Detection of 4 imperiled western North American freshwater mussel species from environmental DNA with multiplex qPCR assays

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Abstract: Four freshwater mussel species native to western North America, *Gonidea angulata*, *Margaritifera falcata*, *Anodonta nuttalliana*, and *Anodonta oregonensis*, have experienced dramatic declines over the last century and are currently threatened in many portions of their ranges. Therefore, improved tools for detecting and monitoring these species are needed. We developed multiplexed, species-specific, quantitative PCR assays for the detection of these species from environmental DNA (eDNA). We empirically tested species specificity and sensitivity of assays in the lab, and we also validated multiplex assays with field-collected eDNA samples. All assays were species specific, sensitive, and effective for detection from eDNA samples collected from streams and rivers. These assays will aid in the detection, monitoring, management, and conservation of these vulnerable mussel species.
Key words: eDNA, freshwater mussels, *Anodonta nuttalliana*, *Anodonta oregonensis*, *Gonidea angulata*, *Margaritifera falcata*

Native freshwater mussel species have experienced dramatic declines in both distribution and abundance in western North America, primarily because of human impacts, such as habitat degradation and introduction of non-native species (Haag 2012, Strayer 2014). As filter feeders, these species provide important ecosystem services, such as improving water clarity, and are a food source for wildlife species. Ongoing population declines highlight the importance of monitoring native freshwater mussels, but traditional sampling requires time-consuming surveys, often necessitating snorkeling or SCUBA diving. Additionally, species identification requires trained expertise and can even, for some species (e.g., *Anodonta* spp.), necessitate genetic analyses. Thus, improved tools are needed for monitoring these species more efficiently and economically.

Western North America is home to 5 native freshwater mussel species: *Gonidea angulata* Lea, 1839; *Margaritifera falcata* Gould, 1851; *Anodonta oregonensis* Lea, 1839; *Anodonta nuttalliana* Lea, 1839; and *Sinanodonta beringiana* Middendorff, 1851. Originally, as many as 6 separate *Anodonta* spp. were described from western North America based on morphology, but genetic analyses have shown that only 3 major genetic lineages exist (Chong et al. 2008). *Sinanodonta beringiana*, which was recently reassigned from *Anodonta* to *Sinanodonta* (Williams et al. 2017), is known to inhabit only Alaska (AK) and Canada, although the southern extent of its range is uncertain. The remaining 2 *Anodonta* lineages in North America include a clade composed of *A. oregonensis* and *Anodonta kennerlyi* Lea, 1860 (here referred to as *A. oregonensis*) and a clade composed of *A. nuttalliana*, *Anodonta californiensis* I. Lea, 1852, and *Anodonta wahlamatensis* Lea, 1839 (here referred to as *A. nuttalliana*). *Anodonta oregonensis* ranges from AK to northern California (CA) (Blevins et al. 2017), and *A. nuttalliana* ranges from Washington (WA) to northern Mexico, extending east into Idaho (ID), Wyoming (WY), Utah (UT), Nevada (NV), and Arizona (AZ) (Blevins et al. 2016a). *Gonidea angulata* occurs in CA, NV, Oregon (OR), WA, ID, and British Columbia

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(BC), and *M. falcata* inhabits these states as well as parts of WY, Montana (MT), UT, and AK (Blevins et al. 2017).

Currently, the International Union for Conservation of Nature (IUCN) lists *A. nuttalliana* and *G. angulata* as vulnerable (Blevins et al. 2016a, c), and some states have recently listed them as species of conservation concern. *Gonidea angulata* has been assessed as endangered in Canada (COSEWIC 2010) and is listed as a species of special concern under Canada's Species at Risk Act. Additionally, a petition has been submitted to list *G. angulata* for protection under the United States Endangered Species Act (Blevins et al. 2020). *Margaritifera falcata* is considered near threatened, and *A. oregonensis* is considered of least concern by the IUCN (Blevins et al. 2016b, d). However, dramatic declines have occurred in portions of the ranges of both species (Blevins et al. 2017). Further, both species are designated as species of concern by some state agencies. Continued monitoring of these species will be essential to document persistence and to locate extant populations for protection.

Environmental DNA (eDNA) has emerged as an efficient and reliable tool for aquatic species monitoring and is more sensitive than traditional methods, under most conditions, for fishes (Wilcox et al. 2016), amphibians (Smart et al. 2015), and reptiles (Hunter et al. 2015) and has recently been used to detect freshwater mussels (Stoeckle et al. 2016, Currier et al. 2018, Dysthe et al. 2018). We designed new, species-specific eDNA assays for *A. nuttalliana*, *A. oregonensis*, and *G. angulata* and developed 3 multiplexed quantitative polymerase chain reaction (qPCR) assays that use these species-specific assays along with an assay previously designed for *M. falcata* (Dysthe et al. 2018). The 1st multiplex assay detects and discerns *A. nuttalliana* and *A. oregonensis*, the 2nd multiplex assay detects and discerns *G. angulata* and *M. falcata*, and the 3rd multiplex assay detects and discerns *A. nuttalliana* and *M. falcata.* We also tested the ability of all 3 multiplex assays to detect species from field-collected filtered water samples. We did not design an assay for *S. beringiana* because this species does not currently appear to face the same conservation challenges as the other 4 North American species (Vinarski and Cordeiro 2011) and because available tissues and sequence data are currently insufficient for the design of a robust assay for this species.

METHODS

Assay design

For assay design, we compiled sequence data for *A. nuttalliana*, *A. oregonensis*, and *G. angulata*, spanning the species' ranges, from the mitochondrial gene cytochrome oxidase subunit 1 (COI). We obtained sequence data from our in-house freshwater mussel sequence database (sequences from 344 samples from 67 populations for *A. nuttalliana*, 95 samples from 23 populations for *A. oregonensis*, and 145 samples from 31 populations for *G. angulata*; Table S1). We included additional sequences from GenBank® (Clark et al. 2016; 27 sequences for *A. nuttalliana*, 11 sequences for *A. oregonensis*, and 6 sequences for *G. angulata*; accessed November 2018; Table S2). For *A. nuttalliana*, we included sequences from WA (n = 25), OR (112), ID (12), UT (32), NV (20), CA (147), AZ (9), and Mexico (11). For *A. oregonensis*, we included sequences from OR (n = 51), WA (31), CA (6), BC (11), and AK (3) (Fig. 1, Tables S1, S2). For *G. angulata,* we included sequences from WA (n = 24), OR (36), NV (10), CA (40), BC (26), and ID (12). We obtained COI sequence data from the non-target native species *S. beringiana* from our in-house sequence database, and we retrieved COI sequence data from GenBank from 5 other potentially sympatric non-target taxa: *Corbicula fluminea*, *Dreissena polymorpha*, *Dreissena bugensis*, *Ferrissia rivularis*, and *Lampsilis siliquoidea* (Table S3).

We used Sequencher® software (version 5.2; Gene Codes, Ann Arbor, Michigan) to align sequence data from all species, and we used the online tool DECIPHER (Wright et al. 2014) to generate species-specific primers. We then used ABI Primer Express™ software (version 3.0.1; Applied Biosystems, Foster City, California) to design TaqMan™ Minor-Groove-Binding (MGB) qPCR probes. For *A. nuttalliana,* we initially tested 2 candidate primer sets and 3 TaqMan probes (1 probe for primer set 1, and 2 probes for primer set 2; Table 1). We later tested an additional probe for use in the Central Valley of CA where we identified a single nucleotide polymorphism (SNP) in the probe region for some populations (see Results). For *A. oregonensis*, we tested 1 candidate primer set and 2 TaqMan probes. For *G. angulata*, we initially tested 3 candidate primer sets in SYBR® green qPCR (Molecular Probes, Eugene, Oregon) and then 1 TaqMan probe (Table 1). For *M. falcata*, our multiplex assays utilized an existing single-species assay described in detail in Dysthe et al. (2018).

Specificity testing

Specificity testing of our assays included both in-silico and in-vitro components. To evaluate primer specificity in silico, we used National Center for Biotechnology Information's (NCBI) Primer-BLAST™ (Ye et al. 2012) against the NCBI non-redundant database to reduce the likelihood that species occurring in western North America could potentially cross-amplify and produce false positives.

In the laboratory, we initially used SYBR green qPCR to test specificity of candidate primer sets. We tested each primer set with 5 tissue DNA extracts from each target species as well as 4 DNA extracts from each non-target species (*S. beringiana*, *A. nuttalliana*, *A. oregonensis*, *G. angulata*, and *M. falcata*, depending on the assay being tested). qPCR reactions included 7.5 μL of Power SYBR-Green Mastermix (Thermo Fisher Scientific, Waltham, Massachusetts), 900 nM

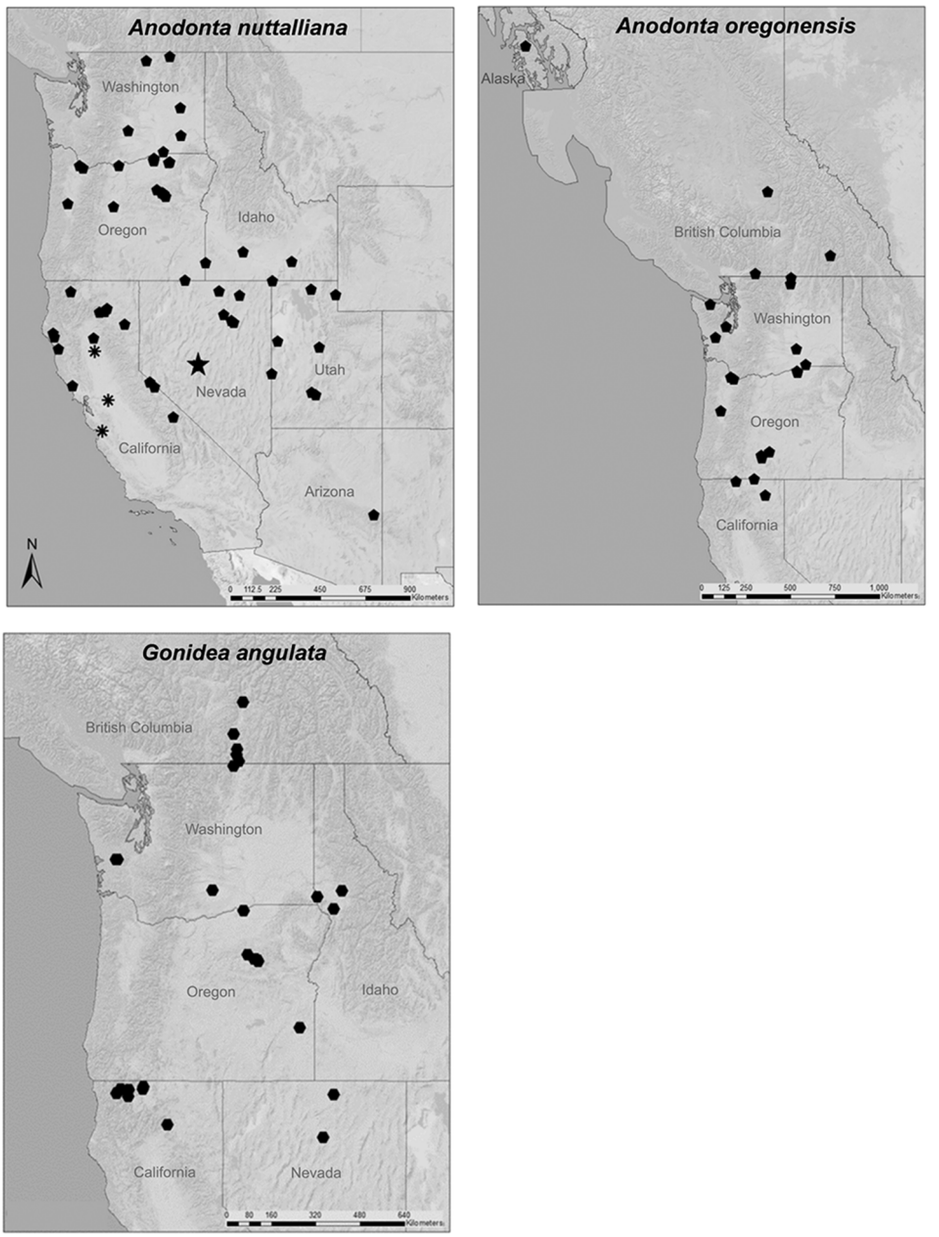

Figure 1. Locations in western North America of sample sequences used to design species-specific qPCR assays for the freshwater mussel species *Anodonta nuttalliana*, *Anodonta oregonensis*, and *Gonidea angulata*. Asterisks (*) designate locations where sample sequences match probe AnuCOI2-Pc. The star (★) designates the location where 1 sample sequence did not match probe AnuCOI2-Pb or AnuCOI2-Pc. At all remaining locations (pentagons, ⬟), sequences were a perfect match to the preferred assay for each species.

of each primer, and 0.1 ng of template DNA in a total reaction volume of 15 µL. Cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min followed by a melt curve.

We then tested primer sets and MGB probes in TaqMan qPCR. As above, analyses included 5 DNA extracts from each target species and 4 DNA extracts from each non-target species (S. *beringiana*, *A. nuttalliana*, *A. oregonensis*, *G. angulata*, and *M. falcata*, depending on the assay being tested). qPCR reactions included 7.5 µL of TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific), 900 nM of each primer, 250 nM of probe, and 0.1 ng of template DNA in a total reaction volume of 15 µL. Cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. For further optimization and sensitivity testing, we selected a single primer set and probe for each species based on performance (see Results). Selected assays included primer set AnuCOI2 and probe AnuCOI2-Pb for

Table 1. Primer and probe sets developed and initially tested for detection of *Anodonta nuttalliana, Anodonta oregonensis,* and *Gonidea angulata*. Primers and probes in bold were chosen as preferable for use in most populations after testing and were used in multiplex assays.

Assay	Primer/probe name	Sequence 5′–3′
A. nuttalliana	AnuCOI1-F	GACGGTATATCCACCTTTATCTGGA
	AnuCOI1-R	CAAACCAGGAGATCGCATGTTT
	AnuCOI1-P	6-FAM-CTTCTGTGGATTTGGCCAT-MGB-NFQ
	AnuCOI2-F	**GATCTCCTGGTTTGGTCGCT**
	AnuCOI2-R	**ACGATCCGTCAACAGCATTG**
	AnuCOI2-Pa	6-FAM-TTTATTTGTTTGGGCTGTTGC-MGB-NFQ
	AnuCOI2-Pb	**6-FAM-ATTAGTTGCTGCTTTACC-MGB-NFQ**
	AnuCOI2-Pc	**6-FAM-ATTGGTTGCTGCTTTAC-MGB-NFQ**
A. oregonensis	**AorCOI-F**	**GGGTCAACCAGGAAGGTTGTTA**
	AorCOI-R	**GAATAAGCCAATTACCAAACCCA**
	AorCOI-Pa	6-FAM-TGTTACAGCTCATGCTT-MGB-NFQ
	AorCOI-Pb	**6-FAM-ACAGCTCATGCTTTTATA-MGB-NFQ**
G. angulata	GanCOI1-F	CCTCTTATGATCGGGGCACCT
	GanCOI1-R	GGACAACGGCGGATACACTGT
	GanCOI2-F	**GGACAACCTGGAAGATTATTAGGTGA**
	GanCOI2-R	**CAGGTGCCCCGATCATAAGA**
	GanCOI2-P	**6-FAM-TAATGTAATTGTTACAGCACATG-MGB-NFQ**
	GanCOI3-F	CGGGTGCTTCTTCTATTTTGGGT
	GanCOI3-R	CCAATAAAACTGCCGTCACAGTC

A. nuttalliana, primer set AorCOI and probe AorCOI-Pb for *A. oregonensis*, and primer set GanCOI2 and probe GanCOI2-P for *G. angulata* (Table 2). We conducted specificity testing for all assays in singleplex with 6-FAM-labeled probes (Table 1) prior to testing sensitivity and field validation with multiplex assays including 6-FAM- and NED-labeled probes (Table 2).

We also tested an additional TaqMan probe (Probe AnuCOI2-Pc; Table 2) for *A. nuttalliana* for use in central CA populations, which possessed an SNP in the initial probe region (see Results). We tested this modified probe on tissue samples from the Pajaro ($n = 1$), Sacramento (1), and San Joaquin (4) Rivers (sites ASA, ASJ, ASM, ASV, ASQ, and APJ; Table S1). In addition, we tested the above tissue samples, as well as tissue samples from ID, OR, and UT, with both probes AnuCOI2-Pb and AnuCOI2-Pc combined at 125 nM each.

Primer concentration optimization

We optimized primer concentrations by running varying concentrations of each forward and reverse primer (100, 300, 600, and 900 nM, each in triplicate) with 100 copies of target-species MiniGene™ synthetic plasmid DNA (Integrated DNA Technologies, Coralville, Iowa) per reaction. We selected primer concentrations with the greatest peak fluorescence and lowest threshold cycle (C_t) value for subsequent analyses (Table 2).

Sensitivity testing

To test assay sensitivity, we used MiniGene synthetic plasmids containing the assay sequence for each assay. Plasmids were suspended in 100 μL of IDTE (10 mM Tris, 0.1 mM EDTA) buffer, linearized by digestion with the enzyme Pvu1, and then purified with a PureLink™ PCR Micro Kit (Invitrogen, Carlsbad, California) following standard protocols. We quantified the resulting products on a Qubit™ (Thermo Fisher Scientific) fluorometer and converted estimated concentrations to copy number based on molecular weight (Wilcox et al. 2013). We then diluted these products in IDTE buffer to create quantities of 2, 5, 10, 20, 50, and 100 copies/reaction, and we analyzed each quantity in 10 qPCR replicates for each species to determine assay sensitivity for each multiplex.

Field validation

To test all 3 multiplex assays in vivo, we used eDNA samples collected from water bodies where 1 or more of our target species were historically present. We did not conduct traditional sampling at most sites, but our dataset included a subset of positive control sites where live mussels were documented either at the time of, or within 1 year of, eDNA sampling (16 sites for *Anodonta*, 15 sites for *M. falcata*, and 5 sites for *G. angulata*; Tables S4, S5). For the *Anodonta* multiplex, we analyzed 70 eDNA samples from 36 water bodies from 7 states (Table S4). For the *M. falcata*/*G. angulata*

Table 2. Primers and probes used in multiplex assays for detection of *Anodonta nuttalliana, Anodonta oregonensis,Gonidea angulata*, and *Margaritifera falcata* from environmental DNA. Tm = primer melting temperature.

Assay	Primer/probe name	Sequence 5′–3′	Tm (°C)	Optimal concentration (nM)
A. nuttalliana/A. oregonensis multiplex	AnuCOI2-F	GATCTCCTGGTTTGGTCGCT	57.8	300
	AnuCOI2-R	ACGATCCGTCAACAGCATTG	58.2	600
	AnuCOI2-Pb	6-FAM-ATTAGTTGCTGCTTTACC-MGB-NFQ	68.0	250 (125)[a]
	AnuCOI2-Pc	6-FAM-ATTGGTTGCTGCTTTAC-MGB-NFQ	69.0	250 (125)[a]
	AorCOI-F	GGGTCAACCAGGAAGGTTGTTA	59.6	600
	AorCOI-R	GAATAAGCCAATTACCAAACCCA	59.5	900
	AorCOI-Pb	NED-ACAGCTCATGCTTTTATA-MGB-NFQ	68.0	250
G. angulata/M. falcata multiplex	GanCOI2-F	GGACAACCTGGAAGATTATTAGGTGA	59.6	600
	GanCOI2-R	CAGGTGCCCCGATCATAAGA	59.5	900
	GanCOI2-P	6-FAM-TAATGTAATTGTTACAGCACATG-MGB-NFQ	68.0	250
	Mfa-F[b]	GGGTTTTGGTAATTGRCTTATTCCACT	59.8–63.1	600
	Mfa-R[b]	ACAAGAAAAGAGCAGGCACAAGC	60.9	900
	Mfa-P[b]	NED-CCTTAACAATTTGAGGTTTTGATT-MGB-NFQ	70	250
A. nuttalliana/M. falcata multiplex	AnuCOI2-F	GATCTCCTGGTTTGGTCGCT	57.8	300
	AnuCOI2-R	ACGATCCGTCAACAGCATTG	58.2	600
	AnuCOI2-Pb	6-FAM-ATTAGTTGCTGCTTTACC-MGB-NFQ	68.0	250
	Mfa-F[b]	GGGTTTTGGTAATTGRCTTATTCCACT	59.8–63.1	600
	Mfa-R[b]	ACAAGAAAAGAGCAGGCACAAGC	60.9	900
	Mfa-P[b]	NED-CCTTAACAATTTGAGGTTTTGATT-MGB-NFQ	70	250

[a] AnuCOI2-Pb and AnuCOI2-Pc can be used individually or in combination depending on location of sample collection.
[b] From Dysthe et al. (2018).

multiplex, we analyzed 31 eDNA samples from 24 water bodies from 6 states (Table S5). For the *A. nuttalliana*/*M. falcata* multiplex, we analyzed 29 eDNA samples from 22 water bodies from 6 states (all of which were positive for either *A. nuttalliana*, *M. falcata*, or both with the other multiplex assays; Table S6).

eDNA samples were either collected specifically for this project or they were repurposed from other previously collected samples. One benefit of eDNA sampling is that a sample can be re-analyzed to detect additional taxa without further field effort even when the eDNA was initially collected to detect a different species (Dysthe et al. 2018, Franklin et al. 2018). We collected all samples following the protocol outlined in Carim et al. (2016a). We used a peristaltic pump to pump up to 5 L of water through a 1.5-µm glass microfiber filter, and we stored filters in silica desiccant until laboratory processing. We additionally collected and analyzed field blank filter samples ($n = 7$) consisting of 1 L of distilled water brought from the lab and filtered in the field to act as a negative control to monitor for field-based cross contamination. We used the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California) and followed a modified protocol described in Carim et al. (2016b) to extract samples in a room dedicated for this purpose. Each round of eDNA extraction ($n = 8$) included 1 extraction blank negative control consisting of a clean filter. We analyzed all samples in triplicate qPCR with 4 µL of eDNA extract, 7.5 µL of TaqMan Environmental Mastermix 2.0, optimized primer and probe concentrations (Table 2), and cycling conditions described above. Each qPCR run included 3 to 6 no-template negative control reactions to monitor for cross contamination. We conducted eDNA extractions in a dedicated clean lab space physically separated from post-PCR spaces and tissue DNA extraction spaces, and we set up all qPCR reactions under a dedicated PCR hood sterilized with ultraviolet radiation prior to each qPCR run (Goldberg et al. 2016).

RESULTS

Assay design

The species-specific assays we developed target 125, 128, and 148 bp fragments of COI in *A. nuttalliana*, *A.*

oregonensis, and *G. angulata*, respectively. For *A. oregonensis* and *G. angulata*, the assays were a perfect match at both primers and the probe for all haplotypes from our database and GenBank across the species' ranges. For *A. nuttalliana*, we were not able to design a single assay that perfectly matched all haplotypes across the species range because of high levels of haplotype diversity. However, we were able to design 1 assay that perfectly matched all sequences in our database from AZ, ID, OR, UT, and WA. One *A. nuttalliana* individual from 1 site in NV (Reese River, site ARR; Table S1) displayed a single SNP in probe AnuCOI2-Pb, but this sequence was a perfect match to probe AnuCOI2-Pa. Thus, use of probe AnuCOI2-Pa may be preferable in this population. A 2nd sample from the Reese River, and all other haplotypes from NV, were a perfect match to both probes. In CA, 2 *A. nuttalliana* samples possessed single SNPs in the reverse primer. A single sample from the San Joaquin River (site ASV; Table S1) possessed 1 SNP in the reverse primer 6 bp from the 3′ end, but 7 other samples collected at the same location were a perfect match to the primer. Likewise, a single sample from the Owens River (site AOR; Table S1) also possessed 1 SNP in the reverse primer 14 bp from the 3′ end, but 9 other samples from the same location were a perfect match to the primer. Such mismatches far from the 3′ end typically have minimal effects on assay sensitivity (Lefever et al. 2013). Of the 11 *A. nuttalliana* reference sequences on GenBank from Mexico (Table S2), 5 were a perfect match to the assay, whereas the remaining 6 possess 1 SNP 13 bp from the 3′ end of the forward primer and a 2nd SNP 8 bp from the 3′ end in the reverse primer. Thus, if eDNA work is to be conducted in Mexico, a modification of the assay may be warranted.

Additionally, all *A. nuttalliana* samples from the Sacramento, San Joaquin, and Pajaro Rivers in central CA (sites ASA, ASJ, ASM, ASV, ASQ, and APJ; Fig. 1, Table S1) possessed a single guanine-adenine (G-A) SNP in the probe for AnuCOI2-Pb 15 bp from the 3' end. This SNP appears to be monomorphic in these populations. All 32 samples from these rivers possessed this SNP. Thus, we designed an additional modified probe with a G instead of an A in the position of the SNP (probe AnuCOI2-Pc).

Specificity testing

In-silico evaluation of primer specificity with NCBI Primer-BLAST for *A. nuttalliana* identified just 1 species with the potential to cross-amplify with our chosen primer set: the congener *Anodonta impura*, which is native to central Mexico and is not known to occur in western North America (MolluscaBase 2019). For *A. oregonensis*, Primer-BLAST identified 14 mussel species with the potential to cross-amplify (Table S7), but none of these taxa occur in western North America. For *G. angulata*, no species with a potential to cross-amplify were identified.

The in-vitro evaluation of primer specificity using SYBR green indicated primer sets for all 3 species were species specific, amplifying DNA from all targets at least 10 cycles earlier than DNA from any non-target species, a range which is suitable for specificity once a TaqMan MGB probe is added. For *A. nuttalliana*, all target samples amplified with a mean $C_t = 24.18$ for primer set AnuCOI1 and mean $C_t = 23.49$ for primer set AnuCOI2. All non-target samples amplified at >11 C_t higher than targets for both assays. Because the primer set AnuCOI2 produced lower mean C_t values than primer set AnuCOI1, we proceeded with primer set AnuCOI2 for further testing. For *A. oregonensis*, all target samples amplified with a mean $C_t = 24.98$. All non-target samples either did not amplify or amplified at >12 C_t higher than targets. For all *Anodonta* primer sets, the melt curve produced a single sharp peak indicating no primer–dimer formation or off-target amplification. For *G. angulata*, all target samples amplified with all 3 primer sets with mean $C_t = 22.51$ for primer set GanCOI1, mean $C_t = 22.63$ for GanCOI2, and mean $C_t = 22.41$ for GanCOI3. All non-targets either did not amplify or amplified at >10 C_t higher than targets for all 3 primer sets, but primer set GanCOI2 had the greatest specificity with all non-targets amplifying >12 C_t higher than targets. In addition, primer sets GanCOI1 and GanCOI3 displayed weak primer–dimer formation in melt-curve analyses, whereas primer set GanCOI2 did not. Thus, primer set GanCOI2 was chosen for further work.

The in-vitro evaluation of assay specificity including species-specific TaqMan™ MGB probes indicated that each assay was specific, detecting DNA from only target species. For *A. nuttalliana*, all target samples amplified with both probes with a mean $C_t = 29.68$ for probe AnuCOI2-Pa and mean $C_t = 28.18$ for probe AnuCOI2-Pb. No amplification was observed in any non-target samples for either probe. Because probe AnuCOI2-Pb displayed a lower mean C_t value than probe AnuCOI2-Pa, we proceeded with probe AnuCOI2-Pb for sensitivity testing and field validation. For *A. oregonensis*, all target samples amplified with both probes with a mean $C_t = 29.31$ for probe AorCOI-Pa and mean $C_t = 29.09$ for probe AorCOI-Pb. No amplification was observed in any non-target samples for either probe. Because probe AorCOI-Pb displayed a lower mean C_t value than probe AorCOI-Pa, we proceeded with probe AorCOI-Pb for sensitivity testing and field validation. For *G. angulata*, all target samples amplified with probe GanCOI2-P with a mean $C_t = 27.87$, and no amplification was observed for any non-target samples.

Furthermore, the *A. nuttalliana* probe modified for use in the Central Valley of CA (AnuCOI2-Pc) amplified robustly with tissue DNA extracts from sites in the Pajaro, Sacramento, and San Joaquin rivers. DNA extracts from ID, OR, and UT also amplified robustly with both probes AnuCOI2-Pb and AnuCOI2-Pc combined at 125 nM each (Table 2). Thus, for work in the Central Valley of CA, we

recommend using both probes in combination if it is not known which haplotype is found in a particular water body. Because of observed polymorphisms in central-CA populations, if eDNA sampling is conducted in water bodies other than those for which sequence data was available for this study (Table S1), we recommend collecting and sequencing additional local tissue samples to ensure the optimal eDNA assay is used.

Sensitivity testing

With the *A. nuttalliana*/*A. oregonensis* multiplex, for *A. nuttalliana*, all replicates amplified down to a template concentration of 5 copies/reaction, and 70% (7 of 10) of reactions containing 2 copies/reaction amplified in qPCR. For *A. oregonensis*, all replicates amplified down to 5 copies/reaction, and 40% (4 of 10) of reactions containing 2 copies/reaction amplified. With the *G. angulata/M. falcata* multiplex, for *G. angulata*, all replicates amplified down to 10 copies/reaction, 80% (8 of 10) of reactions with 5 copies amplified, and 90% (9 of 10) of reactions with 2 copies amplified. For *M. falcata*, all replicates amplified down to 10 copies/reaction, 60% (6 of 10) of reactions with 5 copies amplified, and 30% (3 of 10) of reactions with 2 copies amplified. With the *A. nuttalliana/M. falcata* multiplex, for *A. nuttalliana*, all replicates amplified down to 5 copies/reaction, and 70% (7 of 10) of reactions containing 2 copies/reaction amplified. For *M. falcata*, all replicates amplified down to 10 copies/reaction, 70% (7 of 10) of reactions with 5 copies amplified, and 30% (3 of 10) of reactions with 2 copies amplified.

Field validation

In field validation with the *Anodonta* multiplex, we detected *A. nuttalliana* in 25 of 70 eDNA samples from 17 of 36 water bodies in 5 states and *A. oregonensis* in 13 of 70 samples from 7 of 36 water bodies in 2 states (Fig. 2, Table S4). Both *Anodonta* species were detected at 2 locations: Columbia Slough, OR, and Walla Walla River just above its confluence with the Columbia River, WA. Although traditional sampling was not conducted at all locations where eDNA samples were collected, we observed live *Anodonta* at 16 sites either at the time of or within 1 y of eDNA sampling. At all 16 sites where we encountered live *Anodonta*, the corresponding eDNA sample was positive for 1 or both *Anodonta* species except for 1 site: the Chehalis River in WA (Table S4). At the Chehalis site, we found live *Anodonta* ~50 m downstream of where the eDNA sample was collected but did not detect *Anodonta* eDNA.

For the *G. angulata*/*M. falcata* multiplex, we detected *G. angulata* in 6 of 31 eDNA samples from 5 of 24 water bodies in 3 states, and we detected *M. falcata* in 18 of 31 samples from 13 of 24 water bodies in 6 states (Fig. 2, Table S5). We encountered live *G. angulata* individuals at 5 locations where eDNA samples were collected, and 100% of these eDNA samples were positive for *G. angulata.* We encountered live *M. falcata* at 15 locations where eDNA samples were collected, and 100% of these eDNA samples were positive for *M. falcata* (Table S5).

For the *A. nuttalliana*/*M. falcata* multiplex, we detected *A. nuttalliana* in 13 of 29 eDNA samples and detected *M. falcata* in 18 of 29 samples (Table S6). We detected both species in 2 samples: the Middle Fork John Day River, OR, and the Bruneau River, NV. We had 100% agreement in detection of *A. nuttalliana* and *M. falcata* with the *A. nuttalliana*/*M. falcata* multiplex assay and the other 2 multiplex assays. We did not observe amplification in any field blank, extraction blank, or qPCR negative control samples.

DISCUSSION

We designed and validated sensitive species-specific qPCR assays for 3 freshwater mussel species (*A. nuttalliana*, *A. oregonensis*, and *G. angulata*) native to western North America, and we validated these in 3 multiplex assays along with a previously-designed assay for *M. falcata* (Dysthe et al. 2018). These assays performed well at detecting mussels in the field at locations where they occur. When used in tandem, 2 of our multiplex assays can detect and discern all 4 western freshwater mussel species. In addition, we designed a 3rd multiplex assay for use in areas where *A. nuttalliana* and *M. falcata* co-occur but where the other 2 species are not expected, likely a common scenario because *A. nuttalliana* and *M. falcata* have the widest distributions of the 4 species. It should be possible to use different multiplex permutations for specific sampling needs, although the 3 multiplex assays we designed and tested are likely sufficient for most sampling needs. It may be possible to use 4 different fluorescent dyes to multiplex all 4 assays, but this would rarely be necessary or cost effective because, to our knowledge, there are no sites where all 4 species co-occur, and in only a few locations (e.g., Middle Fork John Day River, OR) do any 3 co-occur.

The assays described here will be particularly useful for assessing presence of freshwater mussel populations in locations with suitable habitat, or where populations have been previously described, with costs far lower than traditional stream or river-bed surveys (Stoeckle et al. 2016, Togaki et al. 2019). Further, our assays could be used in systematic monitoring programs to detect life-history events such as glochidial releases or mussel die-offs, both of which would be expected to show dramatic increases in eDNA concentrations. In addition, these assays could be used for species identification of glochidia, the microscopic immature life stage of freshwater mussels, which are notoriously difficult to identify to species and typically require highly trained visual-identification expertise or costly barcode sequencing. qPCR could potentially provide a more accurate and cost-effective solution for glochidia species identification. Because mussel glochidia attach to gills and fins of infected host fish, identifying glochidia could also be used

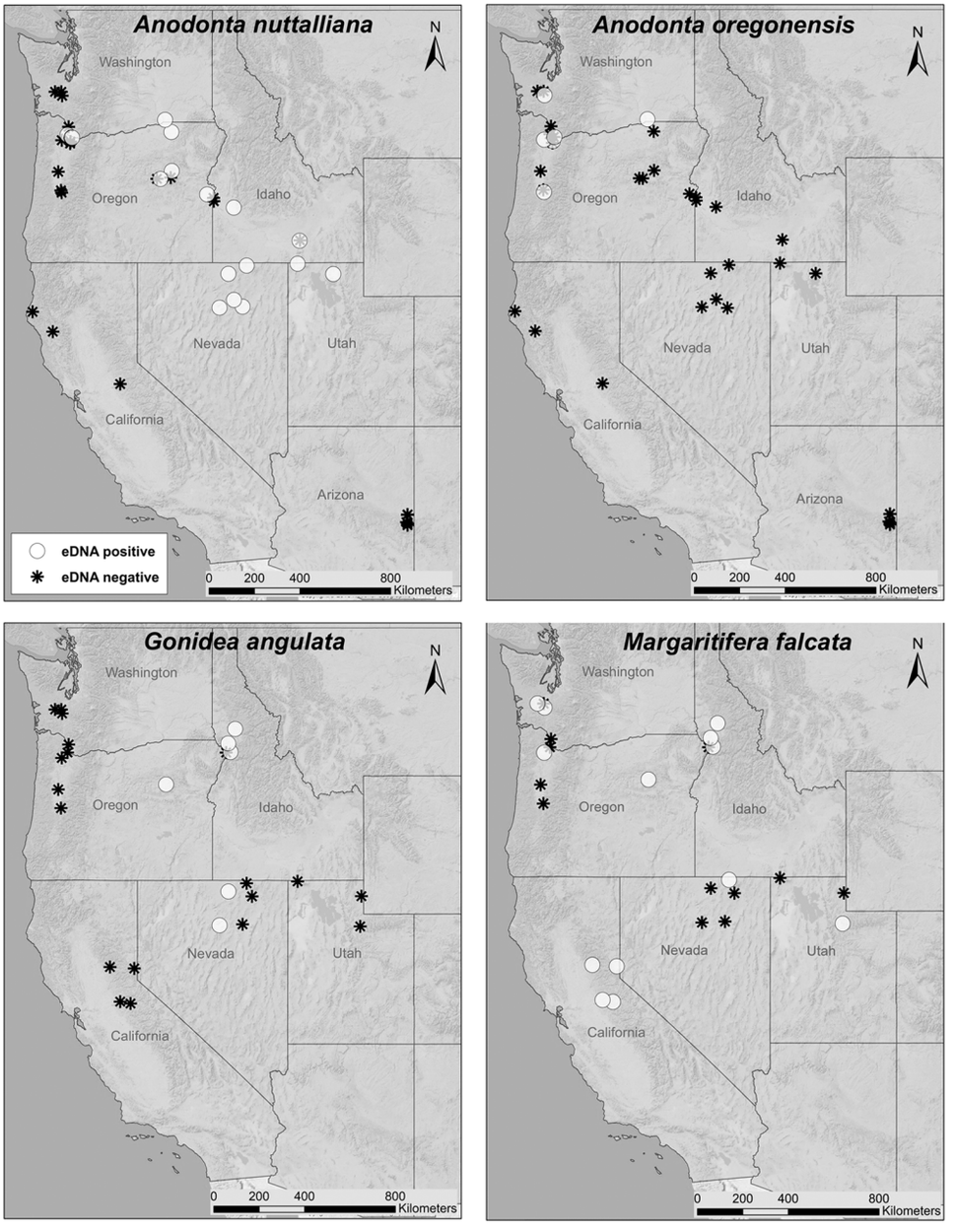

Figure 2. Locations of filtered water samples collected in the western United States for field validation of multiplex environmental DNA assays for *Anodonta nuttalliana*, *Anodonta oregonensis*, *Gonidea angulata*, and *Margaritifera falcata*.

to study species-specific host fish–mussel associations, which could have important implications for mussel transformation and dispersal (Strayer 2008, Maine et al. 2016).

Our *A. nuttalliana*/*A. oregonensis* qPCR assay could also provide low-cost species identification from mature mussel tissue samples. *Anodonta nuttalliana* and *A. oregonensis* are morphologically similar and are often mis-identified as one another based on shell shape. Thus, DNA barcode sequencing is often necessary to properly differentiate these species. Consequently, many historical records based on shell morphology of *A. nuttalliana* and *A. oregonensis* may include incorrect identifications. For example, some historical records indicate that *A. oregonensis* occurred in NV and UT, but neither recent genetic tissue analysis nor re-measurement of museum specimens supports *A. oregonensis* presence in NV or UT (Blevins et al. 2016b, 2017). Likewise, we have not detected *A. oregonensis* in UT or NV with eDNA. Our qPCR assays could be a more cost-effective method of *Anodonta* species identification from tissue samples, and our multiplex assay for *Anodonta* will be useful for resampling

historical *Anodonta* sites with eDNA to determine if original species identifications were correct.

One particular aspect of our eDNA assay design is broadly informative for design of species-specific eDNA assays for other species. For *A. nuttalliana*, we were not able to design a single species-specific assay that was a perfect match to all range-wide haplotypes. This difficulty was likely because *A. nuttalliana* has a wide geographic distribution and relatively-high mitochondrial haplotype diversity caused by prehistoric isolation in distinct hydrologic basins (Mock et al. 2010). The other 3 western mussel species display lower levels of mitochondrial diversity (Mock et al. 2010, 2013). Also, for *A. nuttalliana* we had reference sequence data from a large number of samples for assay design: 344 samples from 67 populations from 7 states. Thus, we likely uncovered sequence diversity that may have been missed had we designed the assay based on a more limited reference sequence library. Many published eDNA assays are based on far more limited data that may not always account for rare haplotypes, which could affect assay sensitivity in certain populations. Our work demonstrates that for species with broad ranges and high mitochondrial haplotype diversity, many reference sequences from many populations are needed to design a robust range-wide assay.

Beyond the need to improve reference databases, future work should focus on establishing best practices for effectively sampling freshwater mussels with eDNA in the field. For example, further research is needed to determine transport distances of eDNA from mussel beds, and the degree to which they are modulated by physical and chemical water properties, to inform the spatial scale of sampling and ensure populations are not missed (e.g., Deiner and Altermatt 2014, Stoeckle et al. 2017, Wacker et al. 2019, Gasparini et al. 2020). Research to determine the optimal sampling season for maximum detection probability will also be important. Western freshwater mussels generally release millions of glochidia into the water column during the spring for dispersal by host fish (Culp et al. 2011, Allard et al. 2017), and these releases may provide an abundance of eDNA for detection at that time (Wacker et al. 2019). Alternatively, late summer through winter low flows may be efficacious for sampling because high water levels in spring can dilute eDNA, making it harder to detect.

As native mussel populations continue to decline throughout North America, efficient and reliable tools for monitoring them across large spatial and temporal scales are important for targeting conservation efforts. The multiplex eDNA assays described here will serve as valuable tools for future surveys of western freshwater mussels and will be more cost-effective than traditional sampling techniques. Additionally, the assays provide an effective means for distinguishing species that can be difficult to identify morphologically, which will aid in the detection, monitoring, and conservation of native freshwater mussels across western North America.

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