populations of Tidewater Mucket. It is not observed in Yellow Lampmussel (Maine Department of Inland Fisheries and Wildlife 2003). However, this colour difference is not evident in Nova Scotia populations within the Sydney River watershed. Tidewater Mucket is also generally smaller than Yellow Lampmussel (Nedeau *et al.* 2000), but this feature is unreliable in practice, as juvenile Yellow Lampmussels can resemble small adult Tidewater Mucket. As such, field identifications based solely on external shell morphology and size remain problematic, particularly in areas of sympatry and for species of conservation concern.

Fig 2 Similarity between Yellow Lampmussel and Tidewater Mucket sampled in Blacketts Lake, Cape Breton, May, 2025. The Tidewater Mucket specimen is shown on the top right. Note the eroded shells. Scale bar = 2cm.

Although internal shell features, such as muscle scars, hinge shape, and hinge teeth, are generally considered more consistent than external characteristics for distinguishing species (Nedeau *et al.* 2000, McAlpine *et al.* 2018), they are only visible in cleaned, opened shells and require lethal sampling. One of the most noted internal differences between Yellow Lampmussel and Tidewater Mucket is the morphology and position of the pseudocardinal teeth relative to the umbo. In Yellow Lampmussel, these teeth are described as stout, conical, and located directly beneath the umbo, while in Tidewater Mucket, they are described as being thinner, more lamellate, and situated farther anterior to the umbo (Johnson 1947, Massachusetts Division of Fisheries and Wildlife, n.d., Nedeau *et al.* 2000, McAlpine *et al.* 2018). Another frequently cited internal trait is nacre coloration: Tidewater Mucket typically has pink or salmon-coloured nacre, while Yellow Lampmussel has white to bluish-white nacre (Nedeau *et al.* 2000, Massachusetts Division of Fisheries and Wildlife, n.d.). As these internal characteristics require destructive sampling or dead specimens to examine, their utility in conservation monitoring is limited. Moreover, these morphological distinctions have not yet been validated using genetically confirmed individuals, so their reliability remains untested.

Molecular genetic approaches, including DNA barcoding and molecular identification keys (MIKs), provide alternative means of

discriminating between species that are similar morphologically, and can often be undertaken without destructive sampling (Gerke and Tiedemann 2001, Kneeland and Rhymer 2007, Boyer *et al.* 2011). Customized molecular tools can also be developed to discriminate between pairs of species that are challenging to tell apart, such as Yellow Lampmussel and Tidewater Mucket. For instance, when DNA sequences are known for a gene region of the two taxa in question, a multiplex Polymerase Chain Reaction (PCR) test can be developed as a quick tool for species-level discrimination (e.g., Marshall *et al.* 2007). In multiplex PCR, custom primers for each species are included in the PCR reaction so that the DNA of each species can be amplified using the same starting mix. Because the positioning of primers is different for each species, the size of the amplification product determines the source DNA. These tests, therefore, only require a simple PCR test for the unknown sample, with subsequent screening for the size of the PCR product using agarose gel electrophoresis. As such, this method can provide a quick and reliable visual guide for differentiating between two species without requiring further downstream processing (e.g., DNA sequencing) of the PCR product (Marshall *et al.* 2007).

To implement an efficient and effective management plan for the Yellow Lampmussel, a reliable method is needed to distinguish this species from the morphologically similar Tidewater Mucket. Misidentifications can lead to overestimating Yellow Lampmussel population sizes, potentially undermining conservation efforts (Shea *et al.* 2011, Willsie *et al.* 2020). Although trained freshwater mussel ecologists may achieve higher rates of correct identification, field surveys are often conducted by individuals with varying levels of expertise (e.g., Willsie *et al.* 2020). Moreover, even with trained observers and quantitative morphometric tools, morphology alone may be insufficient to resolve closely related or cryptic species, further reinforcing the need for molecular confirmation (Keogh and Simons 2019)

Here, we present a customized multiplex PCR assay to reliably discriminate between Yellow Lampmussel and Tidewater Mucket using non-destructive mantle swabs from live mussels. To directly evaluate the reliability of field-based species identifications, we also conducted a survey assessing participants' ability to distinguish these two species based on photographs of external shell features traditionally used to identify mussels under field conditions. Survey results

confirmed that these species are frequently confused, even among participants with field experience, demonstrating the importance of molecular confirmation to ensure accurate species assignments in conservation monitoring.

METHODS

Development of a Customized Multiplex PCR Assay

To develop a multiplex PCR assay, we targeted the mitochondrial (mt) NADH dehydrogenase subunit 1 (ND1) gene – a fast evolving protein-encoding region of the mt genome – using an existing dataset of ND1 sequences encompassing all ten extant species of freshwater mussels in the Canadian Maritimes, including Yellow Lampmussel (*Lampsilis cariosa*), Eastern Lampmussel (*Lampsilis radiata* (Gmelin 1791)), Tidewater Mucket (*Atlanticoncha ochracea*), Eastern Pearlshell (*Margaritifera margaritifera* (Linnaeus 1758)), Brook Floater (*Alasmidonta varicosa* (Lamarck 1819)), Triangle Floater (*Alasmidonta undulata* (Say 1817)), Alewife Floater (*Utterbackiana implicata* (Say 1829)), Eastern Elliptio (*Elliptio complanata* (Lightfoot 1786)), Eastern Floater (*Pyganodon cataracta* (Say 1817)), and Creeper (*Strophitus undulatus* (Say 1817)) (Rawlings and White 2022, Ryan 2022). Our assay comprised two species-specific forward primers positioned within the ND1 gene and a common reverse primer. The reverse primer selected was a variant of the unionid-specific primer, LoGlyR, used broadly in conjunction with unionid-specific primer, Leu-uurF, to amplify the ND1 gene and portions of two neighbouring tRNA genes (Serb *et al*. 2003). To develop the species-specific forward primers, we generated an alignment of all ND1 sequences of Yellow Lampmussel and Tidewater Mucket samples from multiple locations within the Canadian Maritimes (Table 1), and then selected regions spanning 18-22 base pairs that were invariant within each species, but different between species. Candidate primers were then tested against an alignment of ND1 sequences of all ten extant species of freshwater mussels in the Canadian Maritimes to test for non-target primer binding using Geneious Prime (v. 2023.1.1). When selecting custom forward primer sequences, we targeted primers with the following criteria: 1) each was 18-22 base pairs long, with a melting temperature between 50-60°C; 2) primers had a melting temperature within 5°C of each other;

3) each primer had a minimum of 4 mismatches with non-target species of freshwater bivalves, and base mismatches were near the 3' end of the sequence; and 4) PCR products differed in size between the two species when used in conjunction with the common reverse primer (ND1R-FWBIV). For the final vetting of custom forward primers, *in silico* testing was undertaken across all 10 species of freshwater bivalves spanning numerous localities within the Maritimes (Table 1) to determine the number of base-pair mismatches with non-target sequences and the size of PCR products, if amplified. We also tested these primers against the Dwarf Wedgemussel, (*Prolasmidonta heterodon* (I. Lea 1829)), historically present in the Canadian Maritimes, but now believed to be extirpated (COSEWIC 2000).

Validation of Multiplex PCR A*ssay*

Sample selection for *in vitro* testing: To validate the multiplex assay, we tested the assay on 5 Yellow Lampmussel and 5 Tidewater Mucket samples from the Canadian Maritimes, as well as 8 non-target species (Table 1). DNA for these samples was extracted between 2017-2022 from field-collected brush swab samples from sites in Nova Scotia and New Brunswick, as well as ethanol-preserved tissue samples collected across the Maritimes on loan from the New Brunswick Museum. The species identity of each of these samples was confirmed through DNA sequencing of PCR products, with subsequent phylogenetic analyses undertaken with additional sequences from GenBank to confirm the expected placement of these sequences within their species-specific lineages.

In the summer and fall of 2023, we also collected new tissue samples from Yellow Lampmussel and Tidewater Mucket from Blacketts Lake in Cape Breton, NS, in order to compare field-based identifications with species identifications resulting from our multiplex PCR assay. A total of 54 mussels were collected and identified to species in the field using shell and soft tissue features by one of us (Kellie White) with > 25 years of experience working with freshwater mussels in Canada. Specimens were photographed, sampled for DNA using a brush swab (see below) and then returned to the sediment. Of these specimens, 37 were given provisional field identifications of Yellow Lampmussel, and 17 were identified as Tidewater Mucket. Additional mussels were collected and brought back to the lab for photography and species confirmation using our assay. Photographs

Table 1 **Location and source of samples used in the development and testing of multiplex PCR assay for each of the 10 extant freshwater mussel species in the Canadian Maritime, and the Dwaft Wedgemussel (*Prolasmidonta heterodon)* historically present in this region. For each sample, we indicate the type of testing (*in-silico* and/or *in-vitro*) that was undertaken. GenBank numbers for DNA sequences used in *in silico* tests for each species are: *Atlanticoncha ochracea (*EF446103; PQ015210- PQ015217); *Lampsilis cariosa (*EF446096; PQ015218 - PQ015222); *Alasmidonta undulata* (EF446104; PQ015239 - PQ015242); *Alasmidonta varicosa (*EF446103; PQ015243 - PQ015257); *Elliptio complanata (*EF446099; PQ015230 - PQ015231 ; PQ015233 - PQ015238); *Lampsilis radiata* (EF446098; PQ015223 - PQ015229); *Margaritifera margaritifera (*EF446105; PQ015278 - PQ015287); *Prolasmidonta heterodon* (MG905826); *Pyganodon cataracta (*EF446102; PQ015268 - PQ015275); *Strophitus undulatus* (EF446100; PQ015276 - PQ015277); *Utterbackiana implicata (*EF446101; ON952510; PQ015258; PQ015260-PQ015262; PQ015264 - PQ015267). No detailed locality information was available for GenBank samples from Maine and Delaware, and a specimen of *Lampsilis cariosa* from the Saint John River. Abbreviations: NBM - New Brunswick Museum; CBU - Cape Breton University Collection.**

Species	Sample Locality	Province/State	Latitude, Longitude	Sample Source	Test Type
Target Species					
Atlanticoncha ochracea					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Canaan River	NB	45.93906, -65.74627	NBM-010238	*In-Silico & In-Vitro*
	Grand Lake	NB	45.94985202, -66.07231793	CBU	*In-Silico & In-Vitro*
	Middle Island, Saint John River	NB	45.8814, -66.42207	NBM-010227	*In-Silico & In-Vitro*
	Blacketts Lake	NS	46.06939, -60.30711	CBU	*In-Silico & In-Vitro*
	Joe's Lake	NS	46.06758, -60.215123	CBU	*In-Silico & In-Vitro*
Lampsilis cariosa					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Canaan River	NB	45.93906, -65.74627	NBM-010237	*In-Silico & In-Vitro*
	Saint John River*	NB	-	CBU	*In-Silico & In-Vitro*
	Blacketts Lake	NS	46.06939, -60.30711	CBU	*In-Silico & In-Vitro*
	Forresters Lake	NS	46.1412, -60.345	CBU	*In-Vitro*
	Pottle Lake	NS	46.204, -60.291261	CBU	*In-Silico & In-Vitro*

Non-Target Species

Alasmidonta undulata					
	Maine, USA*	ME		GenBank	*In-Silico*
	Miramichi Lake	NB	46.4632, -66.96608	NBM-011251	*In-Silico*
	Petitcodiac River	NB	45.99774, -65.09127	NBM-011061	*In-Silico*
	Gays River	NS	45.03773, -63.369525	CBU	*In-Silico & In-Vitro*
Alasmidonta varicosa					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Bouctouche River	NB	46.35775, -64.899861	NBM-009147	*In-Silico*
	North River	NB	45.96151, -65.19885	NBM-011201	*In-Silico*
	East St. Mary's River	NS	45.391562, -62.253493	CBU	*In-Silico & In-Vitro*
	Gays River	NS	45.03773, -63.369525	CBU	*In-Silico*
Elliptio complanata					
	Maine, USA*	ME	-	GenBank	
	Middle Island, Saint John River	NB	45.8814, -66.42207	NBM-010226	*In-Silico*
	Miramichi Lake	NB	46.45713, -66.95721	NBM-011211	*In-Silico*
	North River	NB	45.93924, -65.1992	NBM-011188	*In-Silico*
	Petitcodiac River	NB	46.0237, -65.0332	NBM-010475	*In-Silico*
	Fredericton, Saint John River	NB	45.946032, -66.631343	CBU	*In-Silico*
	Spednic Lake	NB	45.6202, -67.4308	NBM-010201	*In-Silico*
	Joe's Lake	NS	46.06758, -60.215123	CBU	*In-Silico & In-Vitro*
Lampsilis radiata					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Grand Lake	NB	45.94985202, -66.07231793	CBU	*In-Silico*
	Miramichi Lake	NB	46.4634, -66.9654	NBM-011217	*In-Silico*
	Fredericton, Saint John River	NB	45.946032, -66.631343	CBU	*In-Silico & In-Vitro*
	Fredericton, Saint John River,	NB	45.95286, -66.62533	NBM-010223	*In-Silico*

Table 1 cont'd

Species	Sample Locality	Province/State	Latitude, Longitude	Sample Source	Test Type
Margaritifera margaritifera					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Belleisle Creek	NB	45.6824, -65.73034	NBM-011038	*In-Silico*
	Bouctouche River	NB	46.357806, -64.931306	NBM-009659	*In-Silico*
	Spednic Lake	NB	45.69912, -67.49832	NBM-010203	*In-Silico*
	Midgell River	PEI	46.36109, -62.59462	NBM-010235	*In-Silico*
	Naufrage River	PEI	46.44364, -62.41484	NBM-010239	*In-Silico*
	Balls Creek	NS	46.15179, -60.303632	CBU	*In-Silico*
	Gays River	NS	45.03773, -63.369525	CBU	*In-Vitro*
	Margaree River	NS	46.42591, -61.088691	CBU	*In-Silico*
Prolasmidonta heterodon					
	Delaware River, USA	NY	41.86382, -75.23724	GenBank	*In-Silico*
Pyganodon cataracta					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Cocagne River	NB	46.328611, -64.834917	NBM-009664	*In-Silico*
	Second Fowler Lake	NB	46.81276, -66.45778	NBM-011050	*In-Silico*
	MacNeill's Pond	PEI	46.26521, -63.15782	NBM-011021	*In-Silico*
	Joe's Lake	NS	46.06758, -60.215123	CBU	*In-Silico*
	Loch Lomond	NS	45.75967, -60.599794	CBU	*In-Silico* & *In-Vitro*
	Rotary Drive	NS	46.12183, -60.181694	CBU	*In-Silico*
Strophitus undulatus					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Bouctouche River	NB	46.312333, -64.896222	NBM-009661	*In-Silico* & *In-Vitro*
	Miramichi Lake	NB	46.4632, -66.96608	NBM-011252	*In-Silico*

Utterbackiana implicata					
	Maine, USA*	ME	-	GenBank	*In-Silico*
	Little River	NB	46.02144, -65.02198	NBM-010477	*In-Silico*
	Middle Island, Saint John River	NB	45.8814, -66.42207	NBM-010224	*In-Silico*
	Miramichi Lake	NB	46.4634, -66.9654	NBM-011216	*In-Silico*
	Petitcodiac River	NB	45.99774, -65.09127	NBM-011069	*In-Silico*
	Fredericton, Saint John River	NB	45.946032, -66.631343	CBU	*In-Silico*
	Fredericton, Saint John River	NB	45.96467, -66.669801	GenBank	*In-Silico*
	Midgell River	PEI	46.35931, -62.59206	NBM-010232	*In-Silico*
	East St. Mary's River	NS	45.06272, -61.910788	CBU	*In-Silico* & *In-Vitro*

* No detailed locality information available.

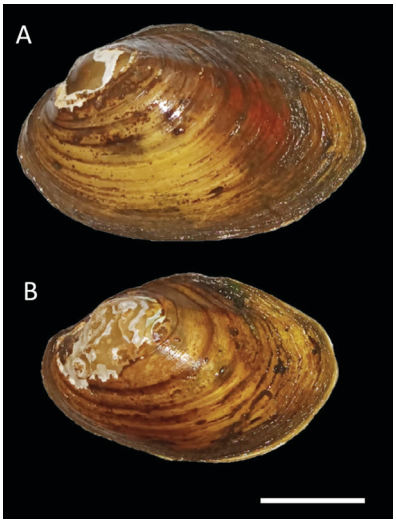

Fig 3 **Left valves of Yellow Lampmussel (A) and Tidewater Mucket (B) collected from Blacketts Lake in September, 2023. These photographs were used in the Mussel Identification Survey. Scale bar: 2cm.**

of these genetically-confirmed specimens were used in our Mussel Identification Survey (Fig 3).

DNA samples were collected from each mussel using a non-destructive technique (e.g., Karlsson *et al.* 2013, Massault *et al.* 2022). A dental brush (GUM Proxabrush®) was inserted into the mussel vertically between the valves and rotated 10 times in each direction. The brush was then removed and preserved in a 1.5 ml Eppendorf tube with 500µl of 95% ethanol. For photography, both in the field and in the lab, the right valve of each mussel was placed down on a whiteboard, so the left valve was facing the camera, and a piece of modelling clay was used to ensure each mussel was in a consistent position relative to the digital camera. A ruler was used for scale.

DNA extraction: DNA extraction for all samples was conducted using a QIAGEN DNeasy Blood and Tissue Kit following the manufacturer's instructions with slight modifications noted below. To prepare brush swabs for DNA extraction, the dental brush was removed from the ethanol and air dried for 2 hours. Brushes were then placed into sterile 1.5 ml Eppendorf tubes with 180µl ATL buffer and 20µl Proteinase K and incubated at 56°C for a minimum of 1 hour. After incubation, samples were spun down, and dental brushes removed from each tube and discarded. Recommended protocols of the kit

were followed from this point onwards, with DNA eluted from each spin column in 75µl of AE Buffer. To ensure the successful extraction of high molecular weight DNA from each sample, 5µl of 1X DNA was mixed with 1.5µl of 6X loading buffer and loaded into a 0.8% TBE agarose gel stained with GelRed. Gel electrophoresis was carried out at 120V for 40 minutes. The gel was then imaged under UV light using AlphaImager™ to visualize the DNA bands associated with each sample.

Multiplex PCR testing on genetically-confirmed samples: To determine the success of our custom-designed multiplex PCR assay, we tested this on genetically-confirmed samples of Yellow Lampmussel, Tidewater Mucket, and Eastern Elliptio from the Canadian Maritimes. PCR amplifications were carried out in 25µl reactions for each DNA sample using GoTaq 2X Master Mix (Promega). Initial reactions contained 10µl of water, 0.5µl of 10µM LC-ND1-F primer, 0.5µl of 10µM AO-ND1-F primer, 0.5µl of 10µM ND1R-FWBIV universal primer, 12.5µl of GoTaq 2X Master Mix, and 1µl of 1X DNA. Samples were amplified using a BioRad T100™ thermal cycler under the following conditions: initial denaturation at 94°C for 30s, followed by 38 cycles of 94°C for 1min, 48-60°C for 30s, 72°C for 1min, and a final step of 72°C for 10min, before being held at 4°C upon completion. Gradient PCRs were conducted over a range of annealing temperatures (48°C, 50°C, 54°C, and 59°C) to establish an optimal annealing temperature in which DNA would amplify the target Yellow Lampmussel and Tidewater Mucket samples only. Negative controls were run with each batch of DNA samples. To visualize the results of each amplified DNA sample, 5µl of each sample was mixed with 1.5µl of 6X loading dye and loaded into its respective well in a 1% TBE agarose gel stained with GelRed. Gel electrophoresis and imaging was carried out as described above.

Multiplex PCR testing on non-target species: To determine if our multiplex PCR assay would amplify non-target species found in the Canadian Maritimes, we conducted additional multiplex PCRs using genetically-confirmed DNA samples of the 8 non-target species currently present in the Maritimes. PCR protocols for non-target taxa and all subsequent multiplex assays were conducted using the Q5® High-Fidelity 2X Master Mix (New England BioLabs) due to limited availability of GoTaq 2X Master Mix for purchase. PCR reactions comprised 8.75µl of water, 1.25µl 10µM LC-NDI-F, 1.25µl

10µM AO-ND1-F, 1.25µl 10µM ND1R-FWBIV, 12.5µl of Q5 Hot Start High-Fidelity 2X Master Mix, and 1µl of 1/10X DNA. Samples were then placed in the thermocycler where they underwent initial denaturation at 98°C for 30s, followed by 30 cycles of 98°C for 10s, 59°C for 30s, 72°C for 30s, and a final step of 72°C for 2 min before being held at 4°C upon completion. After PCR, 5 µl of each sample underwent gel electrophoresis as previously described to determine if a PCR product(s) was evident for each species sampled and to gauge the approximate size.

Multiplex PCR on new Yellow Lampmussel and Tidewater Mucket samples: Once the primer sets had been validated, we applied this assay to DNA extracted from brush swab samples of Yellow Lampmussel and Tidewater Mucket collected from Blacketts Lake in 2023. DNA was amplified using protocols for the Q5® High-Fidelity 2X Master Mix before undergoing gel electrophoresis as described above. Species identifications (either Yellow Lampmussel or Tidewater Mucket) were then made for each sample based on the resulting amplification product size. Although not all samples successfully amplified on the first attempt, all recalcitrant samples eventually amplified using a 1/10X dilution of our original DNA extraction and Q5 Master Mix.

Mussel Identification Survey

Considering the potential challenges of differentiating between Yellow Lampmussel and Tidewater Mucket based on external shell features alone, we developed a Mussel Identification Survey to assess the ability of participants to discriminate between these two species correctly. Twenty photographs of mussel shells with lab-confirmed identifications based on our multiplex PCR assay were compiled into an online quiz via Google Forms and sent to more than 30 biology students/faculty (past and present) who ranged in experience identifying mussels (Fig 3). The survey was voluntary, and participants were asked to provide their level of experience working with freshwater mussels (no experience, some experience, very experienced), and then to identify each photograph as either a Yellow Lampmussel or Tidewater Mucket based on external shell characteristics. Participants were provided with a field guide (McAlpine *et al.* 2018) to help them make this distinction. Results were then compiled, and the percentage of correct and incorrect identifications was determined.

Statistical analyses of survey results were completed using Minitab V21.1.0 to assess the accuracy of identifications using external shell characteristics and to explore any association between the experience level of participants and the accuracy of their identifications. A one-sample t-test was used to determine if there was a significant difference between the mean quiz score and the expectation based on chance (50:50), if each participant was randomly selecting the identity of each photo. A one-way ANOVA was also used to determine if there was a significant difference between experience level in identifying freshwater mussels and accuracy of identifications.

RESULTS

Custom Multiplex Primers and Expected Fragment Sizes

Using an *in-silico* approach, we developed species-specific forward primers for the ND1 region of Yellow Lampmussel and Tidewater Mucket that worked in combination with the common reverse primer, ND1R-FWBIV, to amplify PCR products 460 bp and 758 bp, respectively (Table 2 & 3). *In silico* testing of these primers against non-target species indicated a minimum 4 base mismatch with our custom forward primers (Table 3). Amplification product sizes differed for some of these taxa, providing additional hallmarks of non-target binding (see Table 3).

Table 2 Primer sequences used in the multiplex PCR assay, including a forward primer specific to Yellow Lampmussel (LC-ND1-F), a forward primer specific to Tidewater Mucket (AO-ND1-F), and a universal reverse primer (ND1R-FWBIV). Primers were designed using Geneious Prime (v. 2023.1.1).

Name	Species	Sequence (5'-3')	Size	Direction
LC-ND1-F	*Lampsilis cariosa*	ATT CGC CTA GTT AAC TTC TC	20bp	Forward
AO-ND1-F	*Atlanticoncha ochracea*	TTC CTA CCA TTC ATC CTT AC	20bp	Forward
ND1R-FWBIV	All species	TGC TTG GAA GGC AAY TGT ACT	21bp	Reverse

Table 3 **Minimum number of base pair mismatches associated with species-specific forward primer binding to the ND1 sequence of each of the 11 freshwater mussel species historically recorded in the Canadian Maritimes. Also shown is the predicted size of the amplified product(s) generated using each forward primer in combination with the ND1R-FWBIV primer. Product sizes with primer mismatches of 8bp or more are not shown. *In silico* analyses of primer binding and PCR amplifications were generated in Geneious Prime (2023.1.1).**

Species	Mismatches with LC-ND1-F	Product Size	Mismatches with AO-ND1-F	Product Size
Alasmidonta undulata	8	N/A	5	732bp
Alasmidonta varicosa	7	440bp	6	731bp, 380bp
Atlanticoncha ochracea	7	467bp	**0**	758bp
Elliptio complanata	6	453bp	4	744bp
Lampsilis cariosa	**0**	460bp	4	751bp
Lampsilis radiata	4	460bp	4	751bp
Margaritifera margaritifera	8	N/A	6	747bp, 684bp
Prolasmidonta heterodon	8	N/A	8	N/A
Pyganodon cataracta	9	N/A	6	98bp
Strophitus undulatus	8	N/A	5	731bp
Utterbackiana implicata	7	439bp	6	730bp

In vitro Validation of Multiplex PCR Assay

In vitro validation of our multiplex PCR assay confirmed the expected PCR product sizes when PCR mixes were spiked with DNA from either Yellow Lampmussel or Tidewater Mucket (Fig 4). Using GoTaq 2x Master Mix, the non-target species, Eastern Elliptio, generated a weak PCR product of ~ 750bp at annealing temperatures of 48°C, 50°C, and 54°C, but did not amplify at 60°C. The size of this band suggested that the custom forward primer for Tidewater Mucket, with 4 base differences from the *Elliptio* sequence, was binding to the *Elliptio* ND1 gene at low - moderate annealing temperatures (see Table 3). For this reason, annealing temperatures of 59-60°C were used for all samples moving forward. Further testing of our assay against the 8 non-target freshwater mussel species in the Canadian Maritimes using Q5 High Fidelity 2x Master Mix and an annealing temperature of 59°C did not result in any visible amplification products, except for positive controls.

Multiplex PCR sampling of Yellow Lampmussel and Tidewater Mucket DNA samples from across the Canadian Maritimes (Table 1) yielded successful amplifications for all locations sampled and generated the expected band sizes. Of the 54 specimens from Blacketts Lake, our Multiplex PCR test identified 35 Yellow Lampmussel and

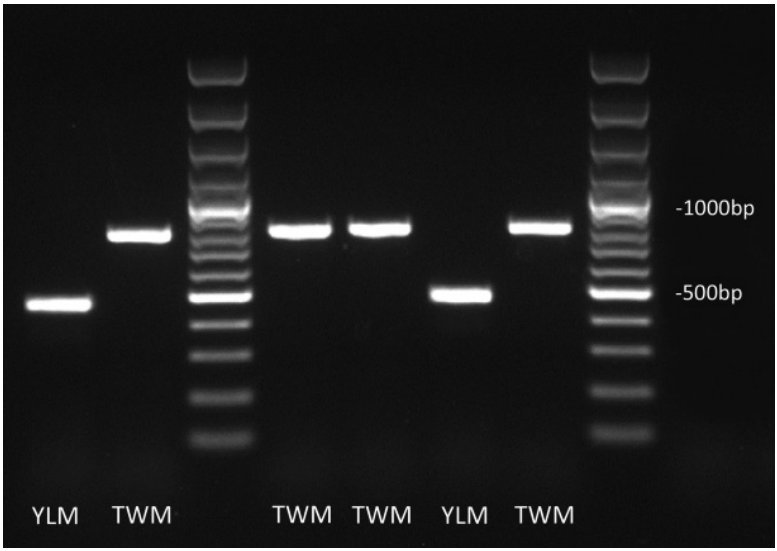

Fig 4 **PCR products resulting from the amplification of the ND1 gene region of Yellow Lampmussel (YLM: lanes 1 and 6) and Tidewater Mucket (TWM: lanes 2, 4, 5 & 7) using LC-ND1-F, AO-ND1-F, and ND1R-FWBIV primers in multiplex PCR. Lanes 3 and 8 include a 100 base pair size standard. As shown, band sizes of samples are a little larger than predicted since GelRed slows the migration of PCR products relative to the standard.**

19 Tidewater Mucket. Of these, 52 (96.3%) of the specimens matched their provisional field-based species identification (37 Yellow Lampmussel, 17 Tidewater Mucket). Of the 2 misidentified mussels, both were Tidewater Mucket specimens that were incorrectly labelled as Yellow Lampmussel.

Mussel Identification Survey

A total of 29 participants completed the Mussel Identification Survey with 15 (51.7%) having no experience in mussel identification, 10 (34.5%) having some experience, and 4 individuals (13.8%) rating themselves as very experienced. The mean quiz score across participants was 12.34/20 (SD= 2.6), indicating that on average, participants were only able to correctly identify Yellow Lampmussel and Tidewater Mucket 61.7% of the time based on external shell features alone. The average accuracy of Yellow Lampmussel identifications was 59.4%, while Tidewater Mucket was 66.0%, suggesting that Yellow Lampmussel was more likely to be misidentified than Tidewater Mucket. A one sample t-test conducted to determine if there

was any significant difference between quiz participants' actual score and the expected score of 10/20 (50.0%) based on guessing correctly by chance alone indicated a significant difference from chance ($t_{(28)} = 4.82$, $p < .05$). This suggests that while not very accurate, external shell characteristics did provide some help in differentiating between Yellow Lampmussel and Tidewater Mucket.

In terms of the benefit of past experience with mussel identification, 15 participants with no experience in identifying freshwater mussels had an average score of 11.93/20 (59.7%) (*SD*= 2.7), 10 participants with some experience had an average score of 12.40/20 (62.0%) (*SD*= 2.7), and 4 experienced participants had an average score of 13.75/20 (68.8%) (*SD*= 2.2). Despite this small increase in score with experience, experience level did not significantly influence a participant's ability to differentiate between the two species of freshwater mussels (ANOVA: $F_{(2, 26)} = 0.75$, $p= 0.48$).

DISCUSSION

The multiplex PCR assay developed in this study provides a rapid, reliable, and non-lethal method for validating field-based species assignments, supporting the conservation and management of Yellow Lampmussel. By enabling the clear differentiation of Yellow Lampmussel and Tidewater Mucket, the assay addresses a key challenge in accurately estimating population sizes and distributions for conservation assessments. Our multiplex PCR assay also demonstrated high specificity, successfully amplifying only the target species even when tested against a range of non-target freshwater mussels from the Maritimes. In addition to its direct application in monitoring efforts, molecular confirmation opens new opportunities to develop improved field identification methods. For example, genetically-verified specimens could support the use of quantitative morphometric approaches to refine shell-based identifications (e.g., Willsie *et al.* 2020, Butler-Doucette 2024). Molecular truthing also enables the systematic evaluation of soft tissue features, such as mantle and siphon morphology, which may offer practical, non-destructive traits for distinguishing species in the field (Fishelson 2000, Martel *et al.* 2010). Although soft tissue characteristics have historically been underutilized in freshwater mussel taxonomy, their potential diagnostic value can now be explored with greater confidence (Fig 5).

Fig 5 **Underwater photographs of a Yellow Lampmussel (A) and Tidewater Mucket (B) *in situ* with soft tissue structures exposed including the mantle, inhalant siphon, exhalant siphon, and papillae. Photographs were taken at Cape Breton University in September, 2023, using an Olympus Tough TG-6 Waterproof Camera. Scale bars: 3mm.**

Misidentifications of freshwater mussels are surprisingly common, even when relying on external and internal shell characteristics and among experienced field biologists (Shea *et al.* 2011, Howells *et al.* 2017, Bolotov *et al.* 2024). In Shea *et al.* (2011), participants at a mussel identification workshop correctly identified only 73.5% of specimens overall, with success rates ranging from 33% to 100% across species, despite prior familiarity with the local fauna. Similarly, Howells *et al.* (2017) reported correct identification of only 24% of specimens among experienced participants in Texas, with particularly low accuracy for threatened species. Both studies found that identification success was positively associated with shell size, sculpture, and years of experience.

Our Mussel Identification Survey, while limited to two species and based solely on external shell photographs, further demonstrates the challenges of field-based mussel identification. Participants correctly identified Yellow Lampmussel and Tidewater Mucket only 61.7% of the time, a rate lower than that observed by Willsie *et al.* (2020) when differentiating Wabash Pigtoe and Round Pigtoe (77% accuracy). Although participants in our study had access to a field guide, most had limited or no prior experience with mussel

identification. Notably, even among those self-reporting as "very experienced," success rates remained below 70%, reinforcing the insufficiency of external shell features alone for reliable species discrimination.

Patterns of misidentification in our survey revealed asymmetries: Yellow Lampmussel was more often misclassified as Tidewater Mucket than the reverse. This raises the possibility that, without molecular verification, Tidewater Mucket may be overrepresented in field surveys where the two species are sympatric. In contrast, provisional identifications of live specimens at Blacketts Lake conducted by experienced personnel aligned with genetic results 96.3% of the time, highlighting the value of extensive field experience. Nevertheless, the occurrence of misidentifications even among trained observers underscores the importance of supplementary molecular verification tools, particularly for species of conservation concern.

It is important to recognize that in typical field settings, biologists may sometimes have access to additional diagnostic features beyond external shell morphology. Empty shells exposing internal structures such as hinge teeth and muscle scars can improve identification accuracy (Nedeau *et al.* 2000, McAlpine *et al.* 2018). However, live surveys, often the only ethical method when working with at-risk species, rely predominantly on external characteristics, and access to internal features is inconsistent. Even when internal features are available, high rates of misidentification have still been documented (Shea *et al.* 2011, Howells *et al.* 2017).

Geographic variation and environmental influences on shell morphology (i.e., phenotypic plasticity) further complicate field identifications (Sheldon 2017). Diagnostic traits that distinguish Yellow Lampmussel and Tidewater Mucket in some regions may be less reliable elsewhere, such as in Cape Breton, where these species appear particularly similar. This highlights the importance of incorporating molecular tools into species identification efforts, especially for conservation-focused surveys.

In conclusion, our findings, combined with previous studies, highlight the persistent challenges of freshwater mussel identification based on morphology alone and reinforce the critical importance of developing complementary molecular approaches. Our multiplex PCR assay provides a practical, accessible tool that can support the conservation and management of Yellow Lampmussel and related

species by substantially improving the accuracy and confidence of species-level identifications in the field.

Acknowledgements We would like to thank Courtney Trask and Sarah Smith for assistance with fieldwork, and Alicia Penney for providing helpful suggestions. Many thanks also to Mary Sollows, Amanda Bremner, and Donald McAlpine at the New Brunswick Museum for the loan of mussel specimens. Our Mussel Identification Survey was approved by the CBU Research Ethics Committee (REB #2024019) prior to implementation. This research was supported by an NSERC USRA to Sophia Butler-Doucette and funding through Fisheries and Oceans Canada (DFO) under the Species at Risk Act (SARA) program. We gratefully acknowledge DFO funding contributions across multiple grants that enabled this work.

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