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Experimental search for forensically useful markers in the genus *Scaphirhynchus*

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Summary

The genus *Scaphirhynchus* is an exclusively freshwater sturgeon that resides in the Mississippi River and Mobile River drainages. Two of the three species in the genus, *S. albus* and *S. suttkusi*, are listed as endangered under the US Endangered Species Act, while the third, *S. platorhynchus*, is of commercial importance. Currently, the USFW Forensics Laboratory uses a 270 bp portion of the mitochondrial cytochrome *b* gene to identify sturgeon products in commercial trade. Although this region of cytochrome *b* is polymorphic in *Scaphirhynchus*, it does not distinguish *S. platorhynchus* from the other two *Scaphirhynchus* species. This study describes an extensive ongoing project designed to identify markers of utility in distinguishing among *Scaphirhynchus* for forensic purposes. A total of 39 different loci, both nuclear and mitochondrial, were screened with varying success. Of these 39 loci, four nuclear loci and one mitochondrial locus display polymorphic sites: two microsatellite markers LS-34 and LS-68; the internal transcribed rRNA spacer one (ITS-1), a zinc fingered protein involved in transcription, Ikaros 3′-UTR and the hypervariable region of the mitochondrial control region. The remaining 34 loci either did not amplify, were not polymorphic, or the resulting sequences could not be verified as the locus in question by a homologous multi-species sequence alignment from GenBank sources. Results indicate that none of the nuclear markers exhibit species specific variation, however, these markers may prove useful in forensic identification if used in conjunction with results from the highly polymorphic mitochondrial control region.

Introduction

Acipenseriformes represent an order of bony fishes that evolved approximately 200 million years ago during the Lower Jurassic period (Bemis et al., 1997). The order consists of 27 species, including nine indigenous North American species in the genera *Acipenser* (5), *Scaphirhynchus* (3) and *Polyodon* (1). Sturgeon are long-lived (> 25 years), generally reach maturity at 5–10 years and are the largest fish found in freshwater. During the last century, habitat perturbation and intensive commercial pressure have severely reduced the abundance of sturgeon and paddlefish throughout their range (Birstein, 1993).

The three species in the genus *Scaphirhynchus*, pallid sturgeon (*S. albus*), shovelnose sturgeon (*S. platorhynchus*) and Alabama sturgeon (*S. suttkusi*), are exclusively freshwater fish that occur in the Mississippi River and Mobile River drainages of North America (Bailey and Cross, 1954; Williams and Clemmer, 1991). Although considered rare and listed as 'endangered' under the US Endangered Species Act (ESA; Federal Register 1990, 55:36641), pallid sturgeon still range throughout the Missouri River and south from its confluence with the Mississippi River into Louisiana. Shovelnose sturgeon occur throughout the greater Mississippi River drainage in sufficient numbers to support sport and commercial fisheries (K. Keenlyne, unpublished data). Alabama sturgeon, initially described as an isolated population of shovelnose sturgeon, are endemic to the Mobile River basin and are isolated geographically from the other two species. Extremely rare, the Alabama sturgeon was listed as an endangered species under the ESA in 2000.

One responsibility of the US Fish and Wildlife Forensic Laboratory is to facilitate sturgeon conservation by providing analytical support for enforcement of the ESA and the Convention on International Trade in Endangered Species. Commercial fisheries currently exist for the shovelnose sturgeon, but pallid and Alabama sturgeon could easily become extinct or extirpated by illegal poaching associated with these fisheries. Knowledge of which species are being traded and the proportion of the trade they comprise is of critical importance for effective conservation and enforcement efforts. To that end, development of a DNA procedure was sought for the forensic identification of the caviar and meat of the river sturgeon, *Scaphirhynchus*.

Initially investigated was the DNA sequence variation in the 5′ end of the cytochrome *b* gene that has been shown to exhibit significant interspecific variation in sturgeon (Schill and Walker, 1994, unpublished data; Birstein et al., 1997) and other fish species (e.g., shark: Martin et al., 1992; rockfish: Rocha-Olivares et al., 1999). Sequence variation was characterized in a 270 bp portion of the cytochrome *b* gene (Brown et al., 1989; Fain et al., 2000a,b) from 19 sturgeon and one paddlefish species. Comparisons among pallid, shovelnose and Alabama sturgeon revealed a total of three unique sequences defined by three nucleotide substitutions for a maximum divergence of 1.1 (Fain et al., 2000b). Among the three species, the 270 bp segment of cytochrome *b* lacked sufficient variation to distinguish shovelnose from pallid and Alabama sturgeon. Consequently, an extensive investigation was mounted for useful markers to distinguish between these three species and to identify possible hybrids. This paper describes the search for forensically useful markers in both mitochondrial and nuclear DNA.

Material and methods

Samples were collected by personnel from the US Fish and Wildlife Service, the US Army Corp of Engineers and state employees from various departments of natural resources (Montana, South Dakota, Iowa and Kentucky). Samples included fin clips, tissue plugs and roe from *Scaphirhynchus* species throughout their range (Fig. 1). DNA was extracted

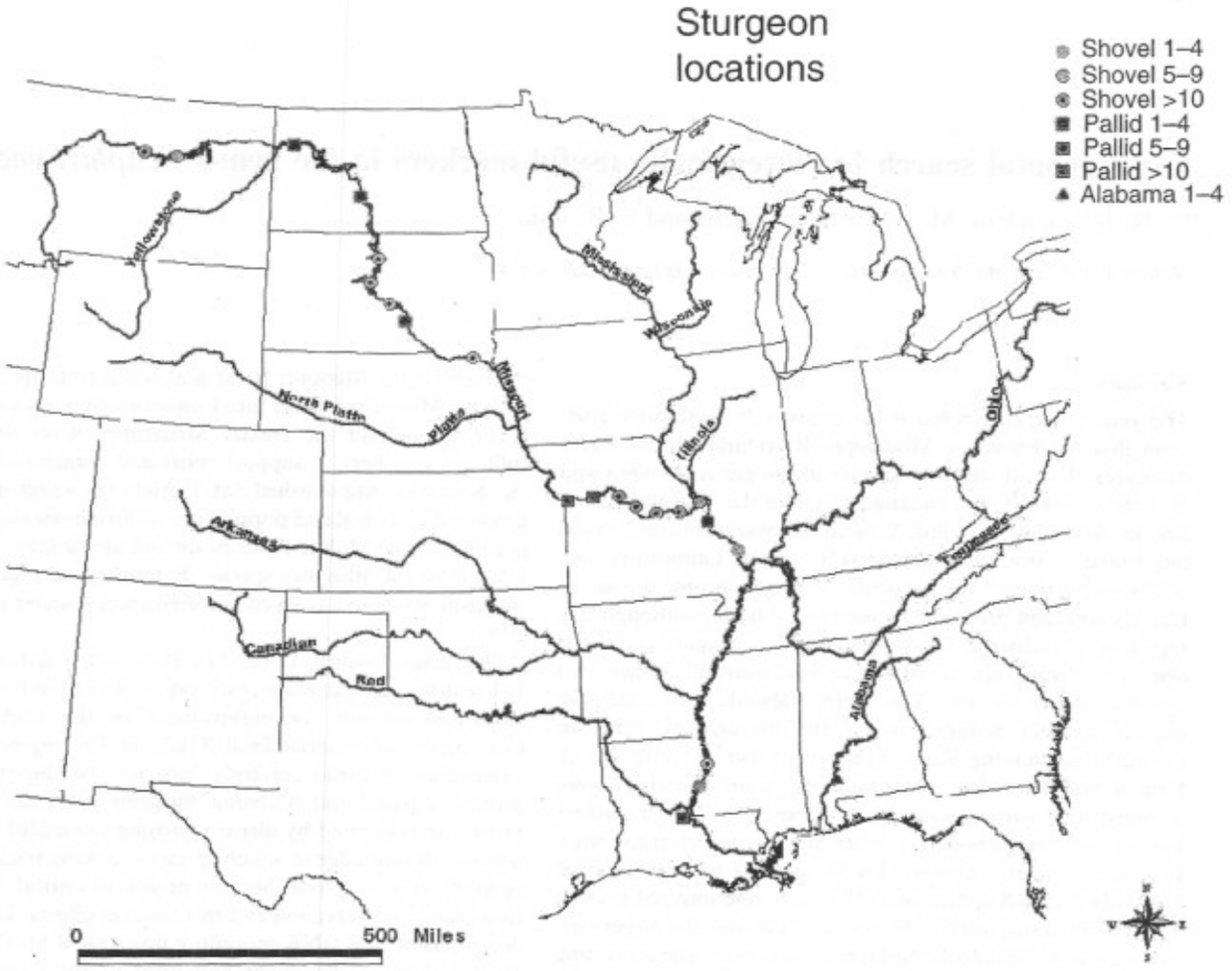

Fig. 1. Location of *Scaphirhynchus* samples used in this study collected by US Federal and State agents and biologists

from four pallid sturgeon and four shovelnose sturgeon using NucleoSpin® Multi-96 Tissue extraction plates (Macherey-Nagel, Düren, Germany)[1]. Twenty nuclear markers, including nine microsatellite loci and four mitochondrial markers, were initially selected as potentially useful forensic markers based upon published phylogenetic studies for other species (Table 1). An additional 22 nuclear markers, originally designed for use in salmonids, were provided by the US National Marine Fisheries Service and screened against a panel of pallid and shovelnose sturgeon (Table 2). With the exception of the mitochondrial control region, samples from Alabama sturgeon were not included in any of the experiments described due to the limited availability of tissue, but at a later date are intended to be analyzed with the applicable markers.

The majority of the primer sets for the chosen markers were not originally designed for use on sturgeon. Polymerase chain reaction (PCR) conditions for each primer set were optimized for *Scaphirhynchus*. Experimental conditions tested included varying the annealing temperature (43–62°C), $MgCl_2$ concentration (1.25–2.5 mM), dinucleotide triphosphate concentration (75–150 μM each), Taq DNA polymerase concentration (0.015–0.05 U μl^{-1}) and primer concentration (0.1–0.2 μM). All reactions were performed in 50 μl total volumes with approximately 100 ng of template DNA, 20 mM Tris (pH 8.4), 50 mM KCl and Bovine Serum Albumin (160 $\mu g\ ml^{-1}$). Positive and negative controls were included in each run.

Several primer sets produced multiple products of different sizes. In the cases where further optimization of PCR conditions could not diminish spurious bands, PCR products were separated by gel electrophoresis and excised from the gel. The selected amplicons were cloned into a plasmid vector using The Original TA Cloning® Kit (Invitrogen, Carlsbad, CA). Successful clones were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing protocol (Perkin Elmer, Foster City, CA). BLAST searches on the NCBI website were performed on all sequences and multi-species alignment was performed, when available, to confirm sequence identity.

Upon verification of the intended loci by a homologous multi-species sequence alignment from sequences obtained from GenBank, species specific primers were designed for *Scaphirhynchus*. These reconfigured primers were used in subsequent PCR amplifications to screen multiple (>50) individuals of both pallid and shovelnose sturgeon (conditions given in Table 3). At the time this manuscript was prepared, no sequences from Alabama sturgeon had been obtained for any nuclear markers. Amplification products from pallid and shovelnose sturgeon were purified to remove unincorporated nucleotides and primers [using the Millipore MultiScreen-FB 96-well filtration and assay plate protocol (Millipore Corp,

[1]Mention of tradenames does not constitute US Government endorsement of commercial products.

Table I
Loci screened on *Scaphirhynchus* during the course of this study. The reference column refers to the article from which the primer sequence was originally cited, while the expected size column refers to the size of the PCR product from the species the primers were originally designed. Primer sets that did not produce an amplicon regardless of experimental conditions are labeled as 'No result' in the Product Size column

Marker	Reference	Primer sequence	Expected size (bp)	Actual size (bp)	Reaction constituents	Cycling conditions
Nuclear Markers						
p53	Park et al. (1996)	p53-F15′-CAG GTG GGA TCA GAG TG-3′	1080	341, 238, 178	150 μM each dNTP's; 120 pM each primer; 1.5 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 60°C/1m; 72°C/1m][40] 72°C/5m
		p53-R1A5′-GGA GAA AGC GAG GAT ATG TG-3′				
C-*mos*	Saint et al. (1998)	G775′-TGG CYT GGT GCW NCA TNG ACT-3′	400–700	650	100 μM each dNTP's; 120 pM each primer; 1.5 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/50s; 55°C/20s; 50°C/5s; 45°C/5s; 40°C/15s; 72°C/50s][30] 72°C/5m
		G785′-AGR GTG ATR WCA AAN GAR TAR ATG TC-3′				
von Willebrand Factor (vWF)	Huchon et al. (1999)	V1 F5′-TGT CAA CCT CAC CTG TGA AGC CTG-3′	1400	850 and 200	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 48°C/1m; 72°C/1.5m][35] 72°C/5m
		W1R5′-TGC AGG ACC AGG TCA GGA GCC TCT-3′				
Internal Transcribed Spacer One (ITS-1)	Booton et al. (1999)	18S3 f5′-gTg AAC CTg Cgg AAg gAT CAT-3′	550	490	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1m][30] 72°C/5m
		28S5 r5′-ATA TgC TTA AAT TCA gCg ggT-3′				
Growth hormone 2 intron C	Oakley and Phillips (1999)	GH2CF:5′-ATC GTG AGC CCA ATC GAC AAG CAG-3′	228–620	No result	n/a	n/a
		GH2CR:5′-GGG TAC TCC CAG GAT TCA ATC AGG-3′				
Phosphoglycerate kinase	Colgan (1999)	p1345:5′-GTC TAT GTC AAT GAT GCT TTT GG-3′	430	600 and 800	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 48°C/1m; 72°C/1m][35] 72°C/5m
		p1772:5′-TGG CTT GGC CAG TCT TTG GCA TTT TCA-3′				
Major histocompatibility complex β-2	Fain, (unpubl).	DRB f5′-GTG ACT GTG TAY CYT GCA AAG-3′		650	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1m][35] 72°C/5m
		DRB r5′-YSC AAG GAW GAR CAG RCC YAG-3′				
Somatolactin	Ford (1998)	SL-ex4-F:5′-ACA AGT GGC TCC TCC ACT CTG-3′ SL-3′ut-R:5′-GAT AAT CAC AAA CGT ACT GTG CC-3′	2000	No result	n/a	n/a
High mobility group protein	Ford (1998)	HMG-in1-F:5′-GAA ACG TGG CGA TAT GTA TTT C-3′	1300	No result	n/a	n/a
		HMG-ex4-R:5′-TGG CCA CAT CAC CGA TAG AC-3′				
Cytochrome p450a	Ford (1998)	CYP-5′-F:5′-ATC GAA TGT TTT ATC CTC CAT G-3′ CYP-ex2-R5′-GTA GTA TCA TGA GAA CCA TTT G-3′	1000	No result	n/a	n/a

Table 1
(Continued)

Marker	Reference	Primer sequence	Expected size (bp)	Actual size (bp)	Reaction constituents	Cycling conditions
Nuclear Markers						
Calmodulin gene intron 3 (CaM-1)	Côrte-Real et al. (1994a)	CAD2 F5′-CCG AAT TCA TCT TNC KNG CCA TCA T-3′	500	120	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 48°C/1m; 72°C/1m]35 72°C/5m
	Côrte-Real et al. (1994b)	CAD3 f:5′-GGA CAG AAT TCV ACN GAR GCN GA-3′				
LS-19	May et al. (1997)	LS-19 f:5′-CAT CTT AGC CGT CTG TGG TAC-3′ LS-19 r5′-CAG GTC CCT AAT ACA ATG GC-3′	100–150	n/a	n/a	n/a
LS-34	May et al. (1997)	LS-34 f5′-TAC ATA CCT TCT GCA AcG-3′ LS-34 r:5′-GAT CCC TTC TGT TAT CAA C-3′	100–150	100–150	150 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/30s; 57°C/30s; 72°C/30s]^{30} 72°C/5m
LS-39	May et al. (1997)	LS-39 f:5′-TTC TGA AGT TCA CAC ATT G-3′ LS-39 r:5′-ATG GAG CAT TAT TGG AAG G-3′	100–150	n/a	n/a	n/a
LS-54	May et al. (1997)	LS-54 f:5′-CTC TAG TCT TTG TTG ATT ACA G-3′ LS-54 r:5′-CAA AGG ACT TGA AAC TAG G-3′	> 200	n/a	n/a	n/a
LS-57	May et al. (1997)	LS-57 f:5′-GCT TGG TTG CTA GTT GC-3′ LS-57 r:5′-GTA CAG ATG AGA CCA GAG GC-3′	150–200	n/a	n/a	n/a
LS-58	May et al. (1997)	LS-58 f:5′-TCG GTT GAG TGA TTG GTA TC-3′ LS-58 r:5′-CTG AAA GGT GAA TCT ACT CCC-3′	150–200	n/a	n/a	n/a
LS-62	May et al. (1997)	LS-62 f:5′-GAT CAG GAG GGC AGA GNA AC-3′ LS-62 r:5′-CCC TGG ATT TGA ATT AAC AG-3′	< 100	n/a	n/a	n/a
LS-68	May et al. (1997)	LS-68 f:5′-TTA TTG CAT GGT GTA GCT AAA C-3′ LS-68 r:5′-AGC CCA ACA CAG ACA ATA TC-3′	100–150	100–150	100 μM each dNTP's; 175 pM each primer; 1.5 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/30s; 58°C/30s; 72°C/30s]^{30} 72°C/5m
LS-69	May et al. (1997)	LS-69 f:5′-ATC TGA ATT GAN TTC GTG-3′ LS-69 r:5′-TTG GAT ACT GTG TTC CAA C-3′	> 200	n/a	n/a	n/a
Mitochondrial Markers						
NADH dehydrogenase 1 (ND-1)	Cronin et al. (1993)	ND-1F5′-ACC CCG CCT GTTTAC CAA AAA CAT-3′ ND-1R5′-GGT ATG AGC CCG ATA GCT TA-3′	2000	No result	n/a	n/a
ND-3/4	Cronin et al. (1993)	tLEU5′-TTT TGG TTC CTA AGA CCA AYG GAT-3′ tGLY5′-TAA YTA GTA CAG YTG ACT TCC AA-3′	2300	1100	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1.5m]^{30} 72°C/5m
ND-5/6	Cronin et al. (1993)	ND515′-AAT AGT TTA TCC RTT GGT CTT AGG-3′ ND615′-TTA CAA CGA TGG TTT TTC ATR TCA-3′	2400	1100	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1.5m]^{30} 72°C/5m
Mitochondrial control region (D-loop)	Campton et al. (2000)	L178-13: 5′-AAT gTT TCA TCT ACC ATC AAA T-3′ H701: 5′-GGT TTG ACA AGC AAT ATA AGG C-3′	500	500	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/45s; 55°C/45s; 72°C/45s]^{30} 72°C/5m

Table 2
List of loci provided by the US National Marine Fisheries Service and screened on *Scaphirhynchus* during this study

Marker	Reference	Actual size (bp)	Reaction constituents	Cycling Conditions
C-myc Protooncogne	Moran (unpubl. data)	550	200 μM each dNTP's; 120 pM each primer; 2 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 45°C/1m; 72°C/1m][40] 72°C/5m
Myelin proteolipid protein (PLP) DM203′ UTR	Moran (unpubl. data)	No result	n/a	n/a
Ependymin 1 (Epd1)	Moran et al. (1997)	700 and 500	150 μM each dNTP's; 175 pM each primer; 2.5 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 42°C/45m; 72°C/1.5m][35] 72°C/5m
Estrogen receptor (ER)	Moran (unpubl. data)	No result	n/a	n/a
Growth hormone 2	Park and Moran (1995)	1300	200 μM each dNTP's; 175 pM each primer; 1.75 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 53°C/1m; 72°C/1.5m][35] 72°C/5m
High mobility group T2	Moran (unpubl. data)	900	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 48°C/1m; 72°C/1m][35] 72°C/5m
Heat shock protein	Unknown	600	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 52°C/1m; 72°C/1m][35] 72°C/5m
Inhibitor of DNA binding/ differentiation gene 1	Moran (unpubl. data)	No result	n/a	n/a
Insulin-like growth factor	Moran (unpubl. data)	No result	n/a	n/a
Ikaros 3′ UTR	aMoran (unpubl. data)	600	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 44°C/45s; 72°C/45s][35] 72°C/5m
Interleukin-8 receptor 3′ UTR	Moran (unpubl. data)	No result	n/a	n/a
Monoamine oxidase 3′ UTR	Moran (unpubl. data)	No result	n/a	n/a
OK5	Unknown	No result	n/a	n/a
Lep-ex1	Unknown	No result	n/a	n/a
Prolactin 2	Moran et al. (1997)	No result	n/a	n/a
Recombination activation gene	Moran (unpubl. data)	No result	n/a	n/a
ras Protooncogene	Moran et al. (1997)	No result	n/a	n/a
SRY-type HMG box 3′ UTR	Moran (unpubl. data)	No result	n/a	n/a
Transport associated protein	Moran (unpubl. data)	No result	n/a	n/a
Vimentin	Moran (unpubl. data)	No result	n/a	n/a
Vitellogenin	Park (unpubl. data)	No result	n/a	n/a
Vitellogenin receptor	Moran (unpubl. data)	No result	n/a	n/a

Beverly, MA)], brought to a final volume of 20 μl in sterile water and sequenced as above.

Results

Nuclear markers

In an initial PCR screen using the 20 nuclear markers listed in Table 1, 16 were successfully amplified. All nine microsatellite loci in the trial produced amplification products, but only LS-34 and LS-68 have been sequenced. Sequences from two other loci, CaM-1 and ITS-1, were both verified by multi-species alignment. New primers were designed for both CaM-1 and ITS-1 (Table 3). A third locus, p53, produced multiple bands of 341, 238 and 178 bp in *Scaphirhynchus*. These bands were used to develop two new species specific primer sets (p53-341 and p53-238). However, multiple species alignment did not verify these p53 amplicons as originating from the *p53* gene. Similarly, the remaining four markers produced amplification products, but could not be verified by multi-species sequence alignment. Additional work was not pursued with these loci.

The nine LS microsatellite primers, originally designed for lake sturgeon (May et al., 1997), had been previously screened by May et al (1997) for use in other sturgeon species. In this study, the focus was on the single copy flanking sequence as a possible source of species specific variation. Direct sequencing of LS-34 on eight pallid and two shovelnose sturgeon, and subsequent alignment of these sequences with the appropriate lake sturgeon sequence (GenBank accession no. U72733), resulted in the observation of two polymorphic sites at positions 34 and 97. Both shovelnose sturgeon exhibited a G at position 34 and an A at position 97, while four pallid exhibited an A and a G at positions 34 and 97. The remaining four pallid sturgeon were clearly heterozygous (i.e. demonstrated both an A and a G at both positions).

Table 3
Primers developed for use on *Scaphirhynchus* as a result of this study Reconfigured PCR

Marker	Primer sequence	Actual size	Reaction constituents	Cycling conditions
Nuclear markers				
p53-c341	p53-c341 f: 5′-GAA CGT GCG AGC AGA AAT T-3′ p53-c341 r:c5′-GTG TGC CCA GAT ATT GAA-3′	294	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/45s; 72°C/45s]^{30} 72°C/5m
p53-c238	p53-c238 f: ACA CTG AAA TGA ACC ACC p53-c238 r: CTG TGC TGG GAT AAA TAG C	196	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/45s; 72°C/45s]^{30} 72°C/5m
Internal Tran scribed Spacer One (ITS-1)	ITS Scal85′-ACC TGC GGA AGG ATC ATT TA-3′ ITS Sca585′-AGT GAT CCA CCG CTA AGA GTT-3′	490	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1.5m]^{30} 72°C/5m
Ikaros 3′ UTR	IK2-F: 5′- TGA TTG ATA CTG ACA GG-3′ IK2-R: 5′-AGC AAG ATG GTCC AG-3′	500	200 μM each dNTP's; 175 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/45s; 72°C/45s]^{30} 72°C/5m
Calmodulin gene intron 3 (CaM-1)	CADS-f5′-AgA TgC TgA Tgg TgA gTC C-3′ CADS r5′-AAY TCW ggg AAR TCR AYK gT-3′	200	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 53°C/1m; 72°C/1m]^{35} 72°C/5m
Mitochondrial Markers				
ND51	ND51s: 5′- AAC TCT Tgg TgC AAC TCC-3′ ND52r: 5′-gTA gCg ATg TTT ATT CAA Tg-3′	265	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/1m; 72°C/1.5m]^{30} 72°C/5m
ND52	ND52-f: 5′-CAT TgA ATA AAC ATC gCT ACC T-3′ ND53r: 5′-AAT gTC CCC TAC TCg gTT-3′	340	200 μM each dNTP's; 200 pM each primer; 2.0 mM $MgCl_2$; 0.03 U/Taq.	95°C/2m [95°C/1m; 55°C/45s; 72°C/45s]^{30} 72°C/5m

An alignment of 10 pallid and four shovelnose sturgeon against the lake sturgeon sequence of LS-68 (GenBank accession no. U72739) revealed an insertion of 8 bp between *Scaphirhynchus* sp. and *Acipenser fulvescens* beginning at position 340. Position 341 in *Scaphirhynchus* is a polymorphic site, two pallid and two shovelnose individuals exhibiting an A at this position, while eight pallid and two shovelnose exhibit a G.

The CaM-1 primers amplify an intron in the highly conserved calmodulin gene. Sequences of individual clones here revealed considerable variation. However, as multiple calmodulin genes occur in several vertebrate species, gene specific primers will be needed to further characterize this locus in *Scaphirhynchus*.

The non-coding first internal transcribed spacer (ITS-1) region is located between the 18S rDNA and 5.8S rDNA coding regions and has been found to be a source of informative variation at the species and population levels for several vertebrate species (Gerbi, 1985; Domanico et al., 1996). Two primers designed for salmonid fishes were used to amplify a 480 bp segment of ITS-1 from eight pallid and eight shovelnose sturgeon. The amplified fragment was verified in multiple species comparisons as the first internal transcribed spacer. A *Scaphirhynchus* primer set was designed to encompass the ITS-1 region from conserved regions in the 3′ end of the 18S rDNA and the 5′ end of the 5.8S rDNA genes (ITS18F, ITS58R; Table 3). Intrapopulation sequence variation was observed and consisted mainly of C-G transitions and insertions in the 5′ end of the ITS-1 segment. The ITS-1 sequence was polymorphic within both species, however none of the sequence differences could discriminate pallid and shovelnose sturgeon.

The *p53* gene is a tumor-suppressor gene that has a role in cell cycle control. Sequence variation in the *p53* gene is often used as a biomarker for aquatic toxins affecting fish species (Bhaskaran et al., 1999). The *p53* primer set described in Table 1 produced three amplification products of 341, 238 and 178 bp in length. New primer sets for each of these amplicons were designed from sequences resulting from clones (Table 3). The p53-c341 primer set amplified a 294 bp product, which displayed no polymorphic sites between eight pallid and 33 shovelnose sturgeon. The p53-c238 primer set amplified a 196 bp product and also displayed no polymorphic sites between seven pallid and 33 shovelnose sturgeon sequenced. Primer set p53-c178 did not amplify well and further work was discontinued.

The 22 primer sets obtained from the National Marine Fisheries Service (Table 2) were designed for use with salmonid species. Six of these produced PCR amplification products in *Scaphirhynchus*. Only one, Ikaros 3′-UTR, has been cloned and sequenced. However, BLAST comparisons of the Ikaros amplicon sequences revealed them to be of anonymous origin, provisionally referred to as Anon-1. Nonetheless, a new primer set (IK-2, Table 3) was designed for use on *Scaphirhynchus*. The new primers produced an amplification product of approximately 500 bp. A 290 bp segment of the IK-2 amplicon was sequenced in 11 pallid and 22 shovelnose sturgeon. The segment exhibited variation at positions 103 and 187. At site 103, five pallid and nine shovelnose display a C, two pallid display a T, while four pallid and 13 shovelnose clearly exhibit both a C and a T. Similarly, at site 187, nine pallid and 17 shovelnose display a C, one shovelnose displays a T, and the remaining two pallid and four shovelnose are heterozygous.

Mitochondrial markers

Two mitochondrial markers, NADH dehydrogenase 5/6 and the control region (D-loop), were successfully amplified from *Scaphirhynchus* (Table 1). Two different primer sets were designed for direct sequencing of the 5′ end of ND-5 in *Scaphirhynchus*. Primer ND5-1 combined with ND5-2R produced a fragment of approximately 265 bp in length, and ND5-2F (reverse complement of ND5-2R) combined with ND5-3R produced a fragment of approximately 340 bp. The combined 634 bp of ND5 were sequenced in 16 pallid and 31 shovelnose sturgeon. Only one A-G transition at nucleotide position 12 was observed in a single shovelnose sturgeon from the Mississippi River, Missouri.

Campton et al. (2000) developed D-loop primers for use in *Scaphirhynchus*. The authors sequenced 496 bp of the control region in 158 individuals: 33 pallid, 123 shovelnose and two Alabama sturgeon. Observed were 51 variable sites, including 18 C–T transitions, 28 G–A transitions, one insertion and four deletions. The 26 haplotypes observed had not been previously reported by Campton et al. (2000). The two characterized Alabama sturgeon also displayed the unique 'I' haplotype initially described by Campton et al. (2000).

All nuclear and mitochondrial sequences discussed above have been submitted to GenBank.

Discussion

The authors designed primers (Table 3) to five different nuclear loci (LS-34, LS-68, CaM-1, ITS-1 and Anon-1) that direct precise and robust amplication sufficient for direct sequence analysis of sturgeon. Also demonstrated was the existence of significant DNA sequence polymorphism at these loci among the species of the river sturgeon, *Scaphirhynchus* (Table 4). New mitochondrial control region polymorphism is described to be added to the catalogue begun by Campton et al. (2000). It is anticipated that these new nuclear markers and additional mitochondrial polymorphism will find significant application in forensic, population genetic, phylogenetic and genome organization investigations of sturgeon.

However, the results indicate that these new nuclear and mitochondrial markers, like the mitochondrial markers before (Campton et al., 2000; Fain et al., 2000b), are polymorphic within pallid and shovelnose sturgeon. As such, they do not appear to be useful for the forensic identification of caviar and meat of shovelnose and pallid sturgeon and their hybrids. Their utility in identifying the Alabama sturgeon is being investigated. Alabama sturgeon continue to be distinguished by the unique 'I' mitochondrial haplotype initially described by Campton et al. (2000).

Table 4
DNA sequence polymorphism demonstrated in this study among the three species of *Scaphirhynchus*

Locus	Sequence length (bp)	No. of variable sites	No. of samples
LS-34	67	2	8 p + 2 s
LS-68	79	1	10 p + 4 s
ITS-1	480	7	8 p + 8 s
CaM-1	70–120	Many between clones	
Anon-1	290	2	11 p + 22 s
ND-5	634	1	16 p + 31 s
CR	496	51 (26 new sites)	33 p + 123 s + 2 a

In general, direct sequencing of amplicons from LS-34, LS-68, ITS-1 and Anon-1 yielded results that were not confounded by the polyploid organization of the *Scaphirhynchus* genome. That is, population samples of pallid and shovelnose sturgeon exhibited both homozygotes and heterozygotes for each polymorphism. This supports the findings of Ludwig et al. (2001) which suggest both *S. albus* and *S. platorynchus* are functionally diploid. However, when sequencing multiple clones generated from the amplification products of CaM-1 and ITS-1, additional variants were detected. In that both of these genes are known to occur in multi-gene families, the attribution of the current observations of these apparently 'non-Mendelian' variants to either polypoidization, intra-gene-family variation, or both remains to be determined.

Only one polymorphic site was observed in a single shovelnose sturgeon from the Mississippi River, Missouri, in comparisons of 16 pallid and 31 shovelnose sturgeon over 634 bp of mitochondrial ND5. These results are consistent with similar comparisons of closely related sturgeon species in the genus *Acipenser*. For example, Birstein et al. (2000) found complete identity between *A. gueldenstaedtii*, *A. persicus* and *A. naccarii* over 643 bp of ND5. The close relationships indicated by so little divergence at ND5 are also supported by low divergence (e.g. 0–1%) at the cytochrome *b* gene (Fain et al., 2000a,b; Birstein et al., 2000).

The authors are proceeding to sequence a collection of 160 *Scaphirhynchus* individuals at LS-34, LS-68, CaM-1, ITS-1 and Anon-1 to further quantify the polymorphism already seen and to characterize its inheritance in populations. Work also continues to document the occurrence of polymorphism for these loci in selected species of *Acipenser* (where considerably more interspecies difference has been observed). These data for *Scaphirhynchus* and preliminary comparisons of species of *Acipenser* add further support to the view that the remarkably similar species of river sturgeon are only very recently diverged.

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