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ORIGINAL PAPER

Genetic discrimination of middle Mississippi River *Scaphirhynchus* sturgeon into pallid, shovelnose, and putative hybrids with multiple microsatellite loci

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Abstract The pallid sturgeon (*Scaphirhynchus albus*), which is protected under the US endangered species act, and shovelnose sturgeon (*S. platorhynchus*), which is legally harvested in some locations, are sympatric throughout the range of pallid sturgeon. There is considerable morphological overlap between the species making discrimination problematic. The inability to reliably differentiate between species across all life stages has hampered pallid sturgeon recovery efforts. Furthermore, the two species are believed to hybridize. This study used allele frequency data at multiple microsatellite loci to perform Bayesian and likelihood-based assignment testing and morphological measures and meristics to discriminate pallid, shovelnose, and putative hybrid sturgeons from the middle Mississippi River. Bayesian model-based clustering of the genetic data indicated that two natural genetic units occur in the region. These units correspond to morphologically identified pallid and shovelnose sturgeon. Some individuals were morphologically intermediate and many of these failed to strongly assign genetically as either pallid or shovelnose sturgeon, suggesting they may be hybrids. These data indicate that pallid sturgeon and shovelnose sturgeon are genetically distinct in the middle Mississippi River (F_{ST} = 0.036, $P < 0.0001$) and suggest that hybridization between pallid sturgeon and shovelnose sturgeon has occurred in this region with genetic distance estimates indicating the greatest distance is between pallid and shovelnose sturgeon, while hybrid sturgeon are intermediate but closer to shovelnose. This study demonstrates that assignment testing with multiple microsatellite markers can be successful at discriminating pallid sturgeon and shovelnose sturgeon, providing a valuable resource for pallid sturgeon recovery and conservation.

Keywords *Scaphirhynchus* · Microsatellite · Assignment testing · Endangered species · Species identification

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Introduction

The pallid sturgeon (*Scaphirhynchus albus*, hereafter abbreviated PS) and shovelnose sturgeon (*S. platorhynchus*, hereafter abbreviated SS) are native to the Mississippi River drainage and are sympatric throughout the range of PS (Forbes and Richardson 1905; Bailey and Cross 1954). PS are protected in the US under the Endangered Species Act (Dryer and Sandoval 1993), while SS are legally harvested in several US states including some bordering the middle Mississippi River (MMR; Keenlyne 1997). What may be described as a morphological continuum exists between the two species making it difficult to reliably identify some specimens (Kuhajda and Mayden 2001; Wills et al. 2002). It is not currently known to what extent the morphological continuum is due to high variability within species or to hybridization between species. PS

do have a distinctly greater maximum adult size of 1.7 m compared to 0.8 m for SS (Birstein 1993). However, in the middle Mississippi River both PS and SS adults are of similar size, and we are unaware of any *Scaphirhynchus* specimens greater than 1.2 m in length from the Mississippi River. Early life history stages of both species are morphologically more similar than are adults.

A recent study using nine microsatellite loci developed in sturgeon of the genus *Acipenser* found that morphologically intermediate *Scaphirhynchus* from the Atchafalaya River (a distributary of the Mississippi) tend to have intermediate genotypes (Tranah et al. 2004). These intermediate individuals have been taken as evidence for hybridization in the Mississippi River. Hybridization is a potential threat to the endangered PS (Allendorf et al. 2001; Rhymer and Simberloff 1996) because the discrete gene pool of the rare PS may be eroded by introgression from the more common SS causing extinction through hybridization. The lack of a reliable differentiation method, effective across regions and life stages, has hampered PS recovery efforts. PS recovery currently relies on stocking of offspring from wild-caught PS broodstock. Any inadvertent selection of hybrids or backcrosses as broodfish with subsequent release of large numbers of backcross offspring may further diminish the integrity of PS. Several morphological and meristic-based identification indices have been created in an attempt to discriminate between the species and their hybrids. However, none of these indices work adequately in all areas or for young sturgeon (Kuhajda and Mayden 2001).

Previous genetic studies using allozyme loci (Phelps and Allendorf 1983), restriction digests of nuclear amplicons (Genetic Analysis 1994 as cited in Wirgin et al. 1997), and DNA sequences (Bischof and Szalanski 2000; Straughan et al. 2002) fail to discriminate PS and SS species. The lack of discrimination is likely due to low levels of genetic variation (allozymes) and an absence of diagnostic differences (mtDNA) between species observed at the markers screened. Significant frequency differences in mitochondrial DNA haplotypes (Campton et al. 2000) and allele frequencies at five heterologous microsatellite markers (Tranah et al. 2001) were reported between PS and SS from the upper Missouri and Atchafalaya Rivers. However, neither genetic marker has detected diagnostic genetic differences among species. Also, it is not known if the absence of diagnostic differences is due to incomplete lineage sorting of ancestral polymorphisms or is due to secondary contact via introgressive hybridization (Campton et al. 2000). Tranah et al. (2004) suggest that multiple microsatellite loci are potentially better suited discriminating PS and SS than other methods. In their study, nine loci, some developed in the genus *Acipenser* could potentially be used to identify 90% of the individuals screened to species.

To date, no genetic study has investigated PS, SS, and putative hybrid sturgeon from the MMR, a region where the presence of hybridization has been suggested (Carlson et al. 1985). This study defines the MMR as the Mississippi River south from its confluence with the Missouri River (River Mile 195) near Saint Louis, Missouri, to the confluence of the Mississippi and Ohio Rivers at Cairo, Illinois (River Mile 0). Additionally, no study has attempted to delineate the two species using genetic assignment testing methods (first developed by Paetkau et al. 1995 and reviewed by Manel et al. 2005 and Hansen et al. 2001) that may discriminate species in the absence of diagnostic genetic differences. It is also possible for assignment testing methods to identify hybrid individuals (Vaha and Primmer 2006). Our objectives were to determine the number of distinct genetic groups within *Scaphirhynchus*, to ascertain if potential hybrid individuals were present in the samples collected, and to estimate the level of genetic differentiation among the identified groups. These objectives were addressed using genetic assignment testing methods with a large panel of microsatellite markers developed in *Scaphirhynchus*. We then compared results from genetic assignment to morphological identification based on a morphological character index (Wills et al. 2002) developed for MMR *Scaphirhynchus*.

Methods

Scaphirhynchus tissue samples ($n = 157$) were collected from the MMR. Putative PS and hybrid surgeon were sampled from multiple locations between the Missouri–Mississippi River confluence south to Cairo, Illinois (RM 195 to RM 0). Putative SS were sampled near Chester, Illinois (RM 110). Individual specimens were preliminarily characterized as PS, SS, and possible hybrid (Table 1) by collectors using the character index (CI) of Wills et al. (2002). The CI is a regression character index that uses five morphometric relationships (outer barbel length/inner barbel length, head length/inner barbel length, head length/mouth to inner barbel base distance, rostrum length/inner barbel length, and rostrum length/mouth to inner barbel distance) and two meristics (dorsal and anal fin ray counts) to discriminate PS and SS. The CI scores typical PS with negative (< –1.48 to –0.46) index values, SS with positive (0.52 to > 1.33) index values, and hybrid sturgeon as intermediate ($-0.08 < CI < 0.36$).

Table 1 Total number of *Scaphirhynchus* specimens screened from the middle Mississippi River reported by the initial morphological character index (CI) identification and the Bayesian genetic assignment test (Q-value) identification

Species	CI	Q-value	Combined
Pallid Sturgeon	44	30	29
Intermediate/Hybrid	19	43	55
Overlap Pallid/Hybrid	3	–	–
Hybrid	8	–	–
Overlap Shovelnose/Hybrid	8	–	–
Shovelnose Sturgeon	94	84	73
Total	157	157	157

Intermediates were defined as those individuals not belonging to either pallid or shovelnose sturgeon for each method. The final species designation was made by syntheses of both morphological and genetic methods

Wills et al. (2002) defined a region of overlap between PS and hybrid (–0.45 to –0.09) and SS and hybrid (0.37–0.51). Sturgeon that fall between the PS/hybrid, and hybrid/SS categories are assigned as PS/hybrid overlap and SS/hybrid overlap respectively.

Genomic DNA was extracted using the DNeasy Tissue Kit[1] (Qiagen, Valencia, CA) and stored at –20°C. Sixteen disomic microsatellite markers developed in *Scaphirhynchus* by McQuown et al. (2000) were optimized for this study (Table 2). Microsatellite loci were initially screened with radiolabeled techniques to determine which markers could be reliably scored. Polymerase chain reaction (PCR) was conducted at a final volume of 10 µl, containing 1 × PCR Buffer (50 mM KCl, 10 mM Tris HCl pH 9.0, and 1% Triton × 100®), 2 mM $MgCl_2$, 200 µM each dNTP, 0.1 unit *Taq* DNA polymerase, 0.14 µM each primer, and 1–20 ng template DNA. Prior to PCR the forward primer was end-labeled with γ ^{32}P adenosine triphosphate using T4 polynucleotide kinase. The PCR thermal profile included a 95°C initial denaturation of 4 m followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 70°C for 30 s. PCR products were separated using 42 cm denaturing polyacrylamide gel electrophoresis for 3 h at 1600 V. Bands were visualized by autoradiography.

Once markers were determined to be polymorphic and amplify reliably, all individuals were screened in multiplex reactions on an ABI 377 (PE Applied Biosystems, Foster City, CA) with fragment analysis software. PCR conditions were identical to those used for the radioactive labeled PCR except, for each locus, fluorescent labeled forward primers (6-FAM, NED, or HEX; PE Applied Biosystems) and unlabeled reverse primers were combined in a primer cocktail (9 µM each primer). Final primer concentration in PCR was 0.15 µM. Thermal profiles for multiplex reactions were: 94°C 2 m, 5 cycles of 94°C 30 s, 54°C 30 s, 70°C 30 s, and 35 cycles of, 95°C 30 s, 56°C 30 s, and 70°C 30 s. PCR products were diluted (1:1) in loading buffer (deionized formamide, blue dextran EDTA, and The Gel Company MRK-400 size standard; The Gel Company, San Francisco, CA), loaded on a 5% Long Ranger (Cambrex Bio Science, East Rutherford, NJ) 36 cm gel, and run at 2500 scans/h for 2.5 h. Gel images were analyzed with Genescan v 3.1.2 (PE Applied Biosystems). Alleles were binned to raw size with Genotyper v 2.5 (PE Applied Biosystems). Alleles defined by Genotyper were scatter-plotted by size to define final bin boundaries and recoded to the inferred number of repeats corresponding to the defined size.

To identify the number of distinct groups and determine individual membership in the defined groups, a Bayesian model-based clustering of the genetic data was performed using STRUCTURE v 2.1 (Pritchard et al. 2000; Falush et al. 2003). To avoid overestimating the number of groups, the ΔK method of Evanno et al. (2005) was used to calculate the second order rate of change in the natural log probability of observing the data given the number of groups. Once the most likely number of groups was identified, another run was performed to identify individual membership in the identified groups. For all runs, the admixture model allowing correlated allele frequencies was used. Q-values were calculated with 0.95 posterior confidence regions (CR) describing the posterior probability of an individual's genotype belonging to each identified group. Analyses were conducted using 30,000 burn-in steps and 1,000,000 post burn-in steps.

The genetic assignment results were compared to the morphological discrimination by scatter-plotting Q-value (y-axis) against CI value (x-axis) for all individuals. This shows the level of concordance between methods and identifies potential hybrids. After comparing the results from both methods we set criteria to define PS and SS (see results), with individuals not meeting the defined criteria treated as intermediate and potential hybrids. The criteria were set to remove any potential hybrids and backcrosses from the baseline data, perhaps at the risk of eliminating some pure PS and SS from the baselines. We then used the baseline data to examine the assignment probabilities of potential hybrids.

GENECLASS v 2.1 (Piry et al. 2004) was used to jackknife the baseline PS and SS samples to reduce bias in self assignment. GENECLASS was also used to test the intermediate/hybrid individuals. The defined PS

[1] The use of trade and product names throughout text does not imply endorsement by the United States Government.

Table 2 Summary statistics for the microsatellite loci screened in pallid, hybrid, and shovelnose sturgeons

Locus	θ	Pallid Sturgeon				Intermediate/Hybrid Sturgeon				Shovelnose Sturgeon			
		N	*R*	H_e	H_o	*n*	*R*	H_e	H_o	*n*	*R*	H_e	H_o
Spl-012	0.034*	29	4.93	0.583	0.345	47	7.08	0.675	0.532	71	6.86	0.732	0.676
Spl-015	0.027*	28	6.00	0.632	0.500	47	9.07	0.793	0.787	71	10.67	0.820	0.761
Spl-018	0.087*	29	5.86	0.485	0.517	47	7.52	0.711	0.638	72	5.50	0.622	0.597
Spl-019	0.011	29	5.00	0.733	0.724	47	6.32	0.767	0.787	72	7.16	0.797	0.819
Spl-030	0.053*	29	3.86	0.347	0.310	47	9.65	0.705	0.745	72	9.30	0.738	0.708
Spl-035	0.040*	29	10.65	0.803	0.793	47	15.92	0.900	0.894	72	16.26	0.918	0.889
Spl-036	0.033*	29	8.86	0.778	0.793	47	12.94	0.868	0.851	72	14.94	0.887	0.764
Spl-040	0.000	27	12.00	0.858	0.741	47	13.75	0.883	0.872	66	14.42	0.892	0.848
Spl-053	0.021*	29	5.00	0.699	0.828	47	10.44	0.794	0.809	72	8.80	0.779	0.819
Spl-056	0.072*	29	8.86	0.796	0.897	47	14.22	0.915	0.915	72	14.85	0.857	0.889
Spl-060	0.029*	29	6.86	0.776	0.897	47	6.72	0.680	0.681	72	5.21	0.575	0.556
Spl-101	0.017	29	5.93	0.751	0.690	47	7.50	0.810	0.830	71	7.97	0.820	0.915
Spl-106	0.014*	29	7.86	0.763	0.759	47	10.33	0.819	0.723	72	9.25	0.819	0.750
Spl-119	0.076*	29	5.93	0.704	0.828	47	9.44	0.814	0.766	72	9.50	0.807	0.667
Spl-158	0.027*	29	7.00	0.801	0.966	47	9.14	0.862	0.936	72	9.83	0.861	0.889
Spl-173	0.031*	29	4.93	0.680	0.759	47	6.99	0.774	0.787	72	7.85	0.851	0.806
Total	0.036*			0.699	0.709			0.798	0.785			0.798	0.772

Estimates of θ among pallid, hybrid, and shovelnose sturgeons are provided for each locus and combined over all loci with an asterisk denoting statistical significance. The number of individuals screened per locus (*n*), allelic richness (*R*), expected heterozygosity (H_e), and observed heterozygosity (H_o) are provided for each locus. No locus in any group was significantly out of Hardy–Weinberg equilibrium, and no loci pair in any group showed significant linkage disequilibrium

and SS individuals were treated as baseline samples from which allele frequencies were drawn for assignment testing, and we tested the assignment probabilities of the intermediate/hybrid individuals to each of these groups. We used the ''assign or exclude individuals'' option with the assignment criteria set at 0.05. The Rannala and Mountain (1997) assignment algorithm was used with probability of assignment calculated following the methods outlined in Paetkau et al. (2004) with 10,000 individuals generated for comparison.

GDA version 1d16c (Lewis and Zaykin 2001) was used to calculate the sample size, number of observed alleles, and observed and expected heterozygosities for each locus within each sample. FSTAT v 2.9.3 (Goudet 2001, an update of Goudet 1995) was used to calculate allelic richness and estimates of F_{ST} on the defined PS, SS, and intermediate/hybrid sturgeon. Allelic richness was estimated based on the lowest observed sample in a group. Weir and Cockerham's (1984) θ estimator of F_{ST} was calculated for each locus and combined across all loci among groups. A 95% confidence interval for θ was calculated by bootstrapping over loci and an exact G-test (Goudet et al. 1996) of genetic differentiation was performed assuming Hardy–Weinberg equilibrium (HWE) within groups. Tests for conformation to HWE and linkage equilibrium (LE) were also performed for each locus (or locus pair) in each sample. All tests used alpha = 0.05 with Bonferroni correction for multiple tests when appropriate (Rice 1989). The shared allele distance (DAS, Chakraborty and Jin 1993) was estimated among PS, SS, and hybrid sturgeon using the software package Populations version 1.2.28 (Langella 2002).

Results

Morphological analysis of all *Scaphirhynchus* specimens with the CI (Fig. 1, Table 1) identified 44 putative PS, 94 putative SS, 3 PS/hybrid overlap, 8 SS/hybrid overlap, and 8 hybrids, using categories defined by Wills et al. (2002). The majority (87.9%) of individuals were placed into either the PS or SS categories, with few individuals (12.1%) scoring as hybrid or hybrid overlap on the CI scale.

Bayesian assignment testing with Structure found evidence for two natural groups of *Scaphirhynchus* in the MMR. When alternately testing the data for the presence of one to four groups, the estimated probability of the data given the number of groups was maximized at two groups. The results showed a unimodal distribution with a sharp increase in probability from one to two groups, followed by increasing variability and lower probabilities with increasing number of groups and the *ad hoc* probability of two groups was near one (Falush et al. 2003; Pritchard et al. 2000). Assuming two groups were present also maximized the second order rate of change function, ΔK (Evanno et al. 2005). Thus the data support the presence of two genetically distinct groups of *Scaphirhynchus* in the MMR.

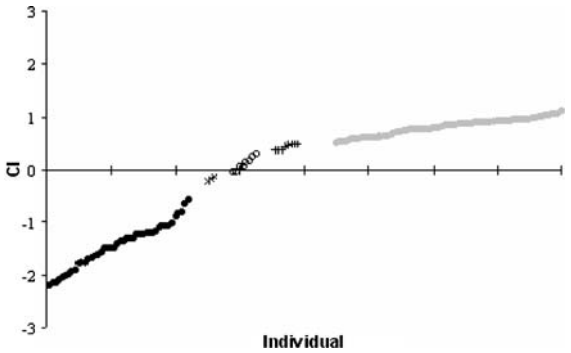

Fig. 1 Character index (CI) values for all *Scaphirhynchus* specimen (n = 157). Each point represents an individual and is coded by CI category (pallid = black circle, pallid/hybrid overlap = X, hybrid = open circle, shovelnose/hybrid overlap = +, and shovelnose = gray circle). Individuals classified as hybrid or in either hybrid overlap category were initially defined as intermediate

When we postulated the presence of two groups and allowed each individual's genotype to be comprised of elements from both groups, most individuals strongly assigned to one of the two groups (Fig. 2). We designated Q-values indicating 100% assignment to the group dominated by morphological PS as Q = 1 while those entirely assigned to the group dominated by morphological SS as Q = 0. A 95% CR is provided for each Q-value (Fig. 2). Individuals were categorized (Table 1) as PS or SS by Q-value, provided their 95% CR did not include 0.50. Individuals whose 95% CR included 0.50 were identified as intermediates.

Synthesizing the morphological (CI) and molecular (Q-value) discrimination results (Fig. 3) finds general agreement between techniques. Individuals were clustered into two major groups, which corresponded to PS (Quadrant II) and SS (Quadrant IV). Several individuals were intermediate, falling near the intersection of the axes, and a small number of individuals (n = 4) showed disagreement between morphological and molecular discrimination. These individuals all had more PS-like morphology and SS-like genotypes. Examining the graph and consulting both morphology and molecular tests allowed boundaries to be defined to identify the more confidently discriminated PS and SS. The intention was to create baseline groups of PS and SS while minimizing the potential of including hybrid individuals in either baseline. Final discrimination was made by combining both methods (Table 1), with PS (n = 29) defined by a CI less than –0.45 and a Q-value with a lower 95% CR bound greater than 0.50. SS (n = 73) were defined by a CI greater than 0.51 and a Q-value with an upper 95% CR bound less than 0.50. All individuals that did not meet both criteria were categorized as intermediate/hybrid (n = 55). Jackknifing the baseline PS and SS groups with GeneClass assigned all individuals back into the expected group with a score greater than 94%.

Testing the intermediate/hybrid samples (i.e. those with 95% CR including Q = 0.5) with GeneClass (Fig. 4) identified 8 individuals with a probability greater than 0.90 of belonging to the SS group. These individuals, which are identified by open boxes on Fig. 3, had morphologies spanning the CI range

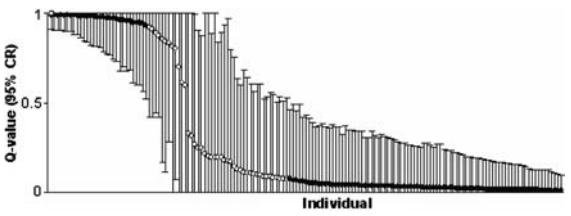

Fig. 2 Bayesian assignment testing results for *Scaphirhynchus* specimen indicating individual membership in each of the two identified groups are presented as Q-values, which indicate the proportion of each individual's genotype that was generated in the two identified natural groups. Each individual's Q-value is represented by a point with 95% credible regions. Values nearer one (1) indicate assignment to the group dominated by pallid sturgeon and values nearer zero (0) indicate assignment to the group dominated by shovelnose sturgeon. Q-values with 95% credible regions not including 0.50 are indicated by closed circles, while open circles indicate Q-value 95% credible regions including 0.50

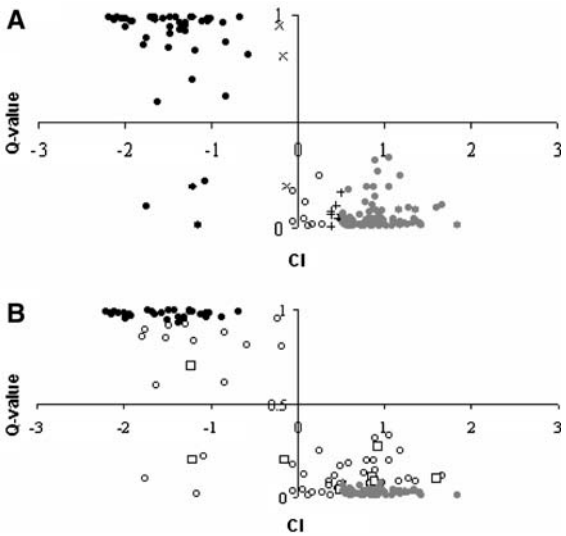

Fig. 3 Synthesis of molecular (Q-value) and morphological (CI) identification of *Scaphirhynchus* specimen. CI values are plotted on the x-axis and Q-values are plotted on the y-axis. Each point represents an individual. In A, the points are coded by CI category (pallid = black circle, pallid/hybrid overlap = X, hybrid = open circle, shovelnose/hybrid overlap = +, and shovelnose = gray circle). In B, the points are coded by final species designation (pallid = black, intermediate = open, and shovelnose = gray) and the eight intermediates that assigned to shovelnose are indicated as an open box

(5 were more SS-like, 1 was intermediate, and 2 were more PS-like). Due to the questionable identification of these 8 fish, which were morphologically and genetically unremarkable except for their inconsistent assignment using Structure and GeneClass, they were excluded from further analyses. The remaining 47 individuals in the hybrid/overlap categories did not assign to either baseline group with a probability greater than 0.90, indicating that their genotypes may have been comprised of elements from both PS and SS. These individuals were retained in the intermediate/hybrid category (hereafter abbreviated IS). The three defined groups, PS, SS, and IS, were used in subsequent analysis.

The microsatellite markers used were highly variable and sufficiently powerful to detect differences in allele frequencies between putative PS and SS (Table 2). Multiple alleles were observed at all markers, with allele richness based on a population size of 27 ranging from 3.86 to 16.26 and expected heterozygosity over all loci ranged from 0.699 to 0.798. Comparing PS to SS (Table 2) shows PS tend to exhibit fewer alleles per locus (lower allele richness at 14 of 16 loci) and lower expected heterozygosity (15 of 16 loci and averaged over loci). No locus in either PS or SS was identified as being significantly out of HWE, and no locus pair in any group was identified as being significantly out of LE. Interestingly, no significant departures from HWE or LE were observed in the intermediate group as well. Low frequency private alleles (Appendix) were observed at all loci except Spl-18 and Spl-53 when comparisons were made between putative PS and SS. PS had private alleles at 2 loci, all occurring as singletons, while SS had private alleles at 13 loci with 19 occurring as singletons and the most frequent observed in 3 of 71 SS. There were 7 private alleles among the 48 intermediates with 5 occurring as singletons, one present in two copies, and one present in five copies.

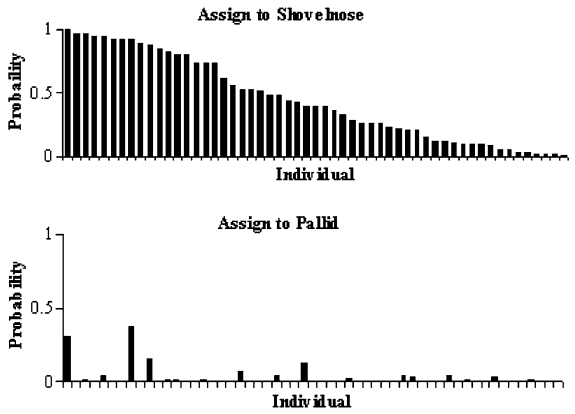

Fig. 4 The results of an assignment test are provided. The test compared intermediate individuals to individuals defined as shovelnose (Assign to Shovelnose) and pallid (Assign to Pallid) sturgeon. The assignment probability to shovelnose and pallid sturgeon for each individual is presented, with individuals plotted in the same order in each graph. Eight individuals assigned to shovelnose sturgeon with probabilities greater than 0.90. All other individuals were assigned to either group with a probability less than 0.90 and were classified as hybrids

Allele frequencies were significantly different between PS, IS, and SS (F_{ST} = 0.036, P = 0.001, Table 2), with a 95% confidence interval of 0.024–0.047. Individual locus estimates of F_{ST} ranged from <0.001 to 0.087 and 13 of the 16 loci exhibited statistically significant genic heterogeneity after Bonferroni correction. Pairwise estimates of F_{ST} among PS, IS, and SS were also statistically significant (Table 3) with the highest F_{ST} value observed between PS and SS (F_{ST} = 0.074) and the smallest value occurring between SS and IS (F_{ST} = 0.011). Estimating genetic distance among PS, IS, and SS (Table 3) with the DAS showed the greatest distance occurred between PS and SS. The IS were intermediate, yet nearer to SS than PS.

Discussion

Analysis of genotypes from 16 microsatellite loci developed in *Scaphirhynchus* resolved two distinct genetic groups of *Scaphirhynchus* in the MMR with most specimens strongly assigning to one of the two groups. Both Structure (Prichard et al. 2000) and ΔK (Evanno et al. 2005) identified two groups as most likely. These groups were largely concordant with morphologically identified PS and SS, with 29 of 44 (66%) genetically identified PS also morphologically PS and 73 of 94 (78%) genetically identified SS also morphologically SS.

Table 3 Pairwise estimates of θ calculated over all loci among pallid, hybrid, and shovelnose sturgeons are provided below the diagonal

	Pallid Sturgeon	Hybrid Sturgeon	Shovelnose Sturgeon
Pallid Sturgeon	***	0.119	0.253
Hybrid Sturgeon	0.035	***	0.036
Shovelnose Sturgeon	0.074	0.011	***

All θ values were significant (P < 0.05). Estimates of the shared allele genetic distance among pallid, hybrid, and shovelnose sturgeons are provided above the diagonal. The greatest observed distance is between pallid and shovelnose sturgeon, with hybrids being intermediate, yet closer to shovelnose sturgeon

Genetically assigned PS and SS exhibited a broad range of CI values (Fig. 3). These results suggest that while the morphological continuum between species may be partly due to hybridization there is also an appreciable amount of variation within each species. There were a relatively large number of morphological intermediates with CI scores between 1 and –1 (especially between 0 and 1) and a smaller number of genetic intermediates. Morphological intermediate tended to be genetically more similar to SS than PS (Fig. 3). Kuhajda and Mayden (2001) found that juvenile hatchery-reared PS × SS scored more similar to SS than PS on the Wills et al. (2002) index. Thus, perhaps some of the SS-like genetic intermediates may be F1 hybrids. We would also expect that if hybrids are fertile the most likely backcross would be between hybrids and the numerically superior SS. Based on the presence of two genetically and morphologically differentiated groups with a number of genetic and morphological intermediates, we believe that distinct PS and SS gene pools remain with some hybrid and perhaps backcross individuals in the MMR.

The number of PS, IS, and SS detected in our study (29, 55, 73) should not be interpreted as representative of the relative abundances of each morph in the MMR. We requested a sample of 100 SS from our collectors and could have acquired many more. We also requested all ''intermediate'' sturgeons our collectors could acquire and had to rely on tissue samples from specimens collected prior to the beginning of our study to sample sufficient numbers of PS. Southern Illinois University researcher Jim Garvey (personal communication in 2006), who heads a field research program that has been extensively sampling sturgeon from the MMR using a variety of gear and who provided most of the specimens used in this study, reports that the relative frequencies of the morphs in the MMR is more on the order of SS >> IS > PS. Carlson et al. (1985) categorized the 4355 sturgeon they collected from the Mississippi River as 4332 SS, 11 PS, and 12 hybrids. The apparently greater abundance of IS relative to PS in the MMR further indicates the dire state of PS stocks and that hybridization is an imminent threat to the survival and recovery of PS.

The current consensus among the PS recovery team is that spawning of wild-caught PS with subsequent release of offspring back into the wild is a critical component of PS recovery. This practice could exacerbate genetic threats to PS if hybrid or backcross *Scaphirhynchus* are used for broodstock. Birstein (1993) reported a maximum adult size of 1.7 m for PS compared to 0.8 m for SS. While very large PS occur in the upper Missouri River and thus any very large specimen of *Scaphirhynchus* can be assumed to be PS, we are unaware of PS longer than 1.2 m from the Mississippi River where both PS and SS have similar adult sizes. In our study, specimens from the three classes were similar in length with PS ranging from 653 to 982 mm, SS between 398 and 873 mm, and IS between 432 and 1190 mm. Thus, size can not be used as an indicator of species identity in the MMR. It is currently a controversial issue among sturgeon biologists whether the large PS in the upper Missouri represent a phenotype that does not occur in the southern part of the range or conversely whether the variation in adult size is entirely due to differences in age structure or growth among regions.

The level of concordance between genetic assignment and the CI shows that synthesis of molecular and morphological data provides additional measures for ensuring the purity of broodstock. Both methods identified two clusters in the data, and these clusters were highly concordant in identifying PS. Assignment testing identified 30 PS, 29 of which were CI PS and 1 was in the PS/hybrid overlap. Of 44 CI identified PS, 29 were genetic PS, 14 were IS, and 1 was SS. Thus, to reduce the likelihood of using hybrid or backcross *Scaphirhynchus* as PS broodstock, both molecular and morphological screening should be employed. While this might reduce the number of potential broodstock available for propagation and deviates from the principle of selecting broodstock as an unbiased sample of the spawning population (Miller and Kapuscinski 2003), we believe that the threat hybridization poses to PS recovery would be greatly exacerbated by stocking large numbers of hatchery-reared hybrid or backcross sturgeon.

Comparisons among defined PS, SS, and intermediate/hybrid sturgeon from the MMR found private alleles occurring among PS and SS. The majority of these private alleles were found within SS. Additionally, PS had fewer alleles and lower heterozygosity per locus. These results suggest that perhaps SS have a larger long term effective population size, which is consistent with current and historical abundance ratios. While a sample of F1 hybrids would be expected to exhibit excess heterozygosity relative to HWE, we did not find significant deviations within the intermediate group. This could be explained by the relatively similar allele frequencies in parent species, the likelihood that some of these intermediates may be introgressed, and the high amount of genetic variation relative to the number of individuals scored resulting in low power for the test of deviation from HWE. Additionally, as noted in the results section, the conservative screening process we used to ensure that no hybrids were retained in

the PS group may have classified some pure PS or SS into the intermediate group. Thus, the excess heterozygosity expected in hybrids may have been offset by the excess homozygosity from mis-assigned pure PS or SS. Significant genetic differentiation was observed among groups. The highest pairwise F_{ST} and the greatest genetic distance was between PS and SS groups, with both tests finding the intermediate/hybrid sturgeon more closely related to SS. Taken together, the morphology and genetic data seem to suggest that introgressive hybridization has occurred between PS and SS, with hybrids more likely backcrossing with SS, as would be expected given the numerical superiority of SS.

This study demonstrates the successful use of assignment testing with microsatellite data to discriminate PS, SS, and putative hybrid sturgeon. Two genetically distinguishable groups within *Scaphirhynchus* in the MMR were observed. These two groups largely correspond to species differences inferred from morphological and meristic characters. The results are compatible with previous genetic studies of PS and SS, which detected significant haplotype and allele frequency differences between the two species in the Missouri and Atchafalaya Rivers using mitochondrial DNA control region sequences (Campton et al. 2000) and heterologous microsatellite markers (Tranah et al. 2001). Additionally, this study finds genetic and morphological intermediate individuals in the MMR suggesting evidence of hybridization similar to the evidence of hybridization found in the Atchafalaya River by Tranah et al. (2004).

The results from this study, in conjunction with additional work being conducted throughout the PS range, will be used to construct a baseline data set to characterize allele frequencies of PS and SS. Assignment testing with these baseline samples should provide valuable information for several recovery and conservation issues. These methods are expected result in a forensic tool, which is capable of identifying PS, SS, and hybrid sturgeon throughout the PS range. Being able to accurately discriminate species should facilitate multiple aspects of PS recovery including forensic identification of sturgeon for law enforcement purposes, estimating stock structure within PS to guide stocking plans, identifying larval and juvenile sturgeons to identify spawning habitat and monitor the effects of changes in flow regime, and to screen potential broodstock for PS propagation. It may also be possible to investigate hybridization among species using a combination of genetic and morphological criteria to determine the relative abundance of hybrid individuals and the potential threat they pose to PS recovery.

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Appendix

Appendix Allele frequencies for each of the sixteen microsatellite loci screened in putative pallid (PS), shovelnose (SS), and intermediate sturgeon (IS)

	Pallid	Shovelnose	Intermediate
Locus: 12			
N	29	71	48
P: 8	0	0.007	0.01
P: 9	0	0	0.01
P: 11	0.034	0.049	0.052
P: 12	0.552	0.282	0.479
p: 13	0.345	0.401	0.281
p: 14	0.052	0.042	0.063
p: 15	0	0.042	0.042
p: 16	0.017	0.162	0.063
p: 17	0	0.014	0
Locus: 15			
N	28	71	48
p: 10	0.036	0.106	0.063
p: 14	0.125	0.225	0.271
p: 15	0.571	0.317	0.344
p: 16	0.036	0.12	0.094
p: 18	0	0.007	0
p: 19	0	0.014	0.031
p: 20	0	0.035	0.021
p: 21	0.054	0.077	0.115
p: 22	0.179	0.028	0.031
p: 23	0	0.007	0.01
p: 24	0	0.021	0
p: 27	0	0.021	0
p: 29	0	0.007	0
p: 31	0	0	0.021
p: 33	0	0.014	0
Locus: 18			
N	29	72	48
p: 9	0	0	0.01
p: 10	0	0	0.01
p: 12	0.017	0	0.01
p: 13	0.086	0.021	0.042
p: 14	0.103	0.042	0.094
p: 15	0.707	0.417	0.479
p: 16	0.069	0.451	0.198
p: 17	0.017	0.028	0.135
p: 18	0	0.042	0.021

Appendix continued

	Pallid	Shovelnose	Intermediate
Locus: 19			
N	29	72	48
p: 18	0	0.014	0.01
p: 20	0.103	0.042	0.021
p: 21	0.172	0.25	0.177
p: 22	0.155	0.201	0.25
p: 23	0.448	0.25	0.344
p: 24	0.121	0.201	0.156
p: 25	0	0.028	0.042
p: 27	0	0.007	0
p: 31	0	0.007	0
Locus: 30			
N	29	72	48
p: 8	0	0.007	0
p: 10	0	0.007	0
p: 11	0	0.007	0
p: 12	0	0.007	0
p: 15	0	0	0.052
p: 16	0	0.007	0.01
p: 17	0.017	0.049	0.052
p: 18	0.793	0.458	0.531
p: 19	0.172	0.035	0.083
p: 20	0	0.181	0.063
p: 21	0	0.069	0.052
p: 22	0.017	0.118	0.094
p: 23	0	0.042	0.042
p: 24	0	0	0.01
p: 25	0	0.014	0.01
Locus: 35			
N	29	72	48
p: 8	0	0.007	0.021
p: 9	0	0.097	0.031
p: 10	0.052	0.021	0.083
p: 11	0.017	0.097	0.125
p: 12	0	0.111	0.094
p: 13	0.224	0.174	0.24
p: 14	0.207	0.021	0.052
p: 15	0.017	0.042	0.052
p: 16	0	0.049	0.063
p: 17	0.034	0.097	0.063
p: 18	0.017	0.021	0.01
p: 19	0	0.056	0.042
p: 20	0.328	0.028	0.042
p: 21	0.017	0.028	0.01
p: 22	0.069	0.007	0.021
p: 23	0.017	0.014	0.01
p: 24	0	0.076	0.021
p: 25	0	0.007	0.01
p: 26	0	0.007	0
p: 27	0	0.035	0
p: 29	0	0.007	0.01
Locus: 36			
N	29	72	48
p: 12	0	0.007	0
p: 14	0	0.007	0.01
p: 17	0	0	0.01
p: 18	0	0.021	0.01
p: 19	0	0.014	0
p: 20	0	0.014	0
p: 21	0.017	0.028	0.031
p: 22	0.121	0.028	0.073

Appendix continued

	Pallid	Shovelnose	Intermediate
p: 23	0.069	0.056	0.094
p: 24	0.172	0.139	0.167
p: 25	0.414	0.139	0.229
p: 26	0.034	0.201	0.188
p: 27	0	0.167	0.031
p: 28	0	0.063	0.021
p: 29	0	0.035	0.021
p: 30	0	0.007	0.021
p: 31	0.017	0.014	0.031
p: 32	0	0.021	0.01
p: 33	0.103	0.021	0.042
p: 34	0.052	0.007	0.01
p: 35	0	0.007	0
p: 36	0	0.007	0
Locus: 40			
N	27	66	48
p: 11	0	0	0.01
p: 12	0	0.008	0
p: 14	0	0.015	0.021
p: 15	0.019	0.015	0.052
p: 16	0.056	0.015	0.01
p: 17	0	0.045	0.042
p: 18	0.019	0.023	0.042
p: 19	0.019	0.045	0.01
p: 20	0.074	0.038	0.042
p: 21	0.241	0.152	0.146
p: 22	0.13	0.159	0.229
p: 23	0.222	0.189	0.135
p: 24	0.148	0.136	0.146
p: 25	0.019	0.045	0.021
p: 26	0.019	0.03	0.052
p: 27	0.037	0.023	0.031
p: 28	0	0.03	0
p: 29	0	0.03	0.01
Locus: 53			
N	29	72	48
p: 9	0	0.014	0.01
p: 10	0	0.014	0.021
p: 11	0.121	0.222	0.219
p: 12	0.034	0.111	0.052
p: 13	0.345	0.375	0.344
p: 14	0	0.021	0.01
p: 15	0	0.028	0.042
p: 16	0	0.014	0.01
p: 17	0.414	0.139	0.208
p: 18	0	0.056	0.021
p: 19	0.086	0.007	0.042
p: 20	0	0	0.01
p: 24	0	0	0.01
Locus: 56			
N	29	72	48
p: 16	0	0.014	0.01
p: 18	0	0.042	0.042
p: 19	0.017	0.049	0.052
p: 20	0	0.16	0.125
p: 21	0	0.319	0.104
p: 22	0	0.069	0.021
p: 23	0	0.028	0.01
p: 24	0.034	0.049	0.063
p: 25	0.017	0.021	0.042
p: 26	0.052	0.049	0.042

Appendix continued

	Pallid	Shovelnose	Intermediate
p: 27	0.034	0.028	0.021
p: 28	0.069	0.042	0.083
p: 29	0.293	0.014	0.115
p: 30	0.293	0.049	0.125
p: 31	0.19	0.035	0.135
p: 32	0	0.021	0.01
p: 33	0	0.007	0
p: 34	0	0.007	0
Locus: 60			
N	29	72	48
p: 21	0.138	0.007	0.01
p: 23	0	0.028	0.021
p: 24	0	0.021	0.021
p: 25	0.259	0.375	0.333
p: 26	0.328	0.535	0.448
p: 27	0.207	0.028	0.125
p: 28	0.034	0.007	0.031
p: 29	0	0	0.01
p: 30	0.017	0	0
p: 33	0.017	0	0
Locus: 101			
N	29	71	48
p: 8	0.017	0.056	0.083
p: 9	0.121	0.12	0.083
p: 10	0.103	0.246	0.292
p: 11	0.121	0.134	0.167
p: 12	0.414	0.282	0.24
p: 13	0.224	0.07	0.094
p: 14	0	0.07	0.031
p: 15	0	0.014	0.01
p: 16	0	0.007	0
Locus: 106			
N	29	72	48
p: 5	0	0.007	0.01
p: 6	0	0	0.01
p: 7	0.017	0	0.01
p: 9	0.017	0.007	0.021
p: 10	0.103	0.014	0.073
p: 11	0	0.118	0.063
p: 12	0.207	0.306	0.26
p: 13	0.19	0.139	0.115
p: 14	0.397	0.201	0.292
p: 15	0.034	0.132	0.073
p: 16	0	0.021	0
p: 17	0.034	0.021	0.042
p: 18	0	0.021	0.021
p: 19	0	0.014	0.01
Locus: 119			
N	29	72	48
p: 7	0.448	0.042	0.146
p: 8	0	0.014	0.01
p: 10	0	0.021	0.021
p: 11	0	0.035	0.01
p: 12	0.259	0.188	0.042
p: 13	0	0.313	0.115
p: 14	0.19	0.229	0.292
p: 15	0.034	0.09	0.26
p: 16	0.017	0.035	0.031
p: 17	0.052	0.021	0.063
p: 18	0	0	0.01
p: 19	0	0.014	0

Appendix continued

	Pallid	Shovelnose	Intermediate
Locus: 158			
N	29	72	48
p: 13	0	0.063	0.01
p: 14	0	0	0.01
p: 15	0.19	0.125	0.125
p: 16	0.086	0.104	0.083
p: 17	0.034	0.056	0.063
p: 18	0.052	0.222	0.156
p: 19	0.345	0.049	0.094
p: 20	0.19	0.215	0.25
p: 21	0.103	0.111	0.135
p: 22	0	0.035	0.063
p: 23	0	0.007	0.01
p: 25	0	0.014	0
Locus: 173			
N	29	72	48
p: 6	0.207	0.083	0.104
p: 7	0.121	0.097	0.073
p: 8	0	0.118	0.073
p: 9	0.5	0.243	0.406
p: 10	0	0.167	0.135
p: 11	0.155	0.167	0.135
p: 12	0	0.097	0.073
p: 14	0	0.028	0
p: 16	0.017	0	0

Alleles are identified by the inferred number of microsatellite repeats and the total number of alleles scored (*N*) is provided

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