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Reproductive Isolation in Sympatric Populations of Pallid and Shovelnose Sturgeon

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Abstract.—Pallid sturgeon *Scaphirhynchus albus* and shovelnose sturgeon *S. platorynchus* are recognized morphologically as separate species. A previous genetic study with allozymes was unable to distinguish between the two species or demonstrate their reproductive isolation in regions of sympatry. Our main objective was to measure the genetic variability within and among populations of pallid and shovelnose sturgeon at the northern and southern extremes of their sympatric ranges to determine if genetic variation within the two species exhibits patterns consistent with reproductive isolation. Additionally, we examined a sample of individuals identified morphologically as hybrids of the two species to determine their genetic relationship to fish identified morphologically as pallid and shovelnose sturgeon. Data from five nuclear DNA microsatellite loci indicated that pallid and shovelnose sturgeon were genetically distinct at three sympatric localities. Pallid sturgeon from two northern populations in the upper Missouri River were genetically distinct from the southern Atchafalaya River population, suggesting that northern and southern populations are reproductively isolated. Shovelnose sturgeon from three populations were genetically indistinguishable and showed no population structure. Sturgeon identified morphologically as hybrids from the Atchafalaya River were genetically distinct from pallid sturgeon but were indistinguishable from shovelnose sturgeon. These latter results are the converse of companion results with mitochondrial DNA published elsewhere. Pallid sturgeon were federally listed as endangered in 1990, and information about their population structure and potential for introgression with shovelnose sturgeon is critical for management and recovery programs for pallid sturgeon.

Based on morphological characteristics, the pallid sturgeon *Scaphirhynchus albus* was first described by Forbes and Richardson (1905) as a variant of shovelnose sturgeon *S. platorynchus.* Recognition of these species depends solely on morphological characters (Bailey and Cross 1954; Carlson et al. 1985; Keenlyne et al. 1994b), although the two forms do have different geographic distributions and microhabitat preferences (Kallemeyn 1989). The shovelnose sturgeon is relatively common and widely distributed throughout the Mississippi, Missouri, Ohio, and Tennessee rivers, including many related major tributaries (Carlson et al. 1985). The pallid sturgeon is less abundant and is restricted to the main channels of the Missouri, Yellowstone, and lower Mississippi Rivers (Carlson et al. 1985; Mayden and Kuhuda 1997) where they occur sympatrically with shov-

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elnose sturgeon. Adult shovelnose sturgeon range from 250 to 900 mm standard length (SL), whereas adult pallid sturgeon attain larger sizes (1,000–1,500 mm SL; Keenlyne et al. 1994b). Pallid sturgeon prefer larger river channels with swift, turbid flows and rocky or sandy substrate, whereas shovelnose sturgeon prefer slower flows and shallow pools associated with sandbars and channel edges (Forbes and Richardson 1905; Carlson et al. 1985; Kallemeyn 1989; Dryer and Sandoval 1993).

Decline in pallid sturgeon abundance since 1960 led to its listing as an endangered species in 1990 under the U. S. Endangered Species Act of 1973 (USFWS 1990). Alterations to the pallid sturgeon's preferred riverine habitat, such as damming and channelization, have been implicated in the species decline (Kallemeyn 1989). Carlson et al. (1985) and Keenlyne et al. (1994a) suggested that natural hybridization with shovelnose sturgeon may also be a threat to pallid sturgeon based on the presence of morphologically intermediate forms in areas of altered habitat.

Although these forms differ morphologically and occupy different ecological niches, previous genetic studies with nuclear markers were unsuccessful in distinguishing between the two species or in demonstrating reproductive isolation in regions of sympatry. Allozyme analysis of pallid, shovelnose, and their suspected hybrid forms could not distinguish between the three groups (Phelps and Allendorf 1983). Of 37 allozyme loci, 34 were identically monomorphic, and allele frequencies at the other three loci were similar among groups. Restriction enzyme analysis of nuclear DNA at five protein coding loci also failed to demonstrate differences between the two species (Morizot 1994). Campton et al. (2000) used mitochondrial DNA (mtDNA) control region (D-loop) sequences and found no fixed nucleotide substitutions that distinguished pallid and shovelnose sturgeon. However, haplotype frequencies differed significantly between the two species, thus providing initial evidence that they are genetically distinct for maternally inherited alleles.

Nuclear microsatellite DNA markers provide many advantages over allozyme loci and complement mtDNA techniques for investigating genetic structure of species (e.g., Estoup et al. 1993, Paetkau and Strobeck 1994, and Pope et al. 1996). Microsatellite loci usually are more polymorphic and have more alleles per polymorphic locus than do allozyme loci. Relatively high rates of mutation, with regard to number of repeat motifs, make this a useful class of markers for fine-scale population structure studies. Microsatellite loci, in contrast to mtDNA, are inherited biparentally and represent multiple independent loci. In addition, microsatellite loci, in contrast to most allozymes, can be more easily scored from tissues sampled nondestructively (e.g., muscle, fin, hair, blood, feces, scale, feather); they can also be preserved by freezing, drying, or alcohol storage. For these reasons microsatellite loci are ideal for studying endangered species. However, shovelnose sturgeon (and presumably, pallid sturgeon) are believed to have a tetraploid origin (Blacklidge and Bidwell 1993), and tetrasomic nuclear loci may complicate interpretation of microsatellite DNA genotypes.

We examined five microsatellite nuclear DNA loci to determine the extent to which pallid and shovelnose sturgeon are genetically or reproductively isolated at the northern and southern extremes of their sympatric ranges. We also examined individuals classified morphologically as hybrids to determine their genetic relationship to individuals classified as pallid and shovelnose sturgeon. Our work with nuclear DNA markers compliments mtDNA analyses of the same populations (Campton et al. 2000). Confirming reproductive isolation between pallid and shovelnose sturgeon with a battery of highly variable nuclear markers would further our understanding of the potential effects of natural hybridization between the parent species. Additionally, detecting population structure within pallid and shovelnose sturgeon would aid our ability to meet the conservation needs of these species.

Methods

Pallid and shovelnose sturgeon were examined from two Montana sites in the upper Missouri River and from a single Louisiana site on the Atchafalaya River (lower Mississippi River drainage) about 5,000 river kilometers downstream (Figure 1; Campton et al. 2000). Sampling was performed by personnel of state and federal agencies in 1992. Each specimen was measured morphometrically and classified as a pallid sturgeon, shovelnose sturgeon, or putative hybrid (morphologically presumed hybrids of pallid and shovelnose sturgeon) based on the criteria of Keenlyne et al. (1994a, b). Fin tissue and blood samples were collected from each fish before release.

At the first upper Missouri River sampling site, located approximately 400 km above Fort Peck Lake (upstream Fort Peck site or UFP), 9 pallid sturgeon (1,207–1,387 mm SL) and 9 shovelnose sturgeon (588–845 mm SL) were collected; at the

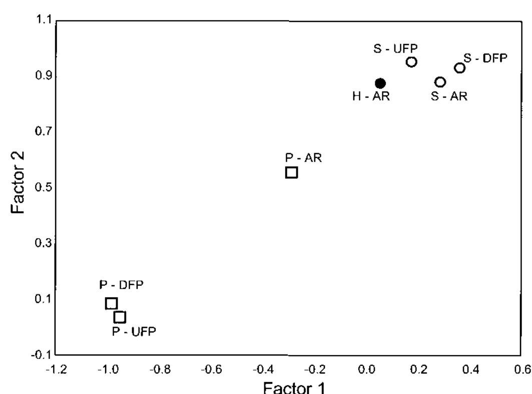

FIGURE 1.—Factor map of the two main factorial axes based on Nei's genetic distances. The first and second axes, extracted by the maximum likelihood method, explained 83% of the total variance contained in the data set. Sample sites for pallid (P; clear squares) and shovelnose (S; clear circles) sturgeon and the putative hybrids (H; shaded circle) included the upstream Fort Peck site (UFP), downstream Fort Peck site (DFP), and Atchafalaya River (AR).

second site, located downstream from Fort Peck Dam at the confluence of the Yellowstone and Missouri rivers (downstream Fort Peck site or DFP), 11 pallid sturgeon (1,016–1,540 mm SL) and 10 shovelnose sturgeon (326–600 mm SL) were collected. Both sites are partially isolated by Fort Peck dam, which is considered to be a barrier to upstream adult migration (Keenlyne at al. 1994b). Individuals sampled from the Atchafalaya River (AR) included 10 pallid sturgeon (567–840 mm SL), 18 shovelnose sturgeon (429–653 mm SL), and 10 putative hybrids (551–689 mm SL) based on the criteria of Keenlyne et al. (1994a, b).

From the three localities, we archived genomic DNA, stored in TE buffer (Sambrook et al. 1989), from sturgeon classified morphologically as pallid, shovelnose, and putative hybrids. We screened all samples for genetic variability using oligonucleotide primers for 19 microsatellite loci, of which 12 were cloned from lake sturgeon *Acipenser fulvescens* (May et al. 1997) and 7 from Atlantic sturgeon *A. oxyrynchus* (Lubinski and King, personal communication). Hereafter, individual primer identifiers, consisting of the species scientific name from which it was cloned (''Afu'' for lake sturgeon and ''Aox'' for Atlantic sturgeon) and the laboratory identification number, are used to identify each locus. Primers are referred to in the text by using a regular font (e.g., Afu19) whereas references to loci use italics (e.g., *Afu19*)

Amplification was done in 200-μL 8-tube strips with 1 unit Taq DNA polymerase (GIBCO), 0.4 μM each primer, 3–10 ng template, 100 or 175 μM dNTPs, and 1.5–2.5 mM $MgCl_2$ in 50 μL of buffer. Reaction mixtures were amplified in an M.J. Research PTC-100 96-V thermocycler with a ''hot bonnet'' lid using the following procedure: The mixture was preheated at 94°C for 3 min, amplified for 35 cycles (denaturing at 94°C for 1 min, annealing at 57°C for 30 s, polymerization at 72°C for 30 s), and a final polymerization was conducted at 72°C for 5 min. Amplified products were run on a 5% denaturing acrylamide gel and visualized with a Molecular Dynamics 595 fluorimager. The amplification products were fluorescently detected using single-primer labeling with fluorescein or by staining with an agarose and Vistra Green overlay (Rodzen et al. 1998). Alleles were sized using FragmeNT Analysis (Molecular Dynamics, Sunnyvale, CA).

Codominant genotypes were classified and recorded for each microsatellite locus. Allele frequencies for each locus, observed heterozygosity (H_O), expected heterozygosity (H_E), and the genetic differentiation index F_{IS} (Weir and Cockerham 1984) were calculated using GENEPOP 3.0 (Raymond and Rousset 1995). All populations were tested for conformance to Hardy–Weinberg expectations with Genes in Populations 2.2 (program designed by B. May and C. C. Krueger; written in C by W. Eng and E. Paul, available from http://animalscience.ucdavis.edu/extension/Gene.htm). Nei's (1978) genetic distance was calculated for all population pairs with Genes in Populations 2.2. A multivariate factor analysis was performed for Nei's genetic distance with Statistica for Windows version 5.1 (Statsoft Inc.). Factors were extracted using a maximum-likelihood method (Harman 1976).

The degree of population subdivision was determined from multilocus estimates of F_{ST} (Weir and Cockerham 1984) for all population pairs using FSTAT 2.9.1 (available from http://www.unil.ch/izea/softwares/fstat.html) updated from Goudet (1995). We assumed no random mating within samples to obtain a more conservative measure of differentiation among populations. Pairwise significance tests using likelihood ratio G (Goudet et al. 1996) were performed by permutation and resampling of multilocus genotypes among pairs of samples. Performing 2,500 randomizations allowed for a tablewide significance at the 1% nominal level after standard Bonferroni corrections.

Results

All 74 sturgeon were screened for polymorphic loci using 19 microsatellite primers yielding six polymorphic loci (*Afu19, Afu34, Afu68, Afu57, Aox27,* and *Aox45*), six monomorphic loci (*Afu21, Afu54, Afu58, Afu62, Aox23,* and *Aox44*), and seven loci that did not amplify (*Afu22, Afu23, Afu69, Afu71, Aox21, Aox46,* and *Aox49*). Individual phenotypes at the six polymorphic loci were either single-banded or symmetrically double-banded, suggesting they were disomic. At polymorphic loci, 2–13 alleles were observed. With 13 alleles, *Aox45* (Lubinski and King, personal communication: 5′-TTGTCCAATAGTTTCCAACGC-3′ and 5′GTGCTCCTGCTTTTACTGTC-3′) was highly variable, and no samples conformed to Hardy–Weinberg expectations for this locus. Therefore, *Aox45* was excluded from subsequent analysis. Observed heterozygosity within samples for the other five loci ranged from 0.00 at *Afu19* and *Aox27* (Lubinski and King, personal communication: 5′-AATAACAATAACGGCAGAACCT-3′ and 5′-TGTGTTGCTCAAGACAGTATGA-3′) to 1.0 at *Afu68* (Table 1). The observed heterozygosity value of 0.0 at *Afu19* was due to fixation of a single allele in Atchafalaya River pallid sturgeon. Also, no heterozygotes at *Aox27* were observed in the collection of Atchfalaya River pallid sturgeon and shovelose sturgeon downstream from Fort Peck. No homozygotes (observed heterozygosity 1.0) were observed at *Afu68* in the collection of Atchafalaya River putative hybrids. Expected heterozygosity per locus ranged from 0.0 at *Afu19* to 0.88 at *Afu68* (Table 1). Only one locus pair (*Afu19* and *Aox27*) in the Athchafalaya River shovelnose sample showed significant genotypic disequilibrium. No alternate alleles were at fixation and, thus, were not diagnostic for species. Each population conformed to Hardy–Weinberg expectations at all loci, except for the following: pallid sturgeon below Fort Peck at *Afu19, Afu34,* and *Afu68*; shovelnose sturgeon below Fort Peck at *Aox27*; and Atchafalaya River putative hybrids at *Aox27*.

Allele frequencies for pallid sturgeon and shovelnose sturgeon differed significantly ($P < 0.01$) in all interspecific, pairwise comparisons (Table 2). Allele frequencies for pallid sturgeon from the two upper Missouri River localities did not differ significantly ($P = 0.36$), although they did differ from those for pallid sturgeon from Atchafalaya River ($P < 0.01$). Allele frequencies for shovelnose sturgeon from the three localities did not differ significantly. Allele frequencies for putative hybrids differed ($P < 0.01$) from those for the three pallid sturgeon populations but were statistically indistinguishable from those for all three samples of shovelnose sturgeon.

Three groups of sturgeon were identified based on factor analysis of Nei's (1978) genetic distances (Table 2; Figure 1). Pallid sturgeon from the two upper Missouri River populations formed a distinct group. Shovelnose sturgeon from both upper Missouri River localities and the Atchafalaya River, including putative hybrids from the latter, formed a second distinct group. Pallid sturgeon from the Atchafalaya River were genetically intermediate to the other two groups. These groups were similar to the patterns revealed with F_{ST} testing. The first and second factorial axes, extracted with the maximum-likelihood method, captured 83% of the total variance in genetic distances among samples.

Discussion

Sympatric populations of pallid and shovelnose sturgeon were genetically distinct at each of the three sites compared in this study. These results, obtained using nuclear microsatellite markers, are similar to those reported for mtDNA by Campton et al. (2000). Our findings contrast with studies reporting an absence of genetic differences between the two species using the more conserved allozyme (Phelps and Allendorf 1983) and protein-coding loci (Morizot 1994). Phelps and Allendorf (1983) explained the close genetic similarity at their allozyme loci ''as due to recent or incomplete reproductive isolation accompanied by rapid morphological differentiation'' and proposed that assortative mating might be reinforced by morphological and ecological preferences, in which selection acts against morphological intermediates. In this scenario, reproductive isolation is incomplete or, more likely, has occurred recently without accumulating any genetically detectable differences at protein-coding loci. The microsatellite loci we used demonstrated higher mutation rates than do previously investigated allozymes and conserved protein-coding sequences and appeared to be more sensitive for detecting divergence between pallid and shovelnose sturgeon. In contrast to mtDNA, microsatellite markers are biparentally inherited and thereby confirm that both male and female sturgeon are assortatively mating at each of the three sample localities.

The comparison of samples within species revealed differing patterns of genetic structure

TABLE 1.—Allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E), genetic differentiation index (F_{IS}), and sample sizes (N) for all sturgeon populations. Sample sites for pallid sturgeon (P), shovelnose sturgeon (S), and putative hybrids (H) included downstream of Fort Peck Dam (DFP), upstream of Fort Peck Dam (UFP), and the Atchafalaya River (AR). Alleles are named by base pair sizes. Asterisks denote deviations from Hardy–Weinberg expectations for which significant ($\alpha = 0.05$) F_{IS} values were obtained.

		Population						
Locus	Allele	P-DFP	P-UFP	S-DFP	S-UFP	P-AR	S-AR	H-AR
Afu19	*122*	0.73	0.50	0.67	0.61	1.00	0.89	0.45
	125	0.27	0.50	0.33	0.39	0.00	0.11	0.55
H_O		0.18	0.33	0.44	0.56	0.00	0.22	0.50
H_E		0.40	0.53	0.47	0.50	0.00	0.20	0.52
F_{IS}		0.54*	0.38	0.59	−0.11	0.00	−0.10*	0.04
N		11	9	9	9	10	18	10
Afu34	*139*	0.23	0.11	0.10	0.17	0.00	0.03	0.15
	142	0.00	0.06	0.00	0.00	0.00	0.00	0.00
	145	0.50	0.39	0.80	0.72	0.80	0.81	0.80
	148	0.00	0.06	0.05	0.00	0.05	0.00	0.00
	151	0.05	0.00	0.00	0.00	0.00	0.00	0.00
	154	0.23	0.33	0.00	0.11	0.15	0.17	0.00
	157	0.00	0.06	0.00	0.00	0.00	0.00	0.00
	160	0.00	0.00	0.00	0.00	0.00	0.00	0.05
	163	0.00	0.00	0.05	0.00	0.00	0.00	0.00
H_O		0.64	0.56	0.40	0.33	0.30	0.28	0.40
H_E		0.65	0.76	0.36	0.47	0.35	0.33	0.35
F_{IS}		0.01*	0.28	−0.11	0.29	0.16	0.17	−0.14
N		11	9	10	8	10	18	10
Afu68	*113*	0.00	0.00	0.00	0.00	0.00	0.03	0.00
	117	0.00	0.00	0.20	0.06	0.10	0.03	0.15
	121	0.00	0.00	0.10	0.00	0.20	0.22	0.15
	125	0.23	0.06	0.35	0.72	0.15	0.36	0.20
	129	0.18	0.13	0.05	0.06	0.20	0.11	0.05
	133	0.00	0.00	0.00	0.00	0.05	0.00	0.00
	137	0.46	0.81	0.15	0.00	0.15	0.06	0.25
	141	0.14	0.00	0.10	0.17	0.15	0.17	0.20
	145	0.00	0.00	0.05	0.00	0.00	0.03	0.00
H_O		0.55	0.38	0.90	0.56	0.90	0.83	1.00
H_E		0.69	0.34	0.83	0.47	0.88	0.79	0.85
F_{IS}		0.21*	−0.11	−0.09	−0.19	−0.19	−0.05	−0.18
N		11	8	10	9	10	18	10
Aox27	*121*	0.05	0.07	0.70	0.39	0.10	0.53	0.39
	125	0.95	0.93	0.30	0.61	0.90	0.47	0.61
H_O		0.10	0.14	0.00	0.33	0.00	0.59	0.78
H_E		0.01	0.14	0.44	0.50	0.19	0.51	0.50
F_{IS}		−0.05	0.00	1.00*	0.35	1.00	−0.15	−0.60*
N		10	7	10	9	10	17	9
Afu57	*144*	0.30	0.33	0.35	0.22	0.50	0.28	0.40
	147	0.65	0.67	0.50	0.50	0.10	0.58	0.30
	150	0.05	0.00	0.15	0.28	0.40	0.14	0.30
H_O		0.70	0.67	0.60	0.78	0.60	0.44	0.90
H_E		0.49	0.48	0.64	0.66	0.61	0.58	0.69
F_{IS}		0.44	−0.30	0.06	−0.19	0.02	0.24	−0.32
N		10	6	10	9	10	18	10
Average H_O		0.43	0.42	0.47	0.51	0.36	0.47	0.72
SE		0.12	0.09	0.15	0.08	0.18	0.11	0.12
Average H_E		0.46	0.42	0.52	0.49	0.39	0.47	0.56
SE		0.11	0.10	0.08	0.03	0.15	0.10	0.08

among populations. Shovelnose sturgeon collected from the upper Missouri River and the lower Mississippi River were genetically indistinguishable. This result was expected because shovelnose sturgeon are widely distributed throughout the Mississippi and Missouri River drainages. In contrast, pallid sturgeon from the Atchafalaya River were genetically differentiated from both upper Missouri populations, a result consistent with mtDNA (Campton et al. 2000). A number of hypotheses could explain these results: polyphyletic origin of pallid sturgeon, sympatric speciation, genetic drift, and introgressive hybridization in the Atchafalaya River (see Discussion of Campton et al. 2000).

TABLE 2.—Estimates of F_{ST} (lower half of matrix) and the Nei's genetic distance (upper half of matrix) between all populations. Significant F_{ST} values (Bonferroni adjusted, $\alpha = 0.01$) are marked with an asterisk. Sample sites for pallid (P), shovelnose (S), or hybrid (H) sturgeon included downstream of Fort Peck Dam (DFP), upstream of Fort Peck Dam (UFP), and the Atchafalaya River (AR).

	Population						
Population	P-DFP	P-UFP	P-AR	S-DFP	S-UFP	S-AR	H-AR
P-DFP		0.04	0.15	0.25	0.16	0.17	0.16
P-UFP	0.01		0.33	0.38	0.33	0.35	0.22
P-AR	0.13*	0.25*		0.25	0.21	0.14	0.16
S-DFP	0.15*	0.22*	0.18*		0.07	0.03	0.06
S-UFP	0.10*	0.21*	0.17*	0.03		0.07	0.08
S-AR	0.13*	0.23*	0.12*	0.01	0.04		0.12
H-AR	0.10*	0.14*	0.12*	0.02	0.04	0.09	

Pallid sturgeon are less abundant than shovelnose sturgeon throughout the Mississippi and Missouri river systems, and their natural absence from the main stem Mississippi River above St. Louis suggests biogeographic limitations to dispersal. The construction of barriers to upstream movement has been implicated as a cause of population decline by preventing migration to historical spawning areas (Kallemeyn 1989). In addition, pallid sturgeon prefer warm turbid rivers with swift, high volume flows. Damming and dredging have altered preferred spawning substrate and water velocity, transforming large, wide channels into relatively clear, cold pools (Dryer and Sandoval 1993; Kallemeyn 1989).

An examination of the microsatellite genotype frequencies does not immediately suggest that the putative hybrids are intermediate to and thus representative of multiple instances of first-generation hybridization of pallid and shovelnose sturgeon. The three observed genotype frequencies of putative hybrids at *Afu57* were intermediate to the observed Atchafalaya River genotype frequencies for pallid and shovelnose sturgeon, as expected for a first-generation hybrid population. However, intermediate genotype frequencies occurred only for this locus. When the putative hybrids were analyzed as a separate population, they were divergent from the pallid sturgeon analyzed in our study but indistinguishable from the three populations of shovelnose sturgeon. In contrast, the distribution of mtDNA haplotypes among the hybrids in the Atchafalaya River were much more similar to those for pallid sturgeon than for shovelnose sturgeon at the same locality (Campton et al. 2000). These results suggest that pallid sturgeon females may have interbred with shovelnose sturgeon males, potentially backcrossing to the latter species, if the hypothesis of natural hybridization is correct. A continued analysis with more microsatellite loci and mtDNA would probably help to confirm the origin of morphologically intermediate putative hybrids.

Building upon previous morphological, genetic, and ecological studies, our study demonstrates genetic differences between sympatric populations of pallid and shovelnose sturgeon. In addition, endangered pallid sturgeon of the lower Mississippi River are genetically differentiated from both upper Missouri River populations. As such, pallid and shovelnose sturgeon warrant treatment as separate species and should be managed accordingly. Additionally, pallid sturgeon in the upper Missouri and Atchafalaya rivers should be managed as genetically distinct populations.

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