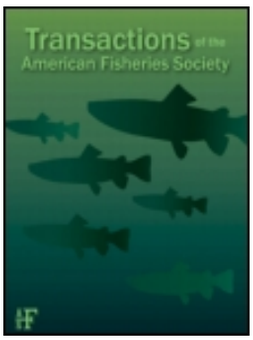

Transactions of the American Fisheries Society

ISSN: 0002-8487 (Print) 1548-8659 (Online) Journal homepage: http://www.tandfonline.com/loi/utaf20

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To cite this article: Ryan M. Lew, Amanda J. Finger, Melinda R. Baerwald, Alisha Goodbla, Bernie May & Mariah H. Meek (2015) Using Next-Generation Sequencing to Assist a Conservation Hatchery: a Single-Nucleotide Polymorphism Panel for the Genetic Management of Endangered Delta Smelt , Transactions of the American Fisheries Society, 144:4, 767-779, DOI: 10.1080/00028487.2015.1037016

To link to this article: http://dx.doi.org/10.1080/00028487.2015.1037016

Transactions of the American Fisheries Society 144:767–779, 2015

ISSN: 0002-8487 print / 1548-8659 online
DOI: 10.1080/00028487.2015.1037016

ARTICLE

Using Next-Generation Sequencing to Assist a Conservation Hatchery: a Single-Nucleotide Polymorphism Panel for the Genetic Management of Endangered Delta Smelt

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Abstract

The Delta Smelt *Hypomesus transpacificus*, listed as threatened under the California Endangered Species Act, has been cultured at a conservation hatchery since 2008 in response to significant declines in the wild. The conservation hatchery relies on accurate, efficacious, and reproducible molecular techniques to help maintain the captive population's overall genetic diversity and to minimize inbreeding. We created a panel of single-nucleotide polymorphisms (SNPs) to support broodstock pedigree reconstruction and improve upon current genetic management. For the SNP discovery, we sequenced 27 broodstock samples from the 2012 spawn by using restriction site-associated DNA sequencing (RAD-seq). We then created a linkage map by genotyping three single-pair crosses at 2,317 newly discovered loci with RAD-seq. We successfully mapped 1,123 loci and identified 26 linkage groups. Fluidigm SNP Type genotyping assays were developed for 104 mapped loci that were selected for minor allele frequencies (MAFs) greater than 0.20, neutrality (Hardy–Weinberg equilibrium), and marker location. Candidates for the genotyping panel were evaluated on a Fluidigm Integrated Fluidic Circuit 96.96 and were tested for marker accuracy and the ability to correctly assign parentage. When applied in conjunction with mating records, we found that a panel of 24 independent SNPs (mean MAF = 0.47) successfully assigned 100% of tested offspring if all of the samples were genotyped at a minimum of 18 loci. Given its capacity to streamline the screening of broodstock candidates, we foresee that the new SNP parentage panel will assume an integral role in genetic management of the Delta Smelt conservation hatchery. Furthermore, genomic resources created for this study have the potential to propel further advances in studying this imperiled species.

Hatchery rearing and supplementation have a long history in fish and wildlife management as a strategy for responding to dwindling fish stocks (Sterne 1995; Lichatowich 1999; Champagnon et al. 2012). Although hatcheries have traditionally been employed to boost the adult census, threats to biodiversity coupled with changing perceptions of stewardship have led to the emergence of conservation hatcheries tasked with safeguarding the persistence of faltering wild populations (Hedrick et al. 2000; Brannon et al. 2004; Waples et al. 2007; Naish et al. 2008). Conservation hatcheries generally take interest in protecting the genetic integrity of their populations (Utter and Epifanio 2002; Herbinger et al. 2006; Fraser 2008; Saltzgiver et al. 2012; Fisch et al. 2013); however, captive breeding carries inherent risks, such as loss of genetic diversity and reduced effective population size, stemming from inbreeding and unintended domestication selection (Utter 1998; Bryant and Reed 1999; Waples 1999; Ford 2002; Utter and Epifanio 2002; Williams and Hoffman 2009; Christie et al. 2012b). Influxes of hatchery-reared fish may reduce the genetic diversity and fitness of the wild population (Ryman

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Received November 29, 2014; accepted March 30, 2015

and Laikre 1995; Araki et al. 2007, 2009; Christie et al. 2012a). Hence, captive breeding programs that fail to both control domestication and maintain natural variation may hinder their own conservation goals.

Pedigree-based management offers a potential breeding strategy for maintaining existing genetic variation in captive-bred populations (Lacy 1989; Ballou and Lacy 1995). With accurate broodstock pedigrees, managers can create crosses that minimize average kinship and equalize family size, thereby delaying the loss of genetic diversity associated with maintaining a population in captivity (Lacy 1989, 1995; Allendorf 1993; Ballou and Lacy 1995; Fernandez and Toro 1999; Ivy and Lacy 2012). A captive-bred population guided by minimal kinship can attain twice the effective population size of a random-mating population by halving the inbreeding rate expected under random mating (Wang 1997; Fisch et al. 2013). However, pedigree-based management depends on accurate pedigrees, which can be a challenge for programs that regularly handle large numbers of offspring with uncertain parentage. In mixed stocks for which individual tagging is impractical, genetic markers can be used to estimate parentage probabilities in order to reconstruct pedigrees (reviewed by Jones et al. 2010, and Fisch et al. 2013). Therefore, reliable genetic markers are essential.

Two types of genetic markers commonly used for parentage analysis are single-nucleotide polymorphisms (SNPs) and microsatellites. Advantages of SNPs over microsatellites include being less prone to genotyping error or spontaneous mutation, allowing greater representation of the genome, and generating genotyping data that can be more easily transferred between laboratories (Vignal et al. 2002; Anderson and Garza 2006; Hauser et al. 2011; Helyar et al. 2011). Nevertheless, multiallelic microsatellites are traditionally preferred for molecular parentage analysis because they can achieve heterozygosities greater than that of any biallelic SNP (Blouin 2003; Liu and Cordes 2004; Jones et al. 2010). With many independent alleles, a single, highly heterozygous microsatellite can be as informative as multiple SNPs. For example, Glaubitz et al. (2003) estimated that 16–20 microsatellites with heterozygosities of 0.75 had greater parentage assignment power than 100 SNPs with minor allele frequencies (MAFs) of 0.20, translating to an approximate 6:1 ratio between the SNPs and microsatellites typically accepted for parentage analysis. An obvious solution to the lower information content of individual SNPs is to exploit the large number of SNPs harbored in most species' genomes to increase panel size or screen for candidates with high MAFs (Morin et al. 2004; Helyar et al. 2011). However, for species that lack a published genome, identifying enough heterozygous SNPs is a significant challenge. Development and application of SNPs to parentage assignment have been impractical for all but well-funded projects with model species (Glaubitz et al. 2003).

Over the past decade, the advent of genotyping-by-sequencing (GBS) methods (e.g., restriction site-associated DNA sequencing [RAD-seq]; Miller et al. 2007; Baird et al. 2008) and the reduction in cost of GBS methods have significantly alleviated the burden of marker development for non-model species. Genotyping-by-sequencing methods can enable the de novo identification of hundreds or even thousands of SNPs without necessitating genome assembly (Davey et al. 2011; Etter et al. 2011; Ogden 2011). By simultaneously genotyping samples at the newly discovered loci, GBS facilitates the estimation of allele frequencies (Davey et al. 2013) and the development of high-density linkage maps (Pfender et al. 2011; Kakioka et al. 2013), which in turn can enable selection of the most informative SNPs for parentage analysis.

As SNP markers become more readily accessible, the emergence of more powerful statistical approaches and high-throughput genotyping technologies has encouraged a renewed interest in applying SNPs to parentage analysis (Morin et al. 2004; Allendorf et al. 2010; Helyar et al. 2011; Ogden 2011). Using a likelihood-based approach, Anderson and Garza (2006) predicted that 60–100 SNPs could capably allocate parentage in returning hatchery salmonids. Since then, low-density SNP panels (25–100 loci) have successfully resolved parentage in both wild and captive-bred populations (Baruch and Weller 2008; Hauser et al. 2011; Abadía-Cardoso et al. 2013; Steele et al. 2013; Clarke et al. 2014). In combination with nanofluidic chips, which are capable of supporting high-throughput genotyping, the operational advantages of low-density SNP panels present an increasingly cost-effective method for estimating parentage, which can then be applied to the genetic management of conservation hatcheries.

STUDY SPECIES

The Delta Smelt *Hypomesus transpacificus* is a pelagic, planktivorous fish endemic to the San Francisco Bay estuary and is protected under federal and state endangered species acts (USFWS 1993; CDFG 2010). The abundance of Delta Smelt, along with many other pelagic fisheries of the San Francisco Bay estuary, has fallen to alarmingly low levels (Feyrer et al. 2007; Sommer et al. 2007; Baxter et al. 2008, 2010; MacNally et al. 2010; Miller et al. 2012; Sommer and Mejia 2013). The Delta Smelt's decline coincides with the anthropogenic-driven transformation of its native San Francisco Bay estuary (Moyle et al. 2010; Cloern and Jassby 2012). These changes include shifts in salinity (Feyrer et al. 2007; Enright and Culberson 2010) and turbidity (Feyrer et al. 2007; Hasenbein et al. 2013), increasing water temperature (Wagner et al. 2011; Cloern and Jassby 2012), food web alterations (Moyle et al. 1992; Moyle 2002; Baxter et al. 2010; Winder and Jassby 2010; Winder et al. 2011; Hasenbein et al. 2013), flow changes (Feyrer et al. 2010), entrainment in water diversion facilities (Grimaldo et al. 2009), and chemical contaminants (Connon et al. 2009). Climate change is expected to further compound these environmental stressors (Wagner et al. 2011).

In response to rising concerns over the long-term viability of Delta Smelt, a captive breeding program was founded in 2008 by the Fish Conservation and Culture Laboratory (FCCL) in collaboration with the Genomic Variation Laboratory (GVL) at the University of California, Davis, and the U.S. Fish and Wildlife Service. The breeding program serves the dual purpose of providing a self-sustaining supply of cultured fish for research and a genetic bank in the event of further declines or extinction (Fisch et al. 2009b, 2010, 2013; Lindberg et al. 2013). Although no cultured Delta Smelt have been released, this "refuge" population could provide a source for supplementation or reintroduction if the species becomes extirpated in the wild.

The Delta Smelt captive breeding program is intensely managed to minimize the loss of genetic diversity by following a minimum kinship strategy (Ballou and Lacy 1995; Lacy 1995). Rearing practices and genetic management are more fully described by Lindberg et al. (2013) and Fisch et al. (2009b, 2010, 2013). In brief, captive breeding is limited to single-pair crosses selected by pedigree to minimize average coancestry and equalize founder contribution in the broodstock. An evaluation of the Delta Smelt program determined that allelic diversity and effective population size had increased overall since the captive population was initiated (Fisch et al. 2013).

Currently, the captive breeding program employs a panel of 12 microsatellite markers for parentage analysis to reconstruct the pedigrees of broodstock candidates (Fisch et al. 2009a, 2010, 2013). Although microsatellites have served reliably since the program's inception, a high-throughput SNP panel offers practical improvements, including easier automation and scoring on a high-throughput platform. Since sampling and mate pairing must be performed in near real-time during the spawning season, high-throughput genotyping could potentially allow management to select from a larger pool of available candidates. Our objective for the present study was to develop a SNP genotyping panel to support the pedigree-based management of Delta Smelt. We also describe (1) the de novo discovery of SNPs that may be used in future assessments of the genetic diversity of the wild Delta Smelt population and (2) the creation of the species' first published linkage map.

METHODS

Sampling design and DNA extraction.—For SNP discovery, we collected adipose fin clips from 28 parents (15 males and 13 females) belonging to the 2012 broodstock. These broodstock samples were drawn from across the spawning season to represent the genetic diversity of the captive population. Each parent had at least one offspring that was genotyped with the microsatellite panel (Fisch et al. 2009a) for the following year's broodstock. All individuals were hatchery-origin fish with hatchery-reared parents.

To create a linkage map, progeny from three single-pair crosses were reared to 25 d posthatch. For each family, we collected tissue samples from both parents and from 46 randomly chosen offspring; thus, a total of 144 individuals were sampled. For the juvenile offspring, DNA was extracted from the caudal half of the whole body while avoiding the stomach to prevent potential cross-amplification with gut content. Eighteen of the sampled fish (6 parents and 12 offspring) were also genotyped by SNP Type assays (Fluidigm, South San Francisco, California) to validate genotyping accuracy.

We designed two test broods that were used to assess the parentage assignment accuracy of the SNP panel. For spawn A, adipose fin clips were collected from 76 fish from the 2012 spawn, including 24 trios, each of which represented a unique parent pair and one of their known offspring. An additional four parents with no offspring were also included in the test. For spawn B, adipose fin clips were sampled from 31 trios ($n = 93$ fish) representing the 2013 spawn.

For all samples, 96× Qiagen DNeasy Kits (Qiagen, Venlo, Limburg, The Netherlands) were used to extract genomic DNA from collected tissue preserved in ethanol. Some of the fin clips used for SNP discovery did not yield the minimum genomic DNA content of 300 ng required for RAD library preparation. To supplement these samples, we extracted DNA from duplicate ethanol-preserved fin clips by using the Axygen extraction kit (Axygen, Union City, California) to increase yields. Table 1 and Supplementary Table S.A.1 (available in the online version of this article) summarize the manner in which each sample was used to develop the SNP panel; Figure 1 provides an overview of the SNP panel's development process.

TABLE 1. Summary of Delta Smelt samples and their numbers used during development of the single-nucleotide polymorphism (SNP) panel; sample group, sample origin (hatchery broodstock or families reared for linkage mapping), genotyping method (restriction site-associated DNA sequencing [RAD-seq] or Fluidigm SNP Type assays), and numbers of fish used for SNP discovery, linkage mapping, panel criteria, and parentage tests are presented.

Group	Origin	Genotyping method	SNP discovery	Linkage mapping	Panel criteria	Parentage testing
BMRL001	2012 spawn	RAD-seq	8		27	
BMRL002	Family 1	RAD-seq		47		
BMRL003	Family 2	RAD-seq		48		
BMRL004	Family 3	RAD-seq		48		
Spawn A	2012 spawn	SNP Type				63
Spawn B	2013 spawn	SNP Type				91

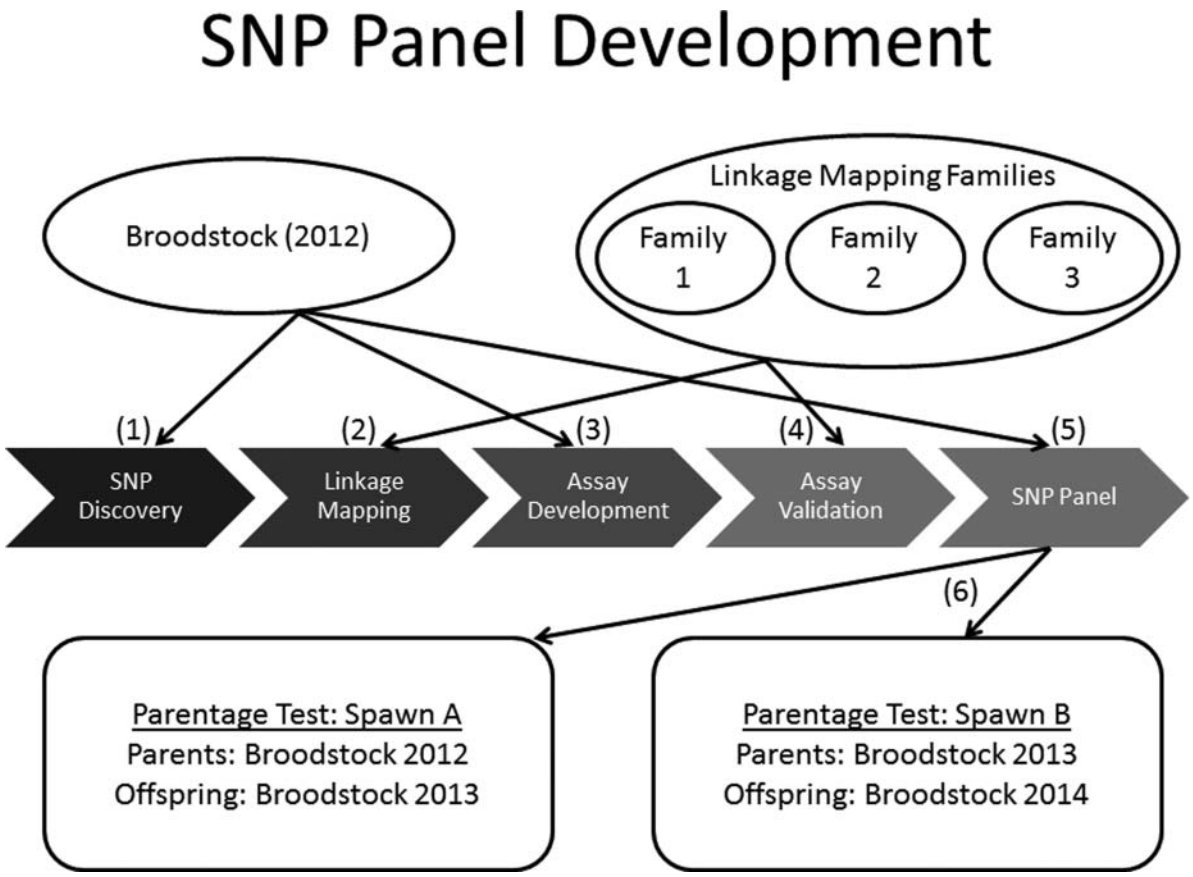

FIGURE 1. Steps used in single-nucleotide polymorphism (SNP) panel development for Delta Smelt: (1) SNP discovery: restriction site-associated DNA (RAD) libraries derived from the conservation hatchery's broodstock were used to identify candidate SNPs; (2) linkage mapping: SNPs were mapped into linkage groups after genotyping-by-sequencing of single-pair crosses with RAD sequencing (RAD-seq); (3) assay development: candidate SNPs were screened for Hardy–Weinberg equilibrium and were ranked by linkage group based on minor allele frequencies (MAFs), as estimated from broodstock samples genotyped by RAD-seq; (4) assay validation: Fluidigm SNP Type genotyping assays were validated by comparing RAD-seq and SNP Type-derived genotypes for mismatches in select samples; (5) SNP panel: the final 24 SNPs were selected from among the remaining candidates for the highest MAF per linkage group; and (6) parentage test: assignment accuracy of the SNP genotyping panel was tested with known parent–offspring triads from hatchery broodstocks.

Sequencing of DNA.—Four RAD libraries were prepared for Illumina HiSeq 2000 sequencing (Table 1): one for SNP discovery with samples representative of the 2012 broodstock (BMRL001); and one for each of the three families that were created for linkage mapping (BMRL002, BMRL003, and BMRL004). Individual samples were quantified by using the Invitrogen Qubit Assay Kit (Life Technologies, Carlsbad, California) and were brought to a uniform concentration. The BMRL001 samples were normalized to 300 ng of genomic DNA for SNP discovery; the BMRL002, BMRL003, and BMRL004 samples were normalized to 400 ng of genomic DNA for linkage mapping.

The RAD-seq libraries were prepared with a protocol adapted from Miller et al. (2012). In brief, samples were digested with 0.25 μL of SbfI-HF restriction endonuclease (NEB R3642L; New England BioLabs, Ipswich, Massachusetts) and were tagged by ligation at the exposed SbfI restriction site with a P1 adapter, which included a nucleotide barcode that was unique by at least two bases to allow subsequent identification of the sample of origin. Once barcoded, 30 μL from each sample were pooled into the respective libraries and were sheared by using a Bioruptor set to high, with 15 s on and 90 s off for at least eight cycles (Diagenode, Denville, New Jersey). Average fragment size was confirmed by gel electrophoresis. Pooled libraries were then purified and concentrated with the Qiagen MinElute PCR Purification Kit. We used AMPure Bead Clean-up to size select fragments that were no larger than 500 bp, and we employed the thermocycling protocol of Miller et al. (2012) to selectively amplify fragments that were tagged with the P1 adapter (RAD tags). After thermocycling and PCR cleanup with AMPure beads, the enriched RAD tag libraries were diluted to 10 nM in tris-HCl containing 0.1% Tween-20.

The RAD libraries were sequenced at the QB3 Vincent J. Coates Genomics Sequencing Laboratory (University of California, Berkeley) on an Illumina HiSeq 2000 platform by using one lane per library. The SNP discovery library (BMRL001) was sequenced to obtain 100-bp paired-end reads to facilitate

primer design. For the linkage mapping libraries (BMRL002, BMRL003, and BMRL004), 100-bp single-end reads were obtained.

Discovery of single-nucleotide polymorphisms.—We adapted Perl scripts from Miller et al. (2012) to identify SNPs from the forward reads of the BMRL001 RAD library. The RAD tags were assigned to individuals by their unique nucleotide barcodes and were trimmed to 92 bp by removing the SbfI restriction site. Discovery of SNPs was limited to eight individuals so as to reduce computation time while still allowing for robust SNP identification. We selected the eight individuals with the highest number of reads (4.62–6.59 million reads), and we subsampled 4.62 million reads from each individual to equalize their contributions to sequence variation identification. Restriction site-associated DNA tags with at least five reads in an individual were included in a sequence alignment with the program Novoalign (Novocraft, Selangor, Malaysia) to align sequences across individuals. Qualifying alleles had to be detected in at least two of the eight samples. To identify loci that were appropriate for the panel, we limited discovery to biallelic SNPs with no more than one polymorphism per 100-bp sequence.

Genotyping-by-sequencing.—Samples sequenced by RADseq were genotyped by using the ratios of their RAD tags for alternate alleles. Sequences were processed with scripts adapted from Miller et al. (2012). Briefly, individually barcoded RAD tags were aligned to the discovered SNPs with the program Bowtie (Langmead et al. 2009) and were converted into individual read counts for alternate alleles. We constructed histograms of the $\log_{10}$ ratios of read counts for alternate alleles to define genotype calling parameters that were appropriate for each RAD library (Figure 2). Genotype calling limits against skewed allele ratios were determined from bin clarity. To reduce genotyping error, samples with allele ratios

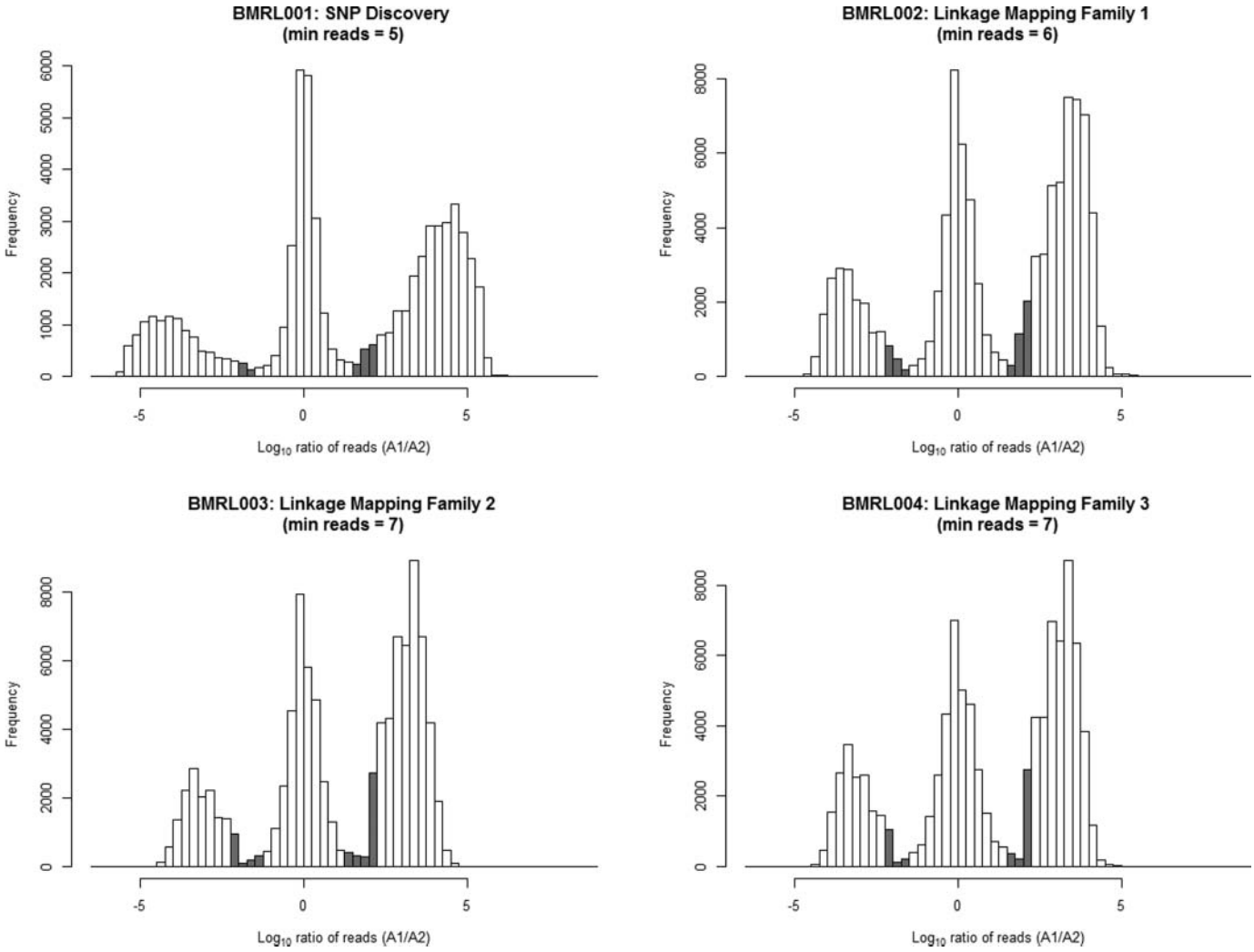

FIGURE 2. Histograms of the log ratios of reads for alternate alleles (A1/A2) in Delta Smelt. Genotype calling parameters were determined from bin clarity and consistency across histograms of varying minimum read depth (SNP = single-nucleotide polymorphism). The shaded regions include the range of allele ratios representing samples that were not genotyped. Samples that did not meet the minimum read depth were also not genotyped.

that fell outside of the genotype bins or that failed to meet the minimum number of reads (5–7 reads) were not genotyped. Loci that were missing from over 20% of individuals were discarded, and individuals that were missing over 20% of the loci were also excluded from further analysis.

Linkage mapping.—We used JoinMap version 4 (Van Ooijen 2006) to create a linkage map of the new SNPs by using the three families genotyped via RAD-seq. Initially, the RAD libraries (BMRL002, BMRL003, and BMRL004) developed from each family were prepared and analyzed separately. Prior to loading genotypes in JoinMap, putative SNPs were quality filtered by screening loci with low information content for linkage mapping. For each family, we discarded any loci that were missing a parent genotype or that were represented in less than 80% of the progeny. We also removed putative loci exhibiting excessive heterozygote offspring, which is indicative of a paralogous sequence variant. We also removed one individual due to low genotyping success. After filtering, the input data set was constructed from 6 parents and 137 progeny.

Linkage maps were assembled with cross-pollination population type codes (CPs), wherein JoinMap assumes that offspring genotypes at a locus are the result of a diploid cross (1) between parents that are both heterozygous or (2) between one heterozygote and one homozygote. The use of CPs makes no prior assumptions about linkage phase. The SNPs were assigned to linkage groups by using a log-of-odds (LOD) score of 7.0 from a test for independence. Within linkage groups, loci were ordered by using a regression mapping algorithm with a recombination threshold of 0.4, a LOD threshold of 0.5, and a jump threshold value of 5.0. A ripple was performed after the addition of each locus. After linkage maps were created for each family, we performed a second round of linkage mapping by joining linkage groups across family-specific linkage maps through their shared loci to create a final consensus linkage map. Graphical displays for the linkage map were generated with Mapchart version 2 (Voorrips 2002).

Assay development and single-nucleotide polymorphism panel selection.—Candidate SNPs for assay development were selected from mapped loci by using population allele frequencies estimated from the 2012 broodstock samples that were genotyped with RAD-seq (BMRL001). Mapped loci were ranked based on MAF and were screened for deviation from Hardy–Weinberg equilibrium (HWE) expectations. For assay development, we selected three to five loci from each linkage group with the highest MAFs and that also demonstrated goodness of fit to HWE (Pearson's chi-square test: $\chi^2 < 3.84$, $P = 0.05$). When possible, candidates were chosen to maximize recombination distances between loci in the same linkage group. To facilitate primer design, we extended the flanking regions of each locus with paired-end sequences to create longer contigs (Senn et al. 2013). We substituted candidates that had extended paired-end sequences with less than 90% consensus among their respective aligned sequences or that had elevated GC content. The selected SNPs and their flanking sequences were then submitted to Fluidigm for development of biallelic SNP Type assays. All candidate loci had MAFs greater than 0.20 to ensure at least a moderate level of informative content (Glaubitz et al. 2003).

The genotyping accuracy of the newly developed SNP Type assays was tested on Fluidigm Integrated Fluidic Circuits (IFCs) by re-genotyping 18 samples that were previously sequenced for linkage mapping. Using our new SNP Type assays, the 18 samples were genotyped at 96 loci as part of a chip run with an IFC 96.96 on the Fluidigm EP1 platform. Twenty-one loci had conflicts between genotypes derived from the RAD-seq and SNP Type methods; those loci were excluded from the final SNP panel. From among the remaining candidates, the loci with the highest MAFs in each linkage group were identified; we subsequently chose the 24 loci with the highest MAFs overall for our SNP genotyping panel.

Parentage analysis and panel validation.—We evaluated the parentage assignment accuracy of the SNP genotyping panel by using two test groups (spawn A and spawn B) derived from the refuge population's spawns in 2012 and 2013, respectively. Both test groups included some parents that had a sibling in a separate cross to evaluate the panel's ability to distinguish between closely related parent pairs when assigning offspring. Parentage in these groups was previously confirmed with the microsatellite panel.

For the parentage test of spawn A, we prepared 24 trios and 4 parents with no offspring for a total of 76 samples. Spawn-A samples were genotyped at 96 loci on an IFC 96.96 with the Fluidigm EP1 platform. For the parentage test with spawn B, we prepared 31 trios for a total of 93 samples. Spawn-B samples were genotyped exclusively at the 24-SNP panel on an IFC 192.24 with the Fluidigm Biomark platform. The IFC 192.24 has the capacity to assay up to 192 samples at 24 loci simultaneously; therefore, we genotyped spawn B twice to empirically determine the panel's genotyping error rate. Prior to parentage analysis of spawn A and spawn B, we screened samples and loci for low genotyping rates. We removed individuals that failed to genotype at a minimum of 75% of the panel's 24 loci ($n \geq 18$). Since candidates for the captive broodstock would never be selected without a full pedigree, we also screened offspring samples for which a parent was removed.

Protocols for determining parentage with SNPs followed those used with microsatellites (Fisch et al. 2013). The parentage assignment software CERVUS version 3 (Kalinowski et al. 2007) was used to identify the most likely parent pairs from among all possible parents. For both broods, we set CERVUS to assign parentage based on LOD scores. We simulated 10,000 offspring from parent genotyping data, with a mistyping rate of 0.001, to estimate the LOD score thresholds for assignment probability. Since both putative parents for each offspring were included under our minimum genotyping requirements, we set the proportion of candidate parents sampled to 1.0.

In conformance with current management practices, we directed CERVUS to report the two most likely pairs of parents based on the trio LOD score. The FCCL and GVL maintain records of all single-pair crosses, providing a further check on parentage assignments. Proposed assignments in CERVUS were only accepted if the putative pair was a known single-pair cross. If the most likely pair proposed by CERVUS was not crossed in the hatchery, then the second most likely pair was reviewed as an alternative. Offspring were left unassigned if neither pair was plausible. We then confirmed whether the proposed trios matched the true parent–offspring relationships that had been determined previously by microsatellite analysis.

RESULTS

Discovery of Single-Nucleotide Polymorphisms

Sequencing with the Illumina HiSeq 2000 yielded a raw total of 135.9 million 100-base forward reads for BMRL001. After quality filtering to discard sequences that were missing an assigned nucleotide barcode, the total sum from all 28 broodstock samples contributing to the BMRL001 RAD library was 95.4 million quality-filtered reads. One individual (YM16) was removed from further analysis due to a low number of reads (<100 filtered reads). The remaining 27 samples had a mean of 3.5 million filtered reads (range = 0.94–6.6 million filtered reads). After screening the RAD tags from eight individuals (as described in Methods), we identified 2,317 high-quality SNP loci that matched our requirements.

Linkage Mapping

For linkage mapping, we genotyped 144 RAD-tagged individuals from three single-pair crosses (BMRL002, BMRL003, and BMRL004) at the 2,317 newly discovered SNPs. Collectively, the mean number of sequencing reads per individual was 1.04 million reads (range = 0.49–1.91 million reads), with 144.4 million RAD-tagged sequencing reads obtained overall. One offspring sample was removed due to genotyping at less than 80% of the loci. We genotyped 1,946 loci in BMRL002, 1,694 loci in BMRL003, and 1,796 loci in BMRL004, from which 904, 793, and 771 loci, respectively, passed all of our filtering criteria; 1,400 loci were analyzed overall (see Methods). JoinMap found genomic positions for 646, 657, and 588 loci, respectively. We used these initial family-specific linkage maps to create a consensus linkage map with 1,123 loci in 26 linkage groups. The map spanned 944.7 cM, with a mean distance of 0.84 cM between markers. Linkage groups ranged from 12 loci (LG-25) to 62 loci (LG-18), with a mean of 43.2 loci. Linkage group mapping lengths ranged from 21.4 cM (LG-05) to 55.9 cM (LG-09; Figure S.B.1).

Single-Nucleotide Polymorphism Panel Design

All candidate loci for the SNP genotyping panel had MAFs greater than 20% and conformed to HWE expectations (Pearson's $\chi^2 < 3.84$, P-value = 0.05; Table S.C.1). After SNP Type assays were screened for mismatches with RAD-seq genotypes, the locus with the highest MAF in each of the 26 linkage groups was selected from among the remaining 75 candidates (Table 2). The final 24 SNPs had a mean MAF of 0.47 and a minimum MAF of 0.41. To estimate genotyping error, the 93 samples belonging to spawn B were genotyped twice on an IFC 192.24. We found no mismatches between duplicate genotypes.

Parentage Analysis

Our parentage test of spawn A evaluated 26 candidate parent pairs and 24 offspring. One candidate father and six candidate mothers had to be removed due to genotyping at fewer than 18 loci. Consequently, we also removed six corresponding offspring from the evaluation. For the remaining 18 offspring samples, potential parents included 20 mothers and 25 fathers. We found that all trios proposed by CERVUS were correct (Figure 3; Table S.D.1).

We anticipate that the low genotyping rates exhibited by some of the spawn-A parents during parentage testing will not typify the SNP panel's performance when implemented according to current practices. During the spawning season, potential broodstock samples are usually genotyped within 1–2 weeks of extraction. To test the parentage assignment capabilities of the SNP panel, we had to use older samples. In particular, spawn-A parents were from the 2012 broodstock, and their samples were extracted over 1 year prior to our parentage test. In contrast, the offspring samples from spawn A and all samples from the linkage mapping study were less than 1 year old and had much higher genotyping rates. Likewise, all samples from spawn B were less than 1 year old, and none was removed due to low genotyping. Hence, the low genotyping rates that necessitated the removal of some spawn-A parents appeared to be a problem limited to older samples. If samples are genotyped promptly as is currently practiced, we expect that genotyping failure will not become a systematic problem.

For our parentage test of spawn B, 31 parent pairs from the 2013 spawn and 31 offspring were genotyped. Since there were no mismatches between duplicated sample genotypes, we ran parentage analysis on the first set of genotypes only. One locus was removed because it failed to genotype at any sample. Using the remaining 23 loci, CERVUS successfully assigned 29 of 31 offspring correctly to the parent pair with the highest LOD score. For the remaining two, the parent pair with the highest LOD score was implausible because the proposed parents were never crossed according to mating records. In accordance with protocol, we assigned those offspring samples to the second most likely pairs after verifying that they

TABLE 2. Delta Smelt loci ($n = 75$ loci) with high-quality Fluidigm single-nucleotide polymorphism (SNP Type) assays; the locus, linkage group (LG), position within the LG (in centimorgans [cM]), major allele, minor allele, minor allele frequency (MAF), Pearson's chi-square test results for neutrality under Hardy–Weinberg equilibrium ($\chi^2 < 3.84$, P-value = 0.05), and inclusion in the SNP genotyping panel for resolving broodstock parentage are presented.

Locus	Linkage	Position (cM)	Major	Minor	MAF	χ^2	Panel?
Htr-GVL-A001799	LG-01	8.2	T	C	0.33	1.18	No
Htr-GVL-A001186	LG-01	15.4	G	A	0.37	1.14	No
Htr-GVL-A001696	LG-01	32.0	A	G	0.43	0.01	No
Htr-GVL-A002185	LG-01	24.0	T	A	0.48	0.04	Yes
Htr-GVL-A000163	LG-02	26.0	T	C	0.46	0.37	Yes
Htr-GVL-A001925	LG-02	4.1	T	A	0.46	1.91	No
Htr-GVL-A000641	LG-03	24.3	C	T	0.41	0.17	No
Htr-GVL-A000988	LG-03	3.8	T	C	0.44	0.27	No
Htr-GVL-A001676	LG-03	14.0	A	G	0.48	2.49	No
Htr-GVL-A000314	LG-03	34.7	G	A	0.48	1.80	Yes
Htr-GVL-A000374	LG-04	23.6	G	A	0.37	0.06	No
Htr-GVL-A001939	LG-04	2.0	A	C	0.38	0.02	No
Htr-GVL-A001918	LG-04	16.7	C	T	0.40	0.04	No
Htr-GVL-A000225	LG-04	10.7	A	G	0.48	0.63	Yes
Htr-GVL-A000431	LG-05	11.2	A	C	0.48	0.94	No
Htr-GVL-A002099	LG-05	3.3	G	T	0.50	0.04	Yes
Htr-GVL-A000914	LG-06	14.1	G	A	0.37	0.20	No
Htr-GVL-A000492	LG-06	30.0	C	T	0.46	0.03	Yes
Htr-GVL-A001235	LG-07	23.0	T	C	0.35	0.31	No
Htr-GVL-A000646	LG-07	31.2	C	T	0.35	0.31	No
Htr-GVL-A000716	LG-07	15.4	A	G	0.39	0.00	No
Htr-GVL-A001734	LG-08	11.8	T	G	0.33	0.00	No
Htr-GVL-A000133	LG-08	35.2	A	G	0.39	0.00	No
Htr-GVL-A001699	LG-08	43.4	C	T	0.48	1.80	Yes
Htr-GVL-A000683	LG-09	8.5	A	C	0.43	0.50	No
Htr-GVL-A000054	LG-09	0.0	A	G	0.46	0.88	No
Htr-GVL-A000612	LG-09	30.9	T	A	0.48	0.94	Yes
Htr-GVL-A001607	LG-10	14.9	T	G	0.39	0.77	No
Htr-GVL-A000419	LG-10	33.3	A	G	0.44	0.75	Yes
Htr-GVL-A001494	LG-10	25.6	C	G	0.44	0.27	No
Htr-GVL-A000144	LG-10	6.3	A	G	0.44	0.27	No
Htr-GVL-A002157	LG-11	37.7	A	C	0.39	2.41	No
Htr-GVL-A001701	LG-11	20.4	A	T	0.50	1.38	Yes
Htr-GVL-A002159	LG-11	31.2	T	C	0.50	0.93	No
Htr-GVL-A001014	LG-12	18.0	G	A	0.43	0.01	No
Htr-GVL-A000505	LG-12	25.4	A	G	0.48	1.80	Yes
Htr-GVL-A000573	LG-13	22.2	A	C	0.44	0.07	No
Htr-GVL-A001677	LG-13	27.3	G	A	0.48	0.33	No
Htr-GVL-A002206	LG-13	8.9	G	T	0.50	0.04	Yes
Htr-GVL-A001430	LG-14	39.5	C	T	0.46	0.18	Yes
Htr-GVL-A001753	LG-14	8.5	G	A	0.46	0.03	No
Htr-GVL-A001921	LG-15	25.9	T	C	0.37	1.14	No
Htr-GVL-A001459	LG-15	20.7	T	C	0.38	0.91	No
Htr-GVL-A001499	LG-15	9.7	G	C	0.48	0.33	Yes
Htr-GVL-A000668	LG-16	15.2	A	G	0.28	0.77	No
Htr-GVL-A000195	LG-16	7.2	A	G	0.46	1.47	Yes
Htr-GVL-A002270	LG-17	42.4	C	G	0.37	1.98	No
Htr-GVL-A000714	LG-17	7.2	C	T	0.48	0.33	Yes
Htr-GVL-A001047	LG-18	4.0	C	G	0.37	0.34	No

TABLE 2. Continued.

Locus	Linkage	Position (cM)	Major	Minor	MAF	χ^2	Panel?
Htr-GVL-A001590	LG-18	12.9	G	T	0.46	0.18	No
Htr-GVL-A001852	LG-18	20.7	G	T	0.48	0.33	Yes
Htr-GVL-A000899	LG-18	26.4	A	G	0.48	0.33	No
Htr-GVL-A000789	LG-19	32.9	A	G	0.31	1.40	No
Htr-GVL-A002242	LG-19	20.4	A	C	0.35	1.96	No
Htr-GVL-A000468	LG-19	10.4	C	T	0.37	0.06	No
Htr-GVL-A000500	LG-19	26.1	A	G	0.44	1.69	Yes
Htr-GVL-A001159	LG-20	14.0	G	T	0.37	1.14	No
Htr-GVL-A001274	LG-20	36.1	A	C	0.43	0.75	No
Htr-GVL-A000078	LG-20	42.3	A	C	0.48	1.80	Yes
Htr-GVL-A000003	LG-21	39.1	T	C	0.31	0.36	No
Htr-GVL-A002161	LG-21	24.4	T	A	0.41	0.15	Yes
Htr-GVL-A001011	LG-22	10.8	T	C	0.35	1.96	No
Htr-GVL-A000903	LG-22	30.6	C	T	0.39	0.00	No
Htr-GVL-A000651	LG-22	0.0	T	G	0.44	0.07	Yes
Htr-GVL-A002165	LG-23	16.9	C	T	0.41	0.15	No
Htr-GVL-A001303	LG-23	22.7	A	C	0.46	1.90	No
Htr-GVL-A001702	LG-23	0.0	T	C	0.48	1.80	Yes
Htr-GVL-A000839	LG-24	27.4	A	G	0.28	0.01	No
Htr-GVL-A001460	LG-24	19.5	G	A	0.33	0.75	No
Htr-GVL-A002107	LG-24	8.8	C	T	0.33	0.75	No
Htr-GVL-A002071	LG-24	14.0	T	C	0.46	1.47	Yes
Htr-GVL-A000366	LG-25	0.0	G	A	0.24	2.71	No
Htr-GVL-A001520	LG-25	22.8	T	C	0.35	1.96	No
Htr-GVL-A001103	LG-26	15.9	G	T	0.40	2.05	No
Htr-GVL-A000850	LG-26	2.9	C	A	0.48	0.33	Yes

were known crosses. In both cases, the corrected assignments matched the parent–offspring relationship that was previously obtained using microsatellites (Figure 3; Table S.D.1).

DISCUSSION

As threats to biodiversity increase, there is a growing need for successful conservation hatcheries like that used for the Delta Smelt (Fisch et al. 2013; Lindberg et al. 2013). With next-generation sequencing, it is now possible to quickly develop new markers that increase our efficiency at managing these populations, even for nonmodel species (Allendorf et al. 2010; Seeb et al. 2011). Over the past decade, the application of SNPs to resolving parentage in managed populations has proliferated, incorporating taxa as diverse as bison (Tokarska et al. 2009), cattle (Fisher et al. 2009; Honda et al. 2009; Karniol et al. 2009), horses (Hirota et al. 2010), Asian Seabass *Lates calcarifer* (Liu et al. 2012), Sockeye Salmon *Oncorhynchus nerka* (Hauser et al. 2011), steelhead *O. mykiss* (Abadía-Cardoso et al. 2013; Steele et al. 2013), Pacific oysters *Crassostrea gigas* (Jin et al. 2013), cocoa (Ji et al. 2012), black tiger shrimp *Penaeus monodon* (Sellars et al. 2014), and sheep (Clarke et al. 2014). Here, we designed a low-density SNP panel for use in pedigree-based management of the Delta Smelt refuge population.

Our genotyping panel consists of 24 neutral, nonlinked loci with high MAFs. During parentage testing, the SNP panel correctly assigned all tested offspring by following management guidelines that were already in place at the conservation hatchery. Although CERVUS proposes candidate pairs from among all parents, access to complete broodstock mating records permits the validation of proposed assignments against known crosses. Any proposed parents that do not constitute a known cross can be excluded until a known cross is identified. During parentage testing, our SNP panel consistently ranked the correct parent pair highest among all known pairs. Furthermore, the correct parent pair was always among the two proposed candidate pairs with the highest likelihood of parentage. Over 95% of the correct parent pairs had the highest LOD score outright. Despite the presence of siblings among potential parents, no offspring sample was assigned to an aunt or uncle over its correct parent.

Although low-density SNP panels were traditionally considered impractical for parentage analysis in nonmodel species (Blouin 2003; Glaubitz et al. 2003; Liu and Cordes 2004; Jones et al. 2010), our study exemplifies two recent trends that

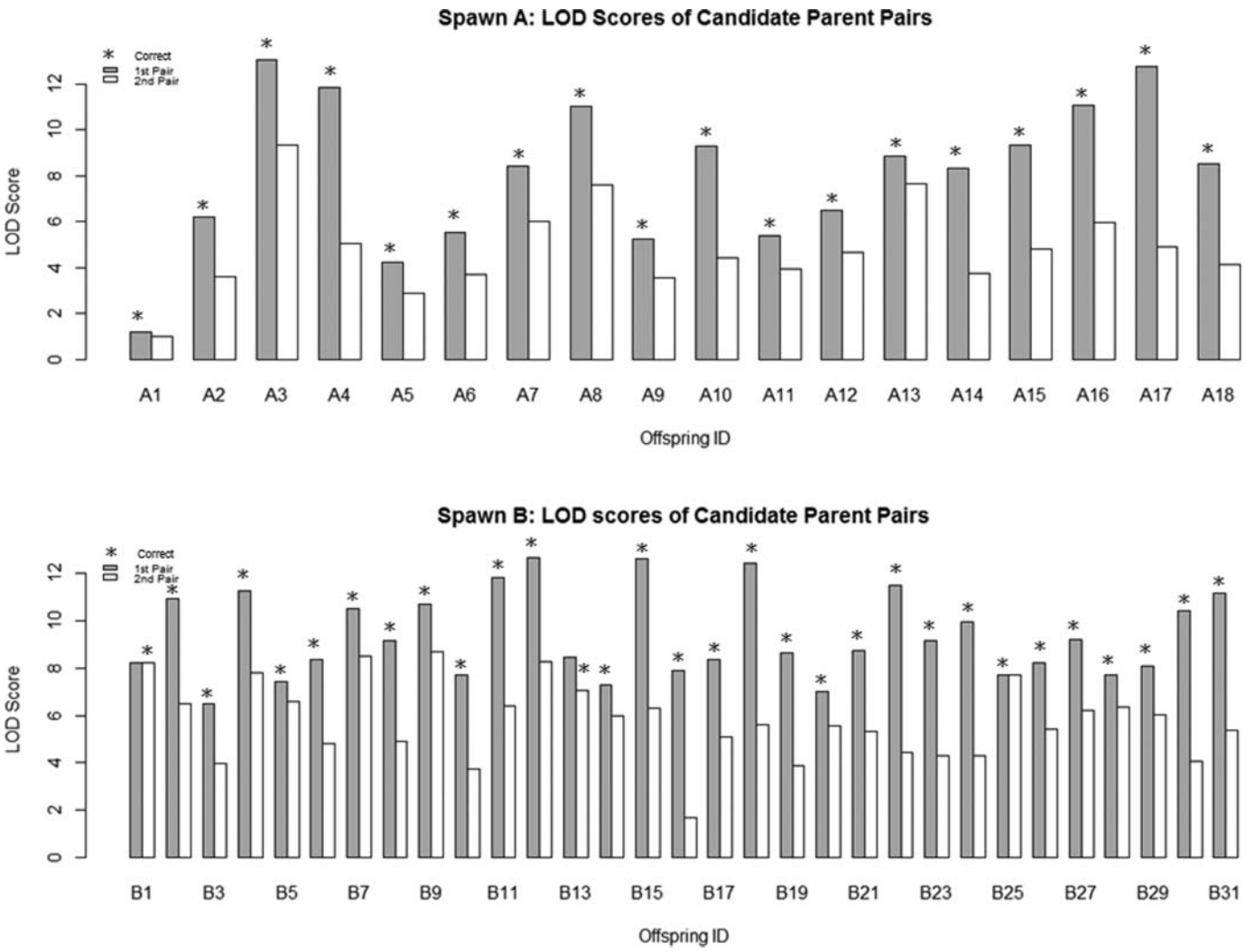

FIGURE 3. Logarithm-of-odds (LOD) scores for the two candidate parent pairs with the greatest likelihood of parentage for each Delta Smelt offspring in spawn A and spawn B. The correct parent pair (indicated by an asterisk) always had either the highest LOD score (gray bar) or the second-highest LOD score (white bar). By cross-referencing the hatchery's breeding records, we were able to exclusively assign the offspring to known parent pairs with the highest LOD scores.

challenge this longstanding assumption. First, identification of loci for a parentage panel is no longer the barrier it once was for organisms lacking an assembled genome. Methods of GBS like RAD-seq can facilitate the rapid identification and mapping of hundreds or even thousands of polymorphisms, which in turn permits the selection of highly heterozygous SNPs. Second, we demonstrated that a low-density SNP panel can effectively assign parentage in an intensively managed population. Our panel selection criteria followed those of previous studies, which found that increasing a prospective SNP panel's minimum and average MAFs can yield a corresponding increase in assignment power (Morin et al. 2004; Baruch and Weller 2008; Fisher et al. 2009; Clarke et al. 2014). Fisher et al. (2009) reported that 40 SNPs with a mean MAF of 0.35 could resolve parentage if applied with breeding records to exclude implausible candidates. Our 24 SNPs had a mean MAF of 0.47 and a minimum MAF of 0.41. By selecting independent loci with MAFs approaching 0.50, we maximized the parentage resolution provided by each locus, and thus we were able to minimize the number of SNPs needed. Although microsatellite markers have reliably resolved broodstock pedigrees since the initiation of the Delta Smelt captive breeding program (Fisch et al. 2013), application of the 24-SNP panel to the conservation hatchery's management offers the potential to double the current sample throughput at a comparable per-sample cost.

These advances may be timely. Over the past decade, there has been growing awareness that supplementation can potentially impair the genetic integrity of a species in the wild (Utter 1998; Waples 1999; Ford 2002; Araki et al. 2007, 2009; Fraser 2008; Naish et al. 2008; Frankham 2010; Champagnon et al. 2012; Christie et al. 2012a,

2012b). To minimize the divergence of hatchery fish from their wild conspecifics (Bryant and Reed 1999; Ford 2002; Williams and Hoffman 2009), hatcheries will need to employ genetic breeding plans that are capable of maintaining high genetic diversity with relatively small broodstocks (Allendorf 1993; Utter and Epifanio 2002; Fraser 2008; Frankham 2010; Ivy and Lacy 2012). Molecular markers will play an important role in managing these populations. Although microsatellites will likely continue to fulfill this function for many studies in the near future, SNPs offer opportunities to access a wealth of genomic information that would otherwise be undetected. Here, we have demonstrated their application with Delta Smelt, but other studies of endangered species that lack genomic resources could implement a similar process. In addition to showing that a low-density SNP panel can accurately assign parentage, we have also provided a framework for development of such a panel in a nonmodel species. Our study also yielded the first linkage map for Delta Smelt as well as additional genotyping assays that may be of use in assessing the genetic diversity of the captive and wild populations. The present work demonstrates that genetic management is increasingly possible for a wide variety of species.

ACKNOWLEDGMENTS

We thank Joan Lindberg, Meredith Nagel, Luke Ellison, Galen Tigan, and the staff at the FCCL for managing the Delta Smelt captive breeding population and raising the Delta Smelt families used for linkage mapping; we also thank Antonia Wong and Melania La Cava for laboratory support. We are grateful to Michael R. Miller, Andrew Whitehead, Nicholas Buckmaster, and Megan Mayo for providing comments in the writing of this manuscript. This work was supported by the U.S. Bureau of Reclamation under Cooperative Agreement Number R10AC20089, CESU Number 3FC810873, to B. May.

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