An experimental assessment of genetic tagging and founder representation in hatchery-reared red drum (*Sciaenops ocellatus*) used in stock enhancement

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Summary

Multiplexed microsatellite markers were evaluated as genetic tags for red drum (*Sciaenops ocellatus*) juveniles used in stock enhancement. Offspring were generated from spawns of nine sets of five broodfish (three dams and two sires) in individual brood tanks. Intensive sampling, by trawling, at 2, 7, 8, 10 and 11 days after release of ~192 500 hatchery-raised fingerlings resulted in recovery of a total of 310 fingerlings. All parents and recovered offspring were genotyped for variation at 30 microsatellites combined for simultaneous assay in six multiplex panels. An optimal combination employing three of the six multiplex panels allowed unambiguous parentage assignment of all recovered offspring. Only 21 of 52 possible dam x sire combinations were represented among recovered offspring. The founder equivalent (f_e) of the recovered offspring was 8.7 vs the expected f_e of 36.0 (95% CI = 33.3–38.4) if reproductive success was randomly distributed among breeders. The significantly lower founder equivalent translated into reduced genetic diversity among the recovered offspring and may reflect differing contributions of individual broodfish to spawning events, differing productivity among brood tanks, and / or variable survival of families during early larval and / or juvenile stages.

Introduction

The red drum (*Sciaenops ocellatus* L.) is an economically important sciaenid fish in the southern USA. Dramatic declines in population numbers in U.S. waters during the 1970s and 1980s led to implementation of harvest restrictions and prohibition of the commercial sale of 'wild' red drum (Matlock, 1990). In parallel, the Texas Parks and Wildlife Department (TPWD) in the 1980s initiated a vigorous stock-enhancement program (McEachron et al., 1993, 1995). Currently 25–30 million hatchery-raised red drum fingerlings (25–30 mm) are released annually into eight Texas bays and estuaries (Vega et al., 2003). Recruitment into the wild population results from 'natural' reproduction and, for a yet unknown proportion, from *S. ocellatus* released during the course of the stock-enhancement program.

Assessment of survival of released red drum fingerlings is essential to program evaluation and optimization but requires development of a reliable method to discriminate hatchery-raised fish from their wild conspecifics. Potential approaches to tagging large numbers of hatchery-raised fish include exposure to oxytetracycline–HCl (OTC), use of coded-wire tags, and deployment of genetic markers. The latter has the advantages that (i) no treatment prior to release is required, (ii) the genetic marks are permanent, (iii) the only limitation to the number of fish that can be tagged is the number of fish produced and (iv) fish do not need be sacrificed in order to detect the marker. In addition, robust identification of hatchery-raised fish can be achieved using genetic markers, provided sufficient allelic diversity is exposed by the marker loci employed (Bernatchez and Duchesne, 2000). Finally, genetic markers also provide assessment of genetic parameters (e.g. levels of genetic variability) thus enabling comparison of hatchery-raised and 'wild' fish and evaluation of potential genetic impacts of stock enhancement on the 'wild' population (Hansen et al., 2000). Assessment of genetic impacts of stock enhancement, along with survival of released fish, is especially critical as information on both topics in marine fishes is limited.

In a previous study (Renshaw et al., 2006a), we used simulations to show that 10–15 nuclear-encoded microsatellites would be necessary to reliably discriminate hatchery-raised from 'wild' red drum, given availability of genotypes of all broodfish present in a hatchery. Subsequently, six multiplex panels were designed that permitted the simultaneous and cost-effective assay of 31 red drum microsatellites (Renshaw et al., 2006b). In this study, we evaluated these multiplex panels for practical genetic identification of hatchery-released red drum juveniles. Genetic assignment followed the logarithm of odds (LOD-) score approach developed by Gerber et al. (2000). Simulations were used to identify a combination of the multiplex panels that optimized assay throughput and assignment power. The method was applied to genetic identification of hatchery-raised fingerlings recovered after an experimental release. The genetic diversity and founder equivalent (f_e) among recovered offspring were compared to expectations based on the ideal case where reproductive success among hatchery broodfish was distributed randomly.

Materials and methods

Offspring production and release

Broodfish used to generate released juveniles were 'wild' red drum (*S. ocellatus*) caught off the south Texas coast by TPWD personnel. Broodfish were maintained in each of nine brood tanks (16 m^3) at the TPWD Marine Development Center (MDC) in Flour Bluff, Texas. Each brood tank contained three dams and two sires. Temperature and photoperiod were manipulated following a 150-day maturation cycle (McCarty, 1987) in order to achieve spontaneous spawning of broodfish. Spawning occurred at night between 4 and 23 April 2002; offspring from 13 spawning events were used in the experiment. The number of spawning events per brood tank was one

(six brood tanks), two (two brood tanks) or three (one brood tank). Following each spawning event, fertilized (buoyant) eggs were collected at the effluent of each brood tank and incubated for ∼72 h under conditions described in Henderson-Arzapalo (1987). Newly hatched larvae were transferred to separate, one- or two-acre, pre-fertilized ponds (Colura, 1987) where they were grown until they reached an average size of ∼30 mm.

Harvest of ponds was conducted 41–57 days post-fertilization and fingerlings from each pond (15 223–29 461 per pond; 227 544 total) were transferred to a single holding pond. On 10 June 2002, the holding pond was harvested and all offspring were transported to Packery Channel Park at the southern end of Corpus Christi Bay, where they were released by TPWD personnel. Corpus Christi Bay is located on the south Texas coast in the northwestern Gulf of Mexico. All offspring were released over a 1-h period and within a 100 m radius of a single release site (a shallow seagrass meadow along an open shoreline, latitude 27°37′43′North, longitude 97°13′2′West). The estimated number of fish released was 192 500; their mean weight was 0.42 g. The out-of-season (for natural spawning of red drum) timing of the release and avoidance of areas within 20 km of release sites in other TPWD stocking activities during the summer of 2002 assured that all early-juvenile red drum taken in the trawl were from the experimental release.

Sampling and recapture of the released offspring

Recapture of released offspring was carried out via trawling suitable habitat (shallow sea grass meadows along the shoreline, Pattillo et al., 1997) at, near, and up to 2 km from the release site, using a hand-pulled 1-m beam trawl with 3-mm mesh. A total of 207, 85-m tows were undertaken over a nine-day sampling period (trawling occurred 2, 7, 8, 10 and 11 days after release); a total of 310 juvenile red drum were recovered. The rate of recovery decreased over time (Table 1) owing to mortality and dispersal of the fish beyond the sampled area. Sampling was terminated at day 11 considering the low number of recaptures at that stage of the experiment. Fish collected were kept on ice during sampling and returned to the laboratory at the end of each sampling day. Fish were placed into individual cryopreservation tubes, flash-frozen in liquid nitrogen, and stored in an ultracold (−80°C) freezer. Fin clips (∼4–5 mm^2) had been removed from all potential parents (i.e. all dams and sires in each of the nine brood tank) and stored in 95% ethanol.

Genetic assays

DNA was extracted from tissue samples by using an alkaline-lysis protocol (Saillant et al., 2002). All broodfish and all recovered offspring were genotyped for variation at 31

Table 1
Number of juvenile red drum (*Sciaenops ocellatus*) recovered in Packery Channel (Corpus Christi Bay, TX) following experimental release of 192 500 hatchery-raised fingerlings on 10 June 2002

Sampling date (days post-release)	No. fish recovered	No. tows
2	110	31
7	85	46
8	68	50
10	33	50
11	14	30

microsatellites included in six multiplexes (Renshaw et al., 2006b). Formal descriptions of each microsatellite may be found in Saillant et al. (2004). One microsatellite (*Soc*201) assayed in multiplex 2 (Renshaw et al., 2006b) could not be scored reliably and was discarded from further analysis.

Data analysis

Parentage analysis (assignment of recovered offspring to parents) based on microsatellite genotypes employed the LOD-score approach described in Gerber et al. (2000), as implemented in the program FAMOZ (Gerber et al., 2003) available at http://www.pierroton.inra.fr/genetics/labo/Software/Famoz/. A LOD score is the logarithm of the ratio of the likelihood of a parent–offspring relationship to the likelihood that the offspring is unrelated to the two parents considered. LOD scores are calculated for all possible parental-pair combinations among hatchery broodfish employed in the experiment; offspring are assigned to the parental pair showing the highest LOD score if the obtained LOD score lies beyond a threshold value. The threshold LOD score for assignment was determined via simulations in FAMOZ. Multilocus genotypes of 100 000 'true' offspring from hatchery broodfish were simulated based on Mendelian principles; multilocus genotypes of 100 000 random individuals from the 'wild' also were simulated based on allele frequencies at each microsatellite in the 'wild' population. Allele frequencies in the wild populations were estimated based on genotypes of 144 adult 'wild' red drum caught by TPWD personnel along the central Texas coast. Temporal genetic variation in the region was assessed by Gold and Turner (2002), who found that allele frequencies were stable over time. The simulation error rate, representing mutational events, was set to 0.0005 considering 'average' mutation rates in microsatellites (Jarne and Lagoda, 1996). The per-locus error rate in LOD-score calculation, representing scoring errors and / or null alleles, was set to 0.01. The distribution of LOD scores of simulated 'true' offspring from hatchery broodfish and random offspring from the 'wild' population were used to determine critical LOD-score values that minimize type I and type II errors in parentage assignment when assignment decisions rely on LOD-score values greater than the threshold (Gerber et al., 2000). Type I error thus is the proportion of 'true' offspring incorrectly excluded as offspring from hatchery broodfish, while Type II error is the proportion of 'wild' offspring incorrectly assigned as offspring from hatchery broodfish. Assignment analysis was conducted using data from individual multiplexes, from combinations of multiplexes, and from all six multiplexes combined.

Homogeneity of the distribution of frequencies of full-sib families recovered at different post-release dates was tested using a likelihood-ratio test (Sokal and Rohlf, 1981). The exact probability value of the likelihood-ratio test was estimated via a Monte Carlo simulation approach in PROC FREQ in SAS® (SAS Institute Inc., Cary, NC), using 10 000 replicates. The founder equivalent (f_e) of the recovered offspring population was estimated for dams, sires, and dams plus sires, using Equation 1 (Lacy, 1989)

$$f_e = \frac{1}{\sum_i f_i^2} \tag{1}$$

where f_i is the relative frequency of offspring from parent i. A bootstrap resampling approach (Efron, 1979) was used to

assess whether the contribution of individual dams and sires to the recovered offspring population was random. POP TOOLS (a free add-in software for Excel, available at: http://www.cse.c-siro.au/poptools/index.htm) was used to generate 310 offspring (the number of released fish recovered) from the broodfish population by randomly sampling a dam and a sire among the 27 dams and 18 sires present in the nine brood tanks. Random sampling was performed 10 000 times and the distribution of the founder equivalents (f_e) for dams, sires, and dams plus sires was summarized as their mean and upper (0.975) and lower (0.025) percentiles. Estimates of the founder equivalents (for dams, sires and dams plus sires) among the 310 recovered offspring were compared to the appropriate simulated distribution.

Summary statistics (number of alleles, allele frequencies, allelic richness, unbiased gene diversity) for each microsatellite were generated for the broodfish and recovered offspring 'populations' using F-STAT (Lausanne, Switzerland) (Goudet, 1995) available at http://www2.unil.cn/popgen/softwares/fstat.htm. Homogeneity of allele distributions between populations was examined for each microsatellite and over all microsatellites via exact tests; the significance of probability values was assessed by a Markov-chain method, as implemented in GENEPOP (Raymond and Rousset, 1995) and using 5000 dememorizations, 500 batches, and 5000 iterations per batch. Homogeneity of allelic richness and gene diversity between the two populations was tested using Wilcoxon signed-rank tests. Departure of genotypic proportions from Hardy–Weinberg (HW) equilibrium expectations within populations was measured for each microsatellite as Weir and Cockerham's (1984) f; probability of significance of f (P_{HW}) was assessed using a Markov-chain method (Guo and Thompson, 1992), as implemented in GENEPOP and using the same Markov-chain parameters as above. Sequential Bonferroni correction (Rice, 1989) was applied to all multiple tests performed simultaneously.

Results and discussion

A total of 310 red drum juveniles was recovered during the five days (207 tows) of sampling. The largest recovery (110 red drum, average of 3.6 per tow, Table 1) occurred during the first day of sampling. The number of red drum recovered thereafter decreased to 14 (0.5 per tow) at day 11 (July 21), when sampling ceased.

The efficiency of the microsatellite multiplexes (individually and collectively) for parental assignment was evaluated from the simulated LOD-score distributions of 100 000 'true' offspring from the broodfish and of 100 000 'wild' offspring. The intersection of the two distributions obtained was taken as an optimal threshold LOD-score value for assignment in order to minimize both type I and type II errors (Gerber et al., 2000). None of the individual multiplexes allowed reduction of assignment errors to a probability lower than 0.05 (Table 2). Use of multiplexes 1 and 4, however, reduced both type I and type II errors to probability values of 0.01 or less. The distributions of LOD scores of simulated 'true' offspring and of 'wild' offspring did not overlap at all when multiplexes 1, 3 and 4 or all six multiplexes were used (Table 2). Threshold LOD scores for the latter two were 9.9 and 10.6 (Table 2) and allowed exclusion of all simulated 'wild' offspring while including all 'true' hatchery offspring. In addition, all simulated 'true' offspring matched a unique pair of hatchery broodfish with LOD scores greater than 13.3. The 310

recovered offspring were then 'assigned', based on genotypes at multiplexes 1, 3 and 4 and at all six multiplexes combined. As expected, unambiguous matching of all recovered offspring to a unique hatchery dam and sire combination was achieved in both instances, with LOD scores greater than the thresholds defined. All LOD scores also fell within the bounds of the simulated LOD-score distribution of 'true' hatchery offspring. These results are consistent with our prior assessment (Renshaw et al., 2006a), based on simulations, that only 10–15 microsatellites are sufficient to achieve robust assignment of hatchery-raised red drum to individual pairs of hatchery broodfish.

Details of the contributions of individual full-sib families (dam × sire combinations) to the recovered offspring are available upon request from the authors. A total of 21 full-sib families, involving 27 breeders (14 dams and 13 sires), were represented among the 310 recovered offspring. The frequencies of the 21 full-sib families recovered did not differ significantly among sampling days ($G = 93.6$, P = 0.08). Data from individual sampling days were therefore pooled for subsequent analyses. Given the mating design, up to 52 full-sib families involving the 45 broodfish (27 dams, 18 sires) could have been generated during the 13 spawning events. The mean (95% CI) founder equivalents among 10 000 simulated sets of 310 offspring, and assuming all broodfish had equal probability of contributing to the offspring population, were 21.7 (19.4–23.5) dams, 14.4 (12.6–15.8) sires, and 36.0 (33.3–38.4) dams and sires combined. In addition, almost all of the simulated sets of 310 offspring included at least one offspring from each of the 27 dams (95% of simulated sets) and each of the 18 sires (99% of simulated sets). Founder equivalents (f_e) of the 310 recovered offspring were 3.9 (dams), 4.9 (sires), and 8.8 (dams plus sires). These values are significantly lower (based on simulated 95% confidence intervals) than those expected under the 'ideal' situation simulated above. The observed reduction in f_e relative to the 'ideal' case was more pronounced for dams (3.9 vs 21.7 effective females) than for the sires (4.9 vs 14.4 effective males) and reflects in large part the lower proportion of contributing dams (14 of 27) compared to the proportion of contributing sires (13 of 18). The reduction in f_e with respect to the 'ideal' number of contributing dams plus sires (8.8 *vs* 36.0) reflects both the lower number of contributing parents (27 vs 45) and the unequal contributions of individual broodfish to the recovered offspring (the contribution of individual broodfish to the offspring varied from one to 139). Unequal contributions of individual broodfish may arise from different egg production among dams and / or fertilization success among sires, differ-

Table 2
Threshold LOD scores for parental assignment and type I and type II error rates observed during simulations based on six microsatellite multiplex panels

	LOD-score value	Type I error (in %)	Type II error (in %)
Multiplex 1	5.8	10	07
Multiplex 2	4.1	50	29
Multiplex 3	5.1	15	14
Multiplex 4	5.3	14	09
Multiplex 5	4.9	18	14
Multiplex 6	3.7	32	24
Multiplexes 1 and 4	8.7	01	0
Multiplexes 1, 3 and 4	9.9	0	0
All six multiplexes	10.6	0	0

Table 3
Summary statistics for 30 microsatellites in the broodfish and recovered offspring

Microsatellite	Broodfish	Offspring	Microsatellite	Broodfish	Offspring	Microsatellite	Broodfish	Offspring
*Soc*11 (4)[a]			*Soc*19 (3)			*Soc*44 (6)		
n	45	297	n	44	294	n	45	283
#A	10	10	#A	15	12	#A	24	21
A_R	9.7	6.7	A_R	14.7	10.0	A_R	23.2	16.0
H_E	0.729	0.667	H_E	0.912	0.861	H_E	0.938	0.914
P_{HW}	0.718	0.197	P_{HW}	0.069	**0.000**	P_{HW}	0.427	**0.000**
f	−0.006	−0.029	f	0.053	−0.118	f	0.052	−0.028
*Soc*60 (2)			*Soc*83 (4)			*Soc*85 (3)		
n	44	306	n	45	299	n	45	292
#A	5	5	#A	12	10	#A	14	13
A_R	4.9	4.6	A_R	11.6	8.7	A_R	13.8	10.3
H_E	0.566	0.508	H_E	0.830	0.797	H_E	0.874	0.827
P_{HW}	0.664	**0.000**	P_{HW}	0.613	**0.000**	P_{HW}	0.049	**0.000**
f	0.157	0.042	f	−0.018	−0.053	f	0.060	−0.031
*Soc*99 (4)			*Soc*138 (3)			*Soc*140 (2)		
n	45	277	n	45	299	n	44	298
#A	22	20	#A	11	9	#A	4	4
A_R	21.4	15.5	A_R	10.7	6.5	A_R	4.0	3.3
H_E	0.933	0.870	H_E	0.809	0.743	H_E	0.618	0.547
P_{HW}	0.781	**0.000**	P_{HW}	0.080	**0.000**	P_{HW}	0.264	0.016
f	−0.024	−0.078	f	−0.043	−0.030	f	−0.250	−0.116
*Soc*156 (3)			*Soc*206 (3)			*Soc*243 (2)		
n	45	310	n	45	306	n	44	281
#A	5	3	#A	5	4	#A	5	5
A_R	4.9	2.1	A_R	4.9	3.0	A_R	4.9	4.8
H_E	0.496	0.383	H_E	0.497	0.505	H_E	0.753	0.699
P_{HW}	0.026	0.240	P_{HW}	0.566	0.048	P_{HW}	0.340	**0.000**
f	−0.210	−0.028	f	−0.162	−0.108	f	0.064	−0.283
*Soc*400 (3)			*Soc*401 (6)			*Soc*402 (5)		
n	45	281	n	45	248	n	43	240
#A	8	7	#A	14	10	#A	16	11
A_R	7.8	6.3	A_R	13.7	8.7	A_R	15.7	9.0
H_E	0.729	0.760	H_E	0.887	0.758	H_E	0.883	0.839
P_{HW}	0.687	**0.000**	P_{HW}	0.095	**0.000**	P_{HW}	0.176	**0.000**
f	0.146	−0.058	f	0.223	0.085	f	0.025	0.052
*Soc*404 (5)			*Soc*407 (4)			*Soc*410 (3)		
n	43	288	n	45	294	n	41	246
#A	26	20	#A	11	10	#A	13	10
A_R	25.7	15.5	A_R	10.9	8.2	A_R	13.0	7.7
H_E	0.933	0.882	H_E	0.864	0.841	H_E	0.798	0.767
P_{HW}	0.404	**0.000**	P_{HW}	0.123	**0.000**	P_{HW}	0.149	**0.000**
f	0.003	−0.003	f	−0.055	−0.160	f	0.114	−0.103
*Soc*412 (1)			*Soc*415 (5)			*Soc*416 (1)		
n	45	302	n	45	295	n	45	298
#A	25	21	#A	17	15	#A	16	15
A_R	23.8	15.1	A_R	16.3	10.2	A_R	15.7	10.9
H_E	0.919	0.865	H_E	0.729	0.728	H_E	0.860	0.805
P_{HW}	0.065	**0.000**	P_{HW}	0.296	**0.000**	P_{HW}	0.319	**0.000**
f	0.081	−0.137	f	0.024	0.004	f	0.121	−0.109
*Soc*417 (1)			*Soc*419 (2)			*Soc*423 (1)		
n	43	302	n	42	293	n	41	309
#A	11	7	#A	11	10	#A	18	15
A_R	10.9	5.1	A_R	11.0	8.0	A_R	18.0	11.7
H_E	0.774	0.667	H_E	0.855	0.769	H_E	0.881	0.865
P_{HW}	0.295	**0.000**	P_{HW}	0.904	**0.000**	P_{HW}	0.535	**0.000**
f	−0.022	−0.053	f	−0.058	−0.119	f	0.004	−0.052
*Soc*424 (4)			*Soc*428 (1)			*Soc*432 (5)		
n	45	301	n	45	305	n	45	295
#A	15	13	#A	28	23	#A	9	10
A_R	14.6	10.6	A_R	27.3	15.6	A_R	8.8	7.6
H_E	0.842	0.772	H_E	0.951	0.875	H_E	0.817	0.814
P_{HW}	0.723	**0.000**	P_{HW}	0.126	**0.000**	P_{HW}	0.867	**0.000**
f	−0.030	−0.076	f	0.019	−0.023	f	−0.061	0.158
*Soc*433 (6)			*Soc*444 (6)			*Soc*445 (1)		
n	44	289	n	43	302	n	45	227
#A	10	12	#A	3	3	#A	11	9
A_R	9.9	9.0	A_R	3.0	2.9	A_R	10.8	6.9
H_E	0.834	0.819	H_E	0.522	0.195	H_E	0.810	0.738
P_{HW}	0.162	**0.000**	P_{HW}	0.544	**0.000**	P_{HW}	0.531	**0.000**
f	0.019	0.012	f	−0.157	0.218	f	0.068	−0.093

n, sample size; #A, number of alleles; A_R, allelic richness; H_E, gene diversity; P_{HW}, probability of conforming to expected Hardy–Weinberg proportions (Bold font indicate significance following Bonferroni correction for multiple tests); f, inbreeding coefficient.
[a]Numbers in parentheses refer to a multiplex panel.

ent contribution of individual brood tanks to the released population, and/or different mortality rates among families during initial rearing prior to the release. Further assessment of the relative role(s) of these factors on reducing f_e in released populations is now in progress.

Summary statistics (number of alleles, allelic richness, and unbiased gene diversity), inbreeding coefficients, and results of tests of HW equilibrium for both broodstock and recovered offspring populations are presented in Table 3. Allele frequencies differed significantly ($P < 0.05$) between broodstock and recovered offspring populations at 28 microsatellites before Bonferroni correction for multiple tests and at 26 microsatellites after correction. The number of alleles per microsatellite ranged from 3 (Soc444) to 28 (Soc428) in the broodfish population and from 3 (Soc444) to 23 (Soc428) in the recovered offspring population. Estimates of allele richness for the broodfish population ranged from 3.0 (Soc444) to 27.3 (Soc428) and from 2.1 (Soc156) to 16.0 (Soc44) in the recovered offspring population. Allelic richness in the recovered offspring population was significantly lower ($Z = -4.78$, $P < 0.0001$) than in the broodfish population. Estimates of gene diversity in the broodfish population ranged from 0.496 (Soc156) to 0.951 (Soc428) and from 0.195 (Soc444) to 0.914 (Soc44) in the recovered offspring population. Gene diversity estimates also were significantly lower in the offspring population ($Z = -4.54$, $P < 0.0001$).

Estimates of the inbreeding coefficient (f) over all 30 microsatellites were 0.013 and -0.05, respectively, for the broodfish and recovered-offspring populations (Table 3). No significant departure of genotype proportions from expectations of HW equilibrium was observed either at individual microsatellites or over all microsatellites for the broodfish population (Table 3). Significant departure from HW equilibrium following Bonferroni correction, however, was observed in the recovered-offspring population at 26 of the 30 microsatellites (Table 3). At 23 of the microsatellites, there was an excess of heterozygotes (overall $f = -0.05$). The significant reduction in genetic diversity and the observation of a general excess of heterozygotes in the recovered-offspring population are consistent with a low effective number of breeders contributing to the offspring (Luikart and Cornuet, 1999). These results highlight the importance of monitoring effective size and genetic diversity among released fish in a stock-enhancement program and of developing procedures that maximize the contribution of all broodfish. Further evaluation of success (and potential genetic impacts) of the TPWD stock-enhancement program will necessitate knowledge of long-term survival and reproductive success of released fish (Ryman and Laikre, 1991). Studies of these issues in red drum are currently in progress.

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