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Genetic variation at microsatellite loci in sturgeon: primer sequence homology in *Acipenser* and *Scaphirhynchus*

Bernie May, Charles C. Krueger, and Harold L. Kincaid

Abstract: Eleven tri- and tetra-meric motif microsatellite loci were identified in a lake sturgeon genomic library and were tested against the six North American species of *Acipenser* (lake sturgeon, *A. fulvescens*; shortnose sturgeon, *A. brevirostrum*; white sturgeon, *A. transmontanus*; green sturgeon, *A. medirostris*; Atlantic sturgeon, *A. oxyrhynchus oxyrhynchus*; gulf sturgeon, *A. o. desotoi*) and the two species of *Scaphirhynchus* (pallid sturgeon, *S. albus*; shovelnose sturgeon, *S. platorhynchus*) using four to six individuals of each species. Eight loci were amplified equally well in all eight species, and the remaining three loci were amplified in two, four, and seven species, respectively. Of the eight loci that worked in all species, one was monomorphic in all species, while the other seven were polymorphic in three to eight species. Single repeat differences in these tri- and tetra-meric repeat motifs can be readily scored on 4% Metaphor agarose gels stained with ethidium bromide. In addition, dosage for those loci exhibiting four gene doses can also be readily scored with this technique. These loci provide a much needed group of genetic markers, detectable with non-invasive sampling (blood, barbel, or fin), that should work well in threatened and endangered species of sturgeon worldwide.

Résumé : Onze loci de microsatellites présentant un motif tri- et tétramérique ont été observés dans une banque génomique d'esturgeon jaune et ont été testés par rapport à six espèces d'*Acipenser* d'Amérique du Nord (esturgeon jaune, *A. fulvescens*; esturgeon à museau court, *A. brevirostrum*; esturgeon blanc, *A. transmontanus*; esturgeon vert, *A. medirostris*; esturgeon noir, *A. oxyrhynchus oxyrhynchus*; esturgeon du golf, *A. o. desotoi*) et les deux espèces de *Scaphirhynchus* (esturgeon pâle, *S. albus*; esturgeon scaphirhynque, *S. platorhynchus*) en utilisant 4-6 individus de chaque espèce. Huit loci ont été bien amplifiés de manière égale, chez les huit espèces, et les trois loci restants ont été amplifiés chez deux, trois et sept espèces respectivement. Des huit loci présents chez toutes les espèces, un était monomorphique chez toutes les espèces, tandis que les sept autres étaient polymorphiques chez trois des huit espèces. Les différences de répétition unique dans ces motifs répétitifs tri- et tétramériques peuvent être facilement observées dans des gels d'agarose Métaphor à 4 % colorés au bromure d'éthidium. De plus, cette technique permet d'établir rapidement le dosage pour ces loci présentant quatre doses géniques. Ces loci fournissent un groupe de marqueurs génétiques dont on avait grand besoin, qui sont détectables par échantillonnage non invasif (sang, barbillon ou nageoire), qui devraient donner un excellent rendement chez les espèces esturgeons menacées et en danger d'extinction dans le monde.

[Traduit par la Rédaction]

Introduction

The order of fishes Acipenseriformes consists of two living families: Acipenseridae (the sturgeons) and Polyodontidae (the paddlefishes). Within Acipenseridae, the genera *Huso*, *Scaphirhynchus*, and *Pseudoscaphirhynchus* consist of two or three species each, while the genus *Acipenser* has more than

15 species. Worldwide nearly all sturgeon species are threatened or endangered (Birstein 1993).

Like other freshwater fish, sturgeon populations have suffered from overfishing, barriers to migration, loss of spawning habitat, and the deterioration of water quality. Recent efforts to improve water quality and aquatic habitats in North America have led to an interest in the restoration of these fishes. The International Conference on Sturgeon Biodiversity and Conservation held at the American Museum of Natural History in July 1994 helped to focus scientific attention on the precarious state of many sturgeon populations and the need for new methods to study these long-lived animals (Birstein et al. 1997).

Genetic research on sturgeons has been limited to a few studies on chromosomal numbers and cellular DNA content (Birstein et al. 1993), allozymes (Phelps and Allendorf 1983; Bartley et al. 1985), and mitochondrial DNA (Ferguson et al. 1993 and references cited therein). The sturgeon's extended age to maturity (5 to more than 20 years), extended period between spawning cycles (2 to more than 10 years), and their residence in deep waters of large lakes and rivers make them difficult species for genetic investigations of behavior, selective breeding, or population structure. Chromosomal and DNA content studies (Birstein and Vasilev 1987; Birstein et al.

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1993; Blacklidge and Bidwell 1993; Fontana 1994) divide the species into three groups of $2N = \sim 120$, ~ 240 , and ~ 360 –500. These results, along with chromosomal data on related taxa, suggest that one polyploid event may have occurred in the evolution of the order Acipenseriformes with several more independent polyploid events in the evolution of the family Acipenseridae.

Codominant Mendelian markers are necessary for definitive studies to be conducted (*i*) to determine the genomic structure of sturgeon species with different chromosome numbers, (*ii*) to describe population structure of species, and (*iii*) to measure the effects of ongoing stocking programs on the genetics of native populations. Traditional allozyme studies are difficult to perform on sturgeon because of the need to sample eye, heart, liver, or kidney tissues, causing mortality of fish that are threatened or endangered.

Recent studies in a number of organisms have shown that the class of variable number tandem repeat (VNTR) loci with simple sequence repeat (SSR) motifs (two to five base pairs (bp); often termed microsatellite loci) offer the advantages of high levels of allelic variation per locus and simplified tissue sampling requirements for their analysis. Microsatellite loci often have more polymorphic loci and more alleles per polymorphic locus than allozyme loci. In addition, microsatellite loci can be scored from tissues non-destructively sampled (e.g., muscle, fin, hair, blood, feces, scale, feather) and preserved by freezing, drying, or stored in alcohol or lysis buffer. These loci have provided a wealth of data for many different types of genetic studies, such as parentage analysis in chimpanzees (Morin et al. 1994) and bees (Estoup et al. 1995), population studies in brown trout (Estoup et al. 1993) and black bears (Paetkau and Strobeck 1994), and genome mapping in mice (Dietrich et al. 1992) and humans (Luty et al. 1990).

Microsatellite loci in lake sturgeon were investigated to find new simple Mendelian markers useful for future population and breeding studies. In this report, 11 microsatellite loci and their respective amplification primers are described that were cloned from a lake sturgeon genomic library. These primers were then tested on extracted DNA from all eight North American species of sturgeon from the genera *Acipenser* and *Scaphirhynchus* to determine the homology of these primers in related taxa.

Methods

Collections

Portions of fin (0.5–1.5 cm²) were excised, placed in 100% ethanol, and either refrigerated or stored at room temperature for 1–6 months. Fin samples were taken from 4 to 11 individuals of lake sturgeon (*Acipenser fulvescens*), shortnose sturgeon (*A. brevirostrum*), white sturgeon (*A. transmontanus*), green sturgeon (*A. medirostris*), Atlantic sturgeon (*A. oxyrhynchus oxyrhynchus*), gulf sturgeon (*A. oxyrhynchus desotoi*), pallid sturgeon (*Scaphirhynchus albus*), and shovelnose sturgeon (*S. platorhynchus*).

Extraction

DNA was extracted from 0.5–1.0 cm² fin samples by the CTAB method of Saghai-Marouf et al. (1984) and Doyle and Doyle (1987) as modified by Grewe et al. (1993). The resultant DNA was highly degraded but most of the DNA was >4 kilobases (kb).

Library screening

Extracted DNA from four lake sturgeon was pooled (6 µg) and digested with *Sau3A*. The plasmid pUC 18 was digested with *Bam*HI, purified with phenol–chloroform, and precipitated with ethanol. Digested pUC 18 (100 ng) was added to non-size-fractionated sturgeon DNA in 1:2, 1:1, and 2:1 weight ratios (50, 100, or 200 ng of sturgeon DNA, respectively) in a ligation mixture containing DNA, buffer, and 1.5 units of T4 DNA ligase. Ligation took place overnight in a thermal cycler at 16°C.

The ligation mix (1 µL) was added to 50 µL of DH5α cells (GIBCO), put on ice for 30 min, incubated for 45 s in a 42°C water bath, and then placed on ice for 2 min. SOC medium (900 µL) was added and the transformed DH5α cells were incubated for 1 h at 37°C. Each transformation (100 µL) was transferred to agar plates that contained ampicillin and allowed to grow overnight yielding from 12 000 to 180 000 colonies. Agar plates then were inoculated to a concentration of about 500–1000 colonies per plate and incubated overnight.

Magnagraph nylon transfer membranes (MSI) were wetted briefly in water and then layered over each plate for 2 min. Membranes lifts were then placed in 10% SDS for 5 min, transferred to denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 20 min, then placed in a neutralizing solution (1.5 M NaCl, 0.5 M Tris; pH 7) for 15 min, and finally transferred to 2 × SSC for 10 min. The membranes were then baked at 80°C for 1 h.

Membrane lifts were hybridized with tetra- and tri-nucleotide repeat probes supplied in the BIOS oligo scan kit (AAAT, AAAG, AAAC, CCG, AAG, AAT, AAC, and CAG) or made at the Cornell DNA synthesis facility (GATA, GACA and GGAT). AAAT, AAAG, AAAC, CCG, AAG, AAT, AAC, and CAG were used to screen “lift set I.” AAAT, AAAG, AAAC, CCG, AAG, GATA, GACA, and GGAT were used to screen “lift set II.” Nucleotide repeats were 3'-end labeled with digoxigenin-11-ddUTP using the Genius 5 Oligonucleotide 3'-end labeling kit (Boehringer-Mannheim). Membrane lifts were prehybridized in Hybaid hybridization bottles in 10 mL hybridization solution (0.5 M sodium phosphate (pH 7.2) 0.5 M EDTA, 20% SDS, and 1% BSA) at 58°C overnight. The next day the hybridization solution was removed and fresh solution containing 1–2 pM of each labeled repeat. Lifts were thus incubated overnight at 58°C. Lifts were then washed three times for 15 min each with 2 × SSC and 0.1% SDS (twice at 21°C and once at 58°C) and once with 0.5 × SSC and 0.1% SDS for 15 min at 21°C. The Genius colorimetric detection kit, which uses NBT and X-phosphate (Boehringer-Mannheim), was used to visualize digoxigenin-labeled repeat probes with the following conditions: Genius buffer 1 for 1 min, Genius buffer 2 for 2.5 h, Genius buffer 2 with FAB α digoxigenin (10 µL/100 mL Genius buffer 2) for 30 min, Genius buffer 1 for 15 min, Genius buffer 1 for 15 min, Genius buffer 3 for 2 min, and 10 mL Genius buffer 3 with 45 µL NBT and 35 µL X-phosphate until development was complete (30 min – 12 h).

Secondary screening

Dot blotting directly

Positively hybridizing colonies were picked from plates with a sterile toothpick and transferred into a 13 × 100 mm culture tube with 5 mL Terrific Broth (TB). After growing overnight at 37°C, 2 µL of the culture was dot blotted onto a piece of Schleicher and Schuell Nytran Plus membrane and allowed to dry. Pretreatment and hybridization of this membrane was identical to that procedure used for the original membrane lifts. Colonies that again were positive were streaked onto an agar plate and grown overnight at 37°C. Four separated colonies from each plate were then picked, used to inoculate 1 mL of TB in 1.5 mL microcentrifuge tubes, and incubated overnight in a heat block at 37°C. Two microlitres were dot blotted from each tube and rescreened.

Secondary plating

Alternatively, colonies in the area of the positively hybridizing colonies were picked and grown overnight. This growth was diluted 1 : 10 000 and 10 μ L were inoculated onto agar plates (usually to a concentration of 50–200 colonies per plate). Colonies were lifted, and lifts were hybridized as described above. Membranes were lined up with plates so individual, positive colonies could be picked. Positively hybridizing colonies then were placed in 5 mL TB with ampicillin and grown overnight at 37°C in 50-mL conical centrifuge tubes. Two microlitres of the overnight growth from each tube was dot blotted and rescreened.

Sequencing of clones

Positive clones were inoculated in 5 mL TB and grown overnight. From this growth, 0.5 mL was added to 30% glycerol in TB and frozen to –70°C for future use and 1.5 mL were purified using the Qiagen plasmid mini-kit to obtain 6–20 μ g of purified DNA. Twelve microlitres of each clone at ~200 ng/ μ L was sent to the Cornell Biotechnology Center's Analytical Chemistry and Peptide/DNA Synthesis Facility (ACPSF) along with 10 μ L M13 forward primers at 5 μ g/mL for sequencing on an ABI 373A automated sequencer.

Primer design, synthesis, and testing

Primers were designed with Generunner and were synthesized at the ACPSF. Primers were first tested with five individual lake sturgeon and subsequently with six lake, four Atlantic, four white, five pallid, four shovelnose, five shortnose, five gulf, and six green sturgeon.

PCR and gel conditions

Amplifications were done in 500- μ L tubes with 1 unit *Taq* DNA polymerase (GIBCO), 0.4 μ M of each primer, 3–10 ng template DNA (prepared by the CTAB method described above), 100 or 175 μ M dNTPs, 1.5–2.5 mM MgCl₂ in 50 μ L of buffer. Reaction mixes were amplified in a Perkin Elmer 4800 cyler as follows: preheating at 94°C for 3 min, 35 cycles of amplification (denaturing at 94°C for 1 min, annealing at 57°C for 30 s, polymerization (elongation) at 72°C for 30 s), and a final polymerization at 72°C for 5 min.

Amplified products were run on a 3.5% agarose gel (Nusieve GTG : SeaKem LE, 1:1; 27.5 \times 22 \times 0.6 cm; 1 \times TBE) at 150 V for 1 h or on a 4% Metaphor agarose gel (27.5 \times 22 \times 0.3 cm; 1 \times TBE) at 460 V for 1.5 h. A buffer (0.5 \times TBE) temperature of 18°C was maintained for the Metaphor gels with a Neslab model RTE100 refrigerated coolant pump, after an initial 7-min loading run at 13°C.

Results

Approximately 61 000 colonies were screened in lift set I yielding 110 positively hybridizing colonies, of which 33 colonies were sequenced that were found positive three times. Among approximately 31 000 colonies screened in lift set II, 71 positive colonies were recognized, of which 24 colonies were sequenced. From the 57 colonies sequenced, (i) primers were made for 14 tri- and tetra-meric microsatellite loci, (ii) 18 possessed a minisatellite repeat of 125 bp, which also contained two trimeric motif microsatellite loci (B. May, C.C. Krueger, and H.L. Kincaid, unpublished data), (iii) five contained multiple clones, (iv) two contained poly As, (v) six contained microsatellites for which primers could not be effectively designed, (vi) one contained a 40-bp repeating sequence too close to the vector, (vii) three inserts were identical to other inserts, (viii) one had a dimeric repeat motif, and (ix) seven had sequencing problems.

Eleven of the 14 microsatellite primer pairs produced an electrophoretically resolvable product when tested on five or more lake sturgeon (Table 1). Four of these loci possessed pure

trimer repeat motifs, five had pure tetramer repeats, one had two back-to-back tetramer repeats, and one had a tetramer repeat followed by a trimer repeat in the sequenced clones. The number of repeats in individual clones varied from 6 to 30 and all were uninterrupted perfect repeats. Locus designation, insert size, repeat motif, number of repeats in the clone, primer sequences, GenBank accession numbers, optimal MgCl₂ and dNTP concentrations, and allele size in the clone for the 11 loci are listed in Table 1. Three of the 14 primer pairs did not produce a consistently resolvable product.

Of the 11 primer pairs that yielded a product in lake sturgeon, eight of the primers produced an electrophoretically resolvable product in all of the other seven species of sturgeon (Table 1). One primer pair (LS-22) functioned only with lake and green sturgeon; another (LS-23) produced products only in lake, Atlantic, shortnose and green sturgeon; while LS-69 produced a product in all but green sturgeon. Though only four to six individuals of each species were tested, alternate allelic forms were present at 9 of the 11 loci in one or more species. Polymorphisms were observed at one locus (LS-68) in all eight species. One locus (LS-58) exhibited a single band identical to the clone for all individuals of all species. Three to eight loci were polymorphic per species. Fifty-six percent of the species–locus combinations that produced amplicons were polymorphic (based on presence of trailing presumptive “heteroduplex” bands, see Discussion) when run on the 3.5% agarose gels.

Discrete bands differing by one or more repeat units were observed when amplified samples were run on Metaphor agarose gels. For example, seven alleles can be observed among two pallid and six shovelnose sturgeon for locus LS-68 (Fig. 1). Each shovelnose sturgeon was heterozygous for a different combination of alleles. While this locus appears to be disomic, other loci clearly displayed four gene doses (Fig. 2). In addition, one or two extra bands with slower mobility were observed in individual heterozygous phenotypes (Figs. 1 and 2).

Discussion

Primer sequence homology in *Acipenser* and *Scaphirhynchus* was observed for 8 of 11 microsatellite loci in eight species. Additionally, variation in microsatellite repeat number may also be conserved as the amplified products of each individual differed in size by the equivalent of <10 repeats among all species examined. Conservation among related taxa of the unique flanking regions around microsatellites has been reported by others. Moore et al. (1991) report that 56% of 48 primer pairs designed for cattle also produced a product in sheep, 6% in horses, and none in humans, indicating that homology of the flanking regions corresponds to relatedness. Complete conservation has been shown for 10 loci for four species within the cat family Felidae (Menotti-Raymond and O'Brien 1995) and for six loci across six species within two turtle families, Cheloniidae and Dermochelyidae (FitzSimmons et al. 1995). These latter studies suggest that a well-developed group of primers in one species may function well in genetic studies of related taxa within families and thus reduce the time and effort required for primer development.

A high level of polymorphism was exhibited at the microsatellite loci resolved, especially considering the few individuals

Table 1. Microsatellite loci in eight species of sturgeon.

Locus	Insert size (bp)	Repeat motif	Primer	Primer sequence	GenBank accession no.	MgCl ₂ (mM)	dNTPs (μM)	Clone size (bp)	LS ^a (6 ^b)	AS (4)	WS (4)	PS (5)	SV (4)	SN (5)	GF (5)	GR (6)
LS-19	267	(TTG) ₉	LS-19F ^c LS-19R	CATCTTAGCCGTCTGTGGTAC CAGGTCCCTAATAACAATGGC	U72730	1.5	175	133	p ^d	P	M	P	P	P	P	P
LS-22	570	(AAAT) ₆ (AAG) ₃₀	LS-22F LS-22R	TCCACAATCCTGAATAATGAC GCACCATCTAATACGAAATTG	U72731	1.5	100	218	P	N	N	N	N	N	N	M
LS-23	278	(GTTT) ₈	LS-23F LS-23R	TGCAGTAGATCATTGGTGG CCTACATCCCACNACGGTAG	U72732	1.5	175	162	M	M	N	N	N	M	N	M
LS-34	448	(GTT) ₁₀	LS-34F LS-34R	TACATACCTTCTGCAACG GATCCCTTCTGTTATCAAC	U72733	2.5	175	137	M	M	M	P	P	P	M	M
LS-39	700	(GTT) ₁₀	LS-39F LS-39R	TTCTGAAGTTCACACATTG ATGGAGCATTATTGGAAGG	U72734	2.0	100	129	P	M	M	M	M	P	P	M
LS-54	500	(GATA) ₆ (GACA) ₇	LS-54F LS-54R	CTCTAGTCTTTGTTGATTACAG CAAAGGACTTGAAACTAGG	U72735	1.5	175	177	M	P	M	P	P	P	P	M
LS-57	1100	(GAA) ₂₉	LS-57F LS-57R	GCTTGGTTGCTAGTTTGC GTACAGATGAGACCAGAGGC	U72736	1.5	100	206	P	M	P	P	P	P	M	P
LS-58	209	(GATA) ₂₀	LS-58F LS-58R	TCGGTTGAGTGATTGGTATC CTGAAAGGTGAATCTACTCCC	U72737	1.5	100	198	M	M	M	M	M	M	M	M
LS-62	190	(GACA) ₇	LS-62F LS-62R	GATCAGGAGGGCAGAGNAAC CCCTGGATTTGAATTAACAG	U72738	2.5	175	85	P	P	P	P	P	P	M	P
LS-68	1500	(GATA) ₁₃	LS-68F LS-68R	TTATTGCATGGTGTAGCTAAAC AGCCCAACACAGACAATATC	U72739	1.5	100	120	P	P	P	P	P	P	P	P
LS-69	300	(TATC) ₁₃	LS-69F LS-69R	ATCTGAATTGANTTCGTG TTGGATACTGTGTTCCAAC	U72740	2.0	100	206	P	M	M	M	M	P	P	N

^a LS, lake sturgeon; AS, Atlantic sturgeon; WS, white sturgeon; PS, pallid sturgeon; SV, shovelnose sturgeon; SN, shortnose sturgeon; GF, gulf sturgeon; GR, green sturgeon.

^b Number of each species examined.

^c F, forward primer; R, reverse primer (5' to 3').

^d M, monomorphic; P, polymorphic; N, no activity.

Fig. 1. Ethidium bromide stained 4% Metaphor agarose gel of LS-68 amplification products of six shovelnose and two pallid (the two 66 individuals) sturgeon individuals. Presumptive numerical genotypes for a single disomic locus (e.g., a 34 indicates an individual heterozygous for alleles 3 and 4) are above each individual and labeled arrows indicate the alleles that produce particular bands. In heterozygous phenotypes the faster (lower molecular weight) bands are the real bands while the slower bands are presumptive heteroduplex bands (marked by an h) composed of complementary strands of the two component alleles. Specific alleles appear to migrate to positions as follows: 1, 116 bp; 2, 120 bp; 3, 124 bp; 4, 128 bp; 5, 132 bp; 6, 136 bp; and 7, 140 bp, as expected for a microsatellite with a motif of 4 bp (GATA).

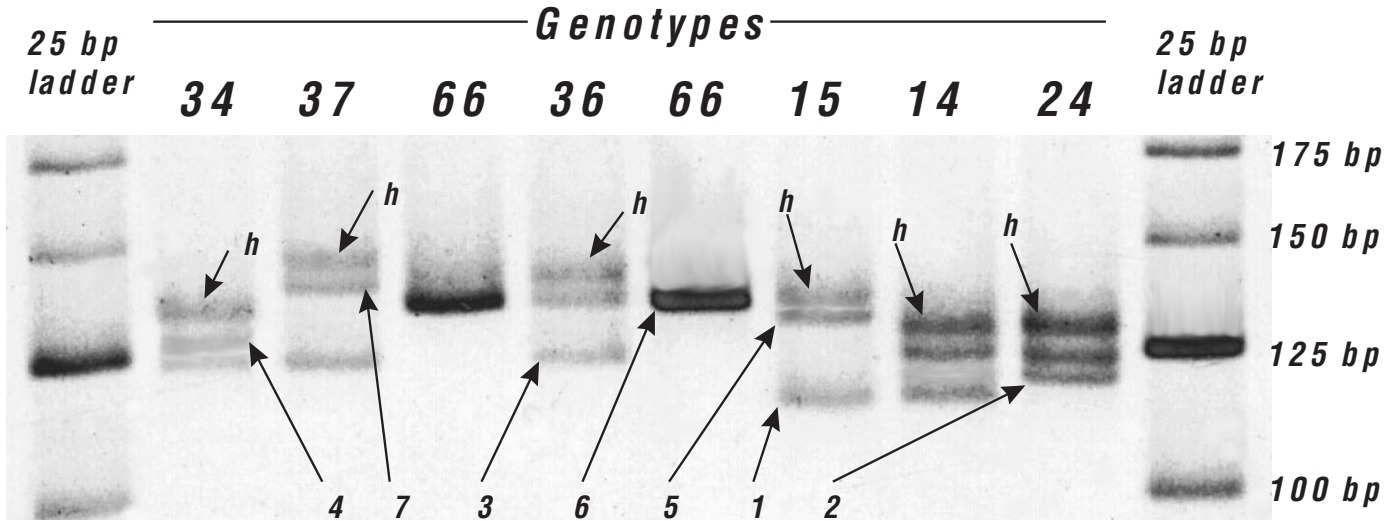
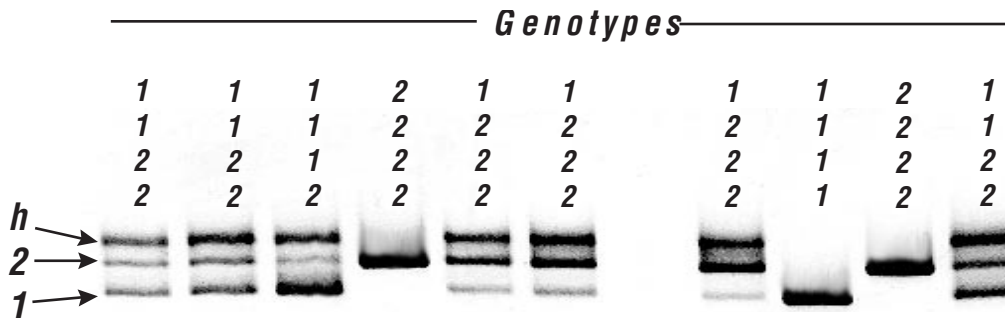


Fig. 2. Ethidium bromide stained 4% Metaphor agarose gel of Atlantic sturgeon amplification products from 10 progeny from a family segregating for LS-19. Bands are coded on left by the alleles 1 and 2 that they represent. Note the extra single slower migrating artifactual heteroduplex band (designated by an h) in the three-banded heterozygous phenotypes. Presumptive genotypes are listed above each respective phenotype, clearly showing the ability to differentiate the three different types of heterozygotes in a four gene dose system with two alleles. The two parental individuals for this family were both 1122 genotypes. Allele 1 has apparent mobility of 131 bp and allele 2 of 143 bp, suggesting a difference of four TTG repeats between these two alleles.



examined and the gel conditions used. Polymorphic loci were detected using 3.5% agarose gels and analyzing samples from four to six individuals of each species, often taken from a single location or population. Only a few locus–species combinations were analyzed on Metaphor agarose gels to demonstrate the resolving power of this medium (Figs. 1 and 2). All microsatellite loci except two (LS-23 and LS-58) contained one or more alternate alleles. The use of higher resolving Metaphor agarose gels and the inclusion of more individuals from different populations likely will reveal new alleles at the polymorphic loci as well as polymorphisms in systems reported here as monomorphic in a particular species. Further, alterations of amplification conditions may permit amplification of those species–locus combinations that failed to amplify for us. LS-58 was fixed for the same allele in all species tested.

Possibly some evolutionary constraint exists for this microsatellite locus that prevents structural change (e.g., it may lie in a transcribed but nontranslated region for a structural gene). In addition, these 4% Metaphor gels clearly permit scoring of gene dosage in heterozygous individuals, a condition of the polyploid ancestry of these fish.

Heterozygous individuals were characterized by the presence of bands that were slower than the allelic bands. Presumably these bands were the result of heteroduplex molecules formed as combinations of complementary strands (from the two different alleles) reannealing after denaturation without amplification. For most loci we saw only one slower band (see Figs. 1 and 2), but occasionally two bands were observed. The presence of one versus two bands may be caused by a difference in annealing and secondary folding (and hence gel

migration rate) between the two different types of heteroduplex molecules.

The microsatellite loci described here provide a much needed group of genetic markers that are detectable with non-lethal sampling (blood, barbel, or fin). Small amounts of tissue may be sampled from individuals of sturgeon populations that are threatened or endangered without causing the death of the fish. Variation at microsatellite loci can be useful in many types of biological studies such as parentage testing, mapping, and population structure (O'Reilly and Wright 1995). These loci are particularly useful because they are codominant in the phenotypic expression of their alleles, exhibit more alleles segregating per locus than allozymes, and have higher mutation rates (Weissenbach et al. 1992) than allozymes. These primers detected genetic variation at microsatellite loci in both *Acipenser* and *Scaphirhynchus*, and thus these primers likely will perform well in sturgeon species worldwide for the analysis of genetic variation at microsatellite loci.

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