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RESOURCE ARTICLE

Rapid CRISPR-Cas13a genetic identification enables new opportunities for listed Chinook salmon management

Melinda R. Baerwald[1] | Emily C. Funk[2] | Alisha M. Goodbla[2] | Matthew A. Campbell[2] | Tasha Thompson[3,4] | Mariah H. Meek[3] | Andrea D. Schreier[2]

[1]Division of Integrated Science and Engineering, California Department of Water Resources, Sacramento, California, USA

[2]Genomic Variation Laboratory, Department of Animal Sciences, University of California Davis, Davis, California, USA

[3]Department of Integrative Biology, AgBio Research, and Ecology, Evolution, and Behavior Programme, Michigan State University, East Lansing, Michigan, USA

[4]Wild Salmon Center, Portland, OR, USA

Correspondence
Melinda R. Baerwald, Division of Integrated Science and Engineering, California Department of Water Resources, Sacramento, CA, USA.
Email: melinda.baerwald@water.ca.gov

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Abstract

Accurate taxonomic identification is foundational for effective species monitoring and management. When visual identifications are infeasible or inaccurate, genetic approaches provide a reliable alternative. However, these approaches are sometimes less viable (e.g., need for near real-time results, remote locations, funding concerns, molecular inexperience). In these situations, CRISPR-based genetic tools can fill an unoccupied niche between real-time, inexpensive, but error-prone visual identification and more expensive or time-consuming, but accurate genetic identification for taxonomic units that are difficult or impossible to visually identify. Herein, we use genomic data to develop CRISPR-based SHERLOCK assays capable of rapidly (<1 h), accurately (94%–98% concordance between phenotypic and genotypic assignments), and sensitively (detects 1–10 DNA copies/reaction) distinguishing ESA-listed Chinook salmon runs (winter- and spring-run) from each other and from unlisted runs (fall- and late fall-run) in California's Central Valley. The assays can be field deployable with minimally invasive mucus swabbing negating the need for DNA extraction (decreasing costs and labour), minimal and inexpensive equipment needs, and minimal training to conduct following assay development. This study provides a powerful genetic approach for a species of conservation concern that benefits from near real-time management decision-making but also serves as a precedent for transforming how conservation scientists and managers view genetic identification going forward. Once developed, CRISPR-based tools can provide accurate, sensitive, and rapid results, potentially without the prohibitive need for expensive specialty equipment or extensive molecular training. Further adoption of this technology will have widespread value for the monitoring and protection of our natural resources.

KEYWORDS
fisheries management, genetic technology, *Oncorhynchus tshawytscha*, San Francisco Bay Delta watershed, SHERLOCK, threatened and endangered species

1 | INTRODUCTION

Accurate taxonomic identification is necessary for effective ecosystem monitoring and species management. However, visual identification of taxa in the field can be complicated by morphological similarity, especially among closely related sympatric species, subspecies, or evolutionary significant units (ESUs). The inability to make accurate field identifications hinders efforts to assess and monitor the distributions and population parameters of listed taxa. Without these metrics, it is impossible to monitor species status and evaluate the effects of conservation and management actions.

The current gold standard for identifying species, subspecies, or ESUs is based on genetic methods. For example, Hebert et al. (2004) used DNA barcoding to identify 10 cryptic species in a neotropical butterfly complex, *Astrapes fulgerator*, in which adults exhibited high phenotypic similarity. Differentiating between similar sympatric species, subspecies, or ESUs becomes particularly critical when the taxa differ in their state, provincial, or federal listing status. In such situations, genetic identification may be necessary to ensure listed entities are being properly managed based on accurate distribution and abundance information. For example, in the San Francisco Bay Delta watershed, some monitoring programmes rely on genetic assays to distinguish between federally threatened delta smelt (*Hypomesus transpacificus*), state threatened longfin smelt (*Spirinchus thaleicthys*) and unlisted invasive wakasagi (*Hypomesus japonicus*) at early life stages, when the taxa are most similar morphologically (Baerwald et al., 2011, 2020; Benjamin et al., 2018; Brandl et al., 2015). In a similar vein, Andree et al. (2018) developed a quantitative PCR (qPCR) assay to distinguish the larvae of an endangered Mediterranean fan mussel (*Pinna nobilis*) from the larvae of other bivalve molluscs in order to understand life history and recruitment dynamics. One drawback to identification using current common genetic approaches (e.g., sequencing or qPCR) is that these methods do not provide quick results (taking hours to days), thereby impeding mangers' abilities to make rapid decisions when necessary. However, CRISPR-based genetic detection platforms (Chen et al., 2018; Gootenberg et al., 2017; Williams et al., 2019) address this drawback and have the potential to revolutionize our ability to conduct rapid and accurate genetic taxonomic identifications when real time decision-making is necessary (Baerwald et al., 2020).

Specific high-sensitivity Eezymatic reporter UnLOCKing (SHERLOCK) is a highly sensitive CRISPR-Cas13a genetic detection platform originally developed for point-of-care diagnostics (Abudayyeh et al., 2017; Gootenberg et al., 2017) that has shown promise for genetic taxonomic identification in ecological studies (Baerwald et al., 2020). SHERLOCK works by detecting and amplifying a target genetic sequence (e.g., taxon-specific). When this target sequence is found, Cas13a activates and cleaves not only the target sequence but any single-stranded RNA molecules present in the reaction. Quenched reporter RNA molecules included in SHERLOCK reactions produce a fluorescent signal when cleaved by Cas13a, indicating the presence of the target sequence. Baerwald et al. (2020) showed that SHERLOCK rapidly distinguished between three morphologically similar smelt fish species. Accurate identifications were obtained from skin mucus in as little as 25 min and could be performed in the field, providing major advantages over more conventional genetic identification approaches. This technology has the power to rapidly improve our ability to conserve and manage species.

Here, we demonstrate the power of this technique using Chinook salmon (*Oncorhynchus tshawytscha*) in the Central Valley of California. Chinook salmon populations throughout the species' range have shown dramatic declines in abundance due to factors such as overfishing, habitat degradation, damming and diversions (Fraidenburg & Lincoln, 1985; Hoekstra et al., 2007; Yoshiyama et al., 1998), and these forces are particularly prevalent in the Central Valley.

Chinook salmon are anadromous and semelparous, meaning they are born in freshwater, then migrate out to the ocean to feed and grow before returning to their natal streams to spawn and die. Within the Central Valley, there are four genetically differentiated "runs" of Chinook salmon (Banks et al., 2000) distinguished and named for the time they return to freshwater to spawn: fall, late fall, winter, and spring. Within these four runs, there are two general migration phenotypes: an "early" migration phenotype (winter and spring runs), which migrates back to freshwater before they are sexually mature and spends time in cool, higher elevation headwaters before spawning, and a "late" migrating phenotype (fall and late fall runs), which migrates to freshwater shortly before spawning and generally use the lower elevation waterways to spawn (Yoshiyama et al., 1998, 2000). However, damming of most river systems in the Central Valley has blocked spawning areas for the winter and spring runs, restricting access to their historic spawning grounds and leading to serious declines in their abundance. Declines have been significant enough to warrant listing of Sacramento River winter-run salmon as Endangered and Central Valley spring-run as Threatened under the Endangered Species Act (National Marine Fisheries Service, 1994; National Marine Fisheries Service et al., 1999). The Central Valley fall and late fall run types, which are designated as a single ESU, were listed as a federal Species of Concern (West Coast Chinook Salmon Biological Review Team, 1997).

The four Chinook salmon runs are morphologically indistinguishable and mix along their migration corridor, both as adults returning to spawn and juveniles during outmigration. The inability to visually distinguish among the four runs hinders accurate assessment of population trends and evaluation of the impacts of anthropogenic activities on the ESA listed runs. The current typical approach for identifying run type of juvenile Chinook throughout the Central Valley relies on a length at date model. However, work has shown that this model is highly error prone (Brandes et al., 2021; Harvey et al., 2014; Merz et al., 2014). Inaccurate run type identification impairs management decision-making as well as the quality of downstream scientific investigations. Genetic marker-based population assignment testing or mixed stock analysis using microsatellites (Banks et al., 2014), single-nucleotide polymorphisms (SNPs; Meek et al., 2016), or microhaplotypes (Barthelemy, 2018) have high

accuracy in assigning juvenile Chinook to run type, but it takes at least 24 h for genetic run type assignments to be returned to managers (Harvey et al., 2014), making near real-time management decisions impossible. The SHERLOCK platform provides a promising path for rapid (<1 h), field genetic identification of Chinook salmon run types that enables near real-time decision making. Accurate run typing capabilities that can be deployed throughout the Chinook salmon migration corridor will improve run-specific monitoring and the integrity of future scientific studies as well as increase the capacity for responsive management actions.

In this study, we demonstrate the power of SHERLOCK to address pressing conservation issues by leveraging polymorphisms in a chromosomal region capable of distinguishing between early (winter and spring) and late (fall and late fall) run types located on Chinook chromosome 28 (Narum et al., 2018; Prince et al., 2017; Thompson et al., 2019, 2020). This region of strong association (RoSA) sensu Thompson et al. (2020) is a relatively small region with two protein-coding genes – *greb1l* and *rock1* – that exhibits high conservation and linkage disequilibrium within run types. We combine newly collected whole genome sequencing data with recently collected sequence data (Thompson et al., 2020) to develop SHERLOCK assays that will distinguish between Chinook salmon run types in the field. To our knowledge, this is the first time SHERLOCK has been used to distinguish units below the species level in an ecological context.

2 | MATERIALS AND METHODS

2.1 | Whole genome sequencing

Whole genome sequencing was performed on 74 fall, 22 late fall, 22 winter, and 74 spring run samples to identify diagnostic or near diagnostic polymorphisms between Central Valley Chinook salmon runs. Please see Appendix S1 for a detailed description of our sequencing methods and SNP discovery results. Whole genome sequencing data is available in the NCBI Sequence Read Archive (accession no. PRJNA871266) at http://www.ncbi.nlm.nih.gov/bioproject/871266.

2.2 | SHERLOCK assay development

2.2.1 | Production of LwCas13a and crRNAs

LwCas13a protein was synthesized and purified by GenScript. CRISPR RNAs (crRNAs) were synthesized as ultramer RNAs (Integrated DNA Technologies) and rehydrated following Dharmacon's synthetic guide RNA resuspension protocol (Dharmacon).

2.2.2 | RPA primer and crRNA design

To distinguish Chinook salmon run types using SHERLOCK, we took a tiered approach because a single diagnostic SNP between the four run types was not identified. Therefore, we first identified early (winter and spring) versus late (fall and late fall) migrating run types. Then, if identified as an early migrating phenotype, we performed a second set of SHERLOCK assays to distinguish between the spring-run and winter-run Chinook salmon. Currently, we do not have assays developed to discriminate between fall and late fall runs because we were unable to identify diagnostic SNPs and fall and late fall-run are considered a single ESU. SNPs and indels distinguishing early and late migrating phenotypes were concentrated on chromosome 28 (NC_037109.1), which contains the RoSA region (Thompson et al., 2020). The SNPs and indels distinguishing spring and winter run types were concentrated on chromosomes 13 (NC_037112.1) and 16 (NC_037124.1). Using the Broad Institute's INTEGRATED GENOMIC VIEWER version 2.8.13 (Robinson, 2011), we aligned our filtered WGS data (Appendix S1) to the appropriate chromosome, and using MEGAX (Kumar et al., 2018), we manually designed several possible crRNAs for each SHERLOCK assay. We then designed recombinase polymerase amplification (RPA) primers flanking those crRNAs using PRIMER3PLUS version 2.4.2 (Table S1; Koressaar & Remm, 2007; Untergasser et al., 2012). Forward primers contained the T7 promoter sequence (TAATACGACTCACTATAGGG) at the 5′ end along with five additional bases to increase binding affinity.

We designed run-specific crRNAs following the guidelines in Gootenberg et al. (2017). Each crRNA was 67 nucleotides in length with a 28-nucleotide spacer sequence and the Cas13a binding sequence. We introduced a mismatch in position 5 of the spacer to increase LwCas13a's specificity (Gootenberg et al., 2017). We used the MULTIPLE PRIMER ANALYSER software (ThermoFisher Scientific) to ensure that both RPA primers and crRNAs that formed self-dimers or cross primer dimers were not taken through to production.

2.3 | SHERLOCK assay screening

We performed 1-pot SHERLOCK detection reactions using the protocol optimized by Arizti-Sanz et al. (2020) with the following modifications based on Baerwald et al. (2020) and Sullivan et al. (2019): 200 nM final concentration polyU reporter, 220 nM final concentration crRNA, 0.48 μM final concentration forward and reverse primers, and 600 μM final concentration DTT (Tables S2 and S3). SHERLOCK reactions occurred in a total volume of 10 μL (excluding DNA input). Template DNA (1 μL) was added to each reaction. We set up reactions in a laminar flow hood to reduce the risk of contamination. We prepared reactions in Bio-Rad white shell qPCR plates and incubated the reaction at 37°C for 1 h with fluorescent plate readings every 5 min for a total of 12 measurements. Fluorescent excitation and emissions were measured using the FAM channel on the Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). We used Bio-Rad CFX MAESTRO Software to obtain relative fluorescent units (RFUs) for each sample across readings. We included four no-template control samples on each plate and used their average RFU to subtract background fluorescence from all samples for each measurement.

We performed initial assay screens using DNA extracted from the caudal fin of two target run type individuals and two of each of the nontarget run types. For example, early migrating SHERLOCK reactions were tested with two winter-run and two spring-run Chinook individuals as well as two fall and two late fall-run individuals. We selected highly specific crRNA/RPA primer pair combinations with the greatest fluorescent intensity and most rapid amplification (one pair per run type) and considerably increased sample sizes for both target and nontarget run types to confirm specificity, as described below.

2.4 | Assay specificity and accuracy

Once we chose our best candidate crRNA and RPA primer pairs for each of the four SHERLOCK assays, we screened a larger subset of individuals, 238 samples, across run types and tributaries collected throughout the Central Valley (Table S4). Fin clips from adult fish were collected at the spawning grounds between 1998 and 2019, and phenotypic run type calls were made by the collector based on spawning location and time. Ten of the winter samples were juveniles with phenotypic run type calls made based on length-at-date. All phenotypic calls were validated using either 11,783 single nucleotide polymorphisms (SNPs) spread throughout the genome and genotyped using RADseq (data set and population assignment described in Meek et al., 2020) or an 81 SNP Fluidigm panel (Table S4; Meek et al., 2016) and assigned to population using ONCOR (Kalinowski et al., 2007; methods described in Meek et al., 2016).

For the early and late migrating SHERLOCK assays, subsequently referred to as Ots28_Early1 and Ots28_Late1, respectively, we tested reaction specificity using 40 winter-run, 65 spring-run, 100 fall-run, and 40 late fall-run samples (Table S4). After distinguishing between early and late migrating samples, those identified as the early migrating phenotype were subjected to a second set of assays, subsequently referred to as Ots16_Spring1 and Ots16_Winter1, to discriminate between spring and winter runs, respectively. We tested the reaction specificity of these two assays using 65 spring-run and 40 winter-run samples (Table S4). DNA was extracted from a 2×2 mm piece of caudal fin tissue using the Qiagen DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol. We ran the SHERLOCK reactions for 1 h at 37°C on the Bio-Rad CFX96 (Bio-Rad). The fluorescence values for the biological replicates were background-subtracted, averaged, and normalized based on the highest fluorescence values across all run types. We graphically displayed these normalized values by creating heatmaps using GGPLOT2 in R version 4.0.3 (R Core Team, 2019).

To determine the accuracy of our SHERLOCK assays, we ran 48 spring-run, 37 winter-run, 50 fall-run, and 39 late fall-run samples in triplicate with the Ots28_Early1 and Ots28_Late1 SHERLOCK assays, and we ran 38 spring-run and 29 winter-run in triplicate with the Ots16_Spring1 and Ots16_Winter1 assays. These samples were run on a BioTek Synergy1 H1 microplate reader (Agilent) at 37°C for 1 h with fluorescent plate readings every 1 min. We included six no template controls on each plate. Accuracy was determined by calculating the total percentage of biological replicates that amplified as expected.

2.5 | Assay sensitivity

To test the reaction sensitivity of Ots28_Early1 and Ots28_Late1 SHERLOCK assays, two gene fragments (gBlocks; Integrated DNA Technologies) were synthesized containing the target regions used to distinguish early and late migrating phenotypes (Table S5) with 20 additional flanking bases on either end and the T7 promoter sequence on the 5′ end. We made 1:10 serial dilutions of the Ots28_Early1 and Ots28_Late1 gBlocks starting with ~600 million copies per reaction and ending with ~0.6 copies per reaction. We ran SHERLOCK detection reactions with three technical replicates per dilution factor. Fluorescence values for these technical replicates were background-subtracted and averaged (±1 SD) after 1 h at 37°C.

Additionally, we compared the sensitivity of our Ots28_Early1 and Ots28_Late1 SHERLOCK assays to that of a genotyping qPCR assay described in Thompson et al. (2019). The genotyping qPCR TaqMan probe targets a SNP within the *greb1l* region (snp640165 from Thompson et al., 2019; position 12277551 on chromosome NC_037124.1) that is 4017 bp from the SNP and indel targeted by our SHERLOCK assays. Therefore, we designed a second set of gBlocks (Integrated DNA Technologies), each comprised of the early and late *greb1l* variants described in Thompson et al. (2019) with the addition of 20 flanking bases on either side, to test the sensitivity of the qPCR assay (Table S5). We created a 10-fold dilution series of qPCR gBlocks from ~478 million copies to ~0.48 copies per reaction. As for the SHERLOCK-targeted region, we analysed three technical replicates. We followed the qPCR protocol described in Thompson et al. (2019) and used the Bio-Rad CFX MAESTRO Software to obtain RFUs for each sample. We included duplicate no-template control samples on each plate and used their average to subtract background fluorescence from all samples for each cycle. Fluorescence values for these technical replicates were background-subtracted and averaged (±1 SD) after 1 h at 37°C.

2.6 | Assay speed

To compare the relative magnitude of fluorescent signal over time between the Ots28_Early1 and Ots28_Late1 SHERLOCK assays, we ran a time-course reaction measuring fluorescence values every 5 min for 1 h at 37°C using 20 winter, 20 spring, 24 fall, and 20 late fall run type samples. We input a standardized 10 ng of tissue-extracted DNA for each target run type per reaction and ran three technical replicates of each. Fluorescence values were background-subtracted and averaged for each replicate (±1 SD).

To compare the rapidity of our SHERLOCK assays to traditional qPCR detection, we ran time-course reactions for both assay types

using the same set of samples. Fluorescence values were measured every 5 min over a 50-min time course for both Ots28_Early1 and Ots28_Late1 SHERLOCK assays and for the genotyping qPCR assay (Thompson et al., 2019). We averaged the resulting background-subtracted fluorescence across samples within each run type.

2.7 | Field deployability

We performed swabbing experiments on wild, juvenile Chinook salmon to test whether using unextracted mucus as the starting material in SHERLOCK reactions would produce run type results with high speed and accuracy comparable to tissue-extracted DNA.

We followed the same SHERLOCK protocol used for tissue samples with the following modifications: the total reaction volume was increased to 20 μL (excluding DNA input) and 2 μL of mucus was added to each reaction. We collected individual fish mucus using a Puritan Rayon swab (Glendora, California) wiped along the lateral line of the body from head to tail five times. We swirled the swabs in 300 μL of 1× phosphate buffered saline (PBS) directly after collection and froze the samples at −20°C until the SHERLOCK detection reaction was prepared in the laboratory. On average, mucus samples were frozen for about 1 week. We ran Ots28_Early1 and Ots28_Late1 SHERLOCK detection reactions on all mucus samples; 18 early migrating (3 winter-run and 15 spring-run) and 15 late migrating (15 fall-run; Table S6), using a portable Axxin T16 ISO instrument (Axxin). Two no-template controls and one positive tissue DNA control were included with each SHERLOCK assay. Reactions were incubated at 37°C for 45 min. The resulting FAM fluorescence values for each sample were background-subtracted using the average RFUs of the no-template controls. We ran a *t*-test comparing the RFU output using mucus DNA versus tissue-extracted DNA as input for both Ots28_Early1 and Ots28_Late1 SHERLOCK assays (R version 4.0.3; R Core Team, 2019).

Relative fluorescence units for all samples and SHERLOCK assays are available on Zenodo (https://doi.org/10.5281/zenodo.7579683).

3 | RESULTS

3.1 | Early versus late-migrating SHERLOCK assays

We identified primer pairs and crRNAs that produced specific and robust fluorescent signals, one targeting the early migrating run types (Ots28_1Early), and the other targeting the late migrating run types (Ots28_1Late; Table S1). Both assays were designed in the same ~400 bp region near *greb1l* on chromosome 28 (NW_020128528.1:2202915–2203344); however, they target different genetic variants, 58 bases apart within that region. The Ots28_1Late assay targets a 28 bp DNA segment that has been deleted from the genome of early migrating individuals, and the Ots28_1Early assay targets two SNP differences between the early and late migration phenotypes.

All spring-run and winter-run samples were detected with the Ots28_1Early assay (Figure 1a,c). With the Ots28_1Late SHERLOCK assay, 95% of fall and 100% of late fall-run samples were detected (Figure 1a,c). Of the five fall-run samples that were not detected by the late migrating assay, three were detected by the early migrating SHERLOCK assay. When tested with the genotyping qPCR assay (Thompson et al., 2019), these three fall samples were also genotyped with the early migrating allele. When considered separately, the Ots28_Early1 assay is 95% accurate, and the Ots28_Late1 assay is 96% accurate. However, when used for management, Ots28_Early1 and Ots28_Late1 assays are always run as a pair, so we can leverage the power of the complementary assay. For the samples that were not detected by their target assay, none of them were detected by the complementary assay either; therefore, no genotype would be called for those sample and no management decisions would be made without further genetic analysis (e.g., qPCR or Fluidigm SNP panel). Additionally, samples detected by both assays would be flagged as potential heterozygotes and undergo further genetic testing.

Four fall-run samples, three spring-run, and two winter-run samples were detected with both the Ots28_Early1 and the Ots28_Late1 SHERLOCK assays suggesting that these samples are potentially heterozygous. Using the qPCR assay previously developed by Thompson et al. (2019), we confirmed the genotype of these potential heterozygous samples. Eight of the nine potential heterozygotes were also genotyped as heterozygotes by the qPCR assay (Table 1). One fall-run sample genotyped as a heterozygote with the SHERLOCK assays was genotyped as homozygous for the early-run (spring or winter) allele with the qPCR assay (Table 1).

Using gBlocks (Table S5) that contain the target region, we tested the sensitivity of the Ots28_Early1 and Ots28_Late1 SHERLOCK assays. By making serial dilutions of the gBlocks, we determined the lowest copy number that can be detected by both the Ots28_Early1 and Ots28_Late1 assays is approximately six copies per reaction (Figure 1e,f). The qPCR genotyping assay (Thompson et al., 2019) is slightly more sensitive than the SHERLOCK assays, being able to detect approximately one molecular copy per reaction for both early and late migrating phenotypes (Figure 2a,b). However, detection of the target region by the Ots28_Early1 and Ots28_Late1 SHERLOCK assays is strong enough to make reliable run type determination within 25 min (Figure 3a), which is twice as fast as qPCR genotyping (Figure 3b). The SHERLOCK time course experiment using a standardized starting DNA input (10 ng) to compare the speed of the four different Chinook SHERLOCK assays also produced reliable run type determinations within 25 min (Figure 3c).

3.2 | Spring versus winter-run SHERLOCK assays

After distinguishing between early and late migrating genotypes, the samples identified as early migrating were tested with a second set of SHERLOCK assays to distinguish between spring-run and winter-run. All spring samples were detected with the

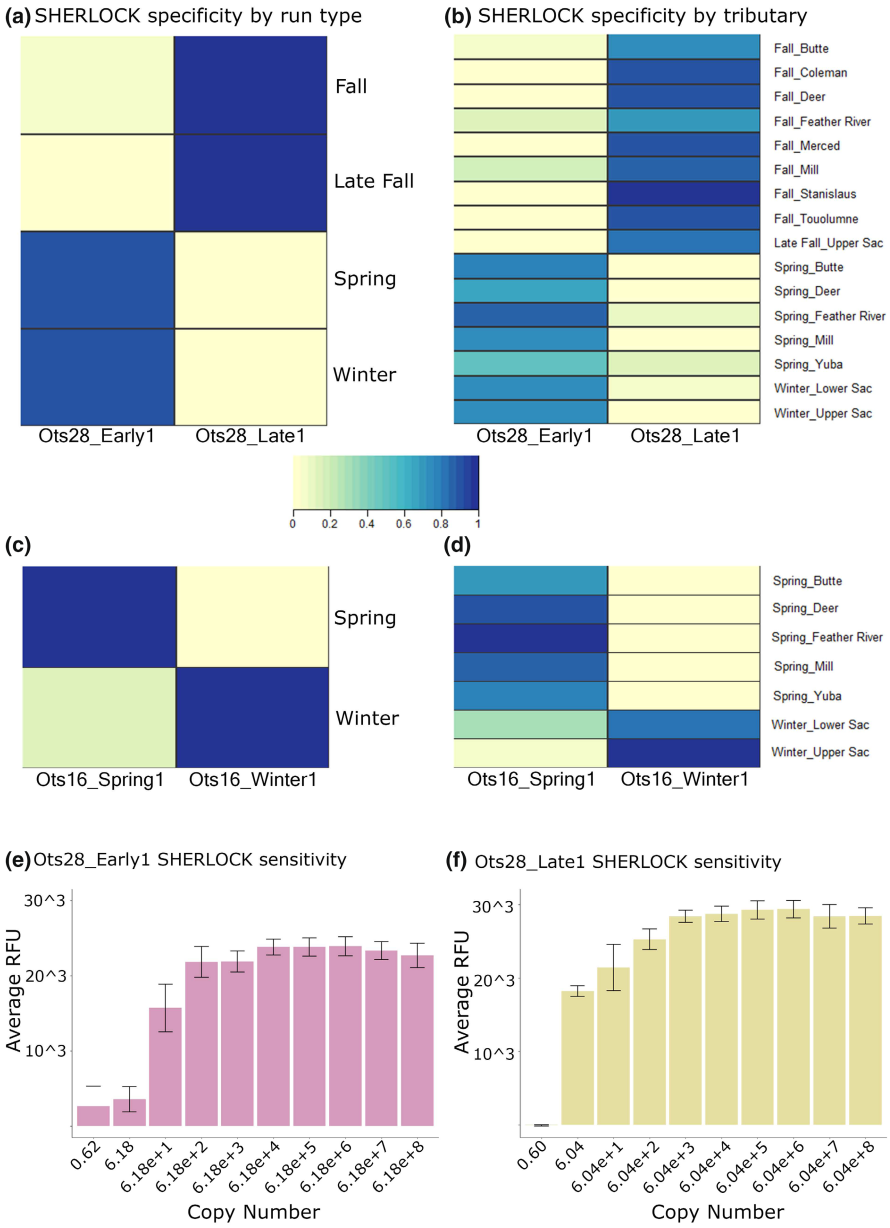

FIGURE 1 Specificity and sensitivity of Chinook run type SHERLOCK assays. (a) Heatmap showing all winter and spring-run samples were detected with the Ots28_Early1 (early migrating) assay. All late fall-run and 95% of fall-run samples were detected with the Ots28_Late1 (late migrating) assay. (b) Heatmap showing the specificity of the Ots28_Early1 and Ots28_Late1 SHERLOCK assays based on tributary origin of the samples. (c) Heatmap showing all spring-run samples were detected with the Ots16_Spring1 assay and 95% of winter-run samples were detected with the Ots16_Winter1 assay. (d) Heatmap showing specificity of Ots16_Spring1 and Ots16_Winter1 assays based on tributary origin. (e, f) Sensitivity of the Ots28_Early1 and Ots28_Late1 SHERLOCK assays after 1 h measured using serial dilutions of synthetically generated fragments (gBlock) of the target region.

Ots16_Spring1 SHERLOCK assay (Figure 1b), while 95% of winter samples were detected with the Ots16_Winter1 SHERLOCK assay (Figure 1c,d). The two winter samples (phenotypically called), C180774LSR and C190779LSR, that were not detected by the Ots16_Winter1 assay were, in fact, detected with the Ots16_Spring1 SHERLOCK assay. One additional winter-run sample (C180775LSR) was detected with both the Ots28_Winter1 and the Ots28_Spring1 SHERLOCK assays. All three samples were from juvenile winter-run fish. C180774LSR and C180775LSR both assigned to winter-run with 100% probability according to the Fluidigm SNP panel (Table S4). Sample C190779LSR assigned to fall-run with 98.4% probability according to the Fluidigm SNP panel (Table S4). When considered separately, the Ots16_Winter1 assay is 99% accurate, and the Ots16_Spring1 assay is 86% accurate. However, when used for management, Ots16_Winter1 and Ots16_Spring1 assays are always run as a pair. For samples not detected by their target assay, none were detected by the complementary assay either; therefore, no genotype would be assigned and no management decisions would be made for those samples without further genetic analysis. Likewise, potential heterozygous samples detected by both Ots16_Winter1 and Ots16_Spring1 assays would undergo further genetic analysis before any management decisions are made.

3.3 | Field deployability

To optimize the SHERLOCK assay for use in the field, we tested the efficacy of unextracted mucus DNA for run type detection. Both the Ots28_Early1 and Ots28_Late1 SHERLOCK assays detected target mucus DNA with relative RFU magnitudes on par with tissue-extracted DNA, *p*-values of .9371 and .4285, respectively (Figure 4).

TABLE 1 Potential heterozygotes identified with Ots28_Early1 and Ots28_Late1 SHERLOCK assays were confirmed with the *greb1l* genotyping qPCR assay (Thompson et al., 2019) and with a SNP panel (Fluidigm or Rad-seq). Those samples with "no call" did not sequence with enough coverage to make high quality genotype calls.

Sample name	Run type (phenotype call)	SHERLOCK call	qPCR call	SNP panel call
F030040MIL	Fall	Heterozygote	Early	Early
F030059MIL	Fall	Heterozygote	Heterozygote	Heterozygote
F040044MIL	Fall	Heterozygote	Heterozygote	No call
F107903FRH	Fall	Heterozygote	Heterozygote	Heterozygote
F100042FRH	Fall	Early	Early	Early
F100043FRH	Fall	Early	Early	Heterozygote
F106580FRH	Fall	Early	Early	Early
S090174FRH	Spring	Heterozygote	Heterozygote	Early
S090265FRH	Spring	Heterozygote	Heterozygote	Early
S090168FRH	Spring	Heterozygote	Heterozygote	Early
C180773LSR	Winter	Heterozygote	Heterozygote	Heterozygote
C180774LSR	Winter	Spring	NA	Early
C190779LSR	Winter	Spring	NA	Early

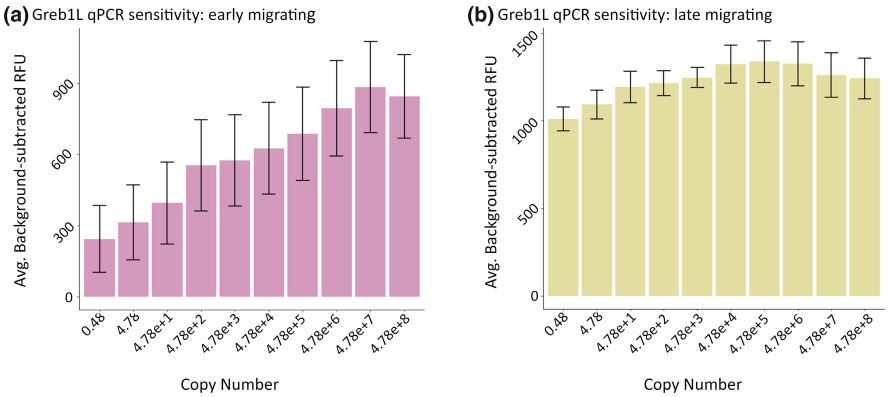

FIGURE 2 Sensitivity of Greb1L genotyping qPCR assay for comparison with SHERLOCK. Sensitivity of the Greb1L qPCR assay (Thompson et al., 2018) using synthetically generated fragments (gBlocks) designed based on (a) early and (b) late migrating variants.

4 | DISCUSSION

CRISPR point-of-care genetic testing is being widely adopted for human healthcare to diagnose and prevent the spread of diseases, including COVID-19 (Joung et al., 2020), and is poised to transform epidemiological surveillance throughout the world (reviewed by Kostyusheva et al., 2022). The technology is being adapted for veterinary pathology and disease ecology applications, such as detection of the economically costly White Spot Syndrome Virus in Pacific white shrimp (*Penaeus vannamei*) aquaculture (Sullivan et al., 2019). It is also being combined with environmental DNA (eDNA) collection to overcome some common hurdles of more traditional sampling approaches (e.g., expensive, labour intensive, handling stress for species) while still providing valuable on-site biodiversity monitoring information for species of management concern, such as Atlantic salmon (*Salmo salar*) (Williams et al., 2019, 2021). Herein, we show how it can be used in a conservation context to inform management decisions for three morphologically indistinguishable yet genetically differentiated ESUs (two with ESA-protected status) within a single species, Chinook salmon. The species' inherent value is well-recognized due to its strong sociocultural significance for tribal nations, contribution to recreational and commercial fisheries, and position as a keystone species of ecosystems it inhabits.

There are several innate characteristics of CRISPR-Cas systems, such as Cas13a or Cas12, that enable major technological advancement for identification of cryptic taxa or ESUs. These include high specificity of target sequence recognition, rapid collateral cleavage of a reporter following target recognition, the ability to work in conjunction with RPA in a single reaction at a single temperature, and highly sensitive detection of minute nucleic acid concentrations (Gootenberg et al., 2017). Here, we show that CRISPR-Cas13a can be used for accurate, rapid, and sensitive intraspecific resolution of Chinook salmon runs in California's Central Valley. From the time a Chinook salmon is collected from the water, we can go from sample-to-answer in under 30 min for each assay. The equipment needed is minimal with field-deployable options that are both portable and affordable (e.g., Axxin's T8 or T16 isothermal instruments or Milenia Biotec's HybriDetect universal lateral flow strips). Because the assays are developed and well-characterized, personnel with minimal training can easily conduct these tests in the field and obtain rapid and easy-to-interpret results. Each of the assays can detect <10 copies of the target locus and for some, ~1 copy, which is similar in sensitivity to that observed with traditional gold-standard qPCR

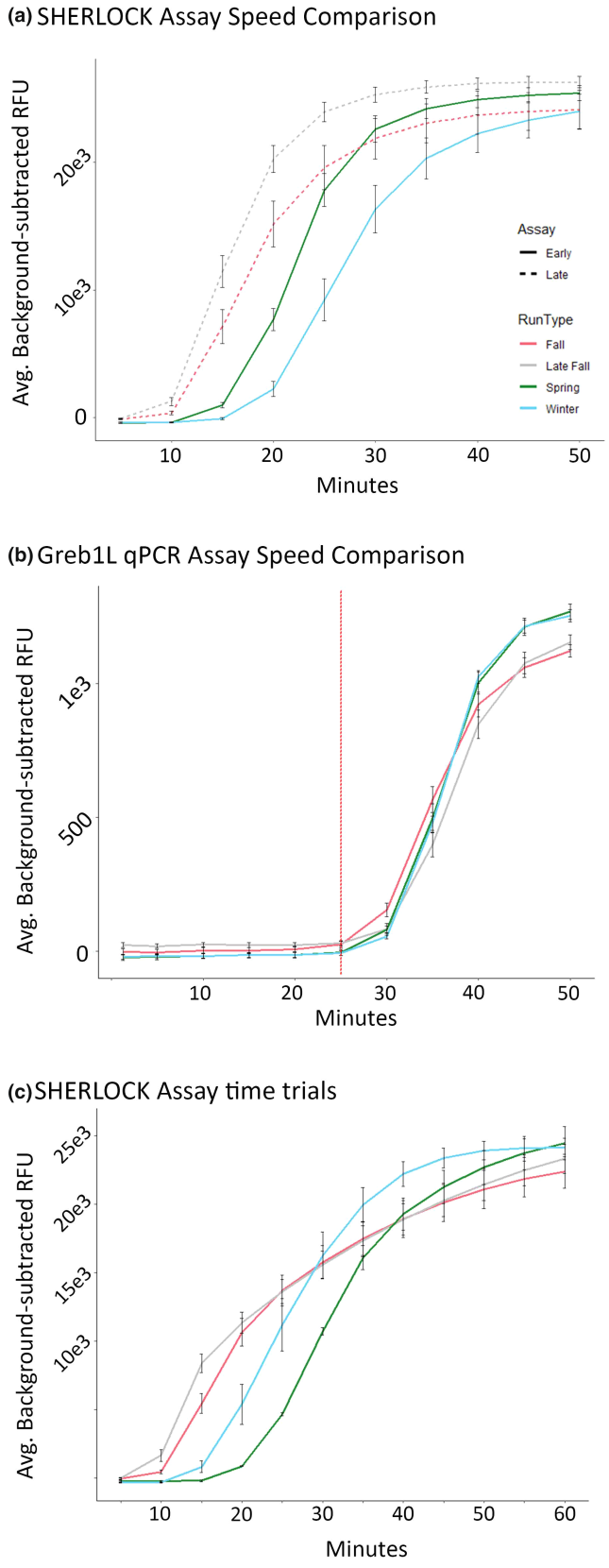

FIGURE 3 Speed of SHERLOCK assays compared to Greb1L genotyping qPCR. (a) Time trials using the Ots28_Early1 and Ots28_Late1 SHERLOCK assays and (b) the Greb1L qPCR assay. Relative fluorescent units (RFUs) were averaged across individuals within each run type, 20 samples each of spring, winter, and late fall-run, and 24 samples of fall-run. Only target samples are plotted for each assay. Negative controls were included in the assays but not detected. The red dotted line marks 25 min, the time at which an accurate run type call can be made using SHERLOCK. (c) Time trials of Ots28_Early1 and Ots28_Late1 SHERLOCK assays using 10 ng of input DNA for all samples to compare speed between the two SHERLOCK assays.

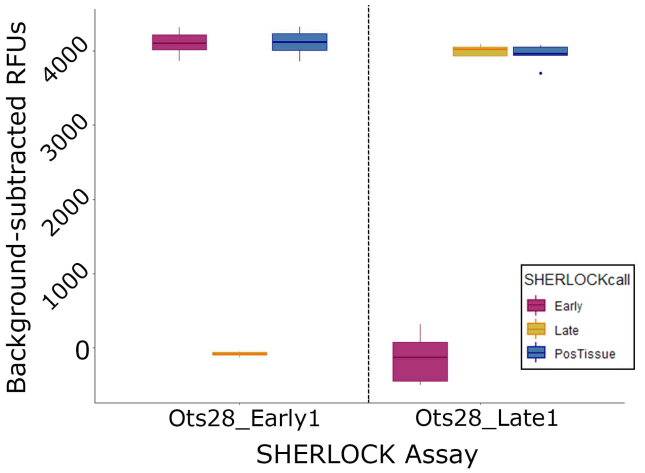

FIGURE 4 Specificity of SHERLOCK using unextracted mucus. Ots28_Early1 and Ots28_Late1 SHERLOCK reactions using unextracted mucus DNA from 18 early migrating and 15 late migrating Chinook salmon. Positive tissue-extracted DNA controls were included alongside the mucus for comparison of relative fluorescent unit (RFU) amplitude.

detection. The use of mucus swabs enables rapid and less invasive collection of genetic material (Tilley et al., 2020) than a traditional fin clip approach. Collectively, these characteristics combine to create a powerful new tool for conservation management of Chinook salmon, as well as other future taxa of conservation concern to which it is applied.

We leveraged the ability of the *greb1l* region to distinguish early and late migration genotypes (Narum et al., 2018; Prince et al., 2017; Thompson et al., 2019, 2020) to create a tiered sequential workflow of salmon run type discrimination (Figure 5). A pair of SHERLOCK assays targeting this region was first used to categorize individuals as either ESA listed (winter or spring run) or nonlisted (fall or late fall run). Assignment accuracy using these assays was high (95% and 96%, respectively). Next, any individual in the ESA listed category was genotyped using a second pair of assays, which distinguish winter and spring run individuals from each other. Assignment accuracy using these assays was also high (99% and 86%, respectively). For all accuracy assignments, there were 0% miscalls (i.e., incorrectly assigning one run type to another) and instead a small percentage of individuals failed to amplify across all replicates, perhaps due to technical errors or samples of insufficient quality. False negatives for each assay are unlikely to be an issue for assignment of homozygous individuals (they would simply undergo further genetic testing) but may cause a small percentage of heterozygous individuals to erroneously be assigned as homozygous. It is worth noting that the current

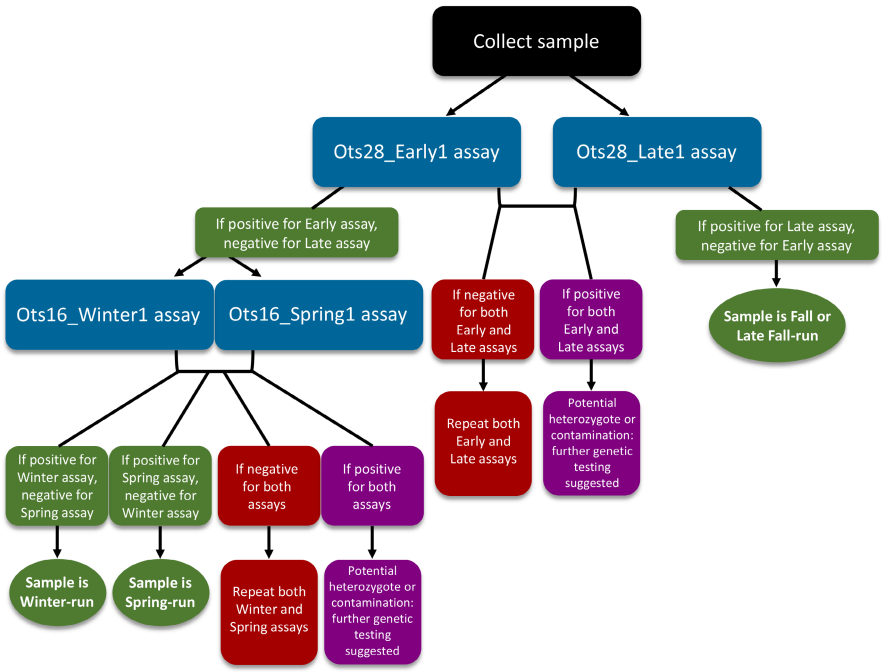

FIGURE 5 Diagram illustrating the workflow from collecting a sample to making a run-type call using the four SHERLOCK assays developed in this study.

assays do not discriminate between fall and late fall runs. Although we found some genomic regions with frequency differences between these run types from our whole genome sequencing analysis, none were diagnostic. Since these two runs are managed within the same unlisted ESU, the inability to distinguish between them does not currently impact ESU-related management decisions.

Genetic and phenotypic assignments of adult Chinook salmon were identical for 98% of the homozygous individuals genotyped using the Ots28_Early1 and Ots28_Late1 assays. When including heterozygous individuals, the concordance was 94%. Samples that were not genetically and phenotypically concordant were genotyped with an alternative method (TaqMan assay), using a SNP located ~4 kb away from the early/late SHERLOCK assays in the RoSA region. The two genetic methods showed nearly identical results and all individuals assigned genotypically to the same run, except one (Table 1). Interestingly, this individual phenotypically classified as a fall-run, genotypically classified as a spring-run (homozygous early) via TaqMan, and genotypically classified as a heterozygous individual via SHERLOCK. For this individual, we would recommend further genetic testing to ascertain run type (following recommended workflow guidelines in Figure 5). The concordance for listed run type samples (i.e., all spring-run and winter-run individuals) tested using the Ots16_Spring1 and Ots16_Winter1 assays was 98% and 97% for homozygous and heterozygous individuals, respectively. As previously mentioned, the concordance between migration phenotype and genetic identification using these assays is high, but not perfect. Prior reports in the literature have found individuals heterozygous for RoSA genotypes have a largely intermediate phenotype in the Klamath River and California's Central Valley (Thompson et al., 2019, 2020) and are thus difficult to bin to a particular run type. Our data showed similar results, with some individuals not genetically assigning to their putative phenotypic run type and instead getting "flagged" as unknown because they genotype as heterozygous. The most likely reasons for the observed low levels of heterozygosity are that discriminatory alternative alleles are not 100% fixed (i.e., they are near-diagnostic but not diagnostic) when comparing the different run types and/or because there is a low level of interbreeding occurring between the run types. Interbreeding between run types may occur at some level in most river systems where more than one run is present, particularly where barriers are blocking historic high elevation spawning habitat (Yoshiyama et al., 2001). In our current study, all samples phenotypically classified as fall- or spring-run that were genotypically classified as heterozygous came from creeks in which fall- and spring-run co-occur. Our results are also consistent with the results of Thompson et al. (2020), which previously identified heterozygotes in Central Valley tributaries that host both run types. Little is currently known about the demographic origins of heterozygotes in the Central Valley, including the extent to which heterozygotes result from recent hybridization between spring- and fall-run fish. Where feasible, further genetic analysis could confirm the demographic origins of heterozygotes. Additionally, phenotypic misclassifications are possible. For example, early migrating individuals may hold in freshwater locations other than their final spawning locations and then finish the migration to their spawning grounds while late migrating individuals are arriving, thus resulting in a field misclassification as late-migrating.

4.1 | Potential management applications

There are several conservation management actions underway or in preparation in California's Central Valley that may benefit from the use of these CRISPR-based assays. These include, but are not limited to, the development of a Juvenile Production Estimate for spring-run

Chinook salmon (Nelson et al., 2022) and assessing loss and salvage of listed Chinook salmon runs due to water exports for urban and agricultural use in California. In addition to enabling more informed decision-making when managing California's water supply, there is potential to use these assays to assess Central Valley Chinook salmon ocean catches from commercial mixed stock fisheries to characterize run distribution patterns in near real-time and ensure that fishing fleets are targeting only unprotected runs for harvest. Currently, there are regulations in place to protect Sacramento River winter-run (location and date restrictions on Chinook salmon ocean fishing) but Central Valley spring-run harvest is not actively managed. Ocean fishing mortality rates of both listed runs are not being estimated (Satterthwaite et al., 2015) and development of these field-deployable and rapid genetic assays provides a technological advancement towards obtaining this valuable data for determining the efficacy of current fishing regulations to protect listed runs.

The applications for CRISPR-based assays in a conservation context are broad in scope. Assays can identify cryptic species or even finer-scale (i.e., intraspecies) taxonomic or ecotypic classifications if there are genetic differences of one or more bases that consistently distinguish them (Gootenberg et al., 2017). This method, which can identify live organisms as well as partial remains, will allow conservation managers and others to confirm the presence of a protected species quickly and accurately outside the laboratory (e.g., remote field sites, boats, hatcheries, fish markets, border crossings) with minimal equipment or staff training. The optional use of colorimetric visual detection of results via lateral flow strips enables rapid genetic identification in virtually any location in the world, including resource-limited settings (Gulati et al., 2021). In addition to identifying protected species, remote identification of nuisance, invasive, or pathogenic species is tremendously valuable when striving to protect entire communities within an ecosystem. For example, it is possible to quantify pathogen viral loads in the field by using a cell phone and low-cost laser illumination to detect fluorescent signal (Fozouni et al., 2021).

It is important for scientists and managers considering the use of CRISPR diagnostics for conservation to be aware of the pros and cons of the technology to determine its appropriateness for a particular application. The CRISPR-based assays developed in this study enable identification of the three Chinook salmon ESUs found in California's Central Valley. This method of identification is accurate, sensitive, rapid, inexpensive (<$1.75/reaction), and obviates the need for extensive equipment or highly trained personnel. Additionally, upstream nucleic acid extraction is not needed when using mucus samples or other sample types with accessible nucleic acid material. However, personnel with molecular training are required for the initial assay development, which can be quite time consuming (e.g., several months of work even with existing genomic resources), as well as for reaction set-up prior to the assays being deployed in the field or elsewhere. Development time will probably decrease as CRISPR protocols becomes more established and more software is developed to assist in primer and crRNA selection. Although SHERLOCK is capable of distinguishing single-base differences, in our experience, not all primer/crRNA combinations tested displayed Chinook salmon run type specificity. The lack of diagnostic SNPs in the mitochondrial genome for distinguishing run types necessitated that we design nuclear assays for our current study, which required further troubleshooting to optimize for minimally invasive sampling (i.e., mucus swabbing) without DNA extraction. The increased development time needed for assay design and reaction optimization may result in higher one-time start-up costs and a longer time until assays are ready for use versus more traditional molecular methods. While we do not believe this will be prohibitive for interspecific assays, one should carefully consider the benefits of applying SHERLOCK versus the drawbacks of development for finer-scale taxonomic resolution (i.e., within species). Another consideration is the level of taxonomic resolution required for a given application. While multiplexing of CRISPR-based assays is possible (Gootenberg et al., 2018; Patchsung et al., 2020), the tool is not as high-throughput in terms of simultaneous assay screening as some other methods commonly used in molecular conservation work (e.g., GT-seq; Campbell et al., 2015). This may make CRISPR-based methods less suitable than other technologies for applications requiring very fine-scale genetic discrimination (e.g., population-specific assignment or parentage assignment). Mixed stock analysis will be most feasible if allele frequencies are highly differentiated between stocks. For Chinook salmon, this translates to using these CRISPR-based assays when managers need to quickly and inexpensively identify ESUs, including in the field or large numbers of individuals in the laboratory, but recognizing that other molecular tools may be more suited to population-specific or finer-scale differentiation. Using the Axxin instrument, we can genetically assay about a dozen individuals in the field with SHERLOCK but recommend transferring significantly larger sample sizes to the laboratory since ~375 individuals can quickly be genetically assayed with SHERLOCK using high-throughput laboratory instruments (e.g., Agilent's BioTek Synergy H1 reader). Lastly, given that CRISPR-based diagnostics are new tools for genetic identification, large-scale comparative analyses with more established methods (e.g., quantitative PCR for SNP genotyping) are required to verify concordance for wide-spread acceptance by the scientific community and its stakeholders.

CRISPR-based diagnostics are well-poised to revolutionize the healthcare industry and the same characteristics that make the technology so attractive for this market can benefit conservation management. The speed, affordability, accuracy, sensitivity, and minimal training and equipment needs can enable genetic identification to be routinely performed in any setting, including remote locations and conservation areas with limited resources. The CRISPR-based Chinook salmon run type assays developed and characterized in this study will improve management for the protected ESUs by providing a reliable and field-deployable alternative to the currently used yet frequently inaccurate length-at-date model, as well as other genetic tools that are more resource intensive in terms of time, labour, training, and equipment. Further adoption of this technology by conservation scientists and managers will have widespread utility and value for the monitoring and protection of our natural resources.

AUTHOR CONTRIBUTIONS

Melinda R. Baerwald and Andrea D. Schreier conceived the presented study. Alisha M. Goodbla designed SHERLOCK crRNAs and primers. Emily C. Funk prepared whole genome sequencing library and performed SHERLOCK experiments. Matthew A. Campbell conducted whole genome bioinformatic analysis. Melinda R. Baerwald, Emily C. Funk, Alisha M. Goodbla, and Andrea D. Schreier contributed to data analysis and interpretation. Mariah H. Meek contributed samples. Mariah H. Meek and Tasha Thompson contributed genomic data. Melinda R. Baerwald, Emily C. Funk, Alisha M. Goodbla, and Andrea D. Schreier prepared the original draft of the manuscript. Matthew A. Campbell, Mariah H. Meek, and Tasha Thompson provided comments and critical revision of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no competing interests.

DATA AVAILABILITY STATEMENT

Whole genome sequencing data have been made available in the NCBI Sequence Read Archive (accession no. PRJNA871266) at http://www.ncbi.nlm.nih.gov/bioproject/871266. Relative fluorescence units for all samples and SHERLOCK assays and intermediate analysis files are available on Zenodo at https://doi.org/10.5281/zenodo.7579683 (Campbell, 2022).

ORCID

Melinda R. Baerwald https://orcid.org/0000-0001-7462-5212
Emily C. Funk https://orcid.org/0000-0002-1237-6385
Mariah H. Meek https://orcid.org/0000-0002-3219-4888

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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