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Research Article

Development and field validation of an environmental DNA (eDNA) assay for invasive clams of the genus *Corbicula*

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Abstract

Early detection is imperative for successful control or eradication of invasive species, but many organisms are difficult to detect at the low abundances characteristic of recently introduced populations. Environmental DNA (eDNA) has emerged as a promising invasive species surveillance tool for freshwaters, owing to its high sensitivity to detect aquatic species even when scarce. We report here a new eDNA assay for the globally invasive Asian clam *Corbicula fluminea* (Müller, 1774), with field validation in large lakes of western North America. We identified a candidate primer pair for the Cytochrome *c* oxidase subunit 1 (COI) gene for *C. fluminea*. We tested it for specificity via qPCR assay against genomic DNA of the target species *C. fluminea*, and synthetic DNA gBlocks for other non-target species within and outside of the genus *Corbicula*. Our best identified primer amplifies a 208-bp fragment for *C. fluminea* and several closely related species within the genus, but was specific for these non-native Asian clams relative to native mollusks of western North America. We further evaluated this assay in application to eDNA water samples for the detection of *C. fluminea* from four lakes in California and Nevada, United States, where the species is known to occur (including Lake Tahoe) relative to seven lakes where it has never been observed. Our assay successfully detected *C. fluminea* in all four lakes with historic records for this species, and did not detect *C. fluminea* from the seven lakes without known populations. Further, the distribution of eDNA detections within Lake Tahoe generally matched the known, restricted distribution of *C. fluminea* in this large lake. We conclude from this successful field validation that our eDNA assay for *C. fluminea* will be useful for researchers and managers seeking to detect new introductions and potentially monitor population trends of this major freshwater invader and other closely related members of its genus.

Key words: *Corbicula fluminea*, invasive species surveillance, invasive species, freshwater clam

Introduction

Non-native invasive species threaten every level of ecological organization, from genes to ecosystems (Sanders et al. 2003; Dextrase and Mandrak 2006). Accompanying these impacts are the often-high economic costs of invasive species control and management (Eiswerth and Johnson 2002; Leung et al. 2002; Pimentel et al. 2005). Effective management of invasive species benefits from early detection of

new populations and surveillance of established or spreading populations (Mehta et al. 2007; Hauser and McCarthy 2009). Thus, there is high interest in developing accurate, sensitive, and cost effective invasive species monitoring techniques. This interest has led to the recent and increasingly widespread application of environmental DNA (eDNA) for tracking aquatic invasive species (Rees et al. 2014). The growing popularity of eDNA – genetic material extracted from bulk environmental collections such as water or sediment (Taberlet et al. 2012; Thomsen and Willerslev 2015) – for faunal surveillance is in part due to its coupling with DNA sequencing technologies to increase detection sensitivity of genetic "signals" left behind by organisms, even at low population abundances (Bohmann et al. 2014; Barnes and Turner 2016). To date, eDNA monitoring protocols have been developed for a number of aquatic invasive species (Ficetola et al. 2008; Jerde et al. 2011; Goldberg et al. 2013; Larson et al. 2017), and may eventually serve as a cost-effective and standardized alternative to traditional surveys for other invaders that currently rely on visual observation and physical collection of whole organisms (DeJean et al. 2012; Lodge et al. 2016).

The invasive Asian clam (*Corbicula fluminea*, Müller, 1774) is a widespread invader that is native to China, Korea and southeastern Russia (McMahon 1983), but has become established in freshwater habitats of 46 states since its first reported introduction into the United States (Strayer 1999; USGS 2014; USGS 2016). Clams of the genus *Corbicula* are native to Asia, Africa and Australia (McMahon 2000), and have invaded regions other than the United States, including South America and Europe (Ludwig et al. 2014). Further, cryptic invasions by other species of this under-studied and taxonomically complex genus have recently been reported from within the United States (Tiemann et al. 2017). As invaders, *C. fluminea* and its related congeners frequently dominate freshwater and brackish ecosystems as the primary source of benthic biomass (Karatayev et al. 2003; Sousa et al. 2005), influence benthic faunal assemblages (Ilarri et al. 2012; Ilarri et al. 2014), alter the flow of organic matter in stream environments (Hakenkamp and Palmer 1999), and reduce phytoplankton abundance (Cohen et al. 1984). Further, *C. fluminea* regularly colonizes intake pipes of power plants and water treatment systems, causing significant economic damage (Isom 1986; Menninger 2013). A combination of life history attributes that include early sexual maturity, high fecundity and both active and passive dispersal (Prezant and Chalermwat 1984; Sousa et al. 2008) help drive the rapid colonization of *C. fluminea*. As such, eDNA may be well suited for both early detection and monitoring of this and other closely related species to help prevent its spread to additional aquatic systems worldwide, and initiate appropriate eradication or containment responses when found (Wittmann et al. 2012).

In the present study, we detail a new eDNA assay for the detection of *C. fluminea* that utilizes a primer pair amplifying a 208-bp amplicon of the mitochondrial (mt) gene Cytochrome *c* oxidase subunit 1 (COI) via quantitative PCR (qPCR). To test the validity of the assay, our study includes field sampling for eDNA from four lakes in California and Nevada, United States, where *C. fluminea* has established populations, and seven lakes where the species has never been observed. Furthermore, of the lakes with established populations, we investigated the distribution of *C. fluminea* eDNA within Lake Tahoe and compared that with previously published information on the within-lake distribution of *C. fluminea*. Accordingly, we add another freshwater invasive species to the list of those for which eDNA surveillance and monitoring may be possible to improve management outcomes in the future.

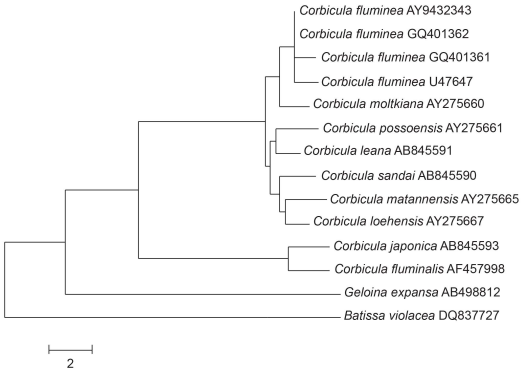
Materials and methods

Primer design and testing

COI sequences for the family Corbiculidae were downloaded from GenBank, including four sequences for the target *C. fluminea* representing the breadth of mtDNA diversity known for this species: AY943243, GQ401362, GQ401361, and U47647. Primer design was accomplished through visual searches for nucleotide variants between in-group and out-group sequences combined with Primer3 (Untergasser et al. 2012). The four in-group sequences were evaluated against sequences from 10 confamilial out-group species: *Geloina expansa* (Mousson, 1849), *Batissa violacea* (Lamarck, 1818), *Corbicula japonica* (Prime, 1864), *C. fluminalis* (Müller, 1774), *C. moltkiana* (Prime, 1878), *C. possoensis* (Sarasin and Sarasin, 1898), *C. sandai* (Reinhardt, 1878), *C. leana* (Prime, 1864), *C. matannensis* (Sarasin and Sarasin, 1898), and *C. loehensis* (Kruimel, 1913; see Figure 1). Four candidate primer pairs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, U.S.).

To evaluate primer performance *a priori*, we collected 268 *C. fluminea* individuals of various sizes from Juday Creek of the St. Joseph River drainage (South Bend, Indiana, United States: Latitude 41.7109; Longitude −86.2170) and transported them in stream water to a laboratory facility at the University of Notre Dame (Indiana, United States). The clams were

(a)



(b)

Scientific name	GenBank	Forward																					Reverse																	% Identity
		T	T	T	A	T	T	A	G	A	T	G	A	T	G	G	G	C	A	G	C	T	G	T	A	T	G	A	T	C	T	A	A	C	C	A	A	C	A	A	A	A	G	C	A	T	A	G	C	
Corbicula fluminea	AY943243	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	100
	GQ401362	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	100
	GQ401361	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	100
	U47647	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	100
C. loehensis	AY275667	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	96
C. matannensis	AY275665	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	94
C. possoensis	AY275661	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	94
C. leana	AB845591	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	C	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	92
C. sandai	AB845590	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•	C	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	•	•	•	•	•	•	•	92
C. fluminalis	AF457998	•	•	•	•	•	•	G	•	•	•	•	•	•	•	T	•	•	•	A	T	•	A	•	•	•	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	88
C. moltkiana	AY275660	•	•	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	A	•	•	•	•	C	•	•	•	•	T	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	88
C. japonica	AB845593	•	C	•	•	•	G	•	•	•	•	•	•	T	•	•	A	T	•	A	•	•	•	•	C	G	•	•	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	81
Geloina expansa	AB498812	G	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	T	•	•	•	•	•	•	A	•	•	A	•	C	•	•	T	•	•	•	•	T	•	A	•	•	•	T	•	•	•	•	•	79
Batissa violacea	DQ837727	G	•	•	G	•	•	G	•	•	•	•	•	•	•	•	•	T	•	•	•	•	•	•	A	•	•	A	•	C	•	•	T	•	•	•	•	C	•	A	•	•	•	•	•	A	•	•	•	77

Figure 1. a) Neighbor-joining tree constructed using 160-bp of the 208-bp Cytochrome *c* oxidase subunit 1 (COI) gene, after primers were removed. Tree illustrates the relationship among members of the family Corbiculidae used for the design of species-specific primers for *Corbicula fluminea*, including four sequences from *C. fluminea* evaluated against 10 confamilial out-group species. Species name is followed by the GenBank accession ID and scale is measured as number of substitutions per nucleotide site. b) Primer matching table for the COI gene compared across *Corbicula fluminea* and 10 confamilial out-group species.

placed in a 265-liter cattle tank with 2 cm of sand sieved to 500 μm grains and smaller. The cattle tank was filled with pre-filtered well water that was constantly aerated by four air stones and allowed to flow-through. A filter prevented *Corbicula* veligers from escaping the system. Each of the four primer pairs were tested in the lab using qPCR for successful amplification with *C. fluminea* DNA, both tissue-derived from collected individuals and water samples filtered from the same tank (methods for DNA extraction and qPCR assays follow those detailed below). The best performing primer pair, Cfluminea_COI_F1 (5'-TTTATTAGATGATGGGC AGCTGTA-3') and Cfluminea_COI_R1 (5'-TGAT CTAACCAACAAAAGCATAGC-3'), produced a 208-bp amplicon that was used for eDNA assays.

We evaluated this primer pair against non-target species (Table 1) by qPCR assay to determine potential amplification of eight congeneric *Corbicula*, and four non-*Corbicula* species chosen to broadly represent mollusk diversity of the western United States consistent with the location of our field validation (below). We used the following mollusks native to the western United States: the Oregon floater (*Anodonta oregonensis,* Lea, 1838), the freshwater limpet (*Ferrissia rivularis,* Say, 1817), the western pearlshell (*Margaritifera falcata,* Gould, 1850), and the pea or fingernail clam (*Pisidium* sp.). Because we could not obtain tissue for many of these species, particularly those of the genus *Corbicula* not known to occur in North America, we performed all primer specificity tests using gBlock fragments manufactured by IDT, based on COI genes available at GenBank (accession numbers are listed in Table 1). A gBlock ("genomic block") is a double-stranded synthetic sequence that matches to a requested sequence (COI gene); due to their similarity to genomic DNA, we used gBlocks in place of tissue-derived genomic DNA for testing. COI sequences for western United States native mollusks were selected from studies in California and Nevada, United States (Mock et al. 2004; Walther et al. 2010; Deiner et al. 2013; Mock et al. 2013). Again, qPCR methods for primer testing followed the protocol in our field validation (below).

We compared qPCR amplification cycle (C_T) and melt curve temperature (°C) between target and non-target taxa across a serial dilution series of gBlock COI fragments, reporting here results of primer

Table 1. Results of primer testing for *Corbicula fluminea* eDNA assay against the target species, non-targets of the same genus that the primer was designed against based on percent sequence identity (see Figure 1), and native clams and mussels representative of taxonomic diversity in vicinity of Lake Tahoe and adjacent California and Nevada, United States study sites. Primer testing was conducted against gBlock fragments synthesized by Integrated DNA Technologies (IDT, Coralville, IA, U.S.) based on GenBank COI sequences for each species, with corresponding accession numbers shown. We report qPCR amplification cycle (C_T) and melt curve temperature (°C) for each primer test for duplicate runs at a mean 1,482 copy number per 1 µl dilution (range 1,228–1,614 copies per 1 µl); results of a broader serial dilution series were consistent with reporting here. Two values for amplification cycle and melt curve temperature represent differing results of duplicate runs, whereas one value indicates agreement between runs.

Species	Accession	Amplification cycle (C_T)	Melt curve (°C)
Corbicula genus			
C. fluminea	AY943243	26.36–29.92	78.05
C. loehensis	AY275667	33.46–34.30	78.37
C. matannensis	AY275665	30.62–30.90	78.37–78.53
C. possoensis	AY275661	30.54–31.17	78.53
C. leana	AB845591	37.01–39.02	78.05
C. sandai	AB845590	36.10–39.74	77.56–78.86
C. fluminalis	AF457998	No amplification	No amplification
C. moltkiana	AY275660	No amplification	No amplification
C. japonica	AB845593	No amplification	No amplification
Native species			
Anodonta oregonensis	AY493472.1	No amplification	No amplification
Ferrissia rivularis	GU391051.1	37.14–No amplification	74.15–No amplification
Margaritifera falcata	KF701441.1	No amplification	No amplification
Pisidium sp.	KF000183.1	No amplification	No amplification

specificity under a mean 1,482 copy number per 1 µl dilution (range 1,228–1,614 copies per 1 µl). Five congeneric species other than *C. fluminea* regularly amplified under our primers at generally delayed cycles, but with similar melt curve temperatures (Table 1). Accordingly, these congeneric species would need to be distinguished from *C. fluminea* using Sanger sequencing confirmation, which we recommend for any field eDNA sample. Three other *Corbicula* species did not amplify under our primers; similarly, gBlock COI fragments for three of four native western U.S. mollusks also did not amplify. *Ferrissia rivularis* experienced occasional but infrequent amplification, with melt curve temperatures highly distinct from *C. fluminea* (Table 1); this species could be distinguished from *C. fluminea* under our assay without difficulty. See discussion for further details on implications of our primer design for monitoring *Corbicula* invasions.

Water sampling for eDNA

We tested our eDNA assay for *C. fluminea* from eleven lakes and reservoirs in the western states of California and Nevada, that included locations with (four locations) and without (seven locations) known records for this species (Figure 2; Table S1). Our sample locations consisted of six natural lakes and five man-made reservoirs, and ranged in size from 32 ha (Gilmore Lake) to 49,620 ha (Lake Tahoe). Visual surveys from 2010–2012 failed to find *C. fluminea* from Boca, Gilmore, Independence, Marlette, Martis Creek, Prosser and Stampede lakes or reservoirs (Caldwell and Chandra 2012). However, these same surveys did find *C. fluminea* present in Donner Lake, and this species is also known from Lake Tahoe, where its distribution is limited primarily to the eastern, southeastern, and southwestern shorelines, including semi-isolated Emerald Bay (Figure 2; Denton et al. 2012; Wittmann et al. 2012). Finally, *C. fluminea* was previously reported from the western shore of Clear Lake (Cummings 2016), and from Camp Far West Reservoir (USGS 2014).

We collected a total of 145 water samples across the eleven lakes described here. We sampled lakes during August 2015, when warm water temperatures should correspond with reproduction by *C. fluminea* (Rajagopal et al. 2000; Mouthon 2001), because life history events like reproduction may increase eDNA detection probability (e.g., de Souza et al. 2016). We used 250 mL sample bottles, which were washed with 10% bleach solution and sterilized via autoclave prior to usage, as per recommended decontamination protocols at the time; 50% bleach solution is now generally recommended (Goldberg et al. 2016). With the exception of sites replicated within Lake Tahoe and Clear Lake, ten total samples containing 250 mL of surface water were collected per lake or reservoir. All collections were made from shore, generally within 100 m of the geographical coordinates given in Table S1. For Lake Tahoe, we retrieved thirty-five 250 mL samples across seven discrete sites (five samples

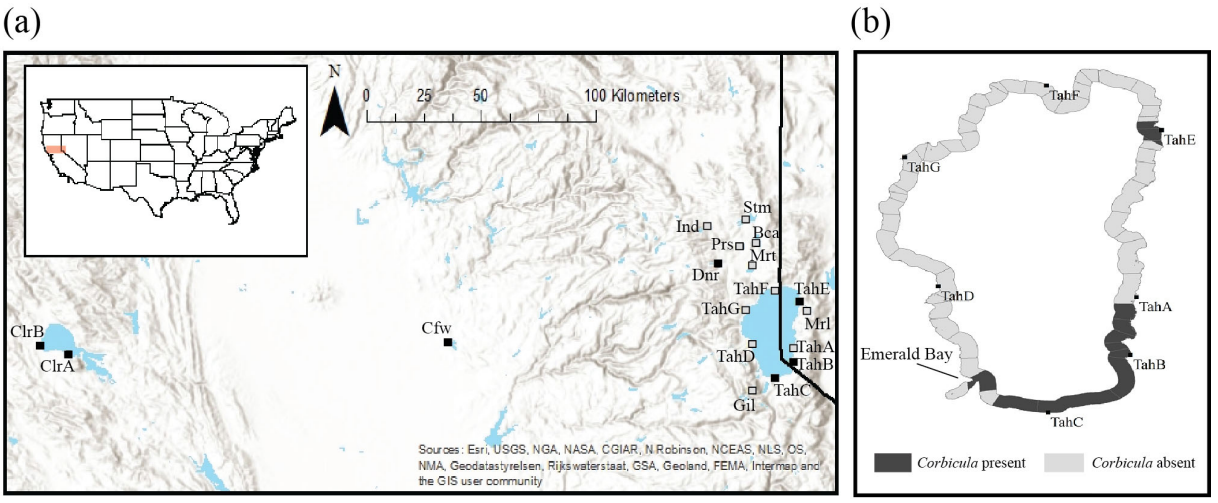


Figure 2. Study locations in California and Nevada, United States (a), with site abbreviations given in Table S1, and study locations specifically in Lake Tahoe (b) as related to the anticipated presence (black) and absence (gray) of *Corbicula fluminea* (Müller, 1774) from visual surveys of this invasive species per Caldwell and Chandra (2012) and other sources.

per site, Table S1). For Clear Lake, ten 250 mL samples were retrieved from two discrete sites at this lake (Figure 2; Table S1). Once collected, samples were immediately processed by filtration through cellulose nitrate filters of 1.2 µm pore size with the aid of a hand vacuum pump (Actron CP7830; Bosch Automotive Service Solutions, Warren, MI, U.S.) attached to a side-arm flask and filter funnel. As samples were processed while in the field, we included a "filtration blank" to test for contamination; the blank consisted of filtering 250 mL of bottled water purchased sealed from a store. We used one filtration blank per every five field samples. After the filtration process, sample filters were placed in 2 mL microcentrifuge tubes (USA Scientific, Ocala, Florida, U.S.) containing 700 µL of Longmire's buffer (Longmire et al. 1997), stored at 4 °C and transported to the laboratory at the University of Notre Dame for eDNA extraction.

eDNA extraction protocol

eDNA was extracted from filters following a modified chloroform-isoamyl alcohol (hereafter "CI") and isopropanol precipitation (see Renshaw et al. 2015). The 2 mL microcentrifuge tubes containing the filters were incubated in a 65 °C water bath for a minimum of 10 minutes, after which 700 µl of CI (24:1, Amresco) was added to each tube. Next, tubes were vortexed for 5 seconds and centrifuged at 15,000 g for 5 minutes. Afterwards, 500 µl of the aqueous (top) layer was transferred to a fresh set of 1.5 mL microcentrifuge tubes and combined with 500 µl of ice cold isopropyl alcohol and 250 µl of 5M NaCl. Tubes were then precipitated at −20 °C overnight. The precipitate was pelleted by centrifugation at 15,000 g at room temperature for 10 minutes and the supernatant was decanted. To wash the pellets, 150 µl of room temperature 70% ethanol was added to each tube prior to centrifugation at 15,000 g (room temperature) for 5 minutes; the liquid was decanted and pellets were washed a second time. Once the liquid was decanted, pellets were dried in a vacufuge at 45 °C for 15 minutes, followed by air drying until no visible liquid remained. Finally, pellets were rehydrated with 100 µl of 1X TE Buffer, Low EDTA (USB). We evaluated the eDNA extraction reagents and technique for contamination by the inclusion of a single extraction blank per each set of eDNA samples that involved just the reagents. All filtration and extraction blanks showed no evidence of contamination.

Quantitative PCR (qPCR) assays

qPCR triplicates were run for each eDNA extract in 20 µl reactions containing 4.85 µl of PCR-grade water, 4 µl of 5X Colorless GoTaq® Flexi Buffer (Promega), 0.4 µl of 10 mM dNTPs, 1.6 µl of 25 mM $MgCl_2$, 1 µl of each 10 µM primer (forward and reverse), 0.15 µl of GoTaq® Flexi DNA Polymerase (Promega), 1 µl of EvaGreen (20X in water; Biotium), 2 µl of 4 µg/µl Bovine Serum Albumin (Amresco), and 4 µl of eDNA extract. Reaction plates were run on a Mastercycler® ep realplex (Eppendorf) under the following cycling conditions: an initial denaturation at 95 °C for 3 minutes; 45 cycles of denaturation at 95 °C for 30

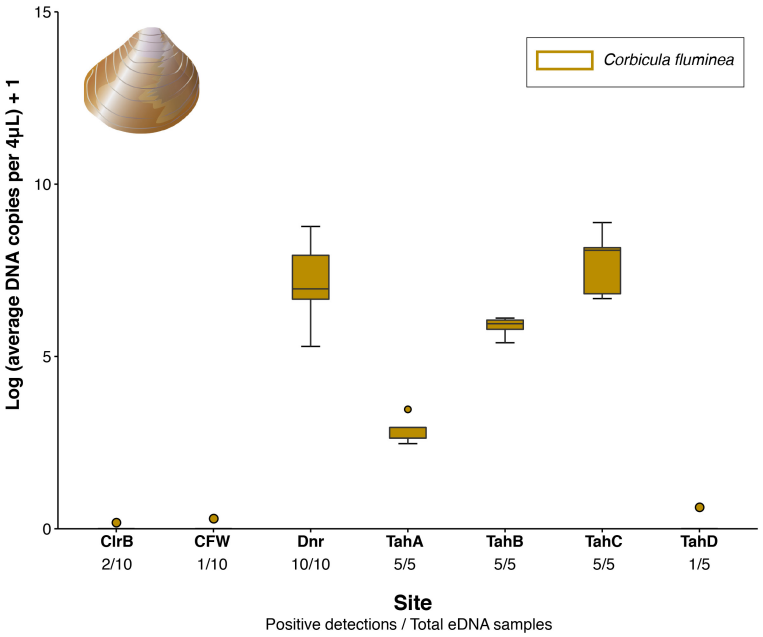


Figure 3. Average eDNA copies per 4 µl + 1 on a log10 axis for study locations in California and Nevada, United States, where we detected the Asian clam *Corbicula fluminea* (Müller, 1774). Sample site abbreviations are given in Table S1 and locations mapped in Figure 1, with more information on laboratory qPCR results in Table 2. On the x-axis, the numbers below the site abbreviations refer to the number of field replicates where eDNA was detected. Image of *C. fluminea* from the Integration and Application Network (Joanna Woerner, http://ian.umces.edu/imagelibrary/).

seconds, annealing at 60 °C for 45 seconds, and extension at 72 °C for 1 minute; followed by a melting curve analysis that transitioned from 60 °C to 95 °C over a span of 20 minutes.

A 500-bp gBlock gene fragment (IDT) was synthesized based on GenBank accession GQ401362 (base 44 to 543) to generate a standard curve for the quantification of eDNA samples. We estimated copy number for the gBlock fragment by multiplying Avogadro's number by the number of moles and a serial dilution of the gBlock fragment provided a range in copy numbers for the quantification of eDNA unknowns while also serving as a qPCR positive control for each plate (Gunawardana et al. 2014; Renshaw et al. 2015; Svec et al. 2015). We note briefly that the absence of inhibitory substances associated with our gBlock serial dilutions could result in inaccuracies in copy number estimates for field samples that might contain inhibitory substances. For each qPCR assay, we evaluated the reagents and technique for contamination with two "no template control" (NTC) wells that included the same Mastermix as the rest of the plate with sterile water in place of the eDNA extract. All NTC showed no evidence of contamination; amplification efficiencies ranged between 0.80 and 0.99, whereas R^2 values ranged between 0.99 and 1.00. Finally, a single qPCR replicate from every positive eDNA amplification was confirmed through unidirectional Sanger sequencing with the reverse primer. For Sanger sequencing confirmation, qPCR products were cleaned with ExoSAP-IT (Affymetrix), cycle sequenced with the Big Dye® Terminator v3.1 kit (ThermoFisher Scientific), cleaned with ethanol precipitation, and run on an AB3730XL at the University of Notre Dame's Genomics and Bioinformatics Core Facility.

Results

Using our assay and subsequent Sanger sequencing, we successfully detected *C. fluminea* eDNA in samples from all four water bodies in which *C. fluminea* had been previously observed: Donner Lake, Camp Far West Reservoir, Clear Lake, and Lake Tahoe (Figure 3). At the remaining locations from which *C. fluminea* had not been observed, we detected no evidence of this species or genus using our eDNA assay. As such, the remaining results will focus on the four water bodies with positive detections of *C. fluminea* eDNA.

The ten water samples collected from Donner Lake were all positive for *C. fluminea* eDNA, with the number of eDNA copies per 4 µl ranging between 521 and 6,452 (Figure 3, Table 2). Despite prior records of *C. fluminea* at Camp Far West Reservoir, we initially did not detect the species from any of the samples collected. We suspected that the lack of eDNA signal was a result of inhibition due to high amounts of suspended sediment observed at this reservoir during sampling; thus, we performed

Table 2. Mean eDNA copy number (N_C) and standard error of triplicate reactions (SE) for locations where Asian clam *Corbicula fluminea (*Müller, 1774) was detected. Positive detections and copy numbers were identified for four sites from Lake Tahoe (TahA, TahB, TahC and TahD), Donner Lake, Camp Far West and Clear Lake (B).

Lake Tahoe								
Sample #	*TahA*		*TahB*		*TahC*		*TahD*	
	N_C	SE	N_C	SE	N_C	SE	N_C	SE
1	12.90	0.65	325.33	21.75	3,487.67	407.06	0.00	0.00
2	30.97	3.07	425.67	29.73	795.00	33.28	0.79	0.79
3	17.90	1.88	451.00	49.92	3,231.67	752.19	0.00	0.00
4	10.83	5.70	220.00	6.80	914.67	100.99	0.00	0.00
5	12.87	1.67	383.00	9.23	7,226.33	271.56	0.00	0.00

	Donner Lake		Camp Far West		Clear Lake (B)	
Sample #	N_C	SE	N_C	SE	N_C	SE
1	6,452.33	1,958.96	0.00	0.00	0.00	0.00
2	197.67	42.21	0.00	0.00	0.00	0.00
3	777.67	96.72	0.00	0.00	<0.01	<0.01
4	1,204.00	165.79	0.17	0.17	0.00	0.00
5	1,118.67	471.93	0.00	0.00	0.00	0.00
6	790.00	162.86	0.00	0.00	0.00	0.00
7	521.33	163.38	0.00	0.00	0.00	0.00
8	3,765.67	2,049.24	0.00	0.00	0.00	0.00
9	3,701.00	1,087.18	0.00	0.00	0.02	0.02
10	993.00	188.98	0.00	0.00	0.00	0.00

serial dilutions of the extracted eDNA samples. After re-running the assay, we identified a positive detection of *C. fluminea* from a dilution of 1/10 the full concentration of eDNA (Table 2). While we obtained no detections from any sample retrieved from the southern shore of Clear Lake (Site A), at the westernmost site in Clear Lake (Site B), two samples were positive for small traces of *C. fluminea* eDNA (Figure 3, Table 2).

Within Lake Tahoe, we obtained positive detections from all samples at Cave Rock (TahA), Marla (TahB) and Tahoe Keys (TahC), while there was a single detection at Sugar Pine (TahD) (Figure 3, Table 2). Furthermore, Tahoe Keys had the highest number of eDNA copies per 4 µl, ranging from 794–7,226.33, followed by Marla (200–451) and Cave Rock (10.83–30.97) (Table 2). Samples for all other Tahoe locations (TahE, TahF and TahG) were negative for *C. fluminea* eDNA. These findings at Lake Tahoe align well with the known distribution of *C. fluminea* along the eastern and southeastern shoreline of this lake (Figure 2). While we failed to detect *C. fluminea* eDNA from a recently discovered, small population at Sand Harbor (TahE), we weakly detected eDNA at Sugar Pine (TahD, Table 2). The Sugar Pine result likely reflects a recently established population in nearby Emerald Bay (Figure 2), which is likely due to the spread of veligers of this species from surface currents from nearby established populations. Finally, all Sanger sequences for each positive eDNA amplification matched 100% identity to *C. fluminea* sequences present in NCBI GenBank database (see Appendix 1).

Discussion

We provide the first eDNA assay, and its field validation, for the detection of the invasive Asian clam *C. fluminea.* Previous researchers have used DNA barcoding methods to differentiate larval *Corbicula* spp. from the morphologically similar *Limnoperna fortunei* (Dunker, 1857) within conventional planktonic samples, by amplifying a 400-bp fragment of the COI gene for identification of these species (Ludwig et al. 2014). In contrast, our eDNA assay produces a shorter (208-bp) COI amplicon useful for revealing presence of *C. fluminea* and other closely related species of the genus *Corbicula* in applications where DNA is scarce and often degraded, such as in bulk environmental samples. As such, our study supports that of Ludwig et al. (2014) in highlighting the value of molecular approaches to detect the presence of invasive freshwater bivalves. However, studies specifically contrasting performance of barcoding conventional planktonic samples relative to eDNA might be useful in determining which of these approaches is best-suited to detect new or expanding populations of invasive clams and mussels.

The eDNA assay detailed here successfully detected the presence of *C. fluminea* at four lakes and

reservoirs where this species is known to occur, while providing no evidence for false positives associated with field or laboratory contamination. For example, we did not detect this species at the seven locations where it has never been documented following previous traditional surveys (Caldwell and Chandra 2012). Similarly, our eDNA detections within Lake Tahoe were largely consistent with the known distribution of *C. fluminea* along the eastern and southern shoreline of this lake (Denton et al. 2012; Wittmann et al. 2012), suggesting that eDNA may accurately represent ranges of organisms within large freshwater lakes. As an exception, we had a weak detection of *C. fluminea* (based on number of detections and copy number) at a western shoreline site where there were no previous records for the species, although we note this was approximately 10 km from an established and expanding population of clams in Emerald Bay (Figure 2). The surface waters of Emerald Bay are connected to the western shores of Lake Tahoe at Sugar Pine (TahD) during summer. Hydrodynamic 3D modeling of currents in the littoral zone and bays from areas with established *C. fluminea* populations in Lake Tahoe suggest small localized pulses of current lead to the dispersal of veligers and likely expansion of clams (Hoyer et al. 2014). As such, the weak *C. fluminea* detection with eDNA at Sugar Pine (TahD) may indicate utility of using eDNA to understand the transport of veligers from established populations to new areas of the lake where populations may be establishing. Additionally, we failed to detect *C. fluminea* eDNA from a smaller, recently discovered population at Sand Harbor (TahE) on the eastern shoreline of Lake Tahoe. The Sand Harbor (TahE) population is highly intermittent, with surveys at the end of 2015 and 2016 identifying very low density to no presence of adult clams, suggesting high mortality in this area of the lake. Ultimately, the ability of eDNA assays to detect target species is affected by how long DNA persists in the environment, and the related issue of DNA transport between source populations and sample collection locations (DeJean et al. 2011; Deiner and Altermatt 2014; Strickler et al. 2015). eDNA persistence and transport is likely dependent on biotic and abiotic parameters including temperature, microbial activity, and target organism eDNA release (Barnes et al. 2014), and we recommend ongoing work on how environmental conditions and organismal biology influence the performance of this tool (Strickler et al. 2015; Barnes and Turner 2016; Goldberg et al. 2016).

We had less ideal performance of eDNA for detection of *C. fluminea* at Camp Far West Reservoir. At this location, an abundance of clam shells was observed on the shoreline during water sampling, which indicated very high densities of *C. fluminea* despite the initial failure of the eDNA assay. The previous historic record for *C. fluminea* in Camp Far West Reservoir also reported the species as highly abundant (USGS 2014). We attributed initial failure to detect *C. fluminea* eDNA to suspended sediment in the water at this location, as soil components such as humic acids have been reported to inhibit PCR amplification of DNA from environmental samples (Tebbe and Vahjen 1993; Jiang et al. 2005). Subsequently, serial dilution of extracted DNA from Camp Far West Reservoir did result in one positive detection for *C. fluminea* with low copy numbers (Table 2), although this result seemingly still did not correspond well with the suspected high abundance of invasive Asian clams. Alternatively, our lack of detections at Camp Far West may also be linked to eDNA not being captured at the exact time point of collection, and repeated water sampling may improve detection rates. Furthermore, we did not conduct serial dilutions at any of our other study sites, which could have resulted in additional *C. fluminea* eDNA detections – although we emphasize that many of our study sites are exceptionally clear, oligotrophic lakes where inhibition was not anticipated to be a problem.

Effectiveness of our eDNA assay may also be influenced by both the generality of our primer to multiple species of the *Corbicula* genus, as well as the complex invasion history and genetics of *C. fluminea* across global non-native ranges. Invasive populations of *C. fluminea* have often been observed to have low mtDNA genetic diversity derived from only clonal or asexual (androgenetic) lineages, relative to more diverse native range populations that also include sexually reproducing lineages (Lee et al. 2005; Pigneur et al. 2014; Gomes et al. 2016; Tiemann et al. 2017). Accordingly, our use of the mitochondrial COI gene should be anticipated to work well for many invasive *C. fluminea* populations with low genetic diversity at this locus. Conversely, some other researchers have observed sympatric invasions of multiple lineages or cryptic species of *Corbicula* (Wang et al. 2014; Peñarrubia et al. 2017). Our assay is non-specific for *C. fluminea,* amplifying several other closely related species in the *Corbicula* genus, any of which would be non-native to North America. Phylogenetic and phylogeographic analyses are needed to continue to untangle the complex invasion history of *Corbicula* spp. globally, but we propose that our more general *Corbicula* eDNA assay might be useful to many researchers – perhaps even in an eDNA meta-barcoding framework (e.g., Comtet et al. 2015). Sanger sequencing confirmation of our relatively large 208-bp eDNA amplicon could be

used following qPCR to identify *Corbicula* species that have co-invaded a region together, or to identify cryptic *Corbicula* species that have been mistaken for *C. fluminea* (e.g., Tiemann et al. 2017). Alternatively, if more specificity in eDNA assay performance is desirable in some *Corbicula* applications, subsequent development of a more specific primer pair, or primer/probe assay (Fukumoto et al. 2015), may be warranted. Finally, the use of high resolution melting curve analysis could perhaps discriminate different *Corbicula* species in our eDNA assay (e.g., Robertson et al. 2009; Šimenc and Potočnik 2011). Our field-validated eDNA assay should provide a foundation for subsequent work by researchers who may desire either more generality or more specificity in a molecular *Corbicula* monitoring tool, a balance that must be navigated in many eDNA applications (Wilcox et al. 2016; Katano et al. 2017).

Successful field validation of our eDNA assay for *C. fluminea* adds to a growing literature that increasingly suggests that this tool is appropriate for early detection of invasive mollusks like the New Zealand mudsnail *Potamopyrgus antipodarum* (Gray, 1843; Goldberg et al. 2013) or the zebra mussel *Dreissena polymorpha* (Pallas, 1771; Egan et al. 2013; Egan et al. 2015). Given the substantial ecological and economic impacts associated with many of these invasive mollusk species (Isom 1986; Strayer 1999; Sousa et al. 2008), our study further demonstrates that eDNA could be used in surveillance and monitoring programs for new biological invasions by these species, to trigger rapid eradication or containment measures (Lodge et al. 2016). Management options are available for *Corbicula* invasions (e.g., Wittmann et al. 2012), and generally accurate performance of our eDNA assay for *C. fluminea* both between and within large lakes and reservoirs indicates that this approach may have high utility in combined invasive species surveillance and management frameworks.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Study locations in California and Nevada, United States, sampled during August 2015.

Appendix 1. *Corbicula fluminea* environmental DNA Sanger sequencing information for confirmation.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2018/Supplements/MBI_2018_Cowart_etal_Table_S1.xlsx
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