Abstract—The evolutionary associations between closely related fish species, both contemporary and historical, are frequently assessed by using molecular markers, such as microsatellites. Here, the presence and variability of microsatellite loci in two closely related species of marine fishes, sand seatrout (*Cynoscion arenarius*) and silver seatrout (*C. nothus*), are explored by using heterologous primers from red drum (*Sciaenops ocellatus*). Data from these loci are used in conjunction with morphological characters and mitochondrial DNA haplotypes to explore the extent of genetic exchange between species offshore of Galveston Bay, TX. Despite seasonal overlap in distribution, low genetic divergence at microsatellite loci, and similar life history parameters of *C. arenarius* and *C. nothus*, all three data sets indicated that hybridization between these species does not occur or occurs only rarely and that historical admixture in Galveston Bay after divergence between these species was unlikely. These results shed light upon the evolutionary history of these fishes and highlight the genetic properties of each species that are influenced by their life history and ecology.

Evolutionary associations between sand seatrout (*Cynoscion arenarius*) and silver seatrout (*C. nothus*) inferred from morphological characters, mitochondrial DNA, and microsatellite markers

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The molecular genetic associations between populations of sand seatrout (*Cynoscion arenarius*) and silver seatrout (*C. nothus*) have not been specifically examined on a large scale with DNA methods despite the close ties between the respective fisheries for the two species. In particular, the possibility of contemporary hybridization or historical admixture between these species remains to be explored by using a large panel of unlinked DNA markers. Sand and silver seatrout are so morphologically similar that they are collectively known as white trout by fishermen (Ginsburg, 1931). Both species are abundant throughout the Gulf of Mexico (hereafter, GOM); the distribution range for sand seatrout extends into the Atlantic Ocean, north to Georgia, and the distribution range for silver trout extends to Massachusetts (Hildebrand and Schroeder, 1928; Cordes and Graves, 2003). These seatrout make up a modest proportion of bycatch in shrimp and other commercial trawl operations (Warren, 1981), although commercial landings have decreased dramatically in the last 30 years (Fig. 1). Weinstein and Yerger (1976) completed perhaps the most comprehensive study of molecular evolution in the genus *Cynoscion*; they assessed protein electrophoresis variants in all four western North Atlantic

species (*C. arenarius*, *C. nothus*, spotted seatrout [*C. nebulosus*], and gray weakfish [*C. regalis*]). Although these methods provided some insight into the evolutionary relationships among the species, the data of Weinstein and Yerger (1976) were insufficient to answer direct questions about rates of contemporary and recent historical gene flow within and among *Cynoscion* species. Enzyme electrophoresis has since been superceded by DNA-based methods on a broad scale. Microsatellite markers are likely more sensitive for studies involving high rates of gene flow and low levels of population identity (Wright and Bentzen, 1994). This is particularly true for marine fishes, whose populations are often characterized by enormous census sizes and higher rates of migration between subpopulations than freshwater and terrestrial organisms (DeWoody and Avise, 2000).

Previous morphological comparisons of sand and silver seatrout have resulted in a suite of distinct characteristics that vary between species in larval (Ditty, 1989) and adult stages (Ginsburg, 1929, 1931; Gunter, 1945; Moshin, 1973; Chao, 2002). However, both display a similar streamlined and fusiform body shape, and the ranges of numerous commonly used morphometric and meristic measures

overlap between the species. Additionally, hybridization, regional differentiation, or a combination of both, may often confound the trademark diagnostics used to distinguish between the two species. In any event, the superficial similarity of these species indicates that morphological divergence has been minimal and the concurrent bimodal timing of spawning indicates overlapping life history parameters (Sheridan et al., 1984). Distributional data seem to indicate that these species exhibit some habitat partitioning (primarily by water depth and distance from the shore) with the result that silver seatrout are found more frequently in deeper water farther from shore (Ginsburg, 1931; Byers, 1981).

These life history and distributional data yield a framework for devising hypotheses to test the influence of niche overlap on historical associations between sand and silver seatrout populations. In particular, if hybridization between these species occurs, it is likely to occur in areas of contact such as nearshore marine waters used commonly by both species. Hybridization in the genus *Cynoscion* has been previously documented on the Atlantic coast of Florida (Cordes and Graves, 2003). In their initial examinations, Cordes and Graves (2003) characterized populations of gray weakfish using genetic techniques and in doing so also identified putative hybrids between gray weakfish and either sand or silver seatrout. This identification was accomplished by using four microsatellite markers and two nuclear intron gene regions (restriction fragment length polymorphisms, or RFLP's). Although these markers were appropriate for identification of hybrids with gray weakfish, they were ineffective for determining conclusively whether the second gametic contribution was made by sand or silver seatrout.

Here, both morphological and molecular (nuclear microsatellites and mitochondrial restriction fragments) data are used to characterize populations of sand and silver seatrout from the nearshore Gulf waters outside of Galveston Bay, Texas. Three competing hypotheses regarding genetic associations between these species are evaluated. First, in the case of contemporary hybridization, hybrids would appear as proportionate admixtures of both parental forms in microsatellite assignment tests. Moreover, the directionality of hybridization could be assessed with the use of mtDNA haplotypes (Wirtz, 1999), and hybrids would likely be found to be intermediate for diagnostic morphological characters (Hubbs, 1955; Campton, 1987). Second, in the case of historical association, such as lineage overlap during speciation or lineage admixture after speciation, mtDNA haplotypes might be shared between the species despite a mutually exclusive assignment of microsatellite genotypes (with the assumption of no contemporary hybridization). In such a case, assignment based on microsatel-
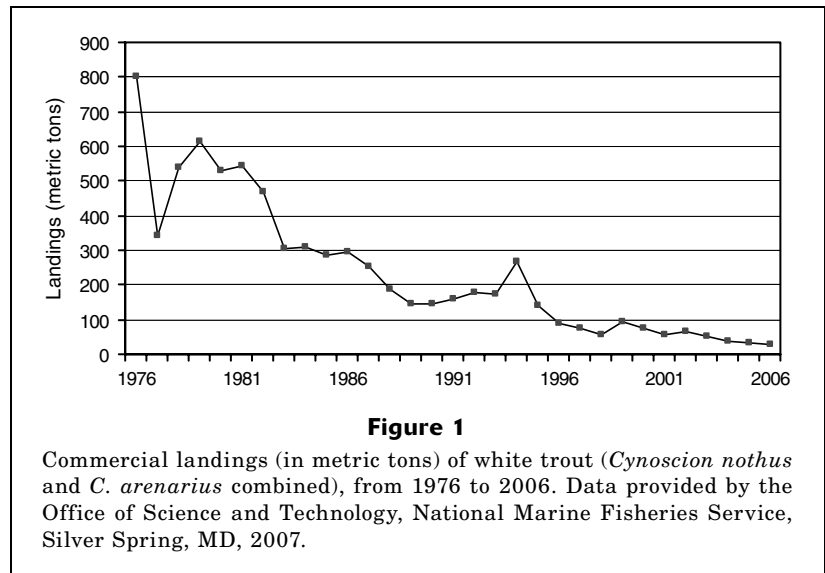

Figure 1

Commercial landings (in metric tons) of white trout (*Cynoscion nothus* and *C. arenarius* combined), from 1976 to 2006. Data provided by the Office of Science and Technology, National Marine Fisheries Service, Silver Spring, MD, 2007.

lite genotypes should be more reliable than assignment by means of mtDNA haplotypes if mtDNA lineages have not been sorted categorically into contemporary populations (species). Third, in the case of no gene flow between the species, microsatellite assignment should be conclusive, mtDNA haplotypes should sort conclusively by species, and specimens should not reveal morphological intermediates for characters previously described as diagnostic among species. Each of these competing hypotheses was examined in light of evidence from the three data sets. The morphological and genetic similarities and differences between the species were examined as evidence for hybridization, and as an aid for future species identification. Finally, aspects of the ecology and life history of each species are invoked to explain the patterns of genetic variability within and between these congeneric species.

Materials and methods

Sample collection and laboratory methods

In July of 2007, whole fish were collected offshore of Galveston Bay, TX, during annual routine monitoring by the Texas Parks and Wildlife, Coastal Fisheries Division. White trout were collected with a 5.7-m otter trawl with 38-mm nylon multifilament mesh stretched throughout. Trawl tows (n=4) were conducted parallel to the fathom curve at a speed of three mph for ten minutes (Fig. 2). After collection, fish were frozen and transported to the Perry R. Bass Marine Fisheries Research Station in Palacios, Texas, for processing.

The sample consisted of 60 young adult sand seatrout and 60 young adult silver seatrout. A single researcher completed all morphological and meristic counts because considerable risk of extraneous variance has been demonstrated in data collected by mul-

tiple individuals (Palmeirim, 1998). Morphometric and meristic measurements were taken on the left side of each fish. In the event that fins or scales were damaged, the right side was used, and no measurements were taken if both sides were damaged. Body weight, standard length (SL), pectoral-fin length, pelvic-fin length, anal-fin base length, and eye diameter were measured in each specimen. Weight was taken in grams (g) and length measurements were taken in millimeters (mm). Unless otherwise indicated, statistical analyses were performed with SAS software (vers. 8.02, SAS Inst., Inc., Cary, NC). Differences in weight and length between the species were assessed with a Satterthwaite *t*-test for unequal variances (after failure of an equality-of-variance test). Length and weight were log-transformed to normalize extreme observations, and the interspecific difference between length-to-weight ratios was tested with a pooled *t*-test of transformed values. Meristic counts were made with a dissecting microscope and included anal-fin soft rays and lateral-line scale counts. For anal-fin soft-ray counts, the last branched soft ray was counted as one ray (McEachran and Fechhelm, 2005). Four commonly used diagnostics were evaluated for identification to species. The ratios of pectoral-fin length to pelvic-fin length (Chao, 2002) and also the ratio of anal-fin base-length to eye-diameter (DeVries and Chittenden, 1982) were calculated, and differences between species were assessed with a pooled *t*-test on untransformed data. Anal-fin soft rays (Ginsburg, 1929) and lateral-line scales (Hoese and Moore, 1998) were counted, and differences between these meristics were assessed with a chi-square test of homogeneity. Following morphological analyses, dorsal-fin soft tissue was excised from each specimen and placed in 70% denatured ethanol.

Total genomic DNA was extracted from each fin-clip with a Puregene® miniprep kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. The mtDNA methods used here were similar to those of Cordes et al. (2001) and Cordes and Graves (2003). A portion of the 12S/16S ribosomal gene locus of the mtDNA was amplified by polymerase chain reaction (PCR) with the primers 12SAL and 16SAH (Cordes et al., 2001). Amplification products were run through a 2% agarose gel next to a size standard spanning the ranges of 100–1500 base pairs (bp) to verify expected fragment length. Each amplicon was then digested with the restriction enzyme RsaI (New England Biolabs, Inc., Ipswich, MA) according to the standard
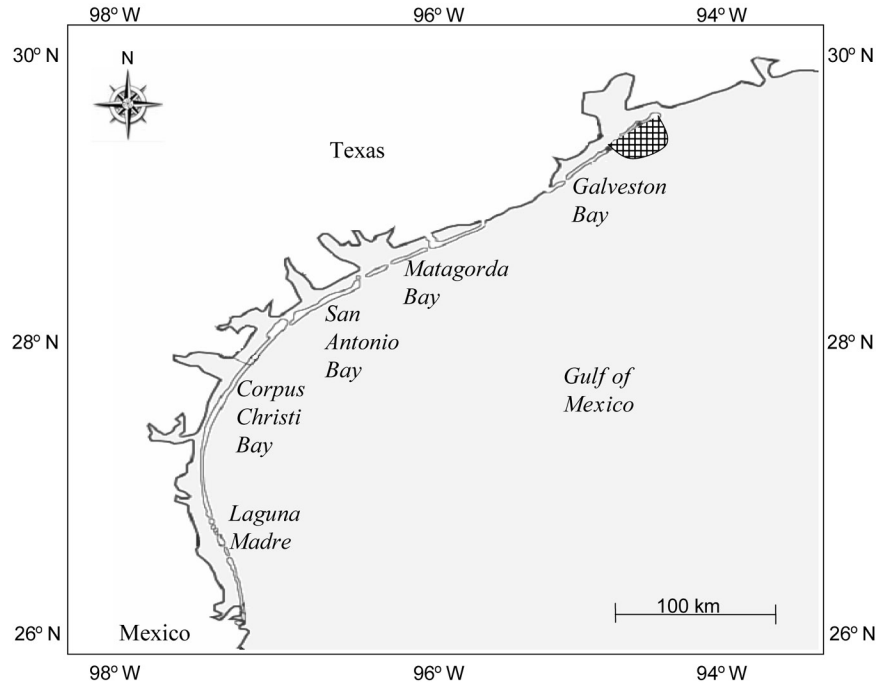

Figure 2

Map of the Texas gulf coast, showing the study area (hatched area), as well as major bays and inlets of Texas provided as points of reference. Trawls were collected in the offshore region of the gulf coast adjacent to Galveston Bay, TX, and sand seatrout (*C. arenarius*) and silver seatrout (*C. nothus*) were retained from each trawl.

protocol of the supplier, and restriction fragments were separated on a 2% agarose gel at 100 volts for 1 hour. A size standard was loaded onto each gel in order to approximate the size RFLPs. Gels were stained with ethidium bromide and RFLP bands were made visible (i.e., fluoresced) under an ultraviolet lamp.

Before initiation of this study, few microsatellite markers had been effectively characterized in the literature for sand or silver seatrout. However, a number of markers had been identified in a genomic library from the closely related sciaenid red drum (*Sciaenops ocellatus*) (Turner et al., 1998; Saillant et al., 2004), and some of these markers have subsequently been used successfully in members of the genus *Cynoscion* (Gold et al., 2003; Ward et al., 2007). Here, sixteen previously described microsatellite markers were chosen for examination: *SOC12*, *SOC50*, *SOC77*, *SOC85*, *SOC125*, and *SOC243* (Turner et al., 1998), *CNE133* (Gold et al., 2003), *SOC410*, *SOC412*, *SOC415*, *SOC416*, *SOC419*, *SOC423*, *SOC424*, *SOC428*, and *SOC432* (Saillant et al., 2004). Eight individuals of each species were genotyped with the suggested primers in each case. Each reverse oligonucleotide was previously labeled with a WellRED dye (Proligo USA LLC, Boulder, CO), and amplified products were produced at each locus by means of PCR. Products from individual reactions were diluted 1:20 with water and separated with a Beckman-Coulter CEQ™ 8000 automated capillary system (Beckman Coulter, Inc., Fullerton, CA), according

Table 1

Allele size ranges, in total DNA base pairs, for the microsatellite markers used to characterize populations of *Cynoscion arenarius* (sand seatrout) and *C. nothus* (silver seatrout) from offshore Galveston, TX, in July 2007. Each marker is listed by name as defined in the reference paper. The size range for *Sciaenops ocellatus* (red drum) was obtained from Saillant et al. (2004) and is included for reference.

Name	Allele range *C. arenarius*	Allele range *C. nothus*	Allele range *S. ocellatus*	Reference
SOC050	161–197	173–191	183–183	Turner et al., 1998
SOC243	100–112	106–127	94–106	Turner et al., 1998
SOC410	301–323	299–305	306–344	Saillant et al., 2004
SOC412	117–171	117–147	102–168	Saillant et al., 2004
SOC415	187–295	171–207	187–235	Saillant et al., 2004
SOC416	134–206	142–170	141–181	Saillant et al., 2004
SOC419	232–268	224–252	238–260	Saillant et al., 2004
SOC428	163–167	165–167	172–242	Saillant et al., 2004
SOC432	98–118	90–128	94–132	Saillant et al., 2004

to the manufacturer's protocol, and a 400-bp internal size standard (Beckman Coulter, Inc., Fullerton, CA) was included for fragment sizing. Each microsatellite locus was evaluated by a prioritized set of criteria: 1) amplification of the PCR product resulted in adequate signal intensity, 2) there were no excessive stepwise "stutter" bands preceding actual allele peaks, and 3) allelic polymorphism was present in both species. From the initial set of 16 loci, nine satisfied these criteria and consistently amplified a product (Table 1). Each experimental individual, for both species, was then genotyped at these nine loci.

Analysis of microsatellite data

The program FSTAT (Goudet, 1995) was used to calculate allele diversity (number of alleles per locus), gene diversity, and conformity with Hardy-Weinberg expectations at each locus within each population. The latter was approximated by testing the significance of the statistic F_{is} (Weir and Cockerham, 1984), which can be described as the within-population inbreeding coefficient. Significant departure of F_{is} from 0 represents significant deviation from Hardy-Weinberg expectations. FSTAT was used to detect the presence of linkage disequilibrium between loci within populations by using a nominal level of $\alpha = 0.05$ and Bonferroni adjustment for multiple tests. Finally, FSTAT was used to estimate genetic divergence between species as θ (Weir and Cockerham, 1984) at each locus and overall. The significance of θ was assessed by using the exact G-test of Goudet et al. (1996) with 1000 randomizations and an arbitrary $\alpha = 0.05$.

In order to determine which microsatellites were the most informative for species assignment, we used the critical population method of Banks et al. (2003) in assessing the discriminatory power of individual markers. The freeware program WHICHLOCI (Banks et al., 2003) was used to generate ten random sand seatrout populations (*n*=1000) based on empirical allele frequency data. These populations were used in simulated assignment procedures with constant assignment stringency (95% correct assignment of group members, 5% mis-assigned to critical population) and two conservative log odds ratio (LOD) assignment scores (LOD 2 and 3). The LOD assignment stringency is the log of the predetermined acceptable ratio of correctly assigned to incorrectly assigned individuals (thus LOD of 2=log10 of the ratio 100:1). The critical population (sand seatrout) used for simulations was also a conservative selection because it was chosen after observation of trial runs to discern which population routinely needed higher numbers of loci for correct assignment. The output from these simulations included a list of loci ranked by discriminatory power of assignment, the locus score based on both type-I and type-II errors, and the relative score of each locus weighted by the overall additive score of the entire microsatellite panel.

To identify hybrids resulting from crosses between these species, we used the Bayesian framework of Pritchard et al. (2000). The freeware program STRUCTURE (Pritchard et al., 2000) attempts to estimate the number of genetic clusters present while simultaneously assigning individuals to groups. This is done in part through progressive minimization of linkage disequilibrium and Hardy-Weinberg disequilibrium in iterative Markov chain Monte Carlo steps. Three sets of data were used in independent runs. In the initial run, we used data from the six highest-ranked microsatellite loci from WHICHLOCI analyses. A second run included data from all nine loci. Finally, a third run included the six highest-ranked loci, but mtDNA haplotype data were used to assign individuals *a priori* and microsatellite data were used to improve assignment. In each case, model parameters and run-times were specified as follows. The burn-in phase was set at 25,000 iterations and runs lasted 175,000 iterations under the admixture model. These values were chosen after inspection of model parameter normalization in preliminary runs. The Dirichlet parameter (α) was inferred from the data and was allowed to vary between populations. Allele

Table 2

Morphometric and meristic differences between two seatrout species, sand seatrout (*Cynoscion arenarius*) and silver seatrout (*C. nothus*), collected in trawls from Galveston Bay, TX, in July 2007. The range includes the overall range of data observations (*n*), followed by the mean and SE (standard error). The significance of each comparison was calculated by using either a standardized *t*-test (indictated as a lower-case a) or a chi-square test of homogeneity (indicated as a lower-case b).

		C. arenarius			*C. nothus*		
	n	Range	Mean ±SE	*n*	Range	Mean SE	*P*-value
Size measurements							
Standard length (mm)	60	83–175	107.9 ±8.3	60	100–163	119.5 ±4.7	<0.0001 [a]
Body weight (g)	60	10–95	26.4 ±7.3	60	20–75	33.4 ±4.1	0.0015 [a]
Ratios							
Pectoral fin/pelvic fin	60	0.9–1.2	1.10 ±0.04	48	0.9–1.3	1.09 ± 0.04	0.5978 [a]
Anal-fin base/eye diameter	60	1.2–2.4	1.59 ± 0.10	60	0.9–1.2	1.01 ± 0.03	<0.0001 [a]
Meristics							
Anal-fin soft rays	60	10–12	10.9 ± 0.2	60	8–9	8.8 ± 0.2	<0.0001 [b]
Lateral-line scales	57	57–61	59.3 ± 0.6	56	57–63	59.5 ± 0.8	0.9151 [b]

frequencies were assumed to be independent between populations. These model parameters were tested with a set $K = 2$, representing the two species as possible genetic contributors for each individual. The program was run under these model conditions for six trials to check for stability of resulting admixture coefficients (*Q*-values).

In order to evaluate the significance of individuals with extreme admixture coefficients values of *Q*, a set of simulated populations of 1000 individuals from each species was generated from allele-frequency data with WHICHLOCI. We analyzed these populations with STRUCTURE using identical model parameters to those in the experimental populations; the probability of obtaining a higher estimated level of admixture for any test individual was estimated as the frequency of higher admixture scores in the simulated population.

Results

Sample statistics and morphological characters

Samples of sand seatrout were collected from a combination of three grids, each of which was roughly one km from shore. The three grids were located at depths of three (one grid) and four (two grids) fathoms. For comparative purposes, trawl data for each species was combined and treated as a single random sample. All individual silver seatrout were collected from a single grid offshore from Galveston Bay. The grid was two km from land and had a depth of seven fathoms. The sand seatrout sample contained specimens that were significantly smaller than those obtained in the silver seatrout sample, in both mean standard length ($t=-4.74$, $P<0.0001$) and mean weight ($t=-3.27$, $P=0.0015$) (Table 2). The difference in mean size was not likely caused by gear selectivity because the size range of both

species combined was from 83 to 175 mm, and larger fish are routinely caught in trawls. The mean ratio of weight to length was not significantly different between the species ($t=0.07$, $P=0.800$), indicating a similarity in growth trajectories between the species at the size examined, despite significant differences in overall size.

Two of four meristic measurements were useful in reliably sorting specimens to species. First, sand seatrout had overall larger anal-fin to eye-diameter ratios ($t=21.32$, $P<0.0001$); the sand seatrout ratio ranged from 1.23 to 2.44, whereas the range in silver seatrout was 0.85–1.19. Second, anal-fin soft rays were significantly different between species ($\chi^2=120$, df=10, $P<0.0001$); sand seatrout possessed an average of 10.9, silver seatrout an average of 8.8 soft rays. In contrast, there was not a significant difference in pectoral-fin to pelvic-fin ratios between species ($t=0.53$, $P=0.5978$) (Fig. 3), nor was there a significant difference in the number of lateral line scales ($\chi^2=7.47$, df=14, $P=0.915$). The anal-fin soft-ray meristic was the most practical morphological character for species discernment because the range of this character did not overlap between species in any specimen (range 10–12 for sand seatrout, 8–9 for silver seatrout), and this character was relatively easy to count.

Mitochondrial DNA

The fragments recovered from each mtDNA amplification were approximately 1500 bp, and this size did not vary between species. Two distinctive RFLP patterns were identified (Fig. 4). The first pattern contained four bands, at approximately 450, 290, 250, and 190 bp. This pattern was identified in each of the 60 sand seatrout assayed. The second pattern also contained four bands, at approximately 400, 290, 200, and 190 bp. This pattern was identified in each of the 60 silver seatrout assayed. Based upon the expected relative intensity of each band,

there was the likelihood that duplicate fragments were present in each haplotype at the 190 band. Additionally, multiple uncharacterized bands appeared that were <100

bp in length. These uncharacterized bands likely account for the remainder of digested DNA (~320 bp expected in *C. arenarius*, and ~420 bp expected in *C. nothus*) that was not accounted for by primary bands, although this assumption was not explicitly tested. Correct assignment of RFLP haplotypes to each species was confirmed by comparison with anal-fin ray counts and anal-fin to eye-diameter ratios.

Microsatellite DNA

The nine microsatellites used for species comparisons had a range of three to 51 alleles overall. One locus (*SOC415*) had a dramatic allele range difference between the species (Table 1), which resulted in almost complete disjunction between allele distributions, possibly the result of a large insertion in sand seatrout, or deletion in silver seatrout. A deletion is most likely the reason because the allele range in sand seatrout is similar to that of the species in which the markers were initially cloned, *S. ocellatus*. Otherwise, the detected allele ranges of the remaining eight loci were similar between *Cynoscion* species and overlapped the range of *S. ocellatus*.

The average allele diversity was 16.44 alleles per locus in sand seatrout and 9.89 alleles per locus in silver seatrout, and diversity was (qualitatively) higher in sand seatrout at eight of nine loci (Table 3). Similarly, gene diversity was higher in sand seatrout at seven of nine loci and ranged from 0.11 to 0.97, compared to a range of 0.02 to 0.90 in silver seatrout. There was no indication of genotypic disequilibrium at any locus in any population because all *P*-values fell at or above the adjusted critical level of $\alpha = 0.0014$. It should be noted, however, that a single combination of loci (*SOC243* and *SOC415*) did show evidence of significant linkage before adjustment for multiple tests ($P \approx 0.0014$). The inbreeding coefficient (F_{is}) was not significantly different from 0 at any locus in either population. As a result, all nine loci were included in downstream assignment assays.

Significant genetic divergence between the species was found at each of the nine loci, resulting in a range for θ of 0.025–0.464, with a mean value of $\theta = 0.117$ (Table 3). Six loci accounted for approximately 86% of the discriminatory power of the panel of microsatellites in WHICHLOCI analyses (Table 4). Of these, only the four highest ranked loci were needed to correctly assign individuals to populations under an assignment stringency LOD-score of two. All six were needed when the assignment stringency was increased to a LOD-score of three. Unsuccessful assignment was due to stringency restrictions rather than to actual mis-assignment of individuals. In every case, all individuals were assigned correctly at a relaxed stringency.
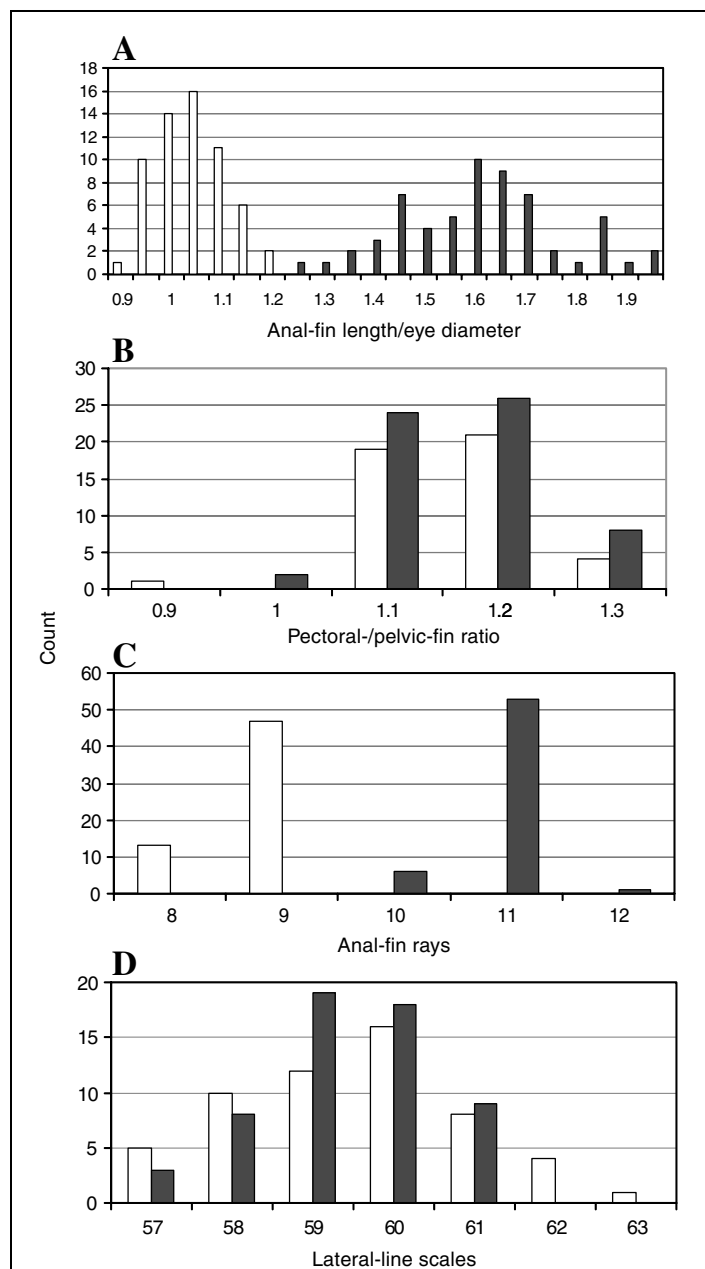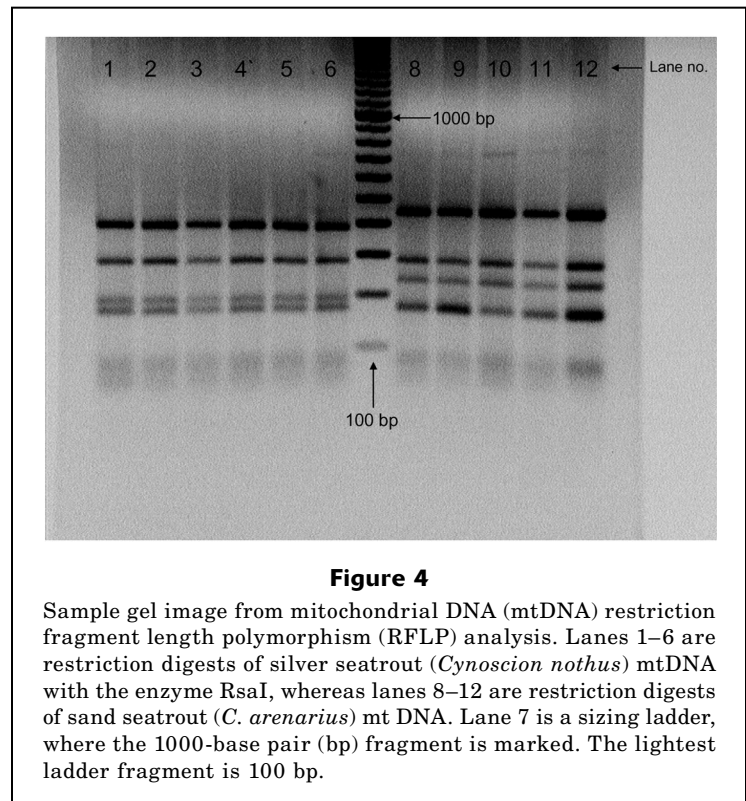

Figure 3

Histograms representing the distribution of meristic and morphometric characteristics of silver seatrout (*Cynoscion nothus*) (white bars) and sand seatrout (*C. arenarius*) (black bars) collected offshore from Galveston Bay, TX, in July 2007. The four main diagnostics examined included (**A**) anal-fin/eye- diameter ratio, (**B**) pectoral-fin/pelvic-fin ratio, (**C**) total anal-fin rays, and (**D**) total lateral-line scales. The abscissa represents each data class (ratio [A and B] or count [C and D] data), whereas the ordinate represents the total membership of each species sample in that class.

Table 3

Genetic allele diversity, gene diversity, and the inbreeding coefficient (F_{is}) for nine microsatellite loci in sand seatrout (*Cynoscion arenarius*) and silver seatrout (*C. nothus*) collected from offshore Galveston Bay, Texas, in July 2007. No value of F_{is} was significantly different from 0. Genetic divergence between species (measured as θ) was significant at each locus. Summary statistics for overall variability are included on the bottom rows (mean above, SE below).

| Locus | *C. arenarius* | | | *C. nothus* | | | |
	Allele diversity	Gene diversity	F_{is}	Allele diversity	Gene diversity	F_{is}	θ
SOC050	11	0.77	−0.095	9	0.54	−0.045	0.059
SOC243	5	0.30	0.038	8	0.52	0.081	0.116
SOC415	40	0.97	0.021	15	0.90	0.107	0.066
SOC410	6	0.60	0.062	4	0.13	−0.048	0.464
SOC412	17	0.85	−0.056	11	0.80	−0.106	0.084
SOC416	30	0.94	0.045	15	0.89	−0.031	0.058
SOC419	16	0.77	0.050	13	0.86	−0.070	0.100
SOC428	3	0.11	−0.046	2	0.02	0.000	0.025
SOC432	20	0.91	−0.024	12	0.81	0.014	0.075
Overall	16.44	0.69	0.000	9.89	0.61	−0.010	0.117
(±SE)	(±8.01)	(±0.20)	(±0.037)	(±3.00)	(±0.22)	(±0.045)	(±0.087)

The performance of each of the three Bayesian assignment models was evaluated by examining the average membership proportion of each species in either of two genetic clusters after assignment. Under the assumption that the best assignment model should result in the highest proportion of membership (POM) of individuals to their correct species cluster (that is, assuming no admixture between the species), each model performed equally well for assignment of sand seatrout (POM=0.978), whereas the six-locus panel of markers (POM=0.989) performed better than the nine-locus panel (POM=0.985) for silver seatrout. The highest average POM of 0.994 and 0.996 for sand and silver seatrout, respectively, was attained by assigning group membership *a priori* based on mtDNA haplotype, and by refining group membership scores with the six highest-rated microsatellite loci. Using the six-locus model without mtDNA information, we found significant coancestry between the species for a single individual identified as belonging to the sand seatrout cluster, and an estimated proportion of silver seatrout ancestry of 0.303 (Table 5). In six successive iterations, the mean *Q* for this individual was 0.297 (range: 0.291–0.305). This value of *Q* was higher than any obtained in simulated populations (*P*<0.05). However, when mtDNA haplotype data were used to improve clustering, probability of admixture was not significant in this individual (*Q*=0.119). Furthermore, morphological evidence indicated no intermediacy at the diagnostic traits (anal-fin to eye-diameter ratio=1.8, anal-fin rays=12); both of these diagnostics indicated this individual was a sand seatrout.

Figure 4

Sample gel image from mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis. Lanes 1–6 are restriction digests of silver seatrout (*Cynoscion nothus*) mtDNA with the enzyme RsaI, whereas lanes 8–12 are restriction digests of sand seatrout (*C. arenarius*) mt DNA. Lane 7 is a sizing ladder, where the 1000-base pair (bp) fragment is marked. The lightest ladder fragment is 100 bp.

Discussion

Morphological and genetic identification of white trout

Two of four commonly used morphological diagnostics are useful in distinguishing conclusively between sand

and silver seatrout. As previously described, the ratio of anal-fin base to eye diameter has a nonoverlapping distribution (DeVries and Chittenden, 1982), and this is due in part to a difference in eye size of each species, for which the mean diameter is lower in sand seatrout than in silver seatrout. Anal-fin soft-ray counts also are diagnostic in these samples, contrary to previous marine fish keys, which indicate overlap of these characters (Robins et al., 1986; Chao, 2002; McEachran and Fechhelm, 2005). The ineffectiveness of the remaining diagnostics (pectoral- to pelvic-fin length ratio, lateral-line scale counts) may be due to differences at various age classes; i.e., the previously described differences may be more apparent in older individuals. Also, it should be noted that several damaged pectoral fins (resulting in shorter fins) in the silver seatrout sample were encountered because of the nature of capturing fish with trawling nets. Therefore there was a smaller sample size for the

pectoral- to pelvic-fin ratio measurement. Nevertheless, the morphological data in conjunction with completely diagnostic mtDNA haplotypes, should prove useful for future species identification issues between sand and silver seatrout.

In contrast to the diagnostic differences in morphological characters and in mtDNA haplotypes, no diagnostic microsatellite loci were identified in the present study. This is likely due to several factors. First, microsatellites have a more elevated rate of mutation than other loci (Ellegren, 2000) and tend to evolve in stepwise fashion (Ellegren, 2000; Xu et al., 2000), such that they may be subject to increased rates of homoplasy (Estoup et al., 2002). The result is that convergent electromorphs can obscure the actual rate of fixation for homologous microsatellite alleles. Second, the fixation of different alleles between the two species may be confounded by recombination, resulting in longer evolutionary timeframes for lineage assortment to occur than what would be expected for mtDNA, which is typically nonrecombinant and clonally inherited. Finally, the enormous population sizes characteristic of marine fish populations may partially mitigate the effects of genetic drift (Allendorf and Phelps, 1981), which is likely the main mechanism for divergence at neutral loci. Collectively these processes have resulted in similarity in allele ranges for all but a single microsatellite locus (*SOC415*), despite significant differences in θ at each individual locus. Thus although no single microsatellite is diagnostic, when used in concert they are adequate for reliable identification to species. Further, because of their high overall between-species divergence and within-species variability, these microsatellites are likely adequate for diagnosis of hybrids, in particular at the F_1 (first generation hybrid) level.

Genetic variability and divergence within and between species

Significant genetic divergence between these species (θ=0.117) is indicated by examination of microsatellite

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Table 4

Locus scores and relative scores from WHICHLOCI analyses, ranked in order of population assignment power. The locus score is the power of each locus as a ratio of correctly assigned to incorrectly assigned individuals in data simulations. The relative score of each locus is obtained by summing all locus scores and then dividing an individual locus score into the total score.

Locus	Score	Relative score (%)
SOC415	0.97	20.8
SOC416	0.70	15.1
SOC419	0.63	13.4
SOC410	0.61	13.1
SOC432	0.58	12.5
SOC412	0.49	10.6
SOC50	0.32	6.8
SOC243	0.31	6.6
SOC428	0.05	1.1

Table 5

Admixture class (proportion of genetic contribution from the opposing species, in rows) membership in a randomized population sample (n=1000), compared to experimental populations (n=60) of silver seatrout (*Cynoscion nothus*) and sand seatrout (*C. arenarius*) collected from offshore Galveston Bay, TX, in July 2007. The admixture classes are defined arbitrarily by Q-score increments of 0.05. The value "*P*" represents the proportion of individuals in each population that fall into a particular admixture class (range of Q), and significance in experimental populations (indicated by a superscript "*a*") was assessed quantitatively by comparing membership in each class to expected values as assessed by examination of the randomized population.

Q-score	Randomized population	P	C. nothus	P	C. arenarius	P
0–0.050	980	0.98	59	0.98	56	0.93
0.060–0.100	13	0.01	0	0.00	2	0.03
0.011–0.150	5	0.01	1	0.02	1	0.02
0.016–0.200	1	0.00	0	0.00	0	0.00
0.210–0.250	1	0.00	0	0.00	0	0.00
>0.250	0	0.00	0	0.00	1[a]	0.02

loci. This divergence is coupled with primarily non-significant estimates of admixture between species, although one individual did exhibit significant evidence of admixture (*Q*>0). Although it is plausible that this individual is an advanced backcross, this result more likely represents an outlier. Evidence for the outlier supposition includes the admixture model with mtDNA haplotypes used to improve clustering; in this scenario, the silver seatrout contribution for this individual is no longer significant. Additionally, no overlap occurred in the mtDNA haplotypes or in the two diagnostic morphological characters examined in this study. Thus, these data indicate that hybrid formation between sand and silver seatrout is either rare or nonexistent in the offshore Galveston Bay area, and enough generations have elapsed since divergence from an ancestral population that contemporary mtDNA haplotypes do not indicate either incomplete lineage sorting or admixture during the time of, or after, speciation. Altough the absence of hybridization seems to be the case in the Galveston Bay area, the present study was limited geographically, and the possibility of hybridization elsewhere within the overlapping ranges of these species must be further explored. Although narrow hybrid zones are rare in marine organisms (Palumbi, 1994), heterogeneity in the rate of hybridization has been demonstrated. For instance, the rate of hybridization in European shads (*Alosa* spp.) tends to be heterogeneous across the range of *A. alosa* and *A. fallax* (Alexandrino et al., 2006).

Estimates of genetic variability were systematically higher in sand seatrout than in silver seatrout, indicating possible demographic differences between populations of these species. For instance, both allelic richness and gene diversity are higher at almost every locus in sand seatrout. This observation is likely of biological significance, rather than a genetic ascertainment bias, because these markers were initially isolated from a species outside the genus *Cynoscion*. The direction of ascertainment bias would therefore be expected to vary from locus to locus, rather than to have comparable effects across a majority of loci. Demographic parameters such as population census size, rates of migration among neighboring populations, and variance in reproductive success can all affect effective population size (N_e), and heterogeneity in these parameters would result in different levels of measured genetic variability between the species. Thus the implication, based on systematically lower levels of genetic variability, is that silver seatrout have a lower N_e than sand seatrout.

The finding of higher genetic variability in sand seatrout is not likely the result of differences in migration rates because both species spawn primarily offshore in the GOM (Shlossman and Chittenden, 1981; DeVries and Chittenden, 1982); the pelagic nature of eggs and larvae in both species allow for long-distance dispersal and gene flow throughout the GOM. It is also unlikely that a difference in census sizes is the cause of elevated genetic variability in sand seatrout. The data of McDonald et al. (2009) indicates dramatically a higher abundance of *C. nothus* in the Galveston Bay

offshore region, and the western GOM in general. The most likely explanation for the elevated genetic variability of sand seatrout is an overall higher level of individual reproductive success than that recorded for individuals in the silver seatrout population. Two key biological differences support this assertion. First, sand seatrout have an approximately 25% higher fecundity (mean 100,900 eggs per spawn than that of silver seatrout (mean 73,900 eggs per spawn) in the GOM (Sheridan et al., 1984). Second, individual sand seatrout are more likely to spawn on multiple occasions during their lifetime. Sand seatrout live longer (2–3 years, Shlossman and Chittenden, 1981) than silver seatrout (1–1.5 years, DeVries and Chittenden, 1982) and have two peak spawns per year in the western GOM, compared to a single relatively definitive peak in silver seatrout (McDonald et al., 2009). Both species mature before age one, and both species experience high mortality at early life stages. Hedgecock (1994) referred to the combination of high fecundity and high mortality at early life stages as a sweepstakes strategy, which is likely common in marine fishes. Such differences in fecundity and longevity between species likely result in a higher variance in reproductive success in silver seatrout, which concurrently results in lower effective population size and a decrease in genetic variability (Hedgecock, 1994; Hedrick, 2005). In any event, one caveat to this result is the fact that while the sand seatrout sample was collected during the course of three trawls, all silver seatrout specimens were collected from a single trawl. Thus sampling error or sample ascertainment bias cannot be ruled out completely as an explanation for differences in diversity estimates.

The morphological and ecological similarities among sand seatrout, silver seatrout, and gray weakfish have resulted in difficulty in distinguishing the taxonomic status of these species (Weinstein and Yerger, 1976). However, there is a fundamental difference between the distributions of the three species; whereas sand seatrout and gray weakfish are functionally parapatric in their distribution, inhabiting primarily the GOM and Atlantic Ocean, respectively, silver seatrout are found in relatively large populations in both areas. The shallow divergence previously reported between sand seatrout and gray weakfish (Weinstein and Yerger, 1976) was likely caused by highly stochastic sea level changes throughout the Pleistocene Epoch, resulting in regional differences between populations in the GOM and Atlantic Ocean. A similar pattern is typical among other marine, freshwater, and terrestrial vertebrates worldwide that diverged during this time period (Avise and Walker, 1998; Avise et al., 1998; Hewitt, 2000), and has been particularly well documented in peninsular Florida (Avise, 1992). However, these episodic sea level changes were likely not long enough for reproductive isolation between sand seatrout and gray weakfish to develop. Therefore, contemporary hybridization between these species is common on the Atlantic coast of Florida (Cordes and Graves, 2003); in contrast, no such patterns have been indicated between populations of sand

and silver seatrout, which have sympatric distributions in the GOM.

This finding supports the monophyly of an assemblage that includes sand seatrout and gray weakfish and may indicate that silver seatrout diverged from this group before the sea level changes of the last glacial maximum. The current data indicate complete divergence in two morphological characters and a single genetic (mtDNA) locus between sand and silver seatrout. In contrast, the morphological data of Aguirre and Shervette (2005) indicate a sister-species relationship between sand seatrout and weakfish. Interestingly, the silver seatrout is the only one of the three species that is not partially estuarine-dependent, indicating that estuarine independence may have evolved after the speciation process. Furthermore, because silver seatrout are not tied to shallow estuarine waters, they may have eluded the significant vicariance effects (that is, genetic divergence caused by the appearance of a transient or permanent boundary) caused by sea level shifts during periods of glacial advance. However, caution must be used in interpreting genetic data in light of contemporary distribution range because extant distributions are not necessarily good indicators of geographic ranges at the time of divergence (Losos and Glor, 2003). Whatever the case, trophic partitioning between the two species likely contributes to the heterogeneous offshore distribution of sand seatrout, in contrast to the relatively consistent offshore distribution of silver seatrout (Ginsberg, 1931; Byers, 1981; McDonald et al., 2009). These data indicate that partitioning may also play a role in the maintenance of reproductive barriers between species, resulting in distinctive genetic profiles, and little evidence of evolutionary association after speciation.

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