Conservation Genet Resour (2013) 5:971–975
DOI 10.1007/s12686-013-9946-0

TECHNICAL NOTE

Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species

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Received: 17 April 2013 / Accepted: 6 May 2013 / Published online: 23 May 2013
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Abstract Thirty-six primer pairs were designed for the detection of rare aquatic species from environmental DNA samples, targeting species-specific binding sequences within the 'barcoding' segment of the mitochondrial cytochrome oxidase I gene. Cross-species amplification tests confirmed the specificity of 18 of 26 tested primer pairs. These primers will be a useful tool for targeted detection of endangered and invasive aquatic species of concern to the Laurentian Great Lakes and help to inform management responses.

Keywords COI · Environmental DNA · Species detection · Species-specific primers

Sensitive detection of rare species is challenging in aquatic environments, but essential for informed conservation of endangered species and proactive management responses for invasive species that threaten native communities. Environmental DNA (eDNA) is a recent technique being used to detect rare aquatic species and determine habitat occupancy from water samples (Ficetola et al. 2008; Goldberg et al. 2011; Jerde et al. 2011). We made use of the growing global DNA barcoding database (BOLD; Ratnasingham and Hebert 2007) to design 36 species-specific primer pairs for eDNA detection of 4 endangered freshwater species and 10 aquatic invasive species that are considered threats to Laurentian Great Lakes ecosystems (Table 1).

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Species-specific primers were designed to target small fragments (80–280 bp) within the 5′ barcoding segment of the COI gene (Hubert et al. 2008). Primer-BLAST (Ye et al. 2012; http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to identify binding sites and verify specificity in silico against available sequence data (GenBank http://www.ncbi.nlm.nih.gov/genbank and BOLD http://www.boldsystems.org). Mitochondrial sequence data from GenBank and BOLD were used for target sequences (Table 1). User-defined primer parameters included product size range of 80–280 bp, 10 candidate primer pairs, and a minimum of 3 mismatches with unintended targets. Primer pair specificity checking was enabled against all sequence data in the nr database (http://www.ncbi.nlm.nih.gov/genbank). Targets with 8 or more mismatches were ignored. Primer-BLAST default parameters were used for all remaining parameters.

Primer pairs were selected for testing based on target position, fragment size, low self complementarity, and no unintended targets in the sampling region. Degenerate bases were incorporated at positions where intraspecific polymorphisms were identified in priming sites (primers: *Dbu*COI3F, *Dpo*COI10R, *Han*COI8R, and *Han*COI9F/R). Forward primers were ordered with an M13 sequence tail for future use on automated sequencers with the exception of 11 primers; *Hno*COI1, *Hno*COI2, *Hno*COI5, *Hmo*COI1, *Hmo*COI2, *Hmo*COI5, *Car*COI2, *Car*COI3, *Cid*COI1, *Cid*COI6, *Cid*COI7.

Genomic DNA was extracted from preserved tissues following an alkali extraction protocol (Malago et al. 2002) and stored at −20 °C until primer testing. Each primer set was screened for amplification using tissue-extracted DNA from 3 to 4 individuals. Preliminary primer screening for amplification, annealing temperature, and specificity (when applicable) was performed in 10 µL PCR reactions. Reaction cocktails contained template DNA, 2 µL 5× PCR

Table 1 Species, accession number, primer sequence, fragment size, annealing and melting temperature (T_A and T_M), % GC content and cross-species amplification results for 36 COI primer pairs developed to detect rare aquatic species from environmental DNA. "Cross species" indicates non-target species tested for amplification; "–" no amplification; "n/a" no species tested

Species common name (scientific name)	Accession #	Primer name	Sequence 5′-3′	Fragment (bp)	T_A (°C)	T_M (°C)	% GC	Cross species amplification results and highest annealing temperature T_A (°C) generating product
American eel	EU523918.1	*Aro*COI1F	TAGCCCACGCCGGAGCATCT	205	64	60.04	65	n/a
(*Anguilla rostrata*)		*Aro*COI1R	GCGGCTAGGACTGGCAGGGA			60.32	70	
		*Aro*COI2F	CGGCGCTCCAGACATAGCGT	166	64	59.3	65	n/a
		*Aro*COI2R	AGATGCTCCGGCGTGGGCTA			60.04	65	
		*Aro*COI9F	ACCAGGCGCCCTTCTTGGAG	248	64	58.7	65	n/a
		*Aro*COI9R	AGCCTGTACCAGCCCCAGCC			60.54	70	
Lake sturgeon	EU524392.1	*Afu*COI2F	CGGCACAGCCCTCAGCCTTC	280	64	60.0	70	n/a
(*Acipenser fulvescens*)		*Afu*COI2R	GGCTCCGGCCTCTACCCCAG			60.3	75	
		*Afu*COI7F	GGGGCCCCAGACATGGCATT	140	64	59.0	65	n/a
		*Afu*COI7R	CCCGCCAGCGGAGGGTAAAC			59.8	70	
		*Afu*COI8F	CTGGGGTAGAGGCCGGAGCC	126	64	60.3	75	n/a
		*Afu*COI8R	TGGACGAAACCCCAGCCAGGT			60.0	62	
		*Afu*COI10F	CTCCCTTCACCTGGCTGGGGT	242	64	59.8	67	n/a
		*Afu*COI10R	TGGGGTCTCCTCCTCCGGCT			60.5	70	
Redside dace	EU524486.1	*Cel*COI3F	TTGGCGCACCAGACATGGCA	104	69	59.5	60	Blacknose dace (*Rhinichthys atratulus*): 68 °C
(*Clinostomus elongatus*)		*Cel*COI3R	ACCGGCCTCAACGCCAGAAG			59.3	65	Creek chub (*Semotilus atromaculatus*): 68 °C
		*Cel*COI5F	CATCGTTACCGCCCACGCCT	205	70	59.5	65	Blacknose dace (*Rhinichthys atratulus*): 70 °C
		*Cel*COI5R	TGTTCCGGCACCGGCCTCAA			61.0	65	Creek chub (*Semotilus atromaculatus*): 70 °C
		*Cel*COI6F	TCGTTACCGCCCACGCCTTC	204	70	59.4	65	Blacknose dace (*Rhinichthys atratulus*): 70 °C
		*Cel*COI6R	CTGTTCCGGCACCGGCCTC			60.0	73	Creek chub (*Semotilus atromaculatus*): 70 °C
		*Cel*COI7F	TTCTGGCGTTGAGGCCGGTG	79	69	59.6	65	Blacknose dace (*Rhinichthys atratulus*): 68 °C
		*Cel*COI7R	TGCTCCTGCATGGGCAAGGT			58.6	60	Creek chub (*Semotilus atromaculatus*): 68 °C
Spotted gar	EU524699.1	*Loc*COI2F	AAGCAGGGGCCGGAACAGGA	260	70	60.1	65	Longnose gar (*Lepisosteus osseus*) eDNA: 70 °C
(*Lepisosteus oculatus*)		*Loc*COI2R	TCCTGCGGCTAGGACAGGCA			59.6	65	
		*Loc*COI3F	CCTCGCACACGCAGGAGCAT	273	64	59.7	65	Longnose gar (*Lepisosteus osseus*) eDNA: 67 °C
		*Loc*COI3R	CCCCTCCTGCGGGGTCAAA			60.4	70	
		*Loc*COI9F	CGGCGCCCCTGACATAGCCT	160	64	61.0	70	Longnose gar (*Lepisosteus osseus*) eDNA: 64 °C
		*Loc*COI9R	TCCTGCGTGTGCGAGGTTGC			60.2	65	

Table 1 continued

Species common name (scientific name)	Accession #	Primer name	Sequence 5′-3′	Fragment (bp)	T_A (°C)	T_M (°C)	% GC	Cross species amplification results and highest annealing temperature T_A (°C) generating product
Bighead carp	HQ682698.1	*Hno*COI1F	ATCACTTCTGGGCGATGACC	120	69	59.8	55	Common carp (*Cyprinus carpio*): –
(*Hypophthalmichthys nobilis*)		*Hno*COI1R	AGTGGCACGAGTCAGTTTCC			60.2	55	Grass carp (*Ctenopharyngodon idella*): –
								Goldfish (*Carassius auratus*): –
								Silver carp *(Hypophthalmichthys molitrix):* 67 °C
		*Hno*COI2F	ACTCGCGGGTAATCTTGCTC	141	65	60.4	55	Common carp (*Cyprinus carpio*): –
		*Hno*COI2R	TGGGAAATGGCTGGTGGTTT			60.4	50	Grass carp (*Ctenopharyngodon idella*): 63 °C
								Goldfish (*Carassius auratus*): –
								Silver carp *(Hypophthalmichthys molitrix):* 63 °C
		*Hno*COI5F	AAACCACCAGCCATTTCCCA	177	66	64.5	60	Common carp (*Cyprinus carpio*): 63 °C
		*Hno*COI5R	TAGGATTGGGTCTCCTCCCC			64.5	50	Grass carp (*Ctenopharyngodon idella*): 65 °C
								Goldfish (*Carassius auratus*): –
								Silver carp *(Hypophthalmichthys molitrix):* 66 °C
Common rudd	HQ960932.1	*Ser*COI6F	CAGGTAATCTAGCCCACGCA	270	63	59.4	55	Grass carp (*Ctenopharyngodon idella*): 67 °C
(*Scardinius erythrophthalmus*)		*Ser*COI6R	GCTGGGTCGAAGAATGTGGT			60.3	55	
		*Ser*COI9F	CCCACCACTCGCAGGTAATC	190	62	60.5	60	Grass carp (*Ctenopharyngodon idella*): 67 °C
		*Ser*COI9R	AGCTGTTACCAGTACGGCTC			59.5	55	
Grass carp	JN988834.1	*Cid*COI1F	AGTTTACCCACCACTCGCAG	151	66	60.0	55	Bighead carp *(Hypophthalmichthys nobilis):* –
(*Ctenopharygodon idella*)		*Cid*COI1R	GGAGATGGCTGGTGGTTTCA			60.0	55	Common carp (*Cyprinus carpio*): 64 °C
								Goldfish (*Carassius auratus*): –
								Silver carp*(Hypophthalmichthys molitrix):* –
		*Cid*COI6F	TGAAACCACCAGCCATCTCC	101	64	60.0	55	Bighead carp *(Hypophthalmichthys nobilis):* –
		*Cid*COI6R	GGCGGCTAGAACTGGTAGAG			59.6	60	Common carp (*Cyprinus carpio*): –
								Goldfish (*Carassius auratus*): –
								Silver carp*(Hypophthalmichthys molitrix):* –
		*Cid*COI7F	ATTGGAGCACCCGACATAGC	141	68	60.1	55	Bighead carp *(Hypophthalmichthys nobilis):*67 °C
		*Cid*COI7R	TGCGAGTGGTGGGTAAACTG			60.2	55	Common carp (*Cyprinus carpio*): 63 °C
								Goldfish (*Carassius auratus*): –
								Silver carp*(Hypophthalmichthys molitrix):* 67 °C
Northern snakehead	JQ358718.1	*Car*COI2F	ACCTCCTGCCATCTCACAATACCA	123	65	64.6	50	Bowfin (*Amia calva*) eDNA: –
(*Channus argus*)		*Car*COI2R	CGGTCCGTGAGCAGCATCGT			66.6	65	
		*Car*COI3F	CGGACATGGCGTTCCCACGA	271	66	66.6	65	Bowfin (*Amia calva*) eDNA: –
		*Car*COI3R	TGGTATTGTGAGATGGCAGGAGGT			64.6	50	

Table 1 continued

Species common name (scientific name)	Accession #	Primer name	Sequence 5′-3′	Fragment (bp)	T_A (°C)	T_M (°C)	% GC	Cross species amplification results and highest annealing temperature T_A (°C) generating product
Round goby	EU524154.1	*Nme*COI5F	CCCTCCCCTGGCAGGCAACT	226	70	60.4	70	Tubenose goby (*Proterorhinus marmoratus*): 70 °C
(Neogobius melanostomus)		*Nme*COI5R	GGCGGCAAGAACTGGGAGGG			59.6	70	
		*Nme*COI6F	CCCTTCACCTGGCCGGCATT	240	70	59.3	65	Tubenose goby (*Proterorhinus marmoratus*): 70 °C
		*Nme*COI6R	TAGGATCCCCTCCCCCGGCA			60.2	70	
		*Nme*COI10F	TGGGGCCCCCGATATGGCATT	150	68	60.0	61.9	Tubenose goby (*Proterorhinus marmoratus*): 66 °C
		*Nme*COI10R	GCCAAGTTGCCTGCCAGGGG			60.5	70	
Silver carp	FJ459502.1	*Hmo*COI1F	GTGCTCGTAACAGCCGTACT	112	68	62.4	55	Bighead carp *(Hypophthalmichthys nobilis)*:66 °C
(Hypophthalmichthys molitrix)		*Hmo*COI1R	CCCCTGCTGGGTCAAAGAAT			62.4	55	Common carp (*Cyprinus carpio*): 66 °C
								Grass carp (*Ctenopharyngodon idella*): 62 °C
								Goldfish (*Carassius auratus*): –
		*Hmo*COI2F	AGTTTATCCACCACTCGCGG	201	70	62.4	55	Bighead carp *(Hypophthalmichthys nobilis)*:70 °C
		*Hmo*COI2R	AGTACGGCTGTTACGAGCAC			62.4	55	Common carp (*Cyprinus carpio*): –
								Grass carp (*Ctenopharyngodon idella*): –
								Goldfish (*Carassius auratus*): –
		*Hmo*COI5F	GAGCCGGAATAGTGGGAACC	121	67	60.4	50	Bighead carp *(Hypophthalmichthys nobilis)*:67 °C
		*Hmo*COI5R	ATTACGAAGGCATGGGCAGT			64.5	60	Common carp (*Cyprinus carpio*): 63 °C
								Grass carp (*Ctenopharyngodon idella*): 65 °C
								Goldfish (*Carassius auratus*): –
Bloody-red shrimp	EU029162.1	*Han*COI8F	GCCCCAGATATAGCGTTTCCTCG	207	70	57.1	56	Opossum shrimp *(Mysis diluviana)*: 67 °C
(Hemimysis anomala)		*Han*COI8R	AGAAGARGCGCCAGCTAAATGCAAA			58.0	44	
		*Han*COI9F	TGCATTTAGCTGGCGCYTCTTCT	166	69	57.3	47	Opossum shrimp *(Mysis diluviana)*: 67 °C
		*Han*COI9R	GCCCCTGCTAAAACAGGTAAAGAYA			57.2	48	
Quagga mussel	U47650.1	*Dbu*COI3F	GGGGTTGAACATTATAYCCACCGTT	164	66	57.0	48	Zebra mussel (*Dreissena polymorpha*): –
(Dreissena bugensis)		*Dbu*COI3R	AAACTGATGACACCCGGCACG			57.7	57	
Spiny waterflea	AF435122.1	*Blo*COI10F	GCTGAGTTGGGACAGGCAGG	248	68	57.2	65	n/a
(Bythrotrephes longimanus)		*Blo*COI10R	CCACTCTCTACGGCCCCTCC			57.6	70	
Zebra mussel	U47653.1	*Dpo*COI3F	GCTAAGGGCACCTGGAAGCGT	254	66	59.1	61	Quagga mussel (*Dreissena bugensis*): –
(Dreissena polymorpha)		*Dpo*COI3R	CACCCCCGAATCCTCCTTCCCT			59.3	63	
		*Dpo*COI10F	GGGAAGGAGGATTCGGGGGTGG	206	66	60.3	68	Quagga mussel (*Dreissena bugensis*): –
		*Dpo*COI10R	TGTGCAGAACAAAGGGACCCGGTW			60.0	54	

Buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP, 0.2 mg/mL BSA (BioShop), 0.2 μM of each primer (Eurofins MWG Operon), 0.25 U of *Taq* polymerase (Promega), and ddH_2O to a final reaction volume of 10 μL. Thermocycler conditions for PCR were 94 °C for 180 s followed by 35 cycles of 94 °C for 45 s; 48–70 °C for 45 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. Amplicons stained with SyBr Green (Cedarlane Laboratories) were visualized by electrophoresis on a 1.5 % agarose gel and size referenced against a 100 bp size standard (BioShop). Positive amplification was recognized as a single band at the expected fragment size (Table 1). Optimal annealing temperature was determined by high DNA yield and amplicon quality. Negative controls were included in all reactions and run on each gel. Select primer pairs were validated for species specificity using tissue derived DNA from closely related and/or co-occurring species as well as the target species (Table 1).

All primer pairs successfully amplified DNA of the expected fragment length from tissue derived DNA from the target organism. Twenty-six primer pairs were selected to be tested for cross-species amplification with DNA (or eDNA) from closely related or co-occurring species. Eighteen of the 26 primer pairs were species specific (Table 1). Non-target species either failed to amplify or amplified only at lower annealing temperatures, with exclusion of non-target amplification at higher annealing temperatures (Table 1). Eight primer pairs consistently amplified non-target species at the expected fragment length at all annealing temperatures. Surprisingly, four of these primers cross amplified non-target species from a different genus (Table 1); the remaining four primer pairs amplified non-target species from within the same genus. Non-specificity may be advantageous when screening for multiple species. For instance, both silver and bighead carp DNA amplified with three primer pairs (two designed to target silver carp and one designed to target bighead carp). The objective of current Great Lakes surveillance efforts is to detect any of three invasive Asian carp species (www.asiancarp.us). Therefore primary amplification with a genus-specific primer set may be more efficient for a widespread screening process, followed by standard sequencing of resulting positives for species verification. Eight primer pairs targeting a total of three species were not tested for cross-species amplification because target species were only distantly related to potential co-occurring species as identified in Hubert et al. (2008).

The development of species-specific primers to detect aquatic species of concern should greatly assist management to address challenges facing Great Lakes biodiversity. In general, species-specific primers targeting the mitochondrial genome are valuable tools for the emerging use of eDNA for detection of rare aquatic species (Ficetola et al. 2008; Darling and Mahon 2011). The global barcoding effort provides the much needed groundwork to efficiently incorporate eDNA analysis into biodiversity monitoring and surveillance efforts.

Acknowledgments Ontario Ministry of Natural Resources (OMNR) and the Canada-Ontario Agreement for Great Lakes Ecosystem (COA) provided funding. Samples provided by Kelly Bowen, Ron Dermott, Michael Fox, Bill Glass, Tim Johnson, Joseph Love, Andrew Mahon, John Odenkirk, OMNR Biodiversity Section and Lake Ontario Management Unit, Kirby Punt, Scott Reid, and the Royal Ontario Museum.

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