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Phylogenetic Relationships Among Four Western Atlantic *Cynoscion* Species Based on DNA Sequences From 11 Nuclear Introns, Two Mitochondrial Genes, and Genotypes From 32 Microsatellite Markers

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Four species of *Cynoscion* occur in the waters off the Atlantic and Gulf coasts of North America, where they are targeted by commercial and recreational fisheries. Previous studies have not resolved the phylogenetic relationships of the four species, largely due to uncertainty as to whether the spotted seatrout, *Cynscion nebulosus*, or silver seatrout, *Cynscion nothus*, is the most divergent member of the North American assemblage. This study used DNA sequences from the nuclear and mitochondrial genes and multilocus genotypes from microsatellite markers to infer relationships among these species. Together, these three techniques strongly suggest that the weakfish, *Cynscion regalis*, and the sand seatrout, *Cynscion arenarius*, are the most closely related species, and that *C. nothus* is the most divergent from all the others.

The genus *Cynoscion* is one of 13 genera in the Sciaenidae (Teleostei) that occur in tropical and subtropical coastal waters of the Americas. The genus comprises 25 species, 13 in the western Atlantic and 12 in the eastern Pacific. Four of the western Atlantic *Cynoscion* species occur naturally off of the eastern United States or in the Gulf of Mexico: sand seatrout, *Cynoscion arenarius*; weakfish, *Cynoscion regalis*; spotted seatrout, *Cynoscion nebulosus*; and silver seatrout, *Cynoscion nothus*. Sand seatrout were long believed to be restricted to the Gulf of Mexico (Robins and Ray, 1986), but a recent genetic study showed that its range extended throughout the inshore waters of the Florida Atlantic coast (Tringali et al., 2011). Similarly, weakfish are generally considered to occur only in Atlantic waters (Robins and Ray, 1986), but they have been reported from the Gulf of Mexico (Weinstein and Yerger, 1976). Spotted seatrout and silver seatrout occur from the waters of the Mid-Atlantic states to the Bay of Campeche in Mexico (Pearson, 1929). All four species fill the ecological niche of midsize predators in inshore habitats and are targeted by commercial or recreational fisheries in portions of their ranges (Van Voorhees et al., 1992).

A comprehensive study by Tringali et al. (2011) documented hybridization among all possible pairs of the four the *Cynoscion* species within the Atlantic coastal waters of Florida, with the most extensive introgressive hybridization occurring between sand seatrout and weakfish, centered in the St. Johns River. Hybridization between these two species has been documented in Georgia waters as well (Cordes and Graves, 2003), which is not surprising considering that sand seatrout and weakfish are sister species that are so morphologically similar that their taxonomic delineation was based on modal differences in a small set of characters (Ginsburg, 1929). The validity of the elevation of *C. arenarius* to distinct species status has been questioned numerous times (Moshin, 1973; Weinstein and Yerger, 1976; Ditty, 1989), and even the original description expressed some uncertainty (Ginsburg, 1929). More recent studies have examined variation in mtDNA sequences and microsatellite allele distributions and have supported the distinctiveness of the two species (Cordes and Graves, 2003; Vergara-Chen et al., 2009; Tringali et al., 2011). Even so, the active hybridization, morphological similarity, and close genetic relationship between the sand seatrout and the weakfish provide strong support for their being the two most closely related of the four *Cynoscion* species of eastern North American waters. The spotted seatrout has been found to be the most morphologically (Ginsburg, 1929), ecologically, and biochemically distinct of the species, all of which suggest that it may be the most evolutionarily divergent (Weinstein and Yerger, 1976). A recent phylogeny, however, based on mitochondrial DNA and including 19 species of *Cynoscion,* found that *C nebulosus*, *C. arenarius*, and *C. regalis* formed a monophyletic group, whereas *C. nothus* was located in a different clade (Vergara-Chen et al., 2009).

In an effort to clarify the relationships among these ecologically and economically important fishes, we evaluated genetic variation within the DNA sequences of 11 introns from five nuclear genes, two regions from the mitochondrial DNA genome (cytochrome oxidase subunit I [COI] and 16S rRNA), and a suite of 32 microsatellite markers for sand seatrout, weakfish, spotted

TABLE 1. Collection locations and sample sizes of specimens of the four *Cynoscion* species and the red drum (*Sciaenops ocellatus*) included in the study of the phylogenetic relationships among the western Atlantic sciaenid fishes.

Species	Collection location	Nuclear intron sequence (n)	mtDNA sequence (n)		Microsatellite genotypes (n)
			COI[a]	16S rRNA	
C. arenarius	Cedar Key, FL	3	3	3	
	Charlotte Harbor, FL	2	2	2	
	Tampa Bay, FL	3	3	3	48
C. nebulosus	Charlotte Harbor, FL	5	5	5	
	Biscayne Bay, FL	2	2	2	
	Tampa Bay, FL				137
C. nothus	Offshore Atlantic FL	4	4	4	30
C. regalis	Delaware				24
	North Carolina	7	7	7	22
S. ocellatus	Tampa Bay, FL	3	3	3	48

[a] Abbreviation: COI, cytochrome oxidase subunit I.

seatrout, silver seatrout, and the cofamilial species red drum, *Sciaenops ocellatus* to serve as an outgroup. This is the first study to investigate interspecifc relationships based on nuclear DNA sequences, mitochondrial DNA regions, and microsatellite genotypes. It provides an opportunity to examine the relationships observed in the mitochondrial and nuclear genomes. Incorporating information from all three techniques should provide well-resolved topologies of the relationships among the four congeners.

METHODS

Sample collection and DNA extraction.—Muscle tissue, fin clips, or whole individuals were collected from a number of coastal areas throughout Florida as well as in North Carolina and Delaware (Table 1) and transported on ice to the Florida Fish and Wildlife Research Institute's Molecular Genetics Laboratory. Total DNA was extracted from small pieces of tissue using the PUREGENE DNA Purification Kit (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

Nuclear and mitochondrial DNA sequencing analyses.—We amplified and sequenced 11 introns from six nuclear DNA genes, mtDNA COI, and mtDNA 16S rRNA in three to seven individuals from each of the four *Cynoscion* species and the red drum (Table 1). Polymerase chain reaction (PCR) was carried out in a Thermo Hybaid Multi Block System (Franklin, MA) using the published primers (Table 2) for four introns from two aldolase genes (three from aldolase B and one from aldolase C), three introns from the gonadotropin-releasing hormone–3 gene, two introns from the S7 ribosomal protein gene, and one intron each from the growth hormone and lactate dehydrogenase A genes. Two regions from the mitochondrial DNA genome were also sequenced: 16S rRNA and COI (Table 2). Each reaction was performed in a final volume of 50 µl that included 5 µl of 10× Promega buffer, 5 µl of 25 mM Promega $MgCl_2$, 0.25 µl of 20-mg/ml bovine serum albumin (Roche Diagnostics, Florence, SC), 0.125 µl of 100 µM solution of each primer, 4 µl of 50 µM premixed dNTPs, 0.25 µl of Promega Taq DNA polymerase, and 2 µl (50 to 100 ng) of DNA template. The reaction mixture was initially subjected to denaturation for 2 min at 94°C followed by 32 cycles of denaturation (94°C, 40 sec), annealing (55°C, 40 sec), and extension (72°C, 45 sec); a final extension (72°C, 15 min) was followed by holding at 4°C. The PCR products were cleaned using the StrataPrep PCR or Gel Purification Kit (Stratagene, La Jolla CA) according to the manufacturer's instructions. Cycle sequencing was performed from both the 5′ and 3′ ends of each fragment using 1 µl (50–150 ng) of the purified PCR product, 2 µl of Big Dye™ Terminator Cycle-Sequencing Ready-Reactions with AmpliTaq FS DNA polymerase (PE Biosystems, Foster City, CA) and 0.0016 pM of each primer, in a total volume of 5 µl. Cycle sequencing PCR conditions were 30 cycles of denaturation (94°C, 30 sec), annealing (55°C, 15 sec), and polymerization (60°C, 4 min), followed by a 4°C hold step. The reaction product was then ethanol-precipitated, resuspended in 22 µl of HiDi formamide (Fisher Scientific, Norcross, GA), denatured at 95°C for 2 min, and cooled on ice. The resuspended product was analyzed on an ABI Prism™ 310 Genetic Analyzer (PE Biosystems). The sequences obtained were aligned and edited using the

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TABLE 2. Nuclear DNA intron and mtDNA regions sequenced for four species of the genus *Cynoscion* and the red drum (*Sciaenops ocellatus*) and GenBank accession numbers.

Gene[a]	Introns	Reference	GenBank accession numbers				
			C. arenarius	*C. regalis*	*C. nebulosus*	*C. nothus*	*S. ocellatus*
Aldolase B	2, 3, 4	Hassan et al., 2002 Friesen et al., 1997 Lessa and Applebaum, 1993	EU180134	EU180135	EU180136	EU180137	EU180138
Aldolase C	1	Hassan et al., 2002	EU180139	EU180140	EU180141	EU180142	EU180143
GnRH3	1, 2, 3	Hassan et al., 2002	EU180149	EU180150	EU180151	EU180152	EU180153
Gh5	1	Hassan et al., 2002	—[b]	EU180154	EU180155	EU180156	EU180157
LDH	A	Quattro and Jones, 1999	AY057091	AY057092	AY057093	EU180158	EU180159
RP	1, 2	Chow and Hazama, 1998	EU180160	EU180161	EU180162	EU180163	EU180133
16S rRNA		Palumbi et al., 1991	DQ179651	DQ179650	EU857949	EU857950	EU857951
COI		Ward et al., 2005	EU180144	EU180145	EU180146	EU180147	EU180148

[a] Abbreviations: GnRH3, gonadotropin-releasing hormone–3; Gh5, growth hormone; LDH, lactate dehydrogenase; RP, S7 ribosomal protein; COI, cytochrome oxidase subunit I.
[b] Polymerase chain reaction did not amplify.

AutoAssembler™ DNA Sequence Assembly Software (PE Biosystems); further electropherogram editing was performed using Chromas version 1.6 (Technelysium Pty, Ltd, Helesville, Queensland, Australia).

Phylogenetic relationships among the DNA sequences were evaluated using both Bayesian and maximum parsimony methods. We performed a series of analyses where (1) the DNA sequences from each gene were analyzed independently, (2) all the sequences from one genome (nuclear vs mtDNA) were analyzed together, and (3) all the nuclear and mtDNA sequences were included in a single analysis. When more than a single nuclear intron or mtDNA gene was included in a Bayesian analysis, we generated mixed models in which each intron/gene was assigned to a partition that had its own model of nucleotide substitution and sequence evolution. The optimal model of sequence evolution for each partition (intron/gene) was assessed with maximum likelihood methods and Akaike information criterion procedures in jModelTest 1.0 (Posada, 2008). The optimal model of sequence evolution for each gene was assigned for the data partitions in MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) using the APPLYTO command, and the associated parameters were allowed to be estimated independently using the UNLINK command. MrBayes was run for 2×10^5 metropolis-coupled Markov chain Monte Carlo generations, the first 5×10^4 of which were considered the burn-in and excluded from the analyses. Trees were sampled every 100 generations during the run. The maximum parsimony analyses were performed using PAUP* 4.0 (Swofford, 2000). Exhaustive searches were conducted and relative clade support was assessed with 1,000 bootstrap pseudoreplicates.

Microsatellite genotype analysis.—Thirty-two microsatellite markers developed for *C. nebulosus* (S. Seyoum, M. D. Tringali, B. L. Barthel V. Villanova and C. Puchulutegui, unpubl., Table 3) were used to genotype specimens of *C. arenarius* from Tampa Bay, Florida (n = 48), *C. regalis* from Delaware and North Carolina (n = 46), *C. nothus* from offshore Atlantic Florida (n = 30), and *S. ocellatus* from Tampa Bay (n = 48). Multiplex PCR reactions containing three sets of labeled microsatellite primers and 100 ng of total DNA were carried out in 12.5-µl volumes using the reaction profile described by Seyoum et al. (S. Seyoum, M. D. Tringali, B. L. Barthel, V. Villanova, and C. Puchulutegui, unpubl.). One microliter of PCR product was mixed with 12 µl of Hi-Di formamide and 0.5 µl of GS-ROX500 size standard and denatured (94°C for 4 min) and snap-frozen. Fragments were visualized on an ABI 3130 genetic analyzer and genotyped using GeneMapper software version 3.7 (Applied Biosystems Inc., Foster City, CA).

For each pair of species we calculated the Cavalli-Sforza–Edwards chord distance (Cavalli-Sforza and Edwards 1967) between species using the microsatellite DNA genotypes. The Seqboot program of the PHYLIP package version 3.69 (Felsenstein, 1989) was used to create 1,000 bootstrap replicates of the gene frequencies of each species. The Gendist program from the PHYLIP package was then used to calculate Cavalli-Sforza–Edwards chord distances for each of the replicate datasets. Finally, a consensus tree was generated from the distance matrices using the Consense program from PHYLIP.

TABLE 3. Characterization of 32 microsatellite loci in four species of the genus *Cynoscion* and the red drum *Sciaenops ocellatus*.

Locus	*C. arenarius* (n = 48)		*C. regalis* (n = 44)		*C. nebulosus* (n = 137)		*C. nothus* (n = 30)		*S. ocellatus* (n = 48)		GenBank accession number
	Size range	K[a]	Size range	K	Size range	K	Size range	K	Size range	K	
Cneb01	161–173	7[b]	161–181	11	161–177	8	161–205	16	—[c]	—	JF495373
Cneb03	143–225	34	139–195	20	147–225	33	135–171	19	155–179	11	JF495374
Cneb04	157–169	4	157	1	163–185	12	153–161	4[b]	139–141	2	JF495375
Cneb06	168–190	5	174–188	2	172–192	5	184–188	3[b]	190–192	2	JF495377
Cneb07	106–156	12	106–132	9	114–130	9	122–132	6	—	—	JF495378
Cneb09	169–199	12	167–203	8	177–199	12	183–207	8	193–227	15	JF495379
Cneb12	159–185	12	159–173	8	159–177	9	157–173	3	157	1	JF495380
Cneb15	98–118	10	102–112	5	96–106	5	98–110	6	100–102	2	JF495382
Cneb16	148–188	16	148–196	18	148–152	2	136–154	10	136–182	13	JF495383
Cneb20	183–209	13	185–199	8	185–191	4	185–189	3	—	—	JF495384
Cneb21	134–158	12	138–162	8	138–168	8	156–168	5	174–176	2	JF495385
Cneb22	103–115	7	111–147	9	103–135	14	103–125	12	97–107	5	JF495386
Cneb23	115–141	10	115–137	3	121–147	11	123–155	13	123–127	3	JF495387
Cneb24	104–120	3	104	1	110–154	20	114–116	2	—	—	JF495388
Cneb25	139–163	12	135–167	11	131–171	19	139–161	10	127–139	7	JF495389
Cneb26	108–198	19	112–212	15	108–192	35	104–112	5	108–122	8	JF495390
Cneb29	100–108	3[b]	100–102	2[b]	104–112	4	102–114	6	100–108	4	JF495392
Cneb30	160–166	4	162	1	164–168	3	158–164	3	156	1	JF495393
Cneb31	85–103	6[b]	83–103	8[b]	74–103	12	89–109	5	81–99	4	JF495394
Cneb32	109–133	11[b]	115–131	7	105–121	7	115–121	2	113–125	5[b]	JF495395
Cneb33	125–139	7	127–161	9	117–157	20	113–157	17	111	1	JF495396
Cneb34	112–116	3	114	1	114–120	4	114–128	6	114–116	2	JF495397
Cneb35	79–155	22	83–125	17	87–131	21	79–123	20	87–177	21	JF495398
Cneb36	120–144	11	124–166	12	118–172	21	118–138	8	118–138	6[b]	JF495399
Cneb37	—	—	—	—	141–193	12	—	—	—	—	JF495400
Cneb38	157–173	7	159–181	9	169–191	11	167–171	3	157–161	1	JF495401
Cneb39	131–157	11	135–171	16[b]	123–153	18	135–145	6	127	1	JF495402
Cneb40	—	—	—	—	147–181	15	139–155	8	—	—	JF495403
Cneb41	156–188	17	160–178	7	152–170	8[b]	162–176	7	166–194	11	JF495404
Cneb05	211–213	2	205–211	2	213	1	211	1	205	1	JF495376
Cneb14	116–124	3[b]	116–126	3	118	1	124	1	124	1	JF495381
Cneb28	135–183	9	153–175	4	169	1	163–175	4	147–157	2	JF495391

[a] K, number of alleles.
[b] Significant departure from Hardy–Weinberg equilibrium.
[c] No amplification.

Each of the specimen genotypes was included in a factorial correspondence analysis (FCA) conducted using the software GENETIX version 4.02 (Belkhir et al., 2000). FCA is a means of investigating relationships in tables, in this case to determine whether there was correspondence between rows (individuals) and columns (alleles). Each allele is considered an independent variable, and axes are generated based on the combinations of alleles that explain portions of the total inertia of the table. These axes are then used to plot the genotypes of individual specimens in multidimensional space. The alleles that exhibit the strongest nonrandom association among individuals contribute most to the axes. In this analysis, the individual genotypes were plotted into three-dimensional space based on the genetic relationships observed at the 32 microsatellite loci. We calculated the distance between the center of mass of members of each pair of the five species that were included in the analysis using the three-dimensional distance formula $d = \mathrm{sqrt}[(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2]$ based on the absolute coordinates from GENETIX.

RESULTS

DNA evolution models and phylogenetic inference.—Alignment of the nucleotide sites from the five nuclear genes produced a total of 3,747 aligned base pairs, and two mtDNA regions produced 1,272 aligned base pairs. Five models of sequence evolution were selected for the 11 nuclear intron data partitions, whereas the two mtDNA regions shared the same model (Table 4). *Cynoscion arenarius* and *C. regalis* were well-supported sister

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TABLE 4. DNA substitution models selected for the data partitions of individual genes using maximum likelihood ratio tests.

Data partition	Base pairs	Substitution model	Substitution types (n)	Substitution rates
Nuclear genes[a]				
AldoB-2	131	K80	2	Equal
AldoB-3	230	GTR	6	Equal
AldoB-4	335	GTR	6	Gamma distribution
AldoC	169	GTR	6	Equal
Gh5	213	GTR	6	Gamma distribution
GnRH3-1	257	HKY	2	Equal
GnRH3-2	250	HKY	2	Gamma distribution
GnRH3-3	392	HKY	2	Equal
LDH	260	GTR	6	Equal
RP-1	791	GTR	6	Gamma distribution
RP-2	719	HKY	2	Equal
mtDNA genes				
16S	617	GTR	6	Gamma distribution
COI	655	GTR	6	Gamma distribution

[a] Abbreviations: Gh5, growth hormone; GnRH3, gonadotropin-releasing hormone–3; LDH, lactate dehydrogenase; RP, S7 ribosomal protein; COI, cytochrome oxidase subunit I.

species in all the analyses (Fig. 1). *Cynoscion nothus* was found to be the most divergent species in 14 of the 22 analyses (Table 5), including extremely well-supported topologies inferred when the nuclear introns and mtDNA genes were analyzed together and when the nuclear intron sequences were included in a single analysis. In the case of the combined mtDNA gene dataset, the Bayesian analysis indicated that *C. nothus* was the most divergent species, whereas the maximum parsimony analysis was not able to resolve the relationships of *C. nebulosus* or *C. nothus*. When phylogenetic analyses were conducted on the individual mtDNA genes, *C. nothus* was found to be the most divergent species in two cases whereas *C. nebulosus* was most divergent in a single maximum parsimony analysis of the COI gene (Fig. 2B).

Microsatellite DNA analyses.—Thirty of the 32 microsatellite loci developed for *C. nebulosus* amplified in the other three *Cynoscion* species, and 25 of them amplified in *S. ocellatus*. The number of polymorphic loci ranged from 30 in the *C. arenarius* samples to 19 in the *S. ocellatus* samples (Table 3). The mean number of alleles per polymorphic locus was greatest in *C. nebulosus* (mean = 11.4, SD = 8.6) and least in *S. ocellatus* (mean = 5.1, SD = 5.2) with *C. arenarius* (mean = 10.1, SD = 6.8), *C. regalis* (mean = 7.8, SD = 5.4), and *C. nothus* (mean = 7.2, SD = 5.2) having intermediate levels of polymorphism (Table 2). None of the loci were found to be out of Hardy–Weinberg equilibrium (HWE) for *C. nebulosus*, but, two to five loci were out of HWE for the other three species (Table 3). The neighbor-joining analysis of the Cavalli-Sforza–Edwards distances produced from the microsatellite genotypes resolved a close genetic relationship only between *C. arenarius* and *C. regalis* (Fig. 3). The analysis did not provide support that either *C. nothus* or *C. nebulosus* was more divergent. In the three-dimensional plots produced from the FCA results (Fig. 4), it was apparent that *C. arenarius* and *C. regalis* were the most closely related species and that *S. ocellatus* was most divergent, since it is the outgroup. The pairwise distances calculated between the centers of mass of each of the species in the plot indicated that *C. nothus* was the most divergent of the *Cynoscion* species included in the study (Table 6).

DISCUSSION

This study has verified that *C. arenarius* and *C. regalis* are the most closely related of the four *Cynoscion* species that coexist in the Atlantic waters of eastern North America. In a study that included 19 *Cynoscion* species, Vergara-Chen et al. (2009) also found these two species to be sister species in a phylogeny based on combined sequences from ATPase 8/6 and cytochrome b genes. The present study is generally consistent with those of Vergara-Chen et al. (2009) and also found these two species to have the closest relationship in the analyses of both the nuclear introns and microsatellite DNA genotypes. *Cynoscion nothus* was found to be the most distantly related *Cynoscion* species in the majority of the

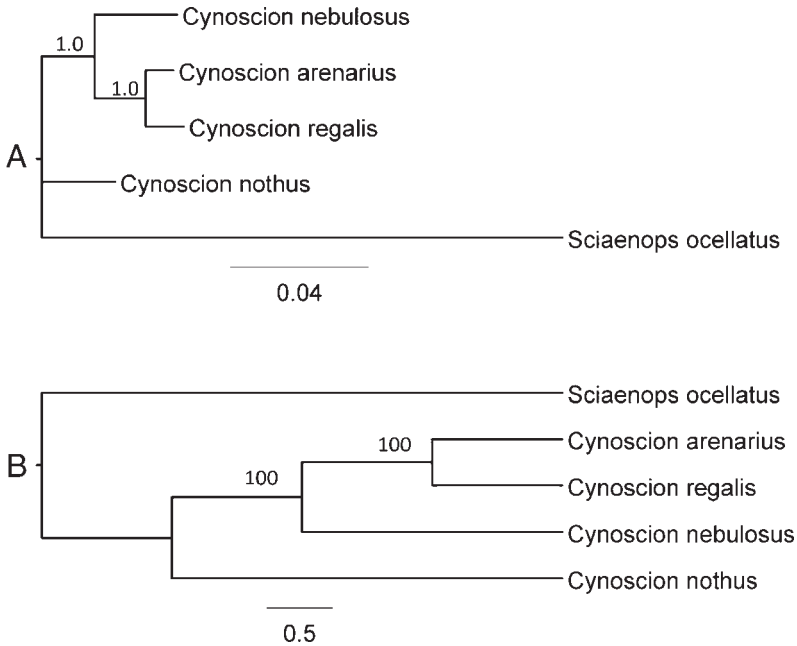

Fig. 1. Majority-rule consensus trees of the five sciaenid species inferred from the Bayesian analysis (A) and maximum parsimony analysis (B) of the combined nuclear intron and mtDNA gene sequences. The numbers presented above the branches represent the posterior probabilities for the Bayesian analysis or the bootstrap values calculated from 1,000 replicates for the maximum parsimony analyses.

TABLE 5. Phylogenetic inference results regarding whether *Cynoscion nebulosus* or *Cynoscion nothus* were the most divergent of the four *Cynoscion* species. In gene descriptions, the numbers in parentheses denote sequence lengths and numbers of variable and parsimony-informative sites, respectively.

Marker	Gene description	Analytical method	Most divergent
All	mtDNA and Nuclear DNA (5019-794-134)	Bayesian	NOT
		Maximum parsimony	NOT
Nuclear	Sequences from the Six Introns (3747-586-69)	Bayesian	NOT
		Maximum parsimony	NOT
mtDNA	16S RNA and COI (1272-208-65)	Bayesian	NOT
		Maximum parsimony	UN
AldoB	Fructose 1,6-bisphosphate aldolase B gene (696-49-15)	Bayesian	NOT
		Maximum parsimony	NOT
AldoC	Fructose 1,6-bisphosphate aldolase C gene (169-8-1)	Bayesian	NOT
		Maximum parsimony	NOT
Gh5	Growth hormone gene (213-17-4)	Bayesian	NOT
		Maximum parsimony	NOT
GnRH	Growth hormone 3 gene (899-52-8)	Bayesian	UN
		Maximum parsimony	UN
LDH	Lactate dehydrogenase gene (260-24-0)	Bayesian	UN
		Maximum parsimony	UN
RP	S7 ribosomal protein gene (1510-420-41)	Bayesian	UN
		Maximum parsimony	NOT
16S	Mitochondrial 16 Ribosomal RNA (617-78-14)	Bayesian	UN
		Maximum parsimony	NOT
COI	Mitochondrial cytochrome *c* oxidase (655-130-51)	Bayesian	NOT
		Maximum parsimony	NEB
MSAT	Microsatellite markers 30 loci	Neighbor-joining	UN
		FCA	NOT

[a] Abbreviations: NOT, *Cynoscion nothus*; NEB, *Cynoscion nebulosus*; UN, unresolved phylogenetic inference; FCA, factorial correspondence analysis.

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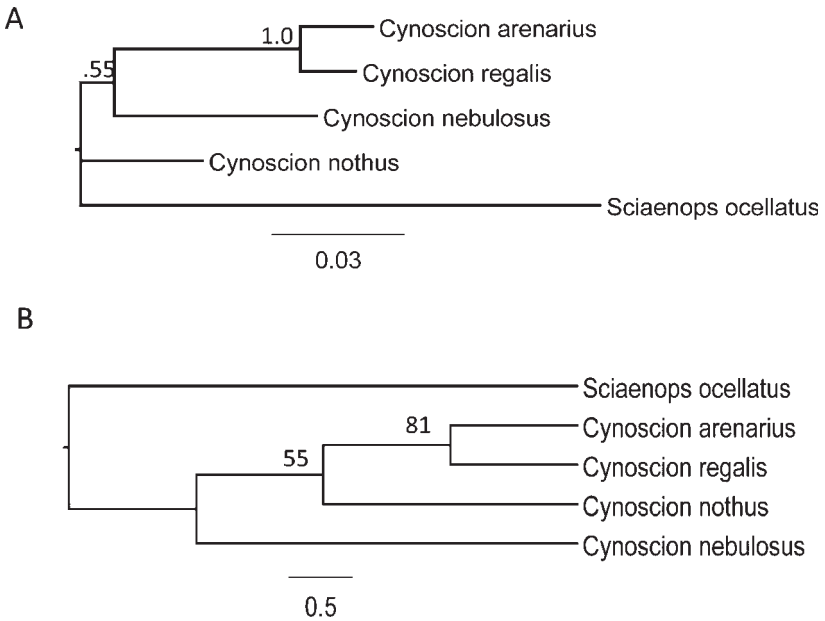

Fig. 2. Majority-rule consensus trees of the five sciaenid species inferred from the Bayesian analysis (A) and maximum parsimony analysis (B) of the cytochrome oxidase subunit I mt DNA sequences. The numbers presented above the branches represent the posterior probabilities for the Bayesian analysis or the bootstrap values calculated from 1,000 replicates for the maximum parsimony analysis.

analyses, whereas *C. nebulosus* was only more divergent than *C. nothus* in the maximum parsimony analysis of the COI region of the mtDNA datasets. However, this noticeable inconsistency in the inferred topology when only COI was used in a parsimony analysis was not strongly supported (55%) and could be considered unresolved. *Cynoscion nebulosus* formed a monophyletic group with *C. arenarius* and *C. regalis* in the mtDNA phylogeny generated by Vergara-Chen et al. (2009), whereas *C. nothus* was assigned to a different clade. Those results are concordant with the majority of the analyses from the present study, particularly the most rigorous analyses, which incorporated both the nuclear and mtDNA sequences into single phylogenetic analyses. This provides strong evidence that *C. nothus* is the most divergent of the four *Cynoscion* species included in this study, contrary to what has been suggested by morphological and biochemical assessments (Ginsburg, 1929; Weinstein and Yerger, 1976).

The phylogenetic trees constructed from the mtDNA sequences were not concordant. *Cynoscion regalis* and *C. arenarius* grouped together with high confidence in all four analyses; *C. nebulosus,* however, was found to be the most divergent species in the maximum parsimony analysis of the COI gene, whereas *C. nothus* was most divergent in two of the three other analyses. Vergara-Chen et al. (2009) conducted a

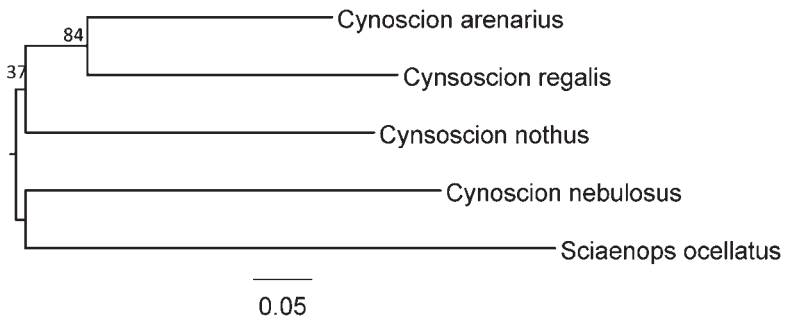

Fig. 3. Majority-rule consensus tree of the five sciaenid species inferred from the neighbor-joining analysis of the Cavalli-Sforza Edwards distances produced from the microsatellite DNA genotypes. Numbers above branches represent bootstrap values based on 1,000 replicate trees.

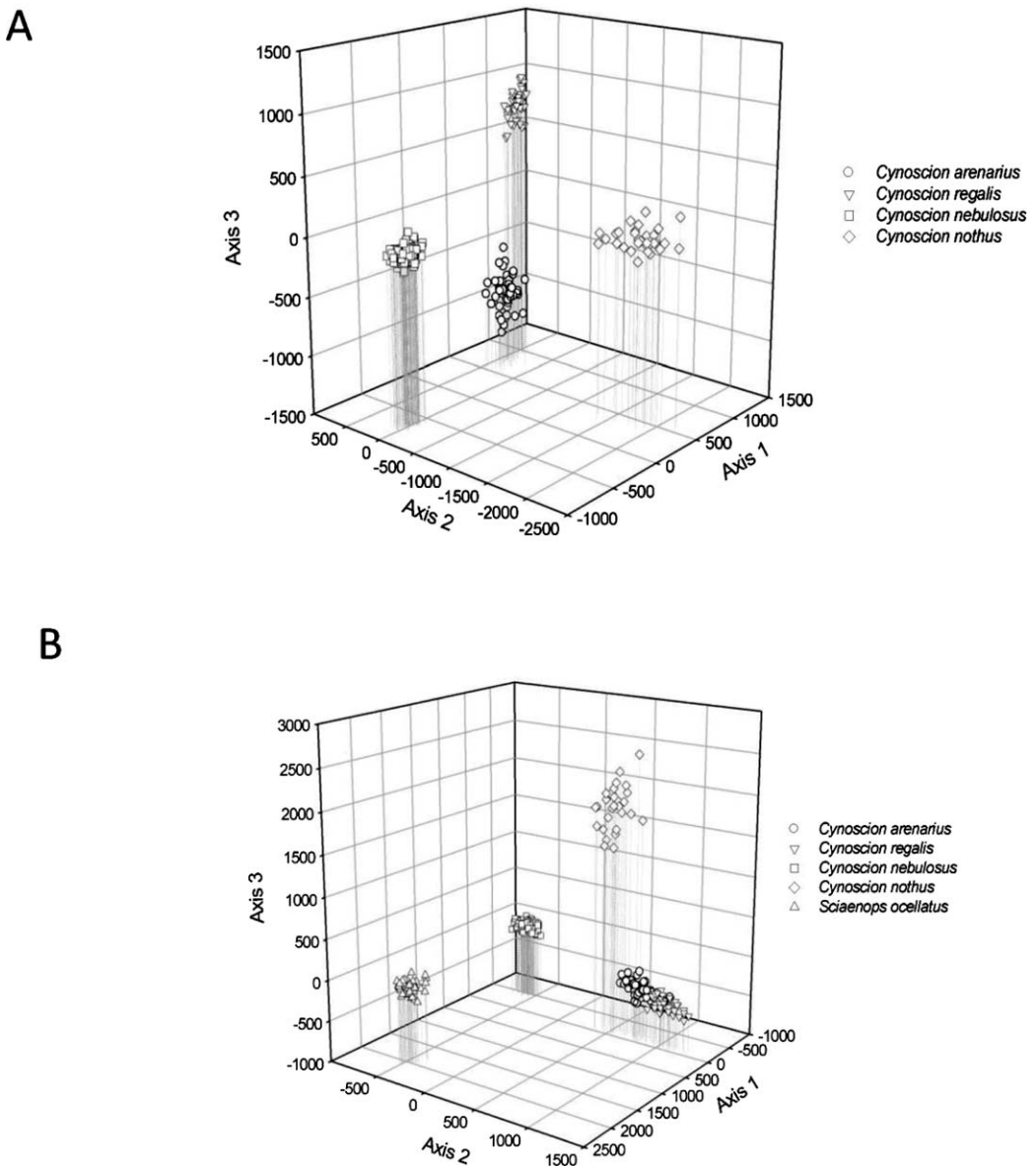

Fig. 4. Three-dimensional plots of factorial correspondence analysis results for five sciaenid species genotyped with 32 microsatellite loci. Analyses were conducted for data sets that included the four *Cynoscion* species (A) and the four *Cynoscion* species plus the red drum (*Sciaenops ocellatus*; B).

phylogenetic analysis using three mitochondrial genes and found that *C. nothus* was the most divergent of the four species considered in the current study. We accessed the DNA sequences (ATP synthase 6 and 8 and cytochrome B) that Vergara-Chen et al. (2009) published in GenBank and conducted Bayesian and maximum parsimony for each gene independently for our four species of interest. In each case *C. nothus* was found to be the most divergent species. The fact that *C. nebulosus* was found to be the most divergent species in only one of the 12 analyses conducted on individual mtDNA gene regions indicates that the maximum parsimony results for the COI gene were not representative of genetic relationships in the mtDNA genome. It is interesting that COI, which is the gene utilized for DNA barcoding efforts (Hebert et al., 2003), appears to have provided misleading information regarding the phylogenetic relationships in this study. This is a further example of the risks of inferring

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TABLE 6. Genetic distances between the four *Cynoscion* species and the red drum (*Sciaenops ocellatus*) calculated from the results of the factorial correspondence analyses conducted on the genotypes from 32 microsatellite markers. Table values represent the distance between the centers of the clusters of each pair of species in the three-dimensional factorial correspondence analysis plot. The distances above the diagonal are the results of the analysis that only included the four *Cynoscion* species, while the lower half of the matrix has the results from the analysis that included the four *Cynoscion* species and the red drum.

	C. arenarius	*C. regalis*	*C. nothus*	*C. nebulosus*
C. arenarius		212	963	663
C. regalis	153		875	466
C. nothus	799	808		932
C. nebulosus	269	213	916	
S. ocellatus	807	850	1,161	680

evolutionary relationships from the sequences of a single gene.

The results of the factorial correspondence analysis conducted on the microsatellite genotypes indicated that *C. nothus* is the most divergent of the *Cynoscion* species, yet the neighbor-joining tree created from the same dataset was inconclusive. This may result, at least in part, from the loss of information that occurs when individual specimen genotypes are consolidated into a set of species-specific allele frequencies that are the basis of the Cavalli-Sforza–Edwards distance estimates. In contrast, the FCA analysis evaluates the alleles carried by each individual at each locus to plot individuals in multidimensional space, maximizing the amount of information used by the analysis. The high mutation rate of microsatellite loci is expected to obscure relationships among more distantly related taxa, but the markers appear to perform relatively well in the present study.

Tringali et al. (2011) documented extensive hybridization between *C. arenarius* and *C. regalis* throughout a natural hybrid zone along Florida's east coast. They concluded that the hybridization was the result of imperfect assortative mating between the species, and an equilibrium between gene flow and one or more opposing forces meant that it was likely that the two species would continue to evolve independently in that area of contact (Tringali et al., 2011). It has also been documented that these two species infrequently hybridize with *C. nebulosus* and *C. nothus* in Florida's coastal waters (S. Seyoum, unpubl. data). At present, it does not appear that introgressive hybridization is challenging the integrity of any of the *Cynoscion* species inhabiting Florida's coastal waters. Yet introgressive hybridization could increase if environmental changes lead to increased rates of genetic flux into hybrid zones or diminish the strength of one or more of the forces opposing introgression.

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