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CHAPTER 5

GENETIC IDENTIFICATION OF FISH HARVESTED FROM OFFSHORE AQUACULTURE: AN EXAMPLE INVOLVING RED DRUM, *SCIAENOPS OCELLATUS*, FROM THE NORTHERN GULF OF MEXICO

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ABSTRACT

A total of 31 nuclear-encoded microsatellites and an ~ 370 base pair fragment from the 'control' region of mitochondrial (mt)DNA were employed to resolve potential forensic issues relating to legal sale of red drum (*Sciaenops ocellatus*) harvested from offshore aquaculture operations in the northern Gulf of Mexico. Exclusion analyses demonstrated that only 16 microsatellites (13 if mtDNA was employed) were necessary to exclude a sample of 101 'wild' red drum from Biloxi Bay, Mississippi, as having been produced by broodfish in a hatchery near Corpus Christi, Texas; the probability of incorrectly assigning a 'wild' fish as having been produced by the broodfish ranged from 2.58×10^{-19} to 1.33×10^{-27}. Probabilities that the most common, hatchery-produced 'composite' genotype would occur in the sample from Biloxi Bay ranged from 1.38×10^{-27} to 2.98×10^{-42}. All probability values were several orders of magnitude smaller than the reciprocal of the total number of adult red drum (10^6–10^7) estimated to occur in the northern Gulf of Mexico. Comparison of results with and without mtDNA indicated that it would be more cost effective to first sequence individuals for the mtDNA fragment and then determine the number of individuals that needed to be assayed for microsatellite genotypes. The study demonstrated that unequivocally distinguishing red drum spawned from broodstock obtained offshore of Corpus Christi, Texas, from the 'wild' stock in Biloxi Bay, Mississippi, is fairly straightforward, given (i) a sufficient number of polymorphic (variable), independent genetic markers, (ii) the genotypes of the broodfish, and (iii) a survey of allelic variation at the genetic markers among representatives of the 'wild' stock. The three 'requirements' essentially would be the same for any offshore aquaculture operation where legal sale of the cultured species could be an issue.

INTRODUCTION

Offshore aquaculture industries marketing 'game fish' species will require methods to identify or distinguish unequivocally harvested products from 'wild' stocks in order to ensure legal sale and alleviate potential conflicts. Identification needs at the market place could arise when fish are harvested (should certification prior to sale be necessary), stored on ice or frozen, or sold or served as fillets. It also may be necessary from time to time to identify escapees from different aquaculture impoundments relative to ownership. Identification methods must thus be accurate and reproducible, capable of deployment on whole fish or fillets (perhaps even when fillets are in the skillet), and have sufficient power to

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identify unambiguously the origin (parentage) of individuals from the same species or population (or even the same hatchery).

Forensic methods used historically to identify origin of animals or animal products have almost exclusively been 'genetic' and primarily have involved analysis of proteins (AOAC 1984; Kim and Shelaf 1986). The most commonly used methods have been electrophoresis (of soluble proteins), high performance liquid chromatography, immunological procedures that rely on antibody-recognition, and isoelectric focusing (Ashoor et al. 1988; Berger et al. 1988). Advantages to using proteins, especially protein electrophoresis, were simplicity, relatively low cost, and low initial start-up costs. However, analysis of proteins is limited generally to tissues that are either fresh or have been frozen fairly soon after procurement. In addition, the relative proportion of hypervariable protein-coding markers (loci) is fairly low in most fishes (Ward et al. 1994) making it very difficult to identify origin of individuals without screening an inordinately large number of different proteins. Direct analysis of genomic DNA polymorphism as a means to discriminate origin of individuals is preferable for a number of reasons. First, DNA is the genetic material and homologous DNA sequences are essentially the same in all tissues and cells of an individual, meaning that any available tissue can be utilized. Second, the information content of genomic or mitochondrial DNA considerably exceeds that of proteins as a large panel of polymorphic markers can be accessed straightforwardly from a reasonably good DNA extract. Third, DNA is remarkably stable and has been successfully extracted from fossilized or mummified tissue (Paabo et al. 1988, 1989) and from meat that has been partially cooked (Bartlett and Davidson 1992; Forrest and Carnegie 1994). Finally, because the approach typically used today to assay DNA markers is based on polymerase chain reaction or PCR amplification (White et al. 1989), the quantity of tissue needed for DNA analysis is far less than that typically required for protein analysis.

The statistical issues involved in DNA analysis to identify parentage (i.e., in this case to discriminate hatchery-produced fish from 'wild' fish) are straightforward, and conceptually were outlined in NRC (1996) and Evett and Weir (1998). Assuming that alleles (forms of genes) and genotypes (allele combinations or genetic constitution) of broodstock in a hatchery are known, and that sufficiently powerful genetic markers are available, genetic profiles can be established that permit unquestionable certification that a given fish was *not* generated from that broodstock or unquestionable certification that a given fish *could* have been generated by that broodstock. The converse, 'proving' that a given fish did *not* come from a 'wild' stock cannot be ascertained unequivocally, but can be stated in terms of acceptable probability levels. Genetic data used forensically might indicate, for example, that there is a probability of 1 that a given fish *could* have originated from known broodstock but that the probability that the same fish was sampled at random from a 'wild' stock was less than one in a billion. Explicit statistical methods of parentage analysis were reviewed recently by Jones and Ardren (2003). Of the methods reviewed, the most appropriate for forensic issues is *exclusion* analysis, where Mendelian expectations are used to reject particular parent—offspring hypotheses. The approach is optimized when the number of candidate parents is small and the genetic markers employed are hypervariable (Jones and Ardren 2003).

In this chapter, we demonstrate the use of hypervariable genetic markers to discriminate hatchery-produced from 'wild' red drum (*Sciaenops ocellatus*). The initial design of the Offshore Aquaculture Consortium (OAC) project was to use red drum as the 'test' species for an offshore aquaculture operation in the northern Gulf of Mexico. Fingerling red drum were to be obtained from hatcheries operated by the Texas Parks and Wildlife Department (TPWD) and ultimately placed into the OAC Ocean Spar Sea Station offshore of Ocean Springs, Mississippi, for grow-out trials. Accordingly, we employed a suite of PCR primers for hypervariable loci in red drum and genotyped the broodfish in one of the TPWD hatcheries and a sample of 'wild' red drum from Biloxi Bay, Mississippi. The hypervariable markers used were 31 nuclear-encoded microsatellites and an ~ 370 base pair fragment from the 'control' region of mitochondrial (mt)DNA. The former (microsatellites) are abundant, short stretches of DNA composed of di-, tri-, or tetranucleotide arrays that are embedded in unique DNA, inherited in a Mendelian fashion, and distributed evenly throughout chromosomes (Wright 1993). Microsatellites are ideal for forensic application because of high levels of polymorphism, codominant inheritance, and Mendelian segregation of alleles (Weber and May 1989; Wright 1993). In addition, because identification of each microsatellite is via amplification, using specific polymerase chain-reaction (PCR) primers, there are few problems associated with homology of alleles from distinct loci (Wright and Bentzen 1994). Mitochondrial (mt)DNA is a haploid genetic molecule inherited through the female parent, meaning that mtDNA, provided there is sufficient polymorphism, can be useful in excluding mother-offspring relationships. Prior studies of red drum mtDNA (Gold et al.1999; Seyoum et al. 2000) had revealed extensive variability of mtDNA among 'wild' red drum; nucleon diversities (the probability that any two fish sampled at random will *differ* in mtDNA genotype) were > 95%, meaning that nearly all female broodstock could be expected to differ in mtDNA genotype from one another and from most 'wild' fish. The specific objectives of the project were to generate a suite of hypervariable DNA markers specific for red drum and then demonstrate how these markers could be employed to distinguish hatchery-produced red drum from 'wild' red drum in Mississippi waters. In a more general way, the project was to serve as a model in terms of using genetic data to resolve forensic issues relating to legal sale of marine products from offshore aquaculture operations.

MATERIALS AND METHODS

Relative small pieces (~ 2–3 cm^3) of upper lobe of the caudal fin were removed from sires (males) and dams (females) in each of nine brood tanks at the CCA/CPL Marine Development Center in Corpus Christi, Texas (hereafter, *Broodstock*). Each brood tank contained two sires and three dams (45 fish total). Fin clips were fixed and preserved in 95% ethanol. Heart tissues, frozen in liquid nitrogen, from a total of 102 age 0 red drum sampled from Biloxi Bay, Mississippi, were kindly provided by J. Franks of the Gulf Coast Research Laboratory in Ocean Springs, Mississippi (hereafter, *Biloxi Bay*). DNA from all individuals was isolated and purified using methods outlined in Gold and Richardson (1991).

Microsatellites were generated from a genomic library of red drum DNA via standard methods described fully in O'Malley et al. (2003). Briefly, size-selected DNA fragments (200–1,200 base pairs in length) were

ligated into cloning vectors and transformed into competent *Escherichia coli* cells. Clones were hybridized with mixtures (cocktails) of synthetic oligonucleotide probes to identify those containing candidate microsatellites. Clones that gave a positive hybridization signal were then sequenced. PCR primers were designed from sequences flanking candidate microsatellites. Optimization of PCR protocols for each designed primer pair was carried out on a panel of DNA from 10–12 individuals. PCR primer sequences, repeat sequence, and optimal annealing temperature for the 31 microsatellites used in the project are given in Appendix Table 1. Details of PCR amplification may be found in O'Malley et al. (2003).

A fragment of ~ 370 base pairs of the mitochondrial DNA control region was amplified in 50 ml reactions. Each reaction contained 1x reaction buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.5 µM of each primer, 2.5 U *Taq* polymerase, approximately 100 ng of template DNA, and ultrapure water. Thermal cycling conditions were: initial denaturation at 94°C (30 sec), 30 cycles of denaturation at 94°C (10 sec), annealing at 55°C (15 sec), and polymerization at 72°C (45 sec). Amplification (and sequencing) primers used were those developed by Seyoum et al. (2000): L15943 (5'-GTAAACCGGATGTCGGGGGTTAG-3') and H16484 (5'-GGAACCAGATACCAGGAATAGT-TCA-3'). Amplification products were purified for sequencing with Montage-96 PCR filter plates (Millipore Inc.) and double-stranded products were sequenced in both directions. Sequencing reactions contained the following (total volume of 10 µl): 1 µl BigDye version 3.0 reaction mix (Applied Biosystems Inc.), 1.5 µl 5x sequencing dilution buffer (400 mM Tris-HCl, pH 9.0, and 10 mM $MgCl_2$), 0.32 µM primer, approximately 50 ng template DNA, and ultrapure water. Cycling conditions were: initial denaturation at 96°C (30 sec), 40 cycles of 96°C (10 sec), 55°C (15 sec), and 60°C (4 min). Sequencing products were purified via precipitation with 95% ethanol and 3 M sodium acetate, washed with 70% ethanol, and dried. Electrophoresis and base-calling were performed with an Applied Biosystems Prism 310 capillary sequencer. Sequences were edited and vector-trimmed with Sequencher (GeneCodes, Inc.).

Genetic variability for nuclear-encoded microsatellites was measured as number of alleles, allelic richness (a measure of the number of alleles independent of sample size), and gene diversity. Genetic variability for mtDNA was measured as number of haplotypes, nucleon diversity (the probability that two individuals will differ in mtDNA haplotype), and nucleotide diversity (the average number of pairwise nucleotide changes per site). Gene and nucleon diversity were estimated after Nei (1987). Deficiency/excess of heterozygotes (F_{IS}) at each nuclear-encoded locus within each sample was estimated via the *f* statistic of Weir and Cockerham (1984). Estimates of allelic richness and gene and nucleon diversity and F_{IS} (*f*) were obtained using F-STAT version 2.9.3.2 <http://www.unil.ch/izea/softwares/fstat.html>. Tests for conformance of genotype proportions (nuclear-encoded loci) to Hardy-Weinberg (HW) equilibrium expectations employed an unbiased estimate of Fisher's exact-test statistic calculated by a Markov-chain procedure (5,000 dememorizations, 500 batches and 5,000 iterations per batch). Genotypic disequilibrium between pairs of nuclear-encoded loci also was tested via exact tests (same Markov-chain parameters as above). Tests of HW and genotypic disequilibrium were carried out using GENEPOP 3.3 (Raymond and Rousset 1995). Homogeneity of allele (genic) and genotype distri-

butions between samples (*Broodstock* versus *Biloxi Bay*) was tested via exact tests (as described above and using GENEPOP). Homogeneity of mtDNA haplotype distributions between samples was assessed via the F_{ST} analogue in ARLEQUIN; the probability that the F_{ST} analogue = 0 was assessed by an exact test (as described above and using ARLEQUIN).

For exclusion analysis, genotype comparisons at the 31 microsatellites were made between *Broodstock* ($n = 45$) and *Biloxi Bay* ($n = 102$). Because there were two sires and three dams in each of the nine TPWD broodtanks, there were a total of 54 different sire x dam combinations possible. As each microsatellite in an offspring has two alleles, one contributed by the sire and one contributed by the dam, genotype comparisons and subsequent exclusion of incompatible individuals from *Biloxi Bay* were based on expected Mendelian segregation from all 54 sire x dam (broodstock) combinations. Any individual from *Biloxi Bay* failing to meet this criterion was excluded and not assigned as an offspring from any of the sire x dam possibilities in *Broodstock*. This analysis essentially asks how many microsatellites are required to exclude the 102 *Biloxi Bay* fish as not having been offspring produced from the TPWD broodfish. Exclusion analysis was carried out using the program PROBMAX-2 (Danzmann 1997; Ferguson and Danzmann 1998). Exclusion analysis, using mtDNA, was even more straightforward. Only 20 of the fish in *Biloxi Bay* possessed an mtDNA haplotype found among the 27 dams from *Broodstock*, automatically excluding the remaining 79 *Biloxi Bay* fish from which mtDNA sequences were recovered as having been produced in the TPWD hatchery. Genotype comparisons, as above, for the 31 microsatellites were then carried out on the 20 *Biloxi Bay* fish with mtDNA haplotypes the same as those found among *Broodstock* dams.

We also estimated exclusion probabilities for each microsatellite and for mtDNA. Exclusion probabilities estimate the probability of individual markers to exclude a given relationship (i.e., a sire x dam cross) based on the number of alleles at the marker and the number of independent markers used in the data set (Gerber et al. 2000). The basic probability formula (after Grundel and Reetz 1981) for excluding parental pairs is:

$$P = 1 + \sum[p_i^2(2 - p_i)]^2 - 2[\sum p_i^2(2 - p_i)]^2 + 4(\sum p_i^3)^2 - 4\sum p_i^6$$

where p_i represents allele frequencies at a given microsatellite. The P value represents the probability that the allele frequency set, estimated from the 102 *Biloxi Bay* fish, will exclude any individual parental pair chosen at random. The value $[1 - P]$ represents the probability of making a mistake and not excluding a pair of non-parents. Estimates of $[1 - P]$ were combined over all microsatellites and mtDNA by multiplying the $[1 - P]$ values from each independent genetic marker.

RESULTS

Summary data of microsatellite variation within the two samples are presented in Appendix Table 2. All 31 microsatellites were polymorphic. Number of alleles sampled per microsatellite averaged 12.6 (*Broodstock*) and 15.0 (*Biloxi Bay*) and ranged from three (*Soc*444, *Broodstock*) to 32 (*Soc*428, *Biloxi Bay*). Allelic richness generally paralleled number of alleles. Gene diversity (expected heterozygosity) per microsatellite averaged 0.793 (*Broodstock*) and 0.787 (*Biloxi Bay*) and ranged between 0.457 (*Soc*156,

Broodstock) and 0.954 (*Soc*44, *Biloxi Bay*). Microsatellite variability in both samples compares favorably with values reported for 32 other fish species (DeWoody and Avise 2000), where the average number of alleles per microsatellite was 13.1 and the average heterozygosity was 0.63. The microsatellites were considerably more variable (polymorphic) than genes encoding proteins. Ward et al. (1994), for example, reported an average heterozygosity of 0.059 for allozyme loci of 57 marine species.

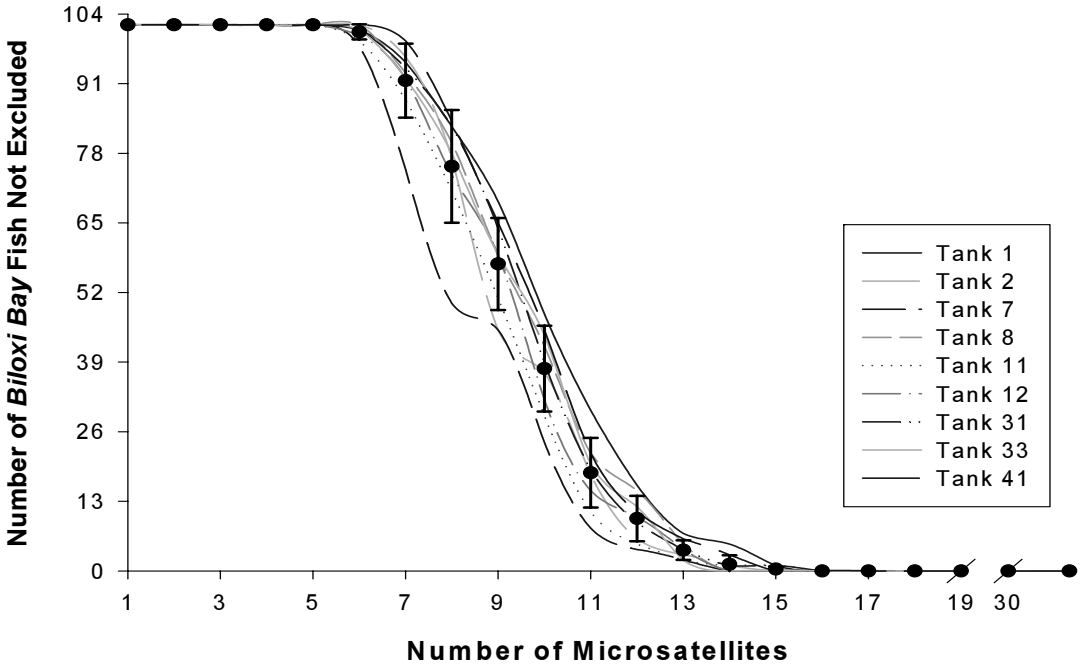
Tests of conformity to Hardy-Weinberg equilibrium expectations, following sequential Bonferroni correction (Rice 1989), were significant for microsatellites *Soc*44, *Soc*201, *Soc*243, *Soc*401, *Soc*404, and *Soc*412 in *Biloxi Bay* and for microsatellites *Soc*404 and *Soc*412 in *Broodstock*. In all but one of these (*Soc*243, *Biloxi Bay*), the inbreeding coefficient (F_{IS}) was positive, indicating a deficit in heterozygotes and possibly reflecting the presence of null alleles. All tests of pairwise genotypic disequilibrium were non-significant ($P > 0.05$) following sequential Bonferroni correction.

A total of 90 unique mtDNA haplotypes (sequences) were detected (Appendix Table 3). These included 29 haplotypes from 45 individuals in *Broodstock* and 73 haplotypes from 99 individuals in *Biloxi Bay*. Twelve haplotypes were common to both samples. The number of polymorphic sites were 52 (*Broodstock*) and 67 (*Biloxi Bay*). Nucleon diversities were 0.963 ± 0.0.016 (*Broodstock*) and 0.992 ± 0.003 (*Biloxi Bay*), and nucleotide diversity values were 0.027 ± 0.002 in both *Broodstock* and *Biloxi Bay*.

Exact tests of homogeneity in allele (genic) distributions between the two samples were significant prior to Bonferroni correction at seven of the 31 microsatellites; only one microsatellite (*Soc*412) remained significant following Bonferroni correction. Similar results were obtained in exact tests of genotype distributions. Fisher's method of combining probabilities from independent (exact) tests of all 31 microsatellites revealed a significant difference ($P = 0.000$) between samples in both allele and genotype distributions. Removal of those microsatellites whose genotype proportions were not in Hardy-Weinberg equilibrium (including *Soc*412) did not change these results appreciably. However, the overall F_{ST} (microsatellites) of 0.003 between samples was of borderline significance ($P = 0.050$), while the distribution of mtDNA haplotypes between samples was homogeneous ($F_{ST} = 0.010$, $P = 0.272$).

Results of the exclusion analysis (microsatellites only) are shown in Fig. 1. Each plot represents the number of individuals from the *Biloxi Bay* sample (*y* axis) that were *not* excluded as offspring from each of the nine broodtanks relative to the number of microsatellites (*x* axis) incorporated into the analysis. The nine plots represent each of the nine broodtanks and the six possible sire x dam combinations in each broodtank. As shown, exclusion profiles for each of the nine broodtanks are fairly similar and only 16 of the 31 available microsatellites were necessary to exclude all 102 fish from *Biloxi Bay* as having been produced from any of the sire x dam combinations in *Broodstock*. Inclusion of mtDNA reduced the number of microsatellites needed to exclude all *Biloxi Bay* fish from 16 to 13 (Fig. 2). However, largely because mtDNA in red drum is highly polymorphic, 77 of the 99 *Biloxi Bay* fish genotyped for mtDNA possessed a haplotype not found among *Broodstock* dams. Thus, the 13 microsatellites were needed for exclusion of only 22 individuals.

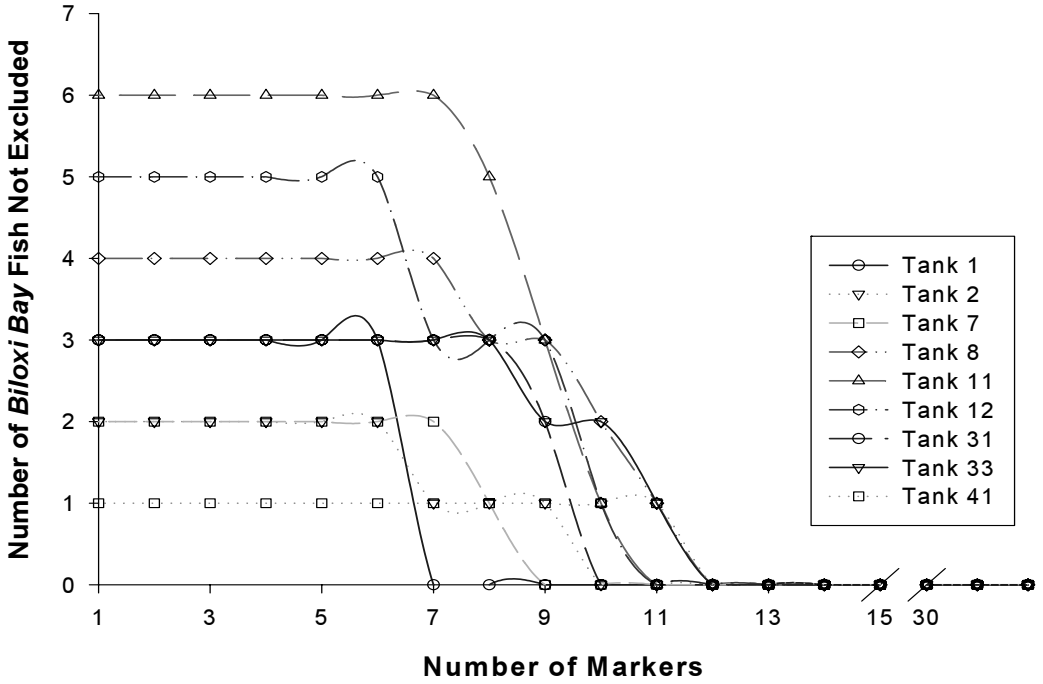
Fig. 1. Exclusion profiles: the number of *Biloxi Bay* fish not excluded (*y* axis) relative to the number of microsatellites required for exclusion (*x* axis). Each plot represents one of nine broodtanks, with six possible sire x dam combinations in each broodtank. Averages and standard deviations (*y* error bars) are indicated.



The heterozygote deficiency observed at five of the microsatellites (*Soc*44, *Soc*201, *Soc*401, *Soc*404, and *Soc*412), if due to null alleles, could potentially generate 'typing errors' and negatively impact genotype exclusion tests (Pemberton et al. 1995; Taylor et al. 1997; Marshall et al. 1998). Errors in 'parental' genotypes (*Broodstock* fish, in this case) can be critical, as they can potentially impact comparisons with every potential 'offspring' genotype; genotyping errors in 'offspring' (*Biloxi Bay* fish, in this case) will only impact assignment of each individual mistyped. PROBMAX-2 enables the user to specify the number of microsatellites at which mistyping may occur. The program then matches genotypes at the remaining microsatellites with all possible combinations of microsatellite alleles. A standard error rate of 5%, for example, would allow for mismatches at two of the 31 microsatellites used here. Incorporating a 5% error rate did not affect the outcome of exclusion analysis, as all *Biloxi Bay* fish were still successfully excluded. Repeated PROBMAX-2 runs with different error rates demonstrated that a typing error rate of nearly 48% (up to 15 microsatellites) could be considered without affecting the exclusion of all *Biloxi Bay* fish.

Exclusion analysis also was carried out without the microsatellites where genotype proportions did not conform to Hardy-Weinberg expectations in either *Broodstock* or *Biloxi Bay*. The results were the same as exclusion profiles when all 31 microsatellites were employed; 16 microsatellites were necessary without mtDNA to exclude all 102

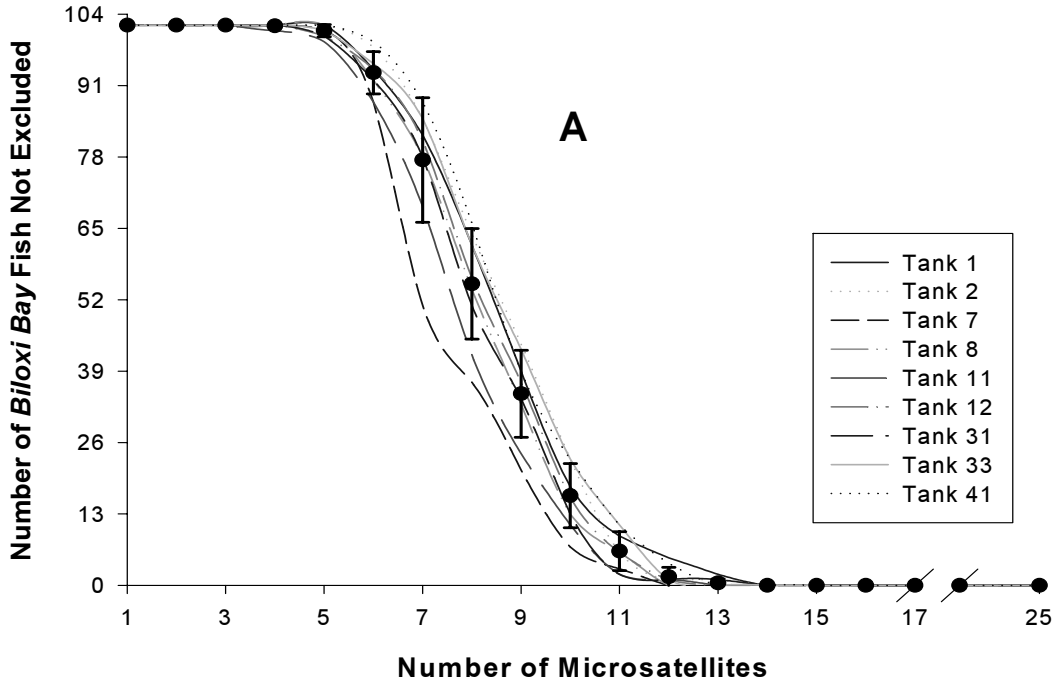
Fig. 2. Exclusion profiles: the number of *Biloxi Bay* fish not excluded (*y* axis) relative to the number of genetic markers (microsatellites and mtDNA) required for exclusion (*x* axis). Each plot represents one of nine broodtanks, with six possible sire x dam combinations in each broodtank.



Biloxi Bay fish (Fig. 3a), while 13 microsatellites were (again) needed when mtDNA was included (Fig. 3b).

Parental pair exclusion probabilities (*P* values) for each microsatellite are given in Table 1. Each *P* value represents the probability that allele frequencies estimated from the 'wild' fish in *Biloxi Bay* will exclude any individual parental pair sampled at random. *P* values for the 31 microsatellites are listed in the table from the largest to the smallest parental-exclusion probability. The highest *P* value (0.980479) is for *Soc*44 and means that ~ 98% of all potential sire x dam combinations are excluded; conversely, the lowest *P* value (0.411053) is for *Soc*206 and means that only ~ 41% of all potential sire x dam combinations are excluded. These exclusion probabilities can then be used to estimate the probability of not excluding a pair of non-parents (simply estimated as $[1 - P]$). The value $[1 - P]$ represents the probability of incorrectly assigning a 'wild' fish as having been produced by any possible sire x dam combinations. Assuming the microsatellites are inherited independently, the estimates of $[1 - P]$ can be combined to yield a cumulative probability. The values for $[1 - P]$ and the cumulative probabilities also are given in Table 1. Using all 31 microsatellites, the cumulative probability of incorrectly assigning one of the 'wild' fish from *Biloxi Bay* to a particular pair of parents is 3.38×10^{-26}. Removing the six microsatellites that failed to conform to Hardy-Weinberg genotype expectations in either sample reduced the probability of incorrect assignment to 2.58×10^{-19}.

Fig. 3. Exclusion profiles with microsatellites failing to conform to Hardy-Weinberg equilibrium expectations (*Soc*44, *Soc*201, *Soc*243, and *Soc*401, *Soc*404, *Soc*412) omitted. (A) Microsatellites (25) only; (B) Microsatellites (25) and mtDNA. Axes are as in Figs. 1 and 2. Averages and standard deviations (y error bars) are indicated for microsatellites (A).



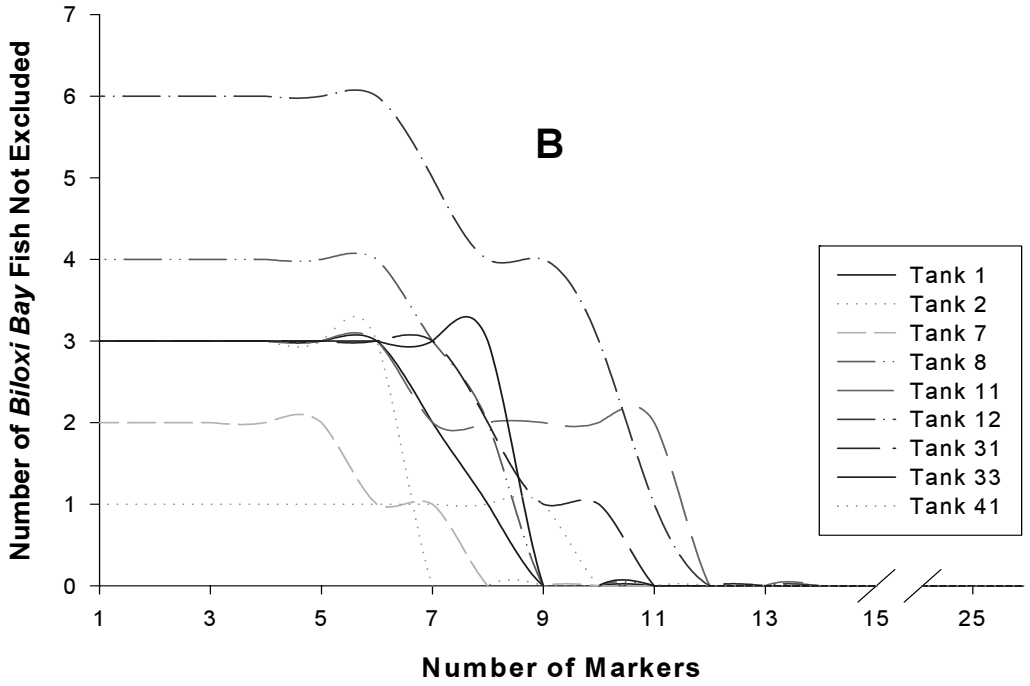


Table 1. Parental pair exclusion probabilities (*P* values) and cumulative probability of incorrectly assigning a *Biloxi Bay* fish [1 – *P*] as having been produced by any sire x dam combination. Probabilities are based on 31 microsatellites. Individual microsatellites are ranked from highest to lowest parent pair exclusion probability.

Microsatellite	Parent Pair (*P*)	Cumulative (1 – *P*)	Microsatellite	Pair Parent (*P*)	Cumulative (1 – *P*)
*Soc*44	0.980479	1.95×10^{-2}	*Soc*445	0.814598	1.44×10^{-19}
*Soc*428	0.980179	3.87×10^{-4}	*Soc*138	0.808236	2.76×10^{-20}
*Soc*404	0.980162	7.68×10^{-6}	*Soc*433	0.806873	5.32×10^{-21}
*Soc*412	0.955382	3.42×10^{-7}	*Soc*410	0.790577	1.11×10^{-21}
*Soc*99	0.947407	1.80×10^{-8}	*Soc*201	0.737766	2.93×10^{-22}
*Soc*19	0.937484	1.13×10^{-9}	*Soc*417	0.737483	7.67×10^{-23}
*Soc*423	0.925199	8.42×10^{-11}	*Soc*400	0.721149	2.14×10^{-23}
*Soc*402	0.921417	6.62×10^{-12}	*Soc*415	0.670224	7.06×10^{-24}
*Soc*401	0.9087 7	6.04×10^{-13}	*Soc*243	0.655686	2.43×10^{-24}
*Soc*416	0.883978	7.01×10^{-14}	*Soc*11	0.633953	8.16×10^{-25}
*Soc*83	0.873138	8.89×10^{-15}	*Soc*140	0.566945	3.54×10^{-25}
*Soc*419	0.871818	1.14×10^{-15}	*Soc*156	0.484788	1.82×10^{-25}
*Soc*85	0.854858	1.65×10^{-16}	*Soc*60	0.454885	9.93×10^{-26}
*Soc*407	0.850088	2.48×10^{-17}	*Soc*444	0.422665	5.73×10^{-26}
*Soc*432	0.826472	4.30×10^{-18}	*Soc*206	0.411053	3.38×10^{-26}
*Soc*424	0.819807	7.75×10^{-19}			

Parental pair exclusion probabilities (*P*) and 'incorrect assignment' probabilities [1 – *P*] when mtDNA is included are given in Table 2. Inclusion of mtDNA decreased the cumulative probability of incorrect assignment (all 31 microsatellites) to 1.33×10^{-27}. Removing the six microsatellites that failed to conform to Hardy-Weinberg genotype expectations in either sample increased the cumulative probability of incorrect assignment to 9.34×10^{-21}.

To further illustrate the power of the exclusion analysis approach, we also generated exclusion profiles (with and without mtDNA) that were based on the 486 possible sire x dam combinations had all 18 sires been crossed randomly with all 27 dams. Only 19 of the 31 microsatellites were necessary to exclude all *Biloxi Bay* fish in the absence of mtDNA data, while only 15 microsatellites were necessary when mtDNA data were included (Fig. 4). The situation where all possible pairwise combinations of broodfish are used to generate progeny is, of course, unlikely, given the facility requirements to hold this many adult red drum. However, the exclusion analysis profiles generated demonstrate quite adequately the power of exclusion analysis.

The foregoing demonstrated first, that all of the 'wild' fish sampled from Biloxi Bay could be excluded unequivocally as having been produced by the TPWD broodstock; and second, that the probability of misidentifying one of the 'wild' fish as having been produced by the broodstock ranged between 2.58×10^{-19} and 1.33×10^{-27}, depending on whether 25 or 31 microsatellites, with and without mtDNA, were used. However, supposing that a fish was *not* excluded as having come from *Broodstock*, the question arises as to whether it *could* have come from the 'wild' population. The 'genetic' approach to this question would be to ask how likely it would be to encounter a hatchery-produced genotype in the 'wild' population. To address this question, we computed the expected frequency of

Table 2. Parental pair exclusion probabilities (*P* values) and cumulative probability of incorrectly assigning a *Biloxi Bay* fish [1 – *P*] as having been produced by any sire x dam combination. Probabilities are based on 31 microsatellites and mitochondrial (mt)DNA. Individual genetic markers are ranked from highest to lowest parent pair exclusion probability.

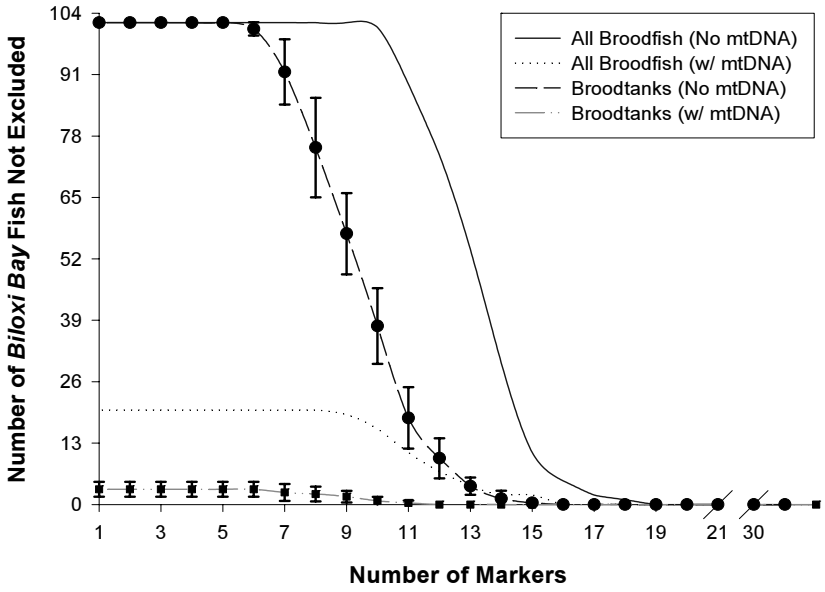
Marker	Parent Pair (*P*)	Cumulative (1 – *P*)	Marker	Parent Pair (*P*)	Cumulative (1 – *P*)
*Soc*44	0.980479	1.95×10^{-2}	*Soc424*	0.819807	2.80×10^{-20}
*Soc*428	0.980179	3.87×10^{-4}	*Soc*445	0.814598	5.19×10^{-21}
*Soc*404	0.980162	7.66×10^{-6}	*Soc*138	0.808236	9.96×10^{-22}
MtDNA	0.963855	2.77×10^{-7}	*Soc*433	0.806873	1.92×10^{-22}
*Soc*412	0.955382	1.24×10^{-8}	*Soc*410	0.790577	4.03×10^{-23}
*Soc*99	0.947407	6.51×10^{-10}	*Soc*201	0.737766	1.06×10^{-23}
*Soc*19	0.937484	4.07×10^{-11}	*Soc*417	0.737483	2.77×10^{-24}
*Soc*423	0.925199	3.04×10^{-12}	*Soc*400	0.721149	7.73×10^{-25}
*Soc*402	0.921417	2.39×10^{-13}	*Soc*415	0.670224	2.55×10^{-25}
*Soc*401	0.908767	2.18×10^{-14}	*Soc*243	0.655686	8.78×10^{-26}
*Soc*416	0.883978	2.53×10^{-15}	*Soc*11	0.633953	3.21×10^{-26}
*Soc*83	0.873138	3.21×10^{-16}	*Soc*140	0.566945	1.39×10^{-26}
*Soc*419	0.871818	4.12×10^{-17}	*Soc*156	0.484788	7.17×10^{-27}
*Soc*85	0.854858	5.98×10^{-18}	*Soc*60	0.454885	3.91×10^{-27}
*Soc*407	0.850088	8.96×10^{-19}	*Soc*444	0.422665	2.26×10^{-27}
*Soc*432	0.826472	1.55×10^{-19}	*Soc*206	0.411053	1.33×10^{-27}

the most common 'composite' genotype in each of the nine broodtanks by multiplying the observed frequencies of the most common observed genotype at each independent genetic marker. We then asked what would be the probability of recovering the most-common, hatchery-produced 'composite' genotype from the population in Biloxi Bay, based on the observed allele frequencies at each genetic marker in the sample from Biloxi Bay. The probabilities of finding a fish with the most common, hatchery-produced genotype (by broodtank) in Biloxi Bay are given in Table 3. These probabilities, by broodtank, ranged from 1.38×10^{-27} (Tank 41, 25 microsatellites, without mtDNA) to 2.98×0^{-42} (Tank 33, 31 microsatellites, with mtDNA). All other genotypes produced from the TPWD broodstock would thus occur among fish from Biloxi Bay at even lower (expected) frequencies. It is an important point to note that the inverse of the highest probability estimate (1.38×10^{-27}) is ~ 20 orders of magnitude larger than the estimated number of 6×10^6 (lower and upper-bound 90% confidence intervals of 4.4×10^6 and 7.7×10^6) adult red drum in the northern Gulf of Mexico (Nichols 1988; Mitchell and Henwood 1999).

DISCUSSION

The exclusion analyses and parental-pair exclusion probabilities indicated, respectively, that (i) only 16 microsatellites (13 if mtDNA was employed) were necessary to exclude all of the red drum sampled from Biloxi Bay as having been produced by TPWD broodfish, and (ii) the probability of incorrectly assigning a *Biloxi Bay* fish as having been produced by TPWD broodfish ranged from 2.58×10^{-19} (25 microsatellites in HW equilibrium) to 1.33×10^{-27} (all 31 microsatellites and mtDNA). Probabilities (with and without mtDNA) that the most-common, hatchery-produced 'composite' genotype would occur in the sample from Biloxi Bay ranged by broodtank from 1.38×10^{-27} to 2.98×10^{-42}.

Fig. 4. Exclusion profiles estimated with all possible combinations of 18 ♂♂ and 27 ♀♀ (486 total) or with the 54 possible combinations in the nine broodtanks (each broodtank with 2 ♂♂ and 3 ♀♀). Axes are as in Figs. 1 and 2. Averages and standard deviations (y error bars) are indicated for broodtanks (no mtDNA).



All of the probability values are several orders of magnitude smaller than the reciprocal of the total number of adult red drum (10^6–10^7) estimated to occur in the northern Gulf of Mexico (Nichols 1988; Mitchell and Henwood 1999).

In part because genotyping expenses, including labor, increase as a function of the number of genetic markers employed, we estimated the minimum number of genetic markers that would be appropriate for the case at hand. We began with the premise that the minimum number of markers would be 16

Table 3. Probabilities for each of nine broodtanks of finding a fish with the most common, hatchery-produced genotype in the 'wild' population from Biloxi Bay. Values are given for all 31 microsatellites (with and without mitochondrial DNA), and for the 25 microsatellites in Hardy-Weinberg equilibrium (with and without mitochondrial DNA).

	31 Microsatellites		25 Microsatellites	
Broodtank	No MtDNA	With MtDNA	No MtDNA	With MtDNA
Tank 1	1.38×10^{-38}	4.19×10^{-40}	8.41×10^{-30}	2.55×10^{-31}
Tank 2	2.27×10^{-38}	6.89×10^{-40}	4.28×10^{-27}	1.30×10^{-28}
Tank 7	8.03×10^{-37}	1.62×10^{-38}	7.76×10^{-28}	1.57×10^{-29}
Tank 8	9.53×10^{-40}	2.89×10^{-41}	3.81×10^{-30}	1.15×10^{-31}
Tank 11	2.45×10^{-38}	4.94×10^{-40}	1.42×10^{-28}	2.87×10^{-30}
Tank 12	2.60×10^{-34}	7.88×10^{-36}	1.43×10^{-26}	4.37×10^{-28}
Tank 31	1.54×10^{-36}	4.68×10^{-38}	2.80×10^{-28}	8.49×10^{-30}
Tank 33	1.48×10^{-40}	2.98×10^{-42}	6.64×10^{-31}	1.34×10^{-32}
Tank 41	1.83×10^{-37}	1.84×10^{-39}	1.38×10^{-27}	1.39×10^{-29}

microsatellites alone or 13 microsatellites plus mtDNA, given that 100% of the red drum sampled from Biloxi Bay were excluded with either marker set. Sequencing ~ 370 base pairs of DNA on an individual-by-individual basis would be more expensive and time consuming than genotyping an additional three microsatellites, particularly if the latter were multiplexed efficiently. However, inclusion of mtDNA alone in this situation resulted in exclusion of 77 of 99 *Biloxi Bay* fish (~ 78%), meaning that only 22 fish needed to be genotyped at 13 microsatellites for 100% exclusion, i.e., in this example, it would be more cost effective to sequence mtDNA fragments, then determine the number of individuals that needed to be assayed for microsatellite genotypes.

For probability levels of either incorrect assignment of *Biloxi Bay* fish as having come from *Broodstock* or for finding in Biloxi Bay the most common 'composite' genotype produced from the broodstock, we suggest that values of 10^{-10} or 10^{-15} would be more than sufficient to insure legal sale and avoid/alleviate potential conflicts. These values are still orders of magnitude smaller than the reciprocal of the estimated number of adult red drum in the northern Gulf of Mexico and are well within the range (10^{-9}–10^{-15}) of match-probability estimates generated for the 13 microsatellite markers validated for forensic use in humans (Chakraborty et al. 1999). The minimum number of microsatellites (from all 31 assayed) needed to attain probabilities of 10^{-10} and 10^{-15} of incorrectly assigning a fish from Biloxi Bay as having come from *Broodstock* were seven and twelve, respectively, without mtDNA, and six and ten, respectively, with mtDNA. Removing the six microsatellites that failed to conform to Hardy-Weinberg genotype expectations in either sample increased the minimum numbers to eight and fifteen (without mtDNA) and seven and thirteen (with mtDNA). The latter thirteen microsatellites are the same 13 microsatellites (plus mtDNA) needed for 100% exclusion. The number of microsatellites (with and without mtDNA) needed to obtaining probabilities of 10^{-10} and 10^{-15} that the most common, hatchery produced 'composite' genotype would occur in the sample from Biloxi Bay ranged by broodtank from three (several broodtanks and with mtDNA) to ten (two broodtanks and without mtDNA). Because different microsatellites were 'informative' across broodtanks, we estimated the minimum number of microsatellites needed over all nine broodtanks. For all 31 microsatellites, the minimum number to obtain probabilities of 10^{-10} and 10^{-15}, respectively, were six and ten (without mtDNA) and five and nine (with mtDNA); for just those microsatellites whose genotypes were in Hardy-Weinberg proportions, the minimum numbers were seven and twelve (without mtDNA) and six and ten (with mtDNA).

The foregoing demonstrates that unequivocally distinguishing red drum spawned from broodstock obtained offshore of Corpus Christi, Texas, from the 'wild' stock in Biloxi Bay, Mississippi, is fairly straightforward, given (i) a sufficient number of polymorphic (variable), independent genetic markers, (ii) the genotypes of the broodfish, and (iii) a survey of allelic variation at the genetic markers among representatives of the 'wild' stock. In the example documented here, thirteen microsatellites and a fragment of mtDNA sufficed to exclude 100% of sampled 'wild' fish from Biloxi Bay as having come from the broodstock, while the probabilities of (i) incorrectly assigning a 'wild' fish as having come from the broodstock, and (ii) finding the most common 'composite' genotype produced from the broodstock in the 'wild' were greater

than 10^{-15}. The three 'requirements' essentially would be the same for any offshore aquaculture operation where legal sale of the cultured species could be an issue. Two other issues remain, however, in terms of the broader applicability of the approach in both red drum and other marine fish species that might be cultured in offshore facilities.

The first issue is the applicability of the findings relative to offshore aquaculture of red drum at other localities in the northern Gulf of Mexico or elsewhere (e.g., the southeast coast of the U.S.). Several previous studies of spatial genetic variation among red drum along both the northern Gulf Coast and the U.S. South Atlantic Coast (Gold et al. 1999; Seyoum et al. 2000; Gold and Turner 2002) have shown that differences in microsatellite and mtDNA allele frequencies accumulate primarily as a function of geographic distance between geographic localities. Consequently, the number of genetic markers needed for forensic exclusion would likely be inversely related to the geographic distance between the location where broodfish were obtained and the location of the offshore aquaculture facility. The minimum number of markers required could then be estimated following a survey of the 'wild' stock at the locality for the same markers used to genotype the broodfish. One advantage in the case of offshore aquaculture of red drum (as opposed to other species) is that the genetic markers and the conditions for their assay already have been developed and tested (this paper).

The second issue is the applicability of findings here to other species. In brief, the approach taken here essentially would be the same for any species of interest. For most marine fish species, appropriate genetic markers, including primer sequences for PCR amplification, would likely need to be generated. The two other species of interest to the OAC were red snapper (*Lutjanus campechanus*) and cobia (*Rachycentron canadum*). Virtually no genetic data are available for cobia, meaning that genetic markers for this species would need to be generated *de nov*o. Both microsatellite (Gold et al. 2001) and mitochondrial DNA (Garber et al. 2004) markers have been developed for red snapper and employed in studies of geographic variation. Levels of variability in red snapper microsatellites were considerably less than those found in red drum. Observed heterozygosity (20 microsatellites) among red snapper sampled from four localities in the northern Gulf of Mexico averaged 0.542–0.609 (Gold et al. 2001), as compared to heterozygosities of 0.793 (*Broodstock*) and 0.787 (*Biloxi Bay*) for red drum studied here. Alternatively, Garber et al. (2004) sequenced an ~ 300 base pair fragment of the mtDNA control region from 140 red snappers from the northern Gulf of Mexico and found a nucleon diversity of 1.00 (each individual possessed a different mtDNA genotype). The lower levels of variability in red snapper microsatellites may indicate that more microsatellites than needed for red drum will likely need to be employed for forensic application in red snapper.

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APPENDIX

Table 1. PCR primer sequences (forward, top; reverse, bottom), repeat sequence (of the cloned allele), and annealing temperature (AT) for 31 microsatellites used to genotype (i) red drum broodstock at the CCA/CPL Marine Development Center in Corpus Christi, TX, and (ii) age 0 red drum sampled from Biloxi Bay, MS.

Micro-satellite	PCR primer sequence (5'→3')	Repeat sequence	AT
*Soc*11	GCCGAGTCACGAAGGAACAGAGAA		
	TGTCGTCTCATCTATCTCCATCTC	$(GA)_{11}$	62
*Soc*19	GGGTACAACTAAACAGACACAATA		
	TTTGAAAATGTTCCTGTGAATCAC	$(GATA)_{16}$	58
*Soc*44	GAGGGTGACGCTAACAGTTGA		
	CACAGCTCCACTCTGATATG	$(CA)_{22}$ $(GT)_5$	62
*Soc*60	TCTATTGAAGCCTGTAAGTTAGTT		
	CAAGGAAGGAGTGGGGAATGACAA	$(AGG)_8$	56
*Soc*83	TGCTGTAATTGAAAAGCAGTGTAC		
	AGCGGAACTAGAATTGGTTTTATA	$(TG)_{19}$	56
*Soc*85	TTTTGGACCTACACTAGAGTAGC		
	CGTGGGAGACTAGCGATGTAGAT	$(AC)_{17}$	58
*Soc*99	CACCCACTGACACACACATACAC		
	GGAACCAATATGTCTGCCATGAT	$(CA)_{29}$	62
*Soc*138	CTGGAGCTTTTCCCTTTCTGT		
	TGGGAGGAGAAGGCAGGAAGG	$(TGTC)_6$	58
*Soc*140	GGTGCAAACACAGCCATACAGT		
	GCAAAATCGAAGACCGAGTTTAG	$(CTGT)_8$	56
*Soc*156	CCTCTCCTTTCTCCATCAGTGC		
	AGCCCGGCTGTCATCTCCTGTA	$(CCT)_6$ $(TCC)_4$	58
*Soc*201	GGAGGAACTGATGAGGGCAGTGT		
	GCACAACACACCTCGCTATATC	$(CCT)_6$	58
*Soc*206	GTTTCCCACATCCCCCAACC		
	AGTTTGGTCGCTTTAAAGGC	$(GCAC)_5$	58
*Soc*243	GACGGGGATGCCATCTGC		
	AATGCGAAAAAGACGAAACAGT	$(CCT)_9$	56
*Soc*400	TGCCATTGTCATTCTACAGAGC		
	TTATAGTGGGGTGAGTGTTTGA	$(CA)_{19}$	52
*Soc*401	ACGTCTTAATCGGTCTCTGTCC		
	ATCTCTGTGTGAAAGGAAAACA	$(TG)_{14}$	52
*Soc*402	CATATTTAACGAGCGACATAGC		
	AAACAGATGAAGCACCTGGACT	$(CA)_{20}$	52
*Soc*404	AGACCCTTTTGTTGATTTCATA		
	ATGACTGCACCATTTCAAAAAG	$(TG)_{23}$	52
*Soc*407	AAAGTCTGCCTCTTACAGCTTC		
	GAGTTAAAGCGTGTGCTAGTCC	$(CA)_{13}$	56
*Soc*410	GTACCAAGTCAGCCAGTGTCAG		
	TCTCTGTGTCCCTCTGTGTTTG	$(TG)_{17}$	56

Appendix Table 1. continued.

Micro-satellite	PCR primer sequence (5'→3')	Repeat sequence	AT
*Soc*412	CACAGAAACTCAGCTCGAGACC		
	AGGAAGAATGTACAAGGTGTTC	$(AC)_{13}$	49
*Soc*415	CTCAGCACCCTCAGACATATGG		
	CACAAGTTAAGTGGTATCGAGT	$(TG)_{15}$	52
*Soc*416	CTCGATACCACTTAACTTGT		
	ATCGACATAATCTGGCACCA	$(GA)_{38}$	49
*Soc*417	CTTACGTGATAAAGTGTGGGTGA		
	ATATGCCAGTAATCCACCGAAG	$(AC)_{24}$	49
*Soc*419	ATTTAGCCAACTGCTCCGCTCA		
	GAGTGCGTGGTGTAGGGGGGTA	$(AC)_{20}$	56
*Soc*423	GTCACCGCACCATGATGGAGAT		
	TACCACTTACACTCAGCAGGTG	$(CA)_{26}$	54
*Soc*424	CACTCTTCATCCCTCACTCGTC		
	TTCGATGGGTGACAGCGTCAGG	$(CA)_{15}$	56
*Soc*428	GACATCGCATTTGTCTACAGAGTCG		
	AACTCCCAGTCATAATATCCCTTT	$(TG)_{38}$	53
*Soc*432	TTTAGGCTACGTCTGGAGGCACA		
	GTGTGTTTGAGGGTCAGCGTAC	$(AC)_{16}$	52
*Soc*433	AGTACGCTGACCCTCAAACACA		
	TTCTCTTTGCCTCCTTTTTCCCTGA	$(TG)_{16}$	52
*Soc*444	TGAACTAATCCAGCCACAGATG		
	CACAGCCGATTAAAGAGAGGGAAT	$(TG)_{17}$	
*Soc*445	ATACAAAGGGACTCTCATACTCTC		
	TTTTAATCCCATTACAGCTTT	$(TCC)_{10}$	

Appendix Table 2. Summary statistics at 31 nuclear-encoded microsatellites in two samples of red drum (Sciaenops ocellatus). N = sample size, #A = number of alleles, AR = allelic richness, H_E = gene diversity (expected heterozygosity), P_{HW} = probability of conformity to Hardy-Weinberg genotypic expectations, and F_{IS} = inbreeding coefficient.

Sample		*Broodstock*	*Biloxi Bay*	Sample		*Broodstock*	*Biloxi Bay*
*Soc*11	N	45	102	*Soc*19	N	45	102
	#A	10	11		#A	15	16
	AR	9.86	8.01		AR	14.82	14.18
	H_E	0.729	0.664		H_E	0.909	0.908
	P_{HW}	0.709	0.932		P_{HW}	0.024	0.774
	F_{IS}	–0.006	–0.019		F_{IS}	0.071	0.050
*Soc*44	N	43	99	*Soc*60	N	45	102
	#A	23	28		#A	5	7
	AR	23.00	24.51		AR	4.96	5.59
	H_E	0.941	0.954		H_E	0.577	0.591
	P_{HW}	0.371	**0.000***		P_{HW}	0.752	0.407
	F_{IS}	0.036	0.195		F_{IS}	0.114	0.021
*Soc*83	N	45	102	*Soc*85	N	45	102
	#A	13	14		#A	14	14
	AR	12.73	12.95		AR	13.91	12.40
	H_E	0.835	0.850		H_E	0.880	0.832
	P_{HW}	0.687	0.413		P_{HW}	0.102	0.764
	F_{IS}	–0.037	0.066		F_{IS}	0.065	-0.014
*Soc*99	N	45	101	*Soc*138	N	45	101
	#A	22	23		#A	12	13
	AR	21.73	19.31		AR	11.90	10.78
	H_E	0.934	0.913		H_E	0.829	0.812
	P_{HW}	0.663	0.251		P_{HW}	0.207	0.991
	F_{IS}	0.000	0.045		F_{IS}	–0.073	–0.024
*Soc*140	N	45	102	*Soc*156	N	45	102
	#A	4	7		#A	4	5
	AR	4.00	6.21		AR	3.96	4.09
	H_E	0.629	0.622		H_E	0.457	0.591
	P_{HW}	0.280	0.794		P_{HW}	0.018	0.007
	F_{IS}	–0.237	–0.040		F_{IS}	–0.166	0.037
*Soc*201	N	43	102	*Soc*206	N	45	102
	#A	10	12		#A	6	5
	AR	10.00	10.05		AR	5.95	4.63
	H_E	0.703	0.739		H_E	0.554	0.541
	P_{HW}	0.401	**0.000***		P_{HW}	0.028	0.083
	F_{IS}	0.107	0.257		F_{IS}	–0.044	0.039

Appendix Table 2. continued.

Sample		*Broodstock*	*Biloxi Bay*	Sample		*Broodstock*	*Biloxi Bay*
*Soc*243	N	45	102	*Soc*400	N	45	102
	#A	5	7		#A	8	10
	AR	4.96	5.81		AR	7.91	8.84
	H_E	0.762	0.726		H_E	0.729	0.742
	P_{HW}	0.244	**0.000***		P_{HW}	0.688	0.663
	F_{IS}	0.037	–0.094		F_{IS}	0.146	0.088
*Soc*401	N	45	101	*Soc*402	N	45	100
	#A	12	16		#A	15	17
	AR	11.86	13.79		AR	14.77	14.55
	H_E	0.859	0.882		H_E	0.885	0.885
	P_{HW}	0.447	**0.000***		P_{HW}	0.250	0.039
	F_{IS}	0.147	0.270		F_{IS}	0.046	–0.051
*Soc*404	N	45	102	*Soc*407	N	45	100
	#A	24	34		#A	10	12
	AR	23.68	28.54		AR	9.91	10.29
	H_E	0.915	0.952		H_E	0.852	0.844
	P_{HW}	0.006	**0.001***		P_{HW}	0.250	0.001
	F_{IS}	0.150	0.135		F_{IS}	–0.069	0.123
*Soc*410	N	45	100	*Soc*412	N	45	100
	#A	14	16		#A	24	26
	AR	13.86	12.87		AR	23.51	22.31
	H_E	0.810	0.753		H_E	0.916	0.921
	P_{HW}	0.131	0.014		P_{HW}	**0.000***	**0.000***
	F_{IS}	0.122	0.031		F_{IS}	0.102	0.175
*Soc*415	N	45	102	*Soc*416	N	45	99
	#A	12	18		#A	16	18
	AR	11.86	13.61		AR	15.86	16.75
	H_E	0.717	0.636		H_E	0.862	0.837
	P_{HW}	0.556	0.140		P_{HW}	0.370	0.149
	F_{IS}	0.070	0.029		F_{IS}	0.150	0.070
*Soc*417	N	45	102	*Soc*419	N	45	100
	#A	11	13		#A	11	14
	AR	10.90	10.61		AR	10.91	10.89
	H_E	0.765	0.740		H_E	0.860	0.857
	P_{HW}	0.258	0.152		P_{HW}	0.888	0.705
	F_{IS}	–0.017	–0.034		F_{IS}	–0.060	0.055
*Soc*423	N	45	101	*Soc*424	N	45	102
	#A	18	19		#A	13	19
	AR	17.69	16.22		AR	12.95	15.29
	H_E	0.891	0.894		H_E	0.850	0.780
	P_{HW}	0.110	0.018		P_{HW}	0.315	0.028
	F_{IS}	0.003	0.092		F_{IS}	0.007	0.082

Appendix Table 2. continued.

Sample		*Broodstock*	*Biloxi Bay*	Sample		*Broodstock*	*Biloxi Bay*
*Soc*428	N	45	101	*Soc*432	N	45	102
	#A	27	32		#A	9	10
	AR	26.77	26.66		AR	8.91	8.74
	H_E	0.957	0.953		H_E	0.817	0.828
	P_{HW}	0.249	0.204		P_{HW}	0.871	0.165
	F_{IS}	0.001	0.065		F_{IS}	–0.061	0.112
*Soc*433	N	45	101	*Soc*444	N	45	102
	#A	10	12		#A	3	5
	AR	9.91	10.21		AR	3.00	4.47
	H_E	0.837	0.804		H_E	0.509	0.555
	P_{HW}	0.188	0.195		P_{HW}	0.526	0.183
	F_{IS}	–0.035	0.003		F_{IS}	–0.179	0.046
*Soc*445	N	45	101				
	#A	10	11				
	AR	9.96	9.80				
	H_E	0.815	0.795				
	P_{HW}	0.132	0.006				
	F_{IS}	0.045	0.153				

* Significant following Bonferroni correction (in boldface).

Appendix Table 3. Distribution of mitochondrial (mt)DNA haplotypes (sequences) in two samples of red drum (*Sciaenops ocellatus*). Data are from 369 base pairs of the mtDNA control region. GenBank Accession Numbers (in sequence) are AY 578986–AY 579075.

Haplotype	*Broodstock*	*Biloxi Bay*	Total	Haplotype	*Broodstock*	*Biloxi Bay*	Total
1	7	3	10	46	0	1	1
2	1	0	1	47	0	1	1
3	1	0	1	48	0	1	1
4	1	0	1	49	0	4	4
5	1	0	1	50	0	1	1
6	1	3	4	51	0	1	1
7	2	0	2	52	0	1	1
8	2	1	3	53	0	1	1
9	2	1	3	54	0	1	1
10	4	2	6	55	0	1	1
11	2	0	2	56	0	1	1
12	1	0	1	57	0	1	1
13	1	1	2	58	0	2	2
14	4	3	7	59	0	1	1
15	1	1	2	60	0	1	1
16	1	0	1	61	0	5	5
17	1	0	1	62	0	1	1
18	1	3	4	63	0	1	1
19	1	0	1	64	0	1	1
20	1	0	1	65	0	1	1
21	1	0	1	66	0	1	1
22	1	0	1	67	0	1	1
23	1	2	3	68	0	1	1
24	1	1	2	69	0	1	1
25	1	0	1	70	0	1	1
26	1	0	1	71	0	1	1
27	1	0	1	72	0	1	1
28	1	1	2	73	0	1	1
29	1	0	1	74	0	1	1
30	0	1	1	75	0	1	1
31	0	1	1	76	0	1	1
32	0	1	1	77	0	1	1
33	0	1	1	78	0	1	1
34	0	2	2	79	0	1	1
35	0	1	1	80	0	1	1
36	0	2	2	81	0	1	1
37	0	1	1	82	0	1	1
38	0	2	2	83	0	1	1
39	0	1	1	84	0	1	1
40	0	3	3	85	0	2	2
41	0	2	2	87	0	1	1
42	0	1	1	87	0	1	1
43	0	1	1	88	0	1	1
44	0	2	2	89	0	1	1
45	0	1	1	90	0	1	1
				Total	29	73	90