Received: 17 December 2019 | Revised: 20 March 2020 | Accepted: 27 March 2020

DOI: 10.1002/edn3.89

ORIGINAL ARTICLE

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada

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Funding information
Funding from the Ministère des forêts de la faune et des parcs du Québec and the Canadian Research Chair in Genomics and Conservation of Aquatic Resourses.

Abstract

Practical applications of environmental DNA (eDNA) are in exponential expansion, especially for the assessment and monitoring of freshwater metazoans. Because eDNA sampling and analysis is noninvasive, it improves the detection of threatened, invasive, and exploited species for which monitoring may be challenging. Species detection efforts using a combination of eDNA and qPCR have been highly successful and, as a result, their use in species monitoring is expanding rapidly. We developed qPCR primers and probes in order to monitor many invasive, threatened, or exploited aquatic species as part of various monitoring eDNA projects in the province of Québec, Canada. Here, we present a total of 60 species-specific qPCR assays (including PCR protocols, primers, and TaqMan probes sequences) developed for the detection of 45 fishes, six amphibians, five reptiles, two mollusks, and two crustaceans. These comprised nine and 27 species, respectively, listed as invasive and threatened in Eastern Canada. These resources should be of broad usefulness not only for monitoring studies based in Québec but throughout the geographic range of the targeted species in North America.

KEYWORDS

amphibians, conservation, crustaceans, environmental DNA, fish, mollusks, primers, reptiles

1 | INTRODUCTION

Freshwater ecosystems rank among the most endangered habitats in the world and due to increasing human pressures conservation of these ecosystems remains a challenge (Chatterjee, 2017; Dudgeon et al., 2006; Reid et al., 2019; WWF, 2018). Among anthropogenic causes, habitat degradation, destruction or modification, unsustainable fisheries, pollution, and invasive species are persistent and significant drivers of population declines in freshwater ecosystems (Dudgeon et al., 2006; Reid et al., 2019). In North America, more than 80% of threats to fish, reptile, and amphibian populations are related to habitat degradation, exploitation, and invasive species (WWF, 2018). Reptilian and amphibian species face the highest proportion of decline among vertebrates (Böhm et al., 2013; IUCN, 2019). In Canada, wood turtle (*Glyptemys insculpta*) and the spiny softshell turtle (*Apalone spinifera*) are examples of species classified as

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threatened and endangered, respectively, by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2007, 2016). The major threats they face include habitat loss and fragmentation, road kills, pesticide exposure, and infectious diseases (Lesbarrères et al., 2014).

Habitat deterioration caused by pollution (i.e., toxic contaminants) organic pollution, and sediment loading, are also responsible for the important extinction rate of North American mollusks, especially for pollution-sensitive species such as freshwater mussels (Lopes-Lima et al., 2018; Ricciardi & Rasmussen, 1999). One example of a nationally imperiled mussel in Canada, the hickorynut, (*Obovaria olivaria*, Unionidea, COSEWIC, 2011), is currently suffering from the population decline of lake sturgeon (*Acipenser fulvescens*), the fish host needed to complete their life cycle. Another major cause for the hickorynut decline is the introduction of aquatic invasive species, such as the zebra mussel (*Dreissena polymorpha*) in the Laurentian Great Lakes and the St. Lawrence River (Hebert, Wilson, Murdoch, & Lazar, 1991; Schloesser, Metcalfe-Smith, Kovalak, Longton, & Smithee, 2006).

The introduction of invasive species, even if they are inconspicuous, can greatly modify freshwater habitats and jeopardize ecosystems integrity. For example, as a consequence of the introduction of the predatory waterflea *Bythotrephes longimanus* in the mid-1980s, the crustacean zooplankton communities of the Laurentian Great Lakes have been drastically modified (Barbiero & Tuchman, 2004; Strecker, Arnott, Yan, & Girard, 2006). This predatory cladoceran also competes directly with larval fish for food resource (Branstrator, 1995).

Effective management of freshwater ecosystems also requires data on the distribution of exploited, rare, or invasive fish species. Expansion of invasive fish species is especially threatening for large interconnected freshwater ecosystems such as the Laurentian Great Lakes, which represent one of most important ecological natural resources as well as being of high socio-economic importance for recreational and commercial fishing industries. For example, the invasion of alewife (*Alosa pseudoharengus*) and sea lamprey (*Petromyzon marinus*) during the 1940s was linked to the decline in native fish abundance including the lake trout (*Salvelinus namaycush*), an important salmonid species for recreational fisheries as well as the lake whitefish *Coregonus clupeaformis* one of the most commercially important freshwater fishes in Canada (Madenjian et al., 2002; Wells & McLain, 1972). A salmonid stocking program was implemented to reduce alewife abundance by introducing a non-native salmonid species, that is, chinook salmon (*Oncorhynchus tshawytscha*), as well as creating interest for recreational fishing of this new species. More recently, the so-called "Asian carps," including the grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*), and black carp (*Mylopharyngodon piceus*) are being thoroughly monitored because of the threat they are representing for the socio-economic and ecological integrity of the Laurentian Great Lakes (Kolar et al., 2005).

For most freshwater species, assessment and monitoring are still mainly conducted using standard sampling methods such as gillnets for fish (Sandstrom, Rawson, & Lester, 2013; SFA, 2011), capture by traps, auditory surveys or visual observation for reptiles and amphibians (Hutchens & DePerno, 2009), and observation with an aqua-scope for mussels (OMNRF, 2018; Stoeckle, Kuehn, & Geist, 2016). However, in many cases, freshwater species may be very difficult to detect using these traditional methods due to their ecology and life-history traits as well as being a cause of habitat and population disturbance. Here, the analysis of environmental DNA (eDNA) may greatly contribute to improve the detection and monitoring of threatened, invasive, and exploited species without disturbing their habitat (Mauvisseau, Tönges, Andriantsoa, Lyko, & Sweet, 2019; Mize et al., 2019). This approach allows tracing DNA from different sources, that is, epidermis, feces, mucus, collected in environmental samples such as water from lakes or rivers. Once filtered and DNA extracted, the presence of several or specific species is confirmed using different methods (e.g., qPCR or metagenomics), and more recently CRIPR-Cas (Williams et al., 2019) depending on the scope and goal of the study. In a metagenomics approach, all species of a targeted taxonomic community can be identified simultaneously while in qPCR or CRIPR-Cas the presence of a single targeted species is normally assessed. (Deiner et al., 2017; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Taberlet, Bonin, Zinger, & Coissac, 2018; Wilcox et al., 2013; Williams et al., 2019).

The use of qPCR for species detection relies on the critical step of developing species-specific primers that only amplify the DNA of the target species, avoiding false-positive results caused by cross-amplification by DNA from sister species. To confirm the absence of cross-amplification, primers must be tested on all related species potentially present in the region of study thus validating that only the target species is amplified by the primers (Wilcox, Carim, McElvey, Young, & Schwartz, 2015; Wilcox et al., 2013).

Over the last years, we have developed qPCR primers and probes in order to monitor invasive, threatened, or exploited aquatic species for various eDNA projects in the province of Québec, Canada. Here, we describe 60 qPCR primer pairs and associated TaqMan probes designed to detect fish (45 species), amphibians (six species), reptiles (five species), mollusks (two species), and crustaceans (two species), as well as their PCR conditions and results of their tests for cross-amplification of related species. As the geographic distribution of essentially all of these species extends throughout northeastern North America and in even more widely in some cases, these qPCR assays should be broadly useful for the detection of these species.

2 | MATERIALS AND METHODS

2.1 | Sequence data for primer development

Reference sequences from mitochondrial genes, either cytochrome oxidase subunit 1 gene (COI), NADH dehydrogenase subunits (NADH), and cytochrome b gene (CYTB) from the targeted and related species were downloaded from BOLD (Ratnasingham & Hebert, 2007; http://www.boldsystems.org) or GenBank (Bensen

et al., 2013; https://www.ncbi.nlm.nih.gov/genbank/) and aligned in Geneious 9.0.5 (https://www.geneious.com/). Primers were designed from the COI sequence for most species; however, NADH or CYTB sequences were chosen when the COI sequences of the targeted species did not have enough mismatches with the related species. All primers and probes were designed in regions with low intraspecific divergence while maximizing mismatches among related species at the extreme 3′end (Wilcox et al., 2013). Sequences were downloaded for 45 targeted fish species from 17 families, for five reptile species from three families, for six amphibian species from two families, for two crustaceans and two mollusks as well as sequences of related species present in Québec (Table S1).

For the alewife floater (mollusk, *Utterbackiana implicata*) and related species, some sequences for the gene of interest were unavailable in the database. Thus, the NADH I sequence was generated by PCR amplification on extracted genomic DNA using primers developed by Serb, Buhay, and Lydeard (2003), Leu-uurF (5′-TGGCAGAAAAGTGCATCAGATTAAAGC-3′) paired with NIJ-12073 (5′-TCGGAATTCTCCTTCTGCAAAGTC-3′) or LoGlyR (5′-CCTGCTTGGAAGGCAAGTGTACT-3′) following these conditions: 34 cycles × [94–98°C, 40 s], 50–58°C for 1 min and 68–72°C for 1.5 min and then Sanger sequenced at the Genomic Analysis Platform, IBIS, Université Laval, QC, Canada.

2.2 | Primer development

Primers were designed to amplify fragments in a range of 101–250 bp to allow for Sanger sequencing in order to be able to validate eDNA detection when necessary. Annealing temperature was validated using Primer Express 3.0 (Life Technologies) and cross-amplification to unrelated species was verified using Primer Blast (Ye et al., 2012; https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All designed primers and probes were validated for amplification of targeted species and for cross-amplification with related species (Table S1) using in-house extracted genomic DNA from various tissues for fish, amphibians, mollusks, and crustaceans using a salt DNA extraction protocol (Aljanabi & Martinez, 1997), and from blood for reptiles using the DNeasy blood and tissue kit (Qiagen). Preliminary primer screening was performed with FAST SYBR Green (Life Technologies). Amplifications were performed on a 7,500 Fast Real-Time PCR System (Applied Biosystems) in a final volume of 20 µl: 10 µl of Fast SYBR® Green Master Mix, 1 µl of each primer (10 µM), 2 µl of DNA (5–10 ng) and 6 µl of UltraPure Distilled Water (DNAse, RNAse, Free, Invitrogen™) following these conditions: 95°C for 20 s, 40 cycles × [95°C for 3 s, 60°C for 30 s]. Finally, selected primers were tested with their probes in a TaqMan assay in a final volume of 20 µl including 1.8 µl of each primer (10 µM), 0.5 µl of probe (10 µM), 10 µl of TaqMan® Environmental Master Mix 2.0 (Life Technologies), 3.9 µl of dH_2O and 2 µl of DNA (10 ng) following these conditions: 50°C for 2 min, 95°C for 10 min 50 cycles × [95°C for 15 s, 60°C for 1 min].

2.3 | Assay sensitivity

A standard curve experiment was performed following the same conditions as described above for the TaqMan assay. A synthetic DNA template of 500 base pairs (Integrated DNA Technologies Inc.) including the target amplicon sequence was designed from the COI, CYTB, or NADH gene sequence depending on the species. From the stock, diluted at 1.00E + 10 copies/µl, a nine-level dilution series (2,000, 1,000, 500, 100, 20, 8, 4, 2, and 1 copies per reaction) was prepared in a sterile yeast tRNA (10 µg/µl) solution. Ten replicates of each dilution were run to determine, for each primer/probe set, the amplification efficiency and the limit of detection defined as the lowest copies per reaction with >95% amplification success (Bustin et al., 2009).

3 | RESULTS

A final set of 60 assays were optimized and validated, one per targeted species which are presented in Tables 1–4. Species used for cross-amplification tests are presented in Table S1 and mismatches to primers with respect to related species are available on DRYAD. Only five tests showed a cross-amplification with the DNA of related species (primer set for *S. namaycush*, *A. rostrata*, *E. lucius*, *M. thompsonii*, and *D. fuscus*), thus confirming assay specificity for practically all primer-probe sets. In addition, 18 assays were tested for efficiency and limits of detection using a standard curve experiment with synthetic DNA, which revealed high amplification efficiency (Table 5). Most assays were developed for detection experiments, not for quantification, therefore no standard curve experiment with synthetic DNA was performed.

3.1 | Exploited fish and monitored fish species

Species-specific primers were designed for 22 key species for recreational fisheries and 20 of these were validated in eDNA studies (Table 1). Two species-specific assays were designed for monitored fish species, brown bullhead (*Ameiurus nebulosus*) and eastern silvery minnow (*Hybognathus regius*). Standard curve experiments were performed for 14 of these species, including six salmonids (*Salmo salar*, *S. trutta*, *Coregonus clupeaformis*, *Prosopium cylindraceum*, *Oncorhynchus mykiss*, *Salvelinus alpinus*), largemouth bass (*Micropterus dolomieu*), striped bass (*Morone saxatilis*), lake sturgeon (*A. fulvescens*), sandlance (*Ammodytes* sp.), Atlantic herring (*Clupea harengus*), capelin (*Mallotus villosus*), rainbow smelt (*Osmerus mordax*), and the redfish (*Sebastes* sp.). Based on the standard curve experiment, the assays for the salmonid species had an amplification efficiency varying between 93.5% and 108.9%, as expected for an efficiency considered as acceptable (Taylor et al., 2019) and a limit of detection varying between 20 mtDNA copies/rxn (*S. trutta*, *M. dolomieu*, *O. mordax*) and two mtDNA copies/rxn (*S. salar*) (Table 5).

TABLE 1 Species-specific primers, probes for exploited and monitored fish species (*)

Scientific and Common name	Primer/Probe Gene	Sequence 5′ > 3′	bp	eDNA
Acipenser fulvescens	ACFU_COIF	GCTGGCGGGAAACCTG	179	v
Lake sturgeon	ACFU_COIR	TGACTAATACAGATCACACAAACAGAGGT		
	ACFU_COI_probe	TACCATTATTAACATGAAACCC		
Ameiurus nebulosus (*)	AMNE_CYTBF	CCCTCGTACAATGAATCTGAGGG	133	–
Brown bullhead	AMNE_CYTBR	GTTTCATGTAAAAAGAGGGCATGTAAA		
	AMNE_CYTB_probe	ACCCGATTCTTCGCATTT		
Ammodytidae sp	AMSP_COIF	GTTGATTTAACAATCTTCTCACTGCATC	143	v
Sandlance	AMSP_COIR	ATTAGCACAGCTCACACAAATAACG		
	AMSP_COI_probe	AACTTCATCACCACAATTA		
Clupea harengus	CLHA_COIF	ACGGTATATCCTCCTCTGTCAGGA	193	v
Atlantic herring	CLHA_COIR	TAACAAGAACGGATCAGACAAACAGA		
	CLHA_COI_probe	CATCAGTTGACCTAACCAT		
Coregonus clupeaformis	COCL_CYTBF	CAAACCTCCTTTCTGCCGTG	198	v
Lake whitefish	COCL_CYTBR	AGTTGATCCCTGCTGGGTTG		
	COCL_CYTB_probe	TTGTGCAGTGAATCTGA		
Cyprinus carpio	CYCA_COIF	CCACTAATAATCGGAGCCCCA	173	v
Common carp	CYCA_COIR	GCTCCTGCGTGGGCTAAG		
	CYCA_COI_probe	ACTGCCCCCATCATT		
Esox lucius	ESLU_COIF	CCATTATTTGTTTGAGCAGTCCTG	152	v
Northern pike	ESLU_COIR	GGTGTTGGTATAGAATAGGGTCTCCA		
	ESLU_COI_probe	TGTACTTCTACTTCTGTCTCTC		
Esox masquinongy	ESMA_COIF	AGGGTTTGGAAACTGACTAATTCCTT	189	v
Muskellunge	ESMA_COIR	GCGTGTGCTAGATTTCCAGCTAGT		
	ESMA_COI_probe	TTTACTGCTGCTGGCC		
Hybognathus regius (*)	HYRE_COIF	GCATCAGTAGACCTTACAATCTTCTCC	204	–
Eastern silvery minnow	HYRE_COIR	CATAGTGATTCCGGCAGCTAAA		
	HYRE_COI_probe	CTGTTCTCCTGCTCCTAT		
Mallotus villosus	MAVI_COIF	GCAATCTCGCTCACGCG	185	v
Capelin	MAVI_COIR	AAGAAGAACGGCTGTAATTAGCACA		
	MAVI_COI_probe	AAACCTCCTGCTATTTCTC		
Microgadus tomcod	MITO_COIF	CTTCTGACTTTTACCCCCGTCA	166	–
Atlantic tomcod	MITO_COIR	TGAAATTCCTGCCAGATGAAGC		
	MITO_COI_probe	CCGGAGCCTCCGTTGA		
Micropterus dolomieu	MIDO_COIF	ACCATCTTCTCTCTTCATCTTGCG	173	v
Smallmouth bass	MIDO_COIR	GCGAGGACTGGGAGCGATAA		
	MIDO_COI_probe	CCCTGTTTGTTTGGTCCGT		
Morone saxatilis	MOSA_COIF	TGGAACTGGCTGAACCGTTTAC	178	v
Striped bass	MOSA_COIR	GGTCTGATATTGGGAGATGGCA		
	MOSA_COI_probe	CATCTGTAGACCTAACAATT		
Moxostoma valenciennesi	MOVA_CYTBF	CTCGAGGATTATACTATGGATCCTACCTATAC	251	–
Greater redhorse	MOVA_CYTBR	GTGAAAGGCGAAGAATCGTGTT		
	MOVA_CYTB_probe	CGCAGTACCTTATGTTGG		

(Continues)

TABLE 1 (Continued)

Scientific and Common name	Primer/Probe Gene	Sequence 5′ > 3′	bp	eDNA
Oncorhynchus mykiss	ONMY_CYTBF	CCTCCCGTGAGGACAAATATCA	125	v
Rainbow trout	ONMY_CYTBR	TGGCGTTGTCAACGGAGAAG		
	ONMY_CYTB_probe	TACGTAGGAGGCGCCCT		
Osmerus mordax	OSMO_COIF	GCAGGCGCCGGGACT	167	v
Rainbow smelt	OSMO_COIR	GCAGGAGGCTTCATATTAATAATGGTT		
	OSMO_COI_probe	CACGCGGGAGCTT		
Perca flavescens	PEFL_COIF	CAGGGGTTTCCTCAATTCTAGGT	157	v
Yellow perch	PEFL_COIR	CCAGCGGCAAGAACAGGTAGT		
	PEFL_COI_probe	CCAATATCAAACTCCCTTGTT		
Prosopium cylindraceum	PRCY_CYTBF	CACTCAAATCCTTACAGGGTTGTTT	176	v
Round whitefish	PRCY_CYTBR	CTCGAGCAATGTGTATATAAATGCAA		
	PRCY_CYTB_probe	TCTGTCGGGATGTAAGCT		
Salmo salar	SASA_COIF	CCCCCGAATGAATAACATAAGTTTT	205	v
Atlantic salmon	SASA_COIR	AATGGCCCCCAGAATTGAA		
	SASA_COI_probe	CTAGCAGGTAATCTTGC		
Salmo trutta	SATR_COIF	GCTTCTGACTCCTCCCTCCG	248	v
Brown trout	SATR_COIR	AAGTGGAGTTTGATATTGGGAGATG		
	SATR_COI_probe	CTAGCAGGTAATCTTGCC		
Salvelinus alpinus	SAAL_COIF	CTTTATAGTCATACCAATTATGATCGGG	164	v
Arctic charr	SAAL_COIR	CGCCAGCTTCAACCCCT		
	SAAL_COI_probe	AATCCCTCTAATAATTGGG		
Salvelinus namaycush	SANA_COIF	GGGCCTCCGTTGATTTAACTATC	101	v
Lake trout	SANA_COIR	GGGCTTCATGTTAATAATGGTTGTG		
	SANA_COI_probe	CTCTCTTCATTTAGCTGGC		
Sander canadensis	SACA_COIF	CGATATGGCATTCCCCCGT	147	v
Sauger	SACA_COIR	GCCAGGTTTCCAGCTAATGGA		
	SACA_COI_probe	AGGGTGGACTGTTTAC		
Sebastes sp.	SESP_COIF	TTACCACAATTATTAATATGAAGCCACC	125	v
Redfish	SESP_COIR	GATGCCGGCAGCAAGAACT		
	SESP_COI_probe	CTGTTCTTCTCCTCCTATCT		

Note: Primer name indicates gene amplified, fragment length (bp) and validation through eDNA studies (v: validated, –: not tested).

Cross-amplification tests revealed co-amplification of *S. namaycush primers* with *S. alpinus*; however, these two species are rarely found in sympatry in North America. Testing for cross-amplification also revealed that *Esox lucius* primers amplified *E. americanus americanus*, with the Canadian distribution range of this latter species being limited in Québec, and hybridization being common throughout this genus (Crossman & Buss, 1965).

3.2 | Threatened or invasive fish species

Specific primers were designed for 15 fish species listed as endangered, threatened, special concern, or susceptible to be special concern by the Species At Risk Act in Canada, by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) or by the Québec's "Loi sur les espèces menacées ou vulnérables" (Table 2); eastern sand darter (*Ammocrypta pellucida*), channel darter (*Percina copelandi*), copper redhorse (*Moxostoma hubbsi*), river redhorse (*Moxostoma carinatum*), American shad (*Alosa sapidissima*), Atlantic sturgeon (*Acipenser oxyrinchus*), American eel (*Anguilla rostrata*), brassy minnow (*Hybognathus hankinsoni*), chestnut lamprey (*Ichthyomyzon castaneus*), deepwater sculpin (*Myoxocephalus thompsonii*), grass pickerel (*Esox americanus vermiculatus*), margined madtom (*Noturus insignis*), northern sunfish (*Lepomis peltastes*), yellow bullhead (*Ameiurus natalis*), rosyface shiner (*Notropis rubellus*); and for six invasive fish species, grass, silver and bighead carps (*Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*), goldfish (*Carassius auratus*), tench (*Tinca*

TABLE 2 Species-specific primers, probes for (a) invasive fish species from the list of invasive species in Quebec, or (b) endangered, threatened, or special concern fish species from the list of the Canadian Species At Risk Act (SARA), under the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) or under the act respecting threatened or vulnerable species of Québec's government

Scientific and common name	Primer/Probe gene	Sequence 5′ > 3′	bp	eDNA
(a) Invasive				
Carassius auratus	CAAU_COIF	GGATTGATGARACACCTGCTAAA	165	–
Goldfish	CAAU_COIR	TTCTTCCCCCATCATTCCTGT		
	CAAU_COI_probe	CATCCGGTGCCAGCT		
Ctenopharyngodon idella	CTID_COIF	TCAACACCAGAAGAGGCTAATAGTAGG	127	v
Grass carp	CTID_COIR	GGTTTGGAAATTGACTCGTACCAT		
	CTID_COI_probe	ACTCATGTTGTTTATTCGTGGGA		
Hypophthalmichthys molitrix	HYMO_COIF	TAGCAGGTGTGTCATCAATTTTAGGA	160	v
Silver carp	HYMO_COIR	CCAGCAGCTAAAACTGGTAAGGATAA		
	HYMO_COI_probe	CGTAACAGCCGTACTTC		
Hypophthalmichthys nobilis	HYNO_COI_F2	TTAGGGGCAATTAACTTCATCACC	124	v
Bighead carp	HYNO_COI_R2	GTAAGGATAGGAGAAGAAGTACGGCC		
	HYNO_COI_probe	ACCAGCCATTTCCCAAT		
Scardinius erythrophthalmus	SCER_COIF	GAGTTTCTGACTTCTCCCTCCG	167	v
Common rudd	SCER_COIR	ATACACCTGCCAGGTGGAGC		
	SCER_COI_probe	ATGAACAGTATACCCACCACT		
Tinca tinca	TITI_CYTBF	CAACCGCATTCTCGTCAGTAAA	244	v
Tench	TITI_CYTBR	CAAAAGGATATTTGTCCTCATGGC		
	TITI_CYTB_probe	TCGCCCGAGGATTAT		
(b) Threatened or special concern				
Acipenser oxyrinchus	ACOX_COIF	TGGTGCCTGAGCAGGCATA	171	–
Atlantic sturgeon	ACOX_COIR	CCGAAGCCGCCGATC		
Threatened COSEWIC	ACOX_COI_probe	TGGCGACGACCAGATT		
Alosa sapidissima	ALSA_COIF	GCGGCTTTGGGAATTGACTG	183	v
American shad	ALSA_COIR	CAAGATTGCCTGCCAAAGGT		
Special concern Quebec	ALSA_COI_probe	CCTCCTCCGGAGTTGA		
Ameiurus natalis	AMNA_COIF	TATGATTGGAGCCCCCGATATA	205	–
Yellow bullhead	AMNA_COIR	TGCAAGGTGAAGTGAAAAGATAGTTAAG		
Susceptible to be special concern Quebec	AMNA_COI_probe	TCTTCTCCTTCTACTAGCCT		
Ammocrypta pellucida	AMPE_COIF	GGGGATTCGGAAACTGACTTGTA	162	v
Eastern sand darter	AMPE_COIR	GGTACACGGTTCATCCGGTG		
Threatened SARA	AMPE_COI_probe	AGACATGGCGTTTCCT		
Anguilla rostrata	ANRO_COIF	GTGCCATTAATAATCGGCGCT	131	–
American eel	ANRO_COIR	CAGCCTGTACCAGCCCCA		
Threatened COSEWIC	ANRO_COI_probe	TAGCCTCCTCTGGAGTAGA		
Esox americanus vermiculatus	ESAMVE_CYTBF	CTTGCCTTACTATTCTCCATTTTAATTCTC	227	–
Grass pickerel	ESAMVE_CYTBR	GGGGTTAGGAGGAGAAAAATGAG		
Special concern COSEWIC	ESAMVE_CYTB_probe	ATTCTTATTCTGACTTCTAGTAGCA		

(Continues)

TABLE 2 (Continued)

Scientific and common name	Primer/Probe gene	Sequence 5′ > 3′	bp	eDNA
Hybognathus hankinsoni	HYHA_COIF	GTTAATTTCATTACTACAATTATTAACATGAAACCT	140	–
Brassy minnow	HYHA_COIR	ATAGTGATCCCGGCAGCTAGC		
Susceptible to be special concern Quebec	HYHA_COI_probe	CTGTTCTCCTGCTCCTA		
Ichthyomyzon castaneus	ICCA_COIF	TCCCTACACCTCGCTGGAATC	169	–
Chestnut lamprey	ICCA_COIR	CGGCTGCCAGTACTGGAAGG		
Special concern SARA	ICCA_COI_probe	CTGCAGTTCTTCTCCTACTAT		
Lepomis peltastes	LEPE_COIF	CTGGCACAGGTTGGACAGTG	222	–
Northern sunfish	LEPE_COIR	GAAGTAAGACGGCAGTGATTAACACA		
Special concern COSEWIC	LEPE_COI_probe	TATCTTCAATCCTCGGAGCTA		
Moxostoma carinatum	MOCA_COIF	TCTTTATAGTAATACCCATTTTAATCGGG	168	–
River redhorse	MOCA_COIR	CGGCACCGGCCTCAACT		
Special concern SARA	MOCA_COI_probe	CATTAATGATCGGAGCCC		
Moxostoma hubbsi	MOHU_CYTBF	TCCGTCCAATCACCCAATTC	163	v
Copper redhorse	MOHU_CYTBR	CATCCGGCTAGTGGAATCAGA		
Endangered SARA	MOHU_CYTB_probe	CATAGTTATTTTGACATGAATTGG		
Myoxocephalus thompsonii	MYTH_COIF	CCTTACATCTAGCAGGAATCTCTTCG	156	–
Deepwater sculpin	MYTH_COIR	CGGGGAGGGAGAGAAGGAGTAAT		
Special concern COSEWIC	MYTH_COI_probe	ATCATTAACATGAAACCC		
Notropis rubellus	NORU_COIF	GACCTAACAATCTTCTCTCTCCACCTT	243	–
Rosyface shiner	NORU_COIR	CCCTGCCGGATCAAAGAAA		
Susceptible to be special concern Quebec	NORU_COI_probe	CAGGTGTATCGTCAATTC		
Noturus insignis	NOIN_CYTBF	TTCCTCCTTCCATTCGCAATC	222	–
Margined madtom	NOIN_CYTBR	GAAGTTTTCTGGGTCGCCG		
Threatened SARA	NOIN_CYTB_probe	CTTAAACTCTGATGCTGATAAA		
Percina copelandi	PECO_COIF	GGAAACTGACTCGTGCCTCTG	168	v
Channel darter	PECO_COIR	CCCAGCCAGAGGTGGGTAT		
Special concern SARA	PECO_COI_probe	TGGAGCTGGAACCGGA		

Note: Primer name indicates gene amplified, fragment length (bp), and validation through eDNA studies (v: validated, –: not tested).

tinca), and common rudd (*Scardinius erythrophthalmus*). Among these, the standard curve experiment was performed only on the grass carp, *C. idella*. The assay had an amplification efficiency of 96.5% and a limit of detection of copies/rxn (Table 5). Cross-amplification tests revealed co-amplification of *A. anguilla* (European eel) with *American eel primers* and amplification of *M. thompsonii* with *M. quadricornis* primers, these species do not co-occur in north America.

3.3 | Threatened and invasive reptiles and amphibians

Primers were successfully designed for four salamanders including three species listed as threatened by the Species At Risk Act in Canada, Allegheny mountain dusky salamander (*Desmognathus ochrophaeus*), northern dusky salamander (*D. fuscus)*, spring salamander *Gyrinophilus porphyriticus*); as well as two frogs, spring peeper (*Pseudacris crucifer)*, boreal chorus frog (*P. maculata*); four turtles species listed as endangered, threatened or special concern by the Species At Risk Act in Canada (endangered: spiny softshell turtle—*Apalone spinifera*; threatened: Blanding's turtle—*Emydoidea blandingii*, wood turtle—*Glyptemys insculpta*; special concern: northern map turtle—*Graptemys geographica*; and considered as invasive species: red-eared slider—*Trachemys scripta*) (Table 3). For all but one of these assays, cross-amplification tests returned negative results. The northern dusky salamander assay showed slight amplification of Allegheny mountain dusky salamander; however, these two species are rarely found in sympatry in Quebec. The standard curve experiment was performed only on the Boreal

TABLE 3 Species-specific primers, probes for reptile and amphibian species

Scientific and Common name	Primer/Probe Gene	Sequence 5′ > 3′	bp	eDNA
Amphibian				
Pseudacris crucifer	PSCR_COIF	TTCTCCTCGCATCAGCAGGT	160	–
Spring peeper	PSCR_COIR	AAATTAATAGCTCCTAGGATGGAAGAGACT		
	PSCR_COI_probe	CTGGCACCGGGTGA		
Pseudacris maculata	PSMA_CYTBF	ATATCCTTCTGAGGAGCCACTGTC	222	v
Boreal Chorus Frog	PSMA_CYTBR	GAGTCCAATTGGGTTGGATGAC		
	PSMA_CYTB_probe	TATTGCCGGGGCATCA		
Eurycea bislineata	EUBI_NADHF	GTGGTATTAATTTATTTCCCACAATTAACTAC	225	–
Northern two-lined salamander	EUBI_NADHR	GATTAGTCATTTTGGTATAAATCCGGAA		
	EUBI_NADH_probe	TACTCAACTTAACATCAACTAGT		
Desmognathus ochrophaeus	DEOC_COIF	CCTTCACTTCTTCTCTTATTAGCCTCA	105	–
Allegheny mountain	DEOC_COIR	AGCTCCCGCGTGAGCC		
Dusky salamander	DEOC_COI_probe	TTGAAGCCGGAGCCGG		
Desmognathus fuscus	DEFU_COIF	AATATCACAATATCAAACACCATTATTTGTC	108	–
Northern dusky salamander	DEFU_COIR	GTTAGAAGTATTGTAATTCCTGCTGCTAAA		
	DEFU_COI_probe	CCGCTATTTTACTATTATTATCACTACC		
Gyrinophilus porphyriticus	GYPO_NADHF	CTTGGATGAATAATTGTTGTATTAACCC	145	–
Spring salamander	GYPO_NADHR	CATGACATGGTTATTTTATTAATATTAGTTGAGG		
	GYPO_NADH_probe	ACCCTAATTAATTTTTCATTGTACCTA		
Reptilian				
Apalone spinifera	APSP_COIF	CTCATGCTGGGGCATCA	161	–
Spiny softshell turtle	APSP_COIR	AATTACTACTGATCACACAAATAATGGG		
	APSP_COI_probe	CCGGAGTATCGTCAAT		
Emydoidea blandingii	EMBL_COIF	ATCATCAGGAATTGAAGCAGGG	179	v
Blanding's turtle	EMBL_COIR	GGGATTTTATGTTAATTGCTGTGGTAATA		
	EMBL_COI_probe	CTGAACTGTATATCCACCACTA		
Glyptemys insculpta	GLIN_COIF	CTGGCCGGTGTATCTTCAATCT	173	–
Wood turtle	GLIN_COIR	AGTATAGTGATGCCTGCAGCTAGTACA		
	GLIN_COI_probe	CCGGCCATATCTCAATA		
Graptemys geographica	GRGE_COIF	GTTATTATTGCTCTTAGCATCATCAGGT	209	v
Northern Map Turtle	GRGE_COIR	GTGATATGGCTGGAGATTTTATGTTAATTA		
	GRGE_COI_probe	TTCTCTTCATTTAGCAGGAGTAT		
Trachemys scripta[a]	TRSC_COIF	GGGAACTGACTCGTGCCATTA	179	v
Red-eared slider	TRSC_COIR	TGGGCTAAATTTCCGGCTAA		
	TRSC_COI_probe	TAGCATCATCAGGAATTGA		

Note: Primer name indicates gene amplified, fragment length (bp) and validation through eDNA studies (v: validated, –: not tested).
[a]Only probes designed by authors, primers from Davy et al. (2015).

chorus frog, *P. maculata*. The assay had an amplification efficiency of 96.9% and a limit of detection of 2 copies/rxn (Table 5).

3.4 | Invertebrate species

Primers for two invasive waterfleas, spiny waterflea (*Bythotrephes longimanus*), and fishhook waterflea (*Cercopagis pengoi*) and two freshwater mussels listed as threatened under the Species At Risk Act (alewife floater-*Utterbackiana implicata* and Hickorynut-*Obovaria olivaria*) were designed (Table 4). Standard curve experiments were performed for the two waterflea species. Assays for *B. longimanus* and *C. pengoi* had an amplification efficiency of 98.1% and 102.7%, respectively, and a limit of detection of 4 copies/rxn for both primer sets (Table 5).

4 | DISCUSSION

The development of the 60 specific assays presented here was requested for specific needs and questions raised by government

TABLE 4 Species-specific primers, probes for invertebrate species

Scientific and Common name	Primer/Probe Gene	Sequence 5′ > 3′	bp	eDNA
Mollusk				
Utterbackiana implicata (*Anodonta implicata*)	ANIM_NADHF	TTTTATGTATTTCTTCACTAGCTGTCTACACT	214	–
Alewife floater	ANIM_NADHR	ATGATGGCTCAAGTCGATATGTTTATA		
	ANIM_NADH_probe	CAAATTCTAAATACGCACTACT		
Obovaria olivaria	OBOL_COI_F2	ATTCTGGGGCTTCGGTGG	200	v
Hickorynut	OBOL_COI_R2	ACAGGCAATGCTGCAACTAGC		
	OBOL_COI_probe	CATCTCTACTGTTGGAAATA		
Crustacean				
Bythotrephes longimanus	BYLO_COIF	GAGACTTATTGGGGACGACCAA	214	v
Spiny waterflea	BYLO_COIR	CCCTCCTACAAGTAGAAGGGTAAGG		
	BYLO_COI_probe	TAATCGGAGGGTTTGGAAA		
Cercopagis pengoi	CEPE_COIF	GGAAATTGACTTGTCCCTCTGATG	188	v
Fishhook waterflea	CEPE_COIR	GCTCCAGCGTGTGCGATA		
	CEPE_COI_probe	ACTGGATGGACAGTGTAC		

Note: Primer name indicates gene amplified, fragment length (bp) and validation through eDNA studies (v: validated, –: not tested).

TABLE 5 Percentage of amplification efficiency, limit of detection, intercept (y-inter), and the coefficient of the linear relation between cycle threshold and log DNA dilution (r^2) corresponding to for each standard curve developed with a synthetic DNA template

Scientific name	Amplification efficiency (%)	Limit of detection (mtDNA copies by rxn)	y-inter	r^2
Exploited fish species				
Acipenser fulvescens	100.1	8	38.4	.975
Ammodytes sp.	102.7	4	39.8	.985
Clupea harengus	103.9	8	39.7	.970
Coregonus clupeaformis	98.4	8	40.0	.971
Mallotus villosus	100.0	8	39.6	.970
Micropterus dolomieu	102.8	20	40.9	.949
Morone saxatilis	101.7	4	40.4	.963
Oncorhynchus mykiss	94.6	8	38.8	.974
Osmerus mordax	103.8	20	42.1	.969
Prosopium cylindraceum	94.3	4	40.3	.969
Salmo salar	98.7	2	38.7	.969
Salmo trutta	108.9	20	43.1	.958
Salvelinus alpinus	98.4	4	39.4	.970
Sebastes spp.	95.5	8	39.9	.981
Invasive fish species				
Ctenopharyngodon idella	96.5	4	40.8	.949
Amphibian				
Pseudacris maculata	96.9	2	37.1	.975
Crustacean				
Bythotrephes longimanus	98.1	4	37.8	.983
Cercopagis pengoi	102.7	4	40.1	.978

agencies, academics, or environmental consulting firms. These species are subject to ongoing monitoring either because they are exploited (e.g., Atlantic salmon, lake sturgeon), because of their invasive status (e.g., grass carp, spiny waterflea) or threatened status (e.g., Atlantic sturgeon, Blanding's turtle, or alewife floater). All of our assays were developed using in silico tests by searching for nonspecific oligonucleotide hybridization using multiple alignments of the target species DNA sequences along with the sequences of related species

that were available in online DNA databases and then predicting probe performance. They were also tested in vitro by amplifying tissue-extracted DNA from both targeted and related species. None of our assays resulted in cross-amplification of DNA for species from the same family, with five exceptions (see Table S1). Since assay development and tests should be specific to a defined geographic area and perhaps population (Goldberg et al., 2016; Wilcox et al., 2015), the cross-amplification tests were done for related species that are present in the same area of the targeted species in Québec. Consequently, before using our assays in other regions, it would be preferable to (a) verify the presence of all related species in the area of interest, (b) verify that cross-amplification tests were done with all related species present in the area of interest and, if not, (c) perform the necessary cross-amplification tests.

The development of eDNA studies is relatively recent and various protocols for eDNA collection, extraction, detection, and analysis have been developed depending on the taxa being studied (Tsuji, Takahara, Doi, Shibata, & Yamanaka, 2019). To the best of our knowledge, qPCR assays targeting the same gene of interest have already been published for 20 of the species addressed in the present study (See Table 6). For ten of them (*A. fulvescens*, *E. lucius*, *G. insculpta*, *H. molitrix*, *M. saxatilis*, *O. mordax*, *P. crucifer*, *S. namaycush*, *S. salar*, and *S. trutta*), the amplicon was less than 100 bp. In addition, for *Trachemys scripta*, only the TaqMan probe was designed by us, and we used the primers developed by Davy, Kidd, and Wilson (2015). Here, all of our assays produce amplicons of at least 101 bp which allows the authentication of the positive amplifications by Sanger sequencing in order to avoid false-positive detections. This is particularly crucial for projects where the objective is to detect threatened or invasive species. In addition, we chose to use a probe-based qPCR to allow for more specific detection and quantification of eDNA (Farrington et al., 2015; Mauvisseau, Burian, et al., 2019; Mauvisseau, Tönges, et al., 2019; Wilcox et al., 2013). The amplification efficiency and detection limit tests are usually performed using purified target molecules such as synthetic DNA or reference DNA from biological samples (Bustin et al., 2009). However, to standardize the analysis, the choice of reference DNA from biological samples requires an important amount of DNA and does not allow estimating the number of DNA copies in qPCRs. For these reasons, we used synthetic DNA to standardize our method for our assay development. The results

TABLE 6 List of species for which a qPCR assay was recently published with its corresponding amplicon length (bp)

Species	Gene	Amplicon length	Reference
Acipenser fulvescens	COI	57	Yusishen, Eichorn, Anderson, and Docker (2020)
Carassius auratus	COI	110	Roy, Belliveau, Mandrak, and Gagné (2018)
Ctenopharyngodon idella	COI	141	Roy et al. (2018)
Desmognathus fuscus	COI	170	Beauclerc, Wozney, Smith, and Wilson (2019)
Desmognathus ochrophaeus	COI	170	Beauclerc et al. (2019)
Esox lucius	COI	94	Olsen, Lewis, Massengill, Dunker, and Wenburg (2015)
Glyptemys insculpta	COI	71	Lacoursière-Roussel, Dubois, Normandeau & Bernatchez (2016)
Hypophthalmichthys molitrix	COI	81	Roy et al. (2018)
Hypophthalmichthys nobilis	COI	117	Roy et al. (2018)
Micropterus dolomieu	COI	147	Hulley, Tharmalingam, Zarnke, and Boreham (2019)
Morone saxatilis	COI	63	Brandl et al. (2015)
Myoxocephalus thompsonii	COI	148	Hulley et al. (2019)
Oncorhynchus mykiss	CytB	153	Minamoto, Hayami, Sakata, and Imamura (2019)
Osmerus mordax	COI	76	Hulley et al. (2019)
Perca flavescens	COI	146	Hulley et al. (2019)
Pseudacris crucifer	COI	99	Beauclerc et al. (2019)
Salvelinus namaycush	COI	101	Lacoursière-Roussel, Côté, Leclerc & Bernatchez (2016)
Salmo salar	COI	74	Atkinson et al. (2018)
Salmo trutta	COI	61	Gustavson et al. (2015)
Trachemys scripta	COI	179	Davy et al. (2015)

obtained for each of the 18 assays that were tested (between 2 and 20 mtDNA copies per reaction) were comparable to previous studies on eDNA fish detection with limit of detection between 2 and 50 mtDNA copies per reaction (e.gCarim et al., 2019; Farrington et al., 2015; Wilcox et al., 2015).

In situ tests were done on 36 of the 60 specific qPCR assays on eDNA studies, which confirmed the assay performance on eDNA samples. Most of these eDNA studies were done at the request of the Province of Québec's government in order to monitor species with a threatened or invasive status. The results required by these studies were either presence/absence detection or relative quantification. For instance, since the first confirmed capture of a female of the invasive grass carp in 2015 in the St. Lawrence River, our qPCR assay has been thoroughly tested on eDNA to monitor the evolving distribution of this species in this river system (https://mffp.gouv.qc.ca/wp-content/uploads/avis-scientifique-carpes-asiatiques-quebec-confirmation-presence.pdf). Validation of sites with positive amplifications was performed by Sanger sequencing and confirmed the assay performance. Another governmental study required the development of a *S. trutta* qPCR assay in order to follow the patterns of eDNA diffusion in the St. Lawrence River (Laporte et al., 2020). This assay has been thoroughly tested and showed the efficiency of these primers to detect eDNA of confined *S. trutta* down to 5 km from the emission point (Laporte et al., 2020). Moreover, some assays developed for exploited fish species such as *S. salar* and *M. dolomieu* were also thoroughly tested on eDNA samples to assess their spatio-temporal distributions and habitat use (O'Sullivan et al., 2020). The performance of these assays was also validated by Sanger sequencing. In addition, qPCR assays developed for other clades showed good performance for detecting the presence or absence of specific species found in Québec. The spiny and fishhook waterfleas are of big concern since their introduction, probably through ballast water or recreational boats. These invasive species are already being monitored in the Laurentian Great Lakes area using nets, sediment, or eDNA analysis (Walsh, Spear, Shannon, Krysan, & Vander Zanden, 2019). Here, our qPCR assay allowed the detection of *B. longimanus* in water samples from diverse regions of the Province of Quebec (Hernandez, Bougas, Perrault-Payette, Normandeau, & Bernatchez, 2018). These results were validated by Sanger sequencing as well as actual specimen collections done in the field in 2018.

5 | CONCLUSION

The use of eDNA analysis is booming and already modifying the design and implementation of biodiversity monitoring programs. The greatest advantage of this tool probably lies in the capacity to monitor threatened and invasive freshwater species without disturbing individuals at risk or their environment. Thus, the costs in terms of both technical resources and ecological impacts in the field are considerably reduced when compared to, for example, methods using gillnets to monitor fish species. eDNA analysis by qPCR is now widely and successfully used to detect a wide range of target species (Tsuji et al., 2019). Despite the challenge to design optimal specific primers throughout a species' geographic range due to differences in co-occurring sister species, rare mitochondrial introgression, or local haplotypic variation, we hope that our 60 qPCR assays will be of broad usefulness not only for monitoring studies in Québec but also wherever these species are present in North America or have been introduced on other continents.

ACKNOWLEDGMENTS

We thank our in-house bioinformatician Eric Normandeau for his assistance in the primer design for problematic species and English revision as well as Charles Babin and Olivier Morissette for their writing assistance. The development of these qPCR assays was made possible through various collaborations which include the Ministère des Forêts, de la Faune et des Parcs du Québec (MFFP), Parks Canada, WWF, University of New Brunswick, Englobe as well as Ressources Aquatiques Québec (RAQ). We are thankful to Associate Editor Margaret Hunter and anonymous reviewers for their useful comments on a previous version of the manuscript.

AUTHOR CONTRIBUTION

C. H and B. B. should be considered joint first author. L. B., G. C. and A. S designed the project. G.C and A. S. shared tissue samples. B.B. and C.H. drafted the manuscript and all authors contributed to the writing and approved the final draft of the manuscript. C.H., B.B., A.P.P realized the primers design and the experiments.

DATA AVAILABILITY STATEMENT

Data has been upload to Dryad: https://doi.org/10.5061/dryad.12jm63xtw.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Hernandez C, Bougas B, Perreault-Payette A, Simard A, Côté G, Bernatchez L. 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. *Environmental DNA*. 2020;2:373–386. https://doi.org/10.1002/edn3.89