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Journal
San Francisco Estuary and Watershed Science, 21(4)

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Publication Date
2023

DOI
10.15447/sfews.2023v21iss4art2

Supplemental Material
https://escholarship.org/uc/item/0n35b5cf#supplemental

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NOTE

Proofing Field and Laboratory Species Identification Procedures Developed for the Non-Native Osmerid Species Wakasagi (*Hypomesus nipponensis*) Using SHERLOCK-Based Genetic Verification

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ABSTRACT

Accurate species identification is critical to monitoring programs because mis-identifications can lead to incorrect assessments of population status and trends. In the San Francisco Estuary, efforts to monitor the imperiled osmerid Delta Smelt (*Hypomesus transpacificus*) using morphology can be challenging because of the presence of the similar-looking non-native osmerid Wakasagi (*Hypomesus nipponensis*). In 2017, the US Fish and Wildlife Service's field office in Lodi implemented a two-stage verification process for Wakasagi to help prevent Delta Smelt from being mis-identified as Wakasagi. Under this process, Wakasagi are initially identified in the field, independently identified a second time by an experienced staff member in the laboratory, then stored on-site where they can be made available for future studies. Using the recently developed Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK) assay for Wakasagi, we evaluated how well verification protocols performed by genetically identifying a subset of Wakasagi collected during routine sampling between 2017 and 2021. Through this study, we found that the protocols have served as an effective quality control measure for over 4 years and across multiple surveys. With the development of field-deployable genetics tools such as SHERLOCK, genetic identification will likely play an increasingly important role in ecological monitoring. We expect that hybrid approaches that combine morphological identifications by trained field crew with application of field-based genetic tools may offer an effective and efficient approach to ensuring data accuracy in the future.

SFEWS Volume 21 | Issue 4 | Article 2
https://doi.org/10.15447/sfews.2023v21iss4art2

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KEY WORDS

Wakasagi, Delta Smelt, monitoring program, morphology, verification protocols, EDSM, SHERLOCK, Osmeridae, isthmus, melanophores

INTRODUCTION

Understanding how populations change over time and space is critical to management objectives such as determining status and trends (Franklin et al. 2021), establishing regulatory take limits

(Zimmerman et al. 2022), and evaluating the effects of management actions (Walters 2002). Ecological monitoring commonly involves collecting and identifying organisms according to morphological traits, but mis-identifications reduce data quality and, if systematic, may lead to inaccurate assessments of population dynamics (Beerkircher et al. 2009; Hull et al. 2010).

In the San Francisco Estuary (the estuary), anthropogenic changes such as habitat loss (Whipple et al. 2012), flow regulation (Monsen et al. 2007), water exports (Luoma et al. 2015), and the introduction of non-native species (Cohen and Carlton 1998) have resulted in the decline of once-abundant fish populations (Sommer et al. 2007). Among these is Delta Smelt (*Hypomesus transpacificus*), an endemic osmerid listed as threatened under the federal Endangered Species Act (Fed Regist 1993) and endangered under the California Endangered Species Act (CFGC 2009). Changes in the relative population size of Delta Smelt have been documented by an extensive network of monitoring surveys in the estuary. However, efforts to monitor Delta Smelt have been complicated by the presence of a similar looking osmerid in the estuary, the Japanese Smelt or Wakasagi (*Hypomesus nipponensis*).

Wakasagi were introduced into reservoirs in northern California in 1959 as forage for trout (Wales 1962; Dill and Cordone 1997) but have since moved downstream into the estuary where they are caught in monitoring surveys with Delta Smelt (Aasen et al. 1998; Davis et al. 2022). To the untrained eye, Delta Smelt and Wakasagi can look nearly identical (Moyle 1995). Even to the trained eye, distinguishing between Delta Smelt, Wakasagi, and potential hybrids can be challenging (Benjamin et al. 2018). Disentangling population trends between Delta Smelt and Wakasagi has been particularly important in recent years as the Delta Smelt population has continued to decline, as indicated by record-low catch densities in long-term monitoring surveys, while Wakasagi presence in the estuary has persisted or possibly increased (Davis et al. 2022).

Genetic methods can help researchers detect identification errors in field data (Benjamin et al. 2018), estimate mis-identification rates, and provide recommendations on how to reduce these rates as needed (Dexter et al. 2010; Shea et al. 2011). Currently, genetic analyses require that samples be transferred to specialized facilities for processing by trained geneticists, imposing additional time and financial costs on the identification process (Baerwald et al. 2020). As a result, genetic methods can be an impractical solution to the problem of resolving identification questions that routinely occur during monitoring in the estuary, particularly when sampling occurs frequently and large numbers of organisms are collected.

Recent advances in technology have led to the development of a rapid, field-deployable method for genetic identification of species. Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK) is a CRISPR-based method that uses a species-specific guide RNA and the enzyme Cas13a to detect a target genetic sequence (Abudayyeh et al. 2017; Gootenberg et al. 2017). When the guide RNA binds to the target sequence, the Cas13a enzyme is activated, cleaving the target molecule and any single-stranded RNA in the reaction. This collateral cleavage is harnessed to identify species by including a quenched RNA reporter molecule in the reaction that produces a fluorescent signal when cleaved. Because the reaction occurs at a single temperature, does not always require DNA extraction (Baerwald et al. 2020), and can be performed in a portable fluorescence reader, the SHERLOCK method is an exciting alternative to identify species in the field. While field-ready genetic tools remain under development, a practical alternative for monitoring programs is to use multiple independent observers to reduce identification errors (Morrison 2016).

Here, we evaluate a two-stage verification protocol designed to prevent mis-identification of Delta Smelt as Wakasagi in monitoring surveys operated by the US Fish and Wildlife Service (USFWS) Lodi Fish and Wildlife Office (LFWO). Under this verification process, Wakasagi are

identified by independent observers in the field and laboratory. We used the SHERLOCK assay developed for Wakasagi by Baerwald et al. (2020) to genetically identify a subset of Wakasagi collected in LFWO monitoring surveys, and estimated identification accuracy rates for Wakasagi that underwent the two-stage verification process. Next we discuss implications and limitations of our study and address future directions for data collection in the estuary.

METHODS

Data Collection and Two-Stage Verification Process

We focused our study on Wakasagi that were collected between July 1, 2017 and November 1, 2021 by five LFWO monitoring surveys: Enhanced Delta Smelt Monitoring (EDSM) Kodiak trawl survey, Chipps Island trawl survey, Mossdale trawl survey, Sherwood Harbor trawl survey, and the beach seine survey (see *https://www.fws.gov/office/lodi-fish-and-wildlife* and IEP et al. [2022] for survey information). All fish were first identified in the field according to standard sampling protocols. Individuals identified as Wakasagi in the field were then retained and later identified a second time by a different staff member, usually within 10 days of capture. Staff used a combination of criteria to distinguish Wakasagi from Delta Smelt. We briefly describe some of these criteria here and provide further details on the collection and identification processes in Appendix A.

We highlight four categories that have been used to help distinguish between Wakasagi and Delta Smelt: odor, body shape, melanophore patterns, and length-at-date. A strong cucumber smell is a key feature of the Delta Smelt captured in the estuary. Wakasagi generally lack this odor, though some individuals caught in areas inhabited by Delta Smelt can have a slight cucumber smell, possibly because of shared environmental factors such as food. In terms of body shape, Wakasagi are tapered and thickest behind the head at the pectoral girdle while Delta Smelt have a more oval appearance and are thickest in the center of the body (Figures 1A and 1B). Relative to Delta Smelt, Wakasagi have a larger eye and a longer

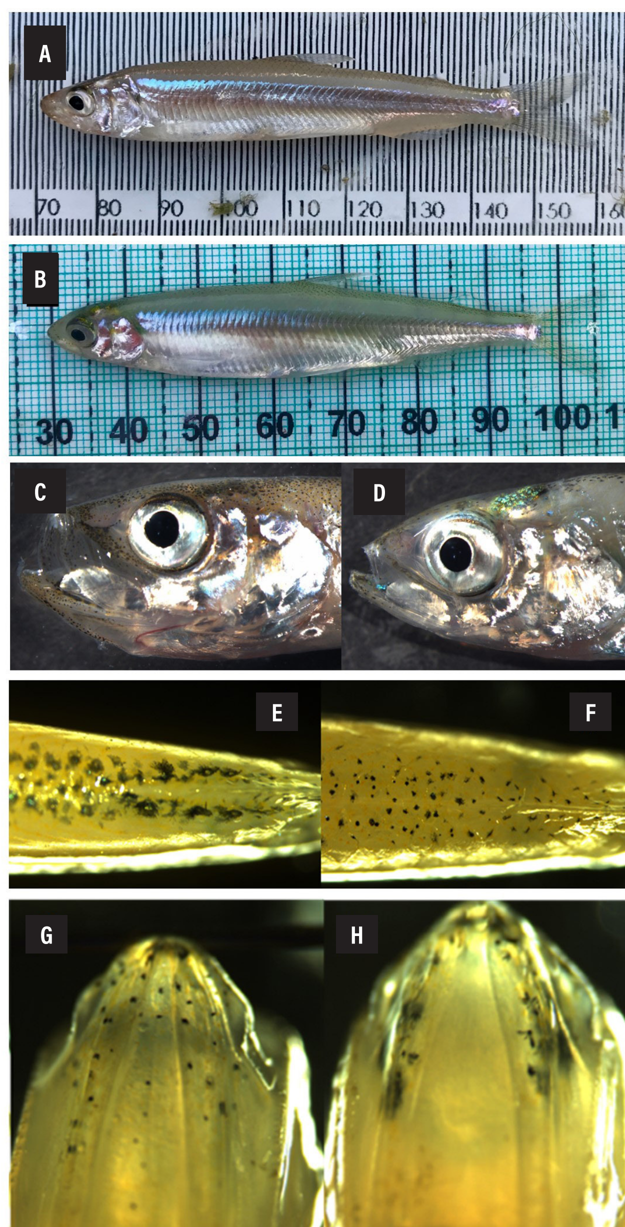

Figure 1 Examples of morphological differences between Wakasagi and Delta Smelt. (**A**) Profile photo of Wakasagi (79 mm FL) showing the tapered body with the thickest area at the pectoral girdle right behind the head. (**B**) Profile photo of Delta Smelt (72 mm FL) showing the oval shape of the body with thickest area at the center of the fish. (**C**) Head shot of Wakasagi (72 mm FL) with relatively large superior mouth. (**D**) Head shot of Delta Smelt (56 mm FL) with relatively small terminal mouth. (**E**) Photo of V-shaped melanophore pattern on the dorsal side of the caudal peduncle of a Delta Smelt (41 mm FL). (**F**) Photo of random peppering melanophore pattern on the dorsal side of the caudal peduncle of a Wakasagi (43 mm FL). (**G**) Photo showing the isthmus of a Wakasagi (43 mm FL) with six melanophores. (**H**) Photo showing the isthmus of a Delta Smelt (41 mm FL) without any melanophores.

https://doi.org/10.15447/sfews.2023v21iss4art2

pointed snout with a superior mouth (Figures 1C and 1D). Melanophore patterns differ between the two species, with Delta Smelt having a distinct V-shaped pattern on the dorsal side of the caudal peduncle around the base of the caudal fin and a helix-like pattern that extends up the back of the fish, whereas Wakasagi tend to have a more scattered melanophore pattern in the same area (Figures 1E and 1F). The number of melanophores on the isthmus can vary widely in Wakasagi, depending on the individual and capture location (Jenkins et al. 2020). In general, Wakasagi have more than one melanophore on the isthmus, while Delta Smelt will typically have one large melanophore or no pigmentation (Figures 1G and 1H). Length-at-date of capture can also provide insight because Wakasagi spawn and hatch earlier in the season and hence tend to be larger than Delta Smelt throughout the year (Figure 2).

Fish positively identified as Wakasagi through the two-stage verification process were stored at −20 °C for archive purposes. Since 2017, four osmerids have been transferred to partner agencies specifically for genetic confirmation, because of remaining uncertainty regarding the species identity after the two-stage verification process. This study focuses on fish that were subject to the two-stage verification process and archived without being transferred for genetic analysis.

Selection of Fish for Genetic Identification

We divided fish into two size groups, small and large, and selected a subset of each group for genetic testing. The small-size group consisted of individuals ≤60 mm fork length (FL) and the large-size group of individuals >60 mm FL. We chose 60 mm as a cut-off based on feedback from field and lab staff that osmerids less than 60 mm are harder to identify and therefore may be identified less accurately than osmerids greater than 60 mm.

Our goal was to estimate the proportion of fish in each size group that were accurately identified as Wakasagi by genetically analyzing a subset

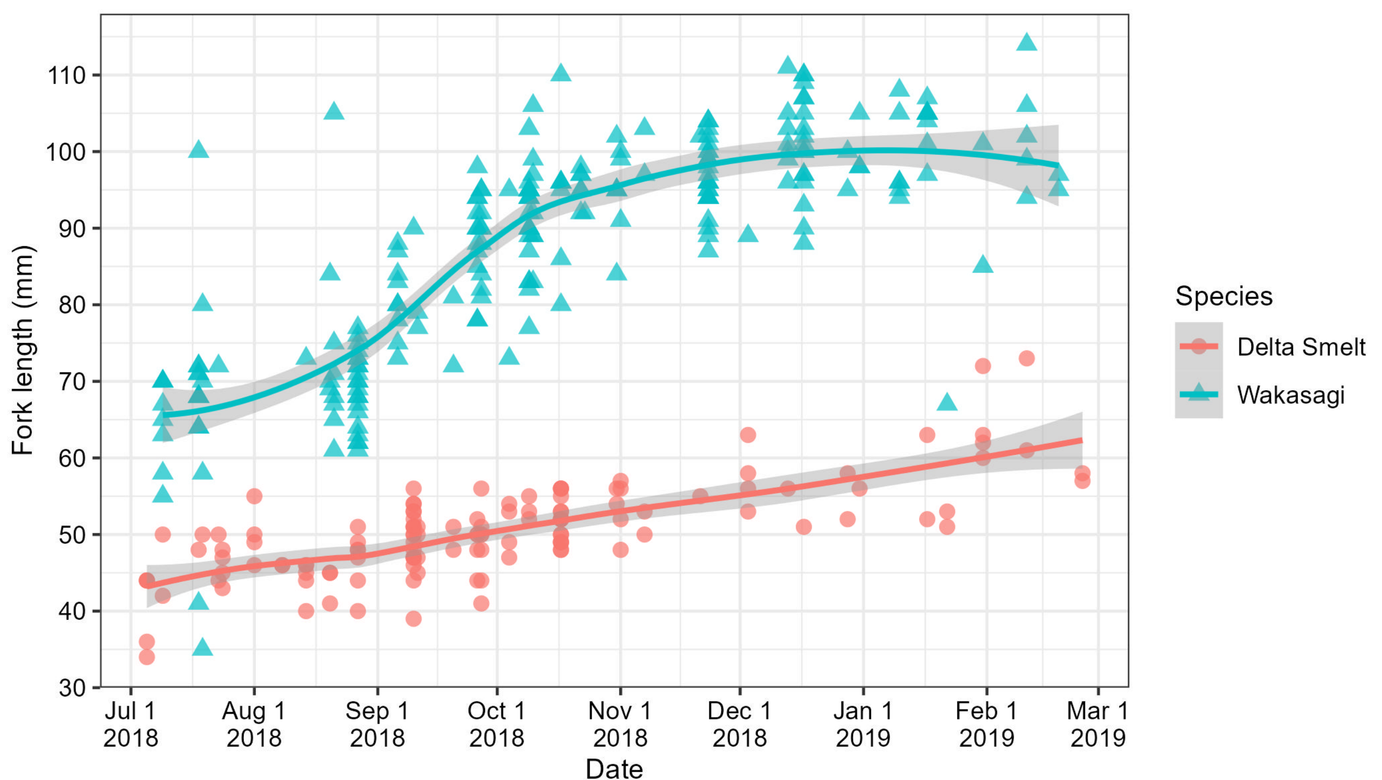

Figure 2 Fork lengths of Delta Smelt (*orange circles*) and Wakasagi (*blue triangles*) caught in the Sacramento Deep Water Ship Channel by LFWO surveys between July 2018 and February 2019, by catch date. Loess fits (*lines*) and 95% confidence intervals (*bands*) are shown for each species.

of fish. We used the sample size formula for estimating a proportion (Thompson 2012) with 5% margin of error and 95% confidence using 0.5 as an estimate of the proportion in the absence of an external estimate. The resulting sample sizes were 281 for the small-size group and 334 for the large-size groups.

For each size group, we generated a list of all individual fish in the group and randomly selected individuals from the list for retrieval from cold storage. However, fish are not stored with unique identifiers. Instead, fish of the same FL collected in the same sampling event (i.e., a given tow of a trawl or haul of a seine) are stored together. For a given combination of sampling event and FL, we randomly selected a subset of fish, without replacement, until we had retrieved the required number. We collected a fin clip from each fish and stored the fin clip in 95% ethanol.

We selected individuals over multiple rounds because we could not locate all selected fish within the storage freezer. We also prioritized fish that had been used in a field test of the Wakasagi SHERLOCK assay in 2019 (Baerwald et al. 2020). Complete details are provided in Appendix A.

Genetic Identification and Estimation of Accuracy Rates

We used SHERLOCK to genetically identify the selected fish with DNA extracted from their fin clips. We extracted DNA from each fin clip sample using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions, and tested each sample with the Wakasagi SHERLOCK assay (Baerwald 2020). All SHERLOCK assays were run on the BioRad CFX96 Touch Real-Time PCR Detection System (BioRad). The resulting relative fluorescent units (RFU) were background subtracted, and samples with RFUs greater than 10,000 were positively identified as a Wakasagi. Any sample with background-subtracted RFUs less than 10,000 were re-run a second time with the Wakasagi SHERLOCK assay and also run with the Delta Smelt SHERLOCK assay for comparison. One low-detection sample was re-run on the Axxin fluorescent reader instead of the BioRad CFX96; when using the Axxin, the threshold for identifying positive detections is 2,500 RFUs after background subtraction. We calculated an accuracy rate estimate for each size group as the proportion of tested fish that were genetically identified as Wakasagi.

RESULTS

A total of 3,607 fish identified as Wakasagi were collected by the five surveys during the study period, with 1,041 in the small-size group and 2,566 in the large-size group. The majority were collected by the EDSM Kodiak trawl survey (Table 1). Fork lengths ranged from 24 mm to 142 mm (Figure 3).

All fish selected for genetic analysis were confirmed to be Wakasagi. Of the 615 total samples, 602 were confirmed in the first round of SHERLOCK testing, with greater than 10,000 RFUs after accounting for background fluorescence. The 13 samples that initially exhibited low-detection RFUs with the Wakasagi SHERLOCK assay were all positively identified as Wakasagi after being run a second time. Additionally, none of these 13 samples were detected with the Delta Smelt SHERLOCK assay. Because all fish were confirmed as Wakasagi, estimated accuracy rates for the small- and large-size groups were both 1, and we were unable to calculate standard errors.

DISCUSSION

Using the SHERLOCK assay to genetically confirm the identity of Wakasagi ≥24 mm FL, we found that the current verification protocols resulted in Wakasagi being identified highly accurately for over 4 years and across multiple surveys. Our results suggest that the protocols have helped prevent or limit mis-identifications that could lead to inaccurate interpretation of changes in the relative population size and distribution of Delta Smelt. In particular, systematic mis-identification of Delta Smelt as Wakasagi is likely not a cause of observed decreases in Delta Smelt catch densities throughout the estuary in recent years. Our findings provide context for ensuring that accuracy rates remain high as the two-stage identification protocols evolve and encompass new technologies. In particular, our

Table 1 Number of Wakasagi caught and identified in LFWO surveys between July 1, 2017 and November 1, 2021. Data are organized by year and size group: (**A**) small fish (≤60 mm FL) and (**B**) large fish (>60 mm FL)

(A) Small fish size group (≤60 mm FL)					
Year	**EDSM Kodiak Trawl Survey**	**Chipps Island Trawl Survey**	**Mossdale Trawl Survey**	**Sherwood Harbor Trawl Survey**	**Beach Seine Survey**
2017	51	0	0	0	1
2018	19	0	2	0	10
2019	407	1	0	15	6
2020	19	0	0	0	0
2021	508	0	0	1	1
(B) Large fish size group (>60 mm FL)					
Year	**EDSM Kodiak Trawl Survey**	**Chipps Island Trawl Survey**	**Mossdale Trawl Survey**	**Sherwood Harbor Trawl Survey**	**Beach Seine Survey**
2017	194	4	0	0	0
2018	245	0	0	2	3
2019	423	3	0	12	5
2020	365	1	0	0	0
2021	1309	0	0	0	0

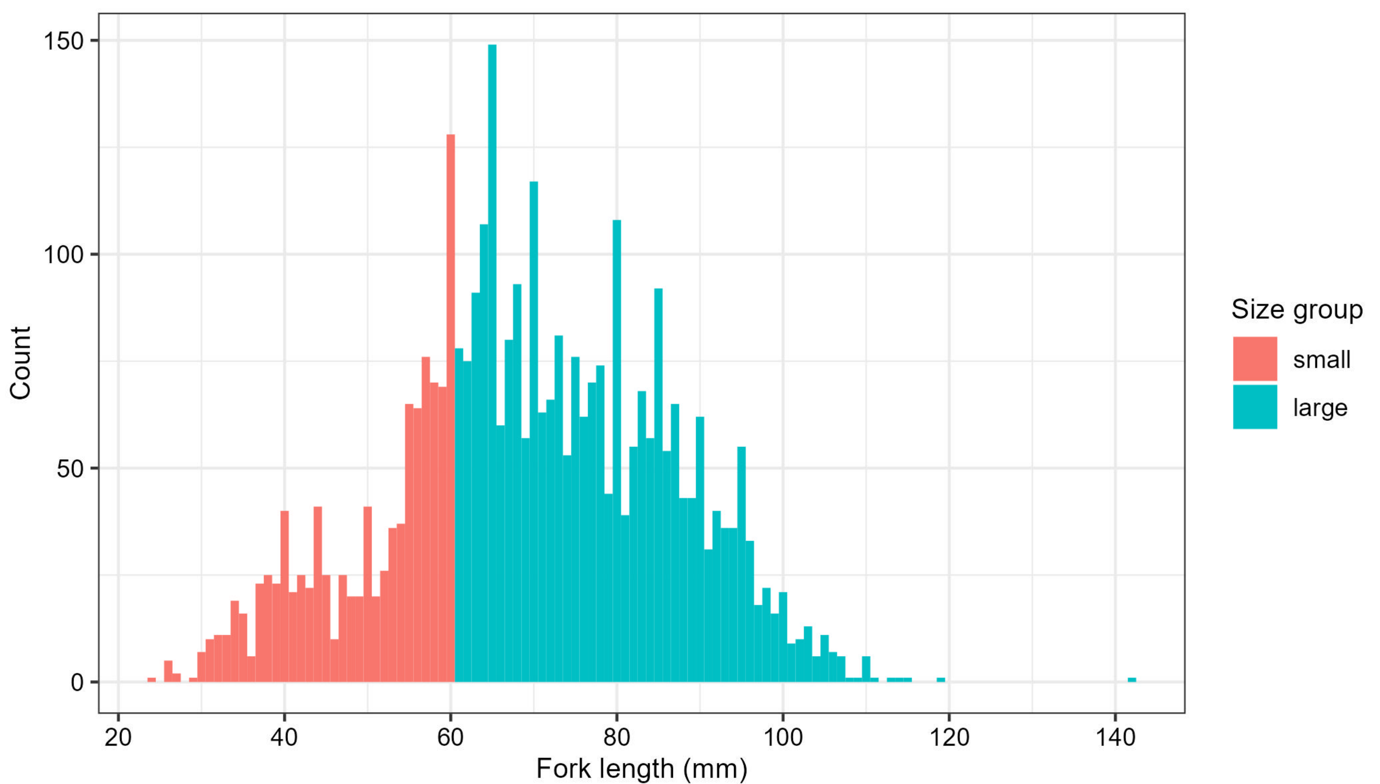

Figure 3 Histogram of fork lengths of Wakasagi caught and identified in LFWO surveys between July 1, 2017 and November 1, 2021. *Bar color* reflects small (≤60 mm FL) and large (>60 mm FL) size groups used in the genetic verification study.

study presents a direct comparison of visual identification methods and the SHERLOCK platform, which may one day serve as an everyday tool for field-based genetic identification. Additionally, this study is the first to demonstrate that SHERLOCK can be used on tissue samples from fish that have been frozen for over 4 years.

A limitation of our study is that it cannot provide accuracy rates for fish identified as Delta Smelt. Although the EDSM program has captured and released Delta Smelt in the past, it has generally retained and transferred Delta Smelt to partner agencies in recent years for a variety of analyses (for example, see Schultz 2019). These collaborations inherently provide a degree of quality assurance because any mismatches between morphological identifications and genetic identifications detected during analyses can be communicated to the LFWO. Furthermore, the identification criteria described here have not been formally quantified. For example, we have not attempted to calculate the percentages of Wakasagi and Delta Smelt that have more than one melanophore on the isthmus. This represents an area for future research in the development of osmerid identification guidelines.

One reason that identification between these two species can be challenging is that both Delta Smelt and Wakasagi display a high degree of phenotypic plasticity. This becomes evident when looking at regional variation in their morphology. For example, Wakasagi in reservoirs and lakes have been noted to have darker pigmentation than those in the estuary (Wang 1995; Jenkins et al. 2020). In contrast, some Wakasagi found in turbid areas favored by Delta Smelt generally have small, pinpoint-shaped melanophores on the isthmus or lack pigmentation on the isthmus at all. As with many other species of fish, there is evidence that these species can change their color patterns to blend into their environment. Delta Smelt raised in captivity have darker more intense pigmentation than wild fish (Jenkins et al. 2020). However, cultivated Delta Smelt that have been released into the estuary as part of experimental supplementation efforts (USFWS 2020) have subsequently changed their pigmentation to resemble that of wild fish (see Appendix A, Figure A1), suggesting that environmental factors play a large role in the species' appearance.

Hybridization between Delta Smelt and Wakasagi may affect visual identification because hybrid smelt display a blending of the key identifying characteristics (Jenkins et al. 2020) highlighted here. Although hybridization between the two species is uncommon and is currently not considered problematic for Delta Smelt, we have a limited understanding of the mechanisms that facilitate their ability to hybridize. Wakasagi and Delta Smelt are known to reproduce freely under laboratory settings (Wang 2007), and F1 hybrid smelt have been documented to produce viable offspring in the wild when spawning with Wakasagi (Benjamin et al. 2018). Current genetic species identification assays are designed for the cytochrome b gene, located in the maternally inherited mitochondrial genome (Baerwald et al. 2011, 2020). Therefore, genetic assays can only identify the maternal parent. To date, all known hybridization events between Delta Smelt and Wakasagi have been male Delta Smelt and female Wakasagi crosses (Trenham et al. 1998; Benjamin et al. 2018). If this observation holds true, all hybrid smelt would be classified as Wakasagi using a cytochrome b gene assay and thus would be managed as Wakasagi.

An area for future research is identification of larval Delta Smelt and Wakasagi (< ~30 mm), which can be more challenging than identification of larger fish from a morphological perspective. Multi-stage Wakasagi verification protocols are applied to larval fish collected in LFWO surveys, but the larval protocols differ greatly from the protocols described here. Furthermore, larval fish are preserved in formalin, which makes genetic identification more difficult (Schultz 2019, Chapter 8). A separate study employing alternative methods would therefore be needed to estimate accuracy rates for larval Wakasagi.

ACKNOWLEDGMENTS

We thank Lodi Fish and Wildlife Office staff members for collecting field data, taking fin

clips, and providing feedback; Evan Carson for his insight on osmerid hybridization; and a reviewer for comments on an earlier version of the manuscript. This work was supported by the Interagency Ecological Program for the San Francisco Estuary. Funding was provided by the U.S. Bureau of Reclamation under Interagency Agreement R21PG00077 and R20PG00036, and by the California Department of Water Resources under Agreement 4600013500. The findings and conclusions in this article are those of the authors and do not necessarily reflect the view of the US Fish and Wildlife Service or other member agencies of the Interagency Ecological Program for the San Francisco Estuary.

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