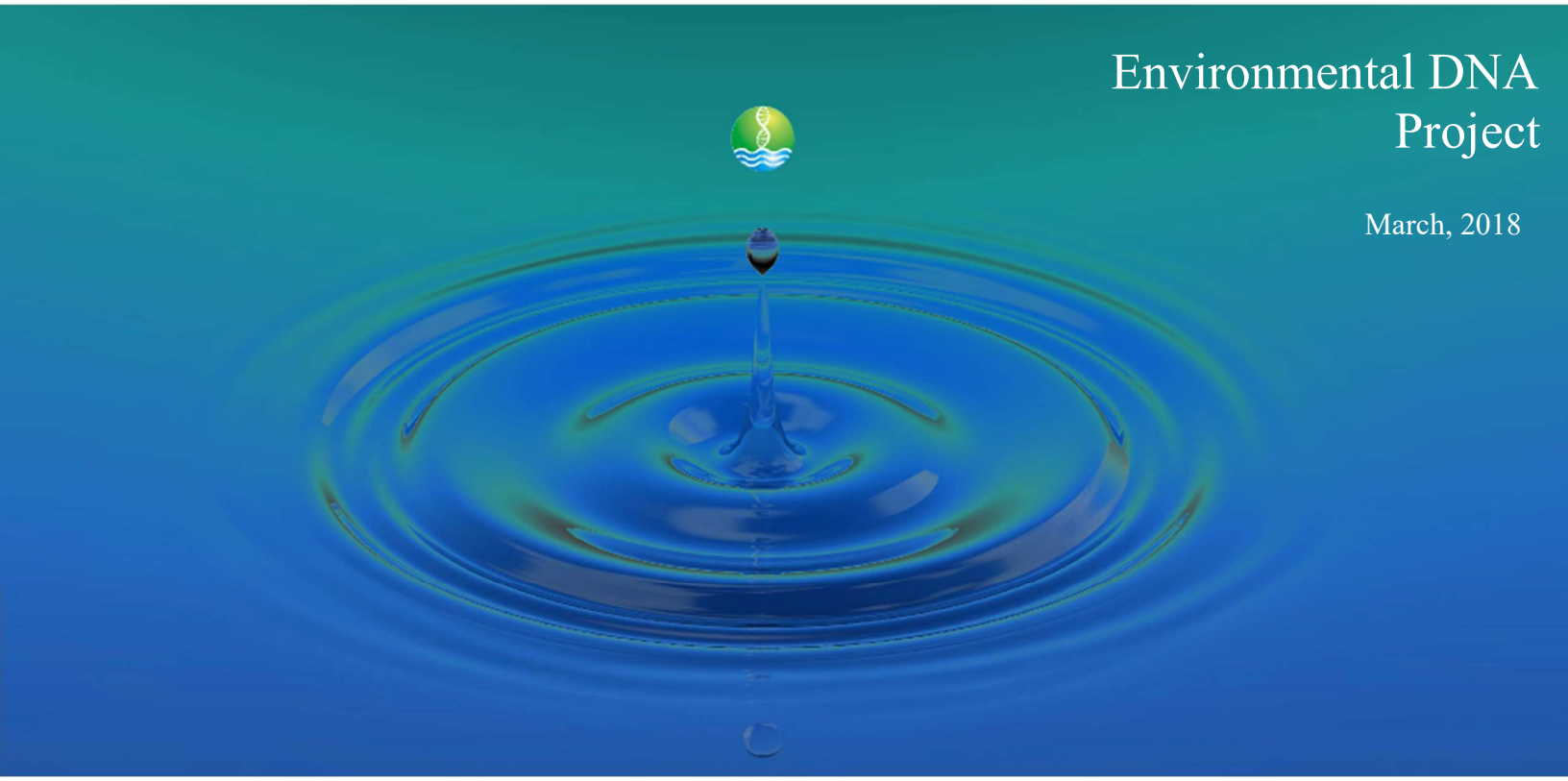

Environmental DNA Project

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Analysis of eDNA samples for Santa Ana Sucker (*Catostomus santaanae*), Arroyo Chub (*Gila orcuttii)* and Rainbow Trout (*Oncorhynchus mykiss)*.

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PROJECT DESCRIPTION

Genidaqs will provide services to HDR Engineering Inc. for training on field collection of eDNA samples, sample processing and testing of eDNA using quantitative Polymerase Chain Reaction (qPCR) hardware platform. Environmental DNA samples will be interrogated for Santa Ana Sucker (*Catostomus santaanae*), Arroyo Chub (*Gila orcuttii)* and Rainbow Trout (*Oncorhynchus mykiss)* using qPCR assays developed by Genidaqs. DNA from water collections will be archived for future use. The goal is to obtain information regarding the presence of the three target species of interest. Detection results will be transmitted to Ben Onanian at HDR Engineering Inc as quickly as possible to inform (adaptively) ongoing survey activities.

QPCR ASSAY DESIGN AND VALIDATION FOR THE ARROYO CHUB (*GILA ORCUTTII*) AND THE SANTA ANA SUCKER (*CATOSTOMUS SANTAANAE*)

CytB DNA Barcoding

DNA barcoding is the process by which a short DNA sequence is identified as being unique to a species. Barcoding pre supposes that tissue samples from vouchered specimen are available for DNA extraction and subsequent DNA sequencing. Tissue specimens from both the Arroyo chub (AC) the Santa Ana sucker (SAS) were not readily available for use as positive DNA controls. Therefore, all DNA sequence data for the purpose of barcoding were drawn from the National Center for Biotechnology Information (NCBI) Nucleotide data base (https://www.ncbi.nlm.nih.gov/nucleotide?cmd=search).

Due to a lack of tissue samples from both species, no internal DNA sequence data was generated and the resulting DNA barcodes were, out of necessity, produced in-silico only. DNA sequence data was mined exclusively from NCBI Nucleotide. All publically available DNA sequence data for the mitochondrial genes Cytochrome Oxidase Subunit I (COI) and Cytochrome B (CytB) were downloaded for both AC and SAS. DNA sequence data was downloaded into Geneious 8.0 (Geneious, Newark, NJ) software package for alignment and analysis. DNA sequences for both genes (COI and CytB) were aligned for each species respectively to identify regions of inner species sequence homology (uniqueness) and intra-species variability. These short unique DNA sequences found on both genes for both species were used as DNA barcodes. These barcodes provided the template upon which quantitative polymerase chain reaction (qPCR) assays were designed.

QPCR assay design and validation

Design

A species-specific qPCR assay consists of a forward and reverse primer and a florescently labelled DNA probe. DNA barcodes, derived from COI and CytB consensus sequences, were used as template in qPCR assay design using the commercially available algorithm Primer Express 3.1 (Thermo Fisher Scientific. Waltham, MA). Primer Express identified assays with the highest probability of detecting the barcode fragments from the target species. Assays targeting COI and CytB fragments from both species were identified by Primer Express. COI and CytB assay sequences were queried for sequence similarity using a BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information (NCBI) Nucleotide database as a means of determining in-silico species specificity. BLAST results for AC indicated that an assay targeting a 93bp region of the COI gene showed the least sequence similarity to any closely related co- existing species. BLAST results for SAS indicated that a 57bp region of the CytB gene gene showed the least sequence similarity to any closely related co-existing species. Assay sequences for both species are listed in Table 1.

Table 1. qPCR assay sequences for the mitochondrial genes COI of *Gila orcuttii* and CytB of *Catostomus santaanae.*

Species	Oligo	Sequence 5'-3'	Reporter	Quencher
Gila orcuttii	AC COI Forward	CTGGGACAGGATGAACGGTATAC		
Gila orcuttii	AC COI Reverse	GCCAGATGTAATGAGAAAATTGTTAGA		
Gila orcuttii	AC COI Probe	CCCCACTCGCAGGC	6 FAM	MGBNFQ
Catostomus santaanae	SAS CytB Forward	GTGACCCCCCCTCACATTAA		
Catostomus santaanae	SAS CytB Reverse	GGAGGATGGCGTAGGCAAA		
Catostomus santaanae	SAS CytB Probe	CCAGAATGGTATTTCC	6 FAM	MGBNFQ

Validation

The primer probe sets were tested for specificity both in-silico and using total genomic DNA of the following common co-existing species: rainbow trout, largemouth bass, small mouth bass, Mississippi silverside, bluegill, Pacific lamprey, and threadfin shad. As there were no tissues available, thus no positive DNA control for either assay, a positive DNA control or standard had to be synthesized. Using the DNA barcode and assay data to identify target DNA strands, species-specific double stranded DNA was synthesized and used as a control for both assays. The synthesis of sequence verified double stranded DNA, or G Block, was done by Integrated DNA Technologies (IDT Inc., Skaoakie IL). G blocks are commonly used as qPCR standards when no other source is available or practical. The qPCR for cross reactivity was performed in triplicate in 5 µl total volume containing 1 µl 20 ng/ µl of DNA template, 2.5 µl TaqMan Universal Master Mix (Thermo Fisher ABI), 0.5 µl /each 900 nM initial concentration of both forward and reverse primers and 1 µl 2.5 µM initial concentration probe. Primer and probe optimization was conducted following Applied Biosystems (Thermo Fisher ABI) guidelines for optimizing primer and probes for amplifying custom target sequences. QPCR for optimization was performed in 5 µl total volume containing 1 µl of DNA template, 2.5 µl TaqMan Universal Master Mix (Thermo Fisher ABI), 0.5 µl /each 50-900nM final concentration of both forward and reverse primers and 1 µl 50-250 nM final concentration probe. Thermocycling for both specificity and optimization PCR reactions were conducted on a BioRad CFX96 (Bio-Rad Laboratories, Inc.) with the following cycle conditions: initial activation 10 min at 95° C followed by 40 cycles of 15 sec denaturation at 95° C and 1 min extension at 60° C. All qPCR reactions were conducted with three no template controls run in parallel. Results of these data were analyzed using the BioRad CFX manager 3.1 (Bio-Rad Laboratories, Inc.).

FIELD COLLECTIONS

HDR Engineering Inc biologists conducted field sampling for eDNA. Collection procedures followed Blankenship and Schumer (2017) and Bergman et al. (2016). The area of survey interest was the Pyramid Reach of Piru Creek (tributary to the Santa Clara River), downstream of Pyramid Dam, with 60 samples and duplicates. Sites were spaced every 500 meters over the 18.5 miles between the Normal Maximum Water Surface Elevation of Lake Piru (1,055 feet) and the base of Pyramid Dam. At each site, the eDNA sample was represented by two sterivex filters. Total water volume filtered at each site was dictated by the water quality at the time of sampling. The water volume filtered was recorded by HDR biologists and is not included as data in Table 1. Field blanks were collected at the beginning and end of each field trip/day, including a total of four sampling trips (three one day trips and one three day trip) for a total of 8 field blanks and a total of 128 samples to be processed and analyzed. Sampling was conducted twice in the spring of 2018.

EDNA ANALYSIS

Total DNA was isolated from each eDNA sample (filter) following Bergman et al. (2016). All samples were analyzed using qPCR assays developed by Genidaqs (Santa Ana Sucker (*Catostomus santaanae*) unpublished, Arroyo Chub (*Gila orcuttii)* unpublished and Rainbow Trout (*Oncorhynchus mykiss)*), where each DNA template (eDNA sample) was interrogated for the presence of Santa Ana Sucker, Arroyo Chub and Rainbow Trout mitochondrial DNA.

Each DNA extract was interrogated in triplicate for each species of interest. QPCR reaction for Santa Ana Sucker, Arroyo Chub and Rainbow Trout included 4 μl of DNA extract in a total volume of 10 μl. Reactions were run using conditions previously established by the Genidaqs lab during assay design and development. All process controls were as expected (Table 1).

Table 1. QPCR analysis of field samples. As described in Bergman et al. (2016), if any one of the three technical replicates tested positive for target species DNA the sample was considered a positive detect (+), otherwise the sample was considered a no detect (ND).

#	Sample_ID	SANTA ANA SUCKER	RAINBOW TROUT (SURROGATE)	ARROYO CHUB
1	FIELD BLANK_031518 AM	ND	ND	ND
2	PC_1A	ND	ND	ND
3	PC_1B	ND	+	ND
4	PC_2A	ND	+	ND
5	PC_2B	ND	+	ND
6	PC_3A	ND	+	ND
7	PC_3B	ND	+	ND
8	FIELD BLANK_031518 PM	ND	ND	ND
9	FIELD BLANK_031418 PM	ND	ND	ND
10	PC_4A	ND	+	ND
11	PC_4B	ND	+	ND
12	PC_5A	ND	+	ND

13	PC_5B	ND	+	ND
14	PC_6A	ND	+	ND
15	PC_6B	ND	+	ND
16	PC_7A	+	+	ND
17	PC_7B	+	+	ND
18	PC_8A	+	+	ND
19	PC_8B	+	+	ND
20	PC_9A	+	+	ND
21	PC_9B	+	+	+
22	PC_BLANK_041918	ND	ND	ND
23	PC_10A	+	+	ND
24	PC_10B	+	+	ND
25	PC_11A	+	+	ND
26	PC_11B	+	+	ND
27	PC_12A	+	+	ND
28	PC_12B	+	+	ND
29	PC_13A	+	+	ND
30	PC_13B	+	+	ND
31	PC_14A	+	+	ND
32	PC_14B	+	+	ND
33	PC_15A	+	+	ND
34	PC_15B	+	+	+
35	PC_16A	+	+	ND
36	PC_16B	+	+	ND
37	PC_17A	+	+	ND

38	PC_17B	+	+	ND
39	PC_18A	+	+	ND
40	PC_18B	+	+	ND
41	PC_19A	+	+	+
42	PC_19B	+	+	ND
43	PC_20A	+	+	ND
44	PC_20B	+	+	ND
45	PC_21A	+	+	ND
46	PC_21B	+	+	ND
47	PC_22A	+	+	ND
48	PC_22B	+	+	ND
49	PC_23A	+	+	ND
50	PC_23B	+	+	ND
51	PC_24A	+	+	ND
52	PC_24B	+	+	ND
53	PC_25A	+	+	ND
54	PC_25B	+	+	ND
55	PC_26A	+	+	ND
56	PC_26B	+	+	ND
57	PC_27A	+	+	ND
58	PC_27B	+	+	ND
59	PC_28A	+	+	ND
60	PC_28B	+	+	ND
61	PC_29A	+	+	+
62	PC_29B	+	+	+

63	PC_30A	+	+	ND
64	PC_30B	+	+	ND
65	PC_31A	+	+	ND
66	PC_31B	+	+	ND
67	PC_32A	+	+	ND
68	PC_32B	+	+	ND
69	PC_33A	+	+	+
70	PC_33B	+	+	ND
71	PC_34A	+	+	+
72	PC_34B	+	+	ND
73	PC_35A	+	+	ND
74	PC_35B	+	+	ND
75	PC_36A	+	+	ND
76	PC_36B	+	+	ND
77	PC_37A	+	+	ND
78	PC_37B	+	+	ND
79	PC_38A	+	+	+
80	PC_38B	+	+	ND
81	PC_39A	+	+	ND
82	PC_39B	+	+	ND
83	PC_40A	+	+	+
84	PC_40B	+	+	+
85	PC_41A	+	+	+
86	PC_41B	+	+	ND
87	PC_42A	+	+	+

88	PC_42B	+	+	ND
89	PC_43A	+	+	ND
90	PC_43B	+	+	ND
91	PC_44A	+	ND	ND
92	PC_44B	+	+	ND
93	PC_BLANK_041618	ND	ND	ND
94	PC_45A	+	+	ND
95	PC_45B	+	ND	ND
96	PC_46A	+	ND	ND
97	PC_46B	+	ND	ND
98	PC_47A	+	+	ND
99	PC_47B	+	+	ND
100	PC_48A	+	+	ND
101	PC_48B	ND	ND	ND
102	PC_49A	+	ND	ND
103	PC_49B	+	+	ND
104	PC_50A	+	+	ND
105	PC_50B	+	+	ND
106	PC_51A	+	+	ND
107	PC_51B	+	+	ND
108	PC_52A	+	+	ND
109	PC_52B	+	+	ND
110	PC_53A	+	+	ND
111	PC_53B	+	+	ND
112	PC_54A	+	+	ND

113	PC_54B	+	+	ND
114	PC_55A	+	+	ND
115	PC_55B	+	+	ND
116	FIELD BLANK_031418	ND	ND	ND
117	PC_56A	+	+	+
118	PC_56B	+	+	+
119	PC_57A	+	+	ND
120	PC_57B	+	+	+
121	PC_58A	+	+	ND
122	PC_58B	+	+	ND
123	PC_59_1	+	+	ND
124	PC_59_2	+	+	ND
125	PC_60_1	ND	+	+
126	PC_60_2	+	+	+
127	FIELD BLANK_031318	ND	ND	ND
128	eDNA Extraction Control 3.15.18	ND	ND	ND
129	eDNA Extraction Control 3.26.18	ND	ND	ND
130	eDNA Extraction Control 3.26.18	ND	ND	ND
131	eDNAExtraction Control 3.27.18	ND	ND	ND
132	eDNAExtraction Control 3.27.18	ND	ND	ND
133	eDNA Extraction Control 4.10.18	ND	ND	ND
134	eDNA Extraction Control 4.10.18	ND	ND	ND
135	eDNA Extraction Control 4.26.18	ND	ND	ND
136	eDNA Extraction Control 4.26.18	ND	ND	ND
137	eDNA Extraction Control 5.04.18	ND	ND	ND

138	No Template Control (qPCR Control)	ND	ND	ND
139	Positive Control	+	+	+

Literature cited

Bergman, P.S., G. Schumer, S. Blankenship, and E. Campbell. 2016. Detection of Adult Green Sturgeon Using Environmental DNA Analysis. PLOS ONE 11(4): e0153500.

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