Genome Duplication Events and Functional Reduction of Ploidy Levels in Sturgeon (Acipenser, Huso and Scaphirhynchus)

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ABSTRACT

Sturgeon (order Acipenseriformes) provide an ideal taxonomic context for examination of genome duplication events. Multiple levels of ploidy exist among these fish. In a novel microsatellite approach, data from 962 fish from 20 sturgeon species were used for analysis of ploidy in sturgeon. Allele numbers in a sample of individuals were assessed at six microsatellite loci. Species with ~120 chromosomes are classified as functional diploid species, species with ~250 chromosomes as functional tetraploid species, and with ~500 chromosomes as functional octaploids. A molecular phylogeny of the sturgeon was determined on the basis of sequences of the entire mitochondrial cytochrome *b* gene. By mapping the estimated levels of ploidy on this proposed phylogeny we demonstrate that (I) polyploidization events independently occurred in the acipenseriform radiation; (II) the process of functional genome reduction is nearly finished in species with ~120 chromosomes and more active in species with ~250 chromosomes and ~500 chromosomes; and (III) species with ~250 and ~500 chromosomes arose more recently than those with ~120 chromosomes. These results suggest that gene silencing, chromosomal rearrangements, and transposition events played an important role in the acipenseriform genome formation. Furthermore, this phylogeny is broadly consistent with previous hypotheses but reveals a highly supported oceanic (Atlantic-Pacific) subdivision within the Acipenser/Huso complex.

STURGEON have been fishes of great public interest for several centuries. They include the most valuable freshwater fish species worldwide, the producer of black caviar. Since the middle ages, Eurasian species (*Acipenser gueldenstaedtii*, *A. stellatus*, *A. sturio*, and *Huso huso*) have been used for production of caviar. Since the colonization of North America by European settlers, North American species (*A. brevirostrum*, *A. oxyrinchus*, and *A. transmontanus*) have also been used for production of caviar. This exploitation resulted in a drastic decline of all populations. All species are currently near extinction or strongly threatened (Birstein 1993a).

All sturgeon species are members of the order Acipenseriformes. Early debate about their classification arose from their unusual mixture of morphologic characters. For this reason, Acipenseriformes occupy a special place in historical ideas about the classification and evolution of fishes (Bemis *et al.* 1997). Sturgeon fossils are known from the Lower Jurassic (200 million years ago; Bemis *et al.* 1997). All fossil and recent taxa arose in the Holarctic. Basic questions such as the chromosomal evolution or the phylogeny of these living fossils are still unresolved. Their evolutionary age, their special place in fish systematics, and their wide distribution in the Northern hemisphere, together with the large public interest, make sturgeon a good model for studies of molecular evolution in fishes.

Previous studies demonstrate that assigning an exact number of chromosomes can be somewhat arbitrary depending on the technique used that may permit observation of varying numbers of microchromosomes. For example, the number of chromosomes reported in *A. transmontanus* ranged from 230 (Hedrick *et al.* 1991) to 248 ± 8 (Fontana 1994) up to 276 (van Eenennaam *et al.* 1998a). Species of the genera Acipenser, Huso, Scaphirynchus, and Polyodon are separable into three different classes of chromosome numbers: (I) species with ~120 chromosomes including all taxa with between 110 and 130 chromosomes; (II) species with ~250 chromosomes including all taxa with between 220 and 276 chromosomes; and (III) species with ~500 chromosomes (reviewed in Birstein *et al.* 1997, Table 1). The ploidy levels of recent acipenseriform species are controversial in the literature.

Some authors believe that all species with ~120 chromosomes are tetraploid (*e.g.*, Ohno *et al.* 1969; Dingerkus and Howell 1976; Birstein and Vasiliev 1987; Birstein *et al.* 1997); others call them functionally diploid (Fontana 1994; Fontana *et al.* 1998a,b; Jenneckens 1999; Tagliavini *et al.* 1999). Divergent opinions

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TABLE 1

Origin of samples from 22 of 24–25 extant Acipenseriform species used in this study

Species (no. of chromosomes)	Common name	Geographic origin of sampled species	Distribution area	Provider of samples
Family Polyodontidae				
Genus Polyodon				
P. spathula	North American paddlefish	Aquaculture (Mississippi River)	Mississippi—Missouri basin, southeastern United States	I. Jenneckens
Family Acipenseridae				
Subfamily Acipenserinae				
Genus Acipenser				
A. baerii	Siberian sturgeon	Siberia, Russia	Rivers of north coast of Russia	I. Jenneckens, P. Williot
A. brevirostrum	Shortnose sturgeon	Aquaculture	North America—Atlantic coast	B. May
A. fulvescens	Lake sturgeon	Menominee River & Wolfe River, Wisconsin; Lake Winnebago	North America—central United States	L. Debus, B. May
A. gueldenstaedtii	Russian sturgeon/osetra[a]	Caspian Sea; Black Sea; Sea of Azov	Ponto-Caspian species	E. N. Artyukhin, M. Chebanov, L. Debus, N. Patriche, M. Pourkazemi
A. medirostris	Green sturgeon	Columbia River	North America—Pacific coast	B. May
A. mikadoi	Sakhalin sturgeon	Tumnin River, Russia	Siberia—Pacific coast	E. N. Artyukhin
A. naccarii	Adriatic sturgeon	Buna River, Albania; Po River, Italy	Adriatic Sea	P. Bronzi, T. Gulyas
A. nudiventris	Ship sturgeon	Caspian Sea; Volga River	Ponto-Caspian species	L. Debus
A. oxyrinchus	Atlantic sturgeon	Delaware River; Hudson River; St. John River	North America—Atlantic coast	J. Gessner, B. May
A. persicus	Persian sturgeon	Caspian Sea; Volga River	Ponto-Caspian species	L. Debus, M. Pourkazemi
A. ruthenus	Sterlet	Volga system, Kuban River, Russia; Danube River, Romania	Ponto-Caspian area and some rivers of north coast of Russia	E. N. Artyukhin, M. Chebanov, L. Debus, N. Patriche
A. schrenckii	Amur sturgeon	Amur River	Amur River drainage, Sea of Okhotsk	V. Svirsky
A. sinensis	Chinese sturgeon	Yangtze River, China	China, South Japan	S.-M. Zhang
A. stellatus	Stellate sturgeon/sevruga[a]	Caspian Sea; Black Sea; Sea of Azov	Ponto-Caspian species	E. N. Artyukhin, M. Chebanov, N. Patriche, L. Debus
A. sturio	European sturgeon	Gironde River, France; North Sea	West European Atlantic coast	J. Gessner, P. Williot
A. transmontanus	White sturgeon	Kootenai River	North America—Pacific coast	L. Debus, B. May; Acc. nos. AF184107, X14944
Genus Huso				
H. huso	Great sturgeon/beluga[a]	Caspian Sea; Black Sea	Ponto-Caspian species	E. N. Artyukhin, L. Debus, D. Mirea
H. dauricus	Kaluga[a]	Amur River	Amur River drainage	V. Svirsky
Subfamily Scaphirhynchinae				
Genus Scaphirhynchus				
S. albus	Pallid sturgeon	Atchafalaya River	Mississippi—Missouri basin, southeastern United States	L. Debus, B. May; Acc. nos. U56983, U56987
S. platorynchus	Shovelnose sturgeon	Mississippi River & Missouri River	Mississippi—Missouri basin, southeastern United States	B. May; Acc. nos. U56984–6, U56988
S. suttkusi	Alabama sturgeon	GenBank U55994	Mississippi—Missouri basin	Acc. no. U55994

[a] Trade name of caviar.

concerning ploidy levels also apply to species with higher chromosome numbers, even in studies that use the same general methods. For instance, on the basis of observed numbers of stained active nuclear organizer regions (NORs) in four different sturgeon species, Birstein and Vasiliev (1987) suggested tetraploidy for species with ~120 and octaploidy for species with ~250 chromosomes. However, using data on the chromosomal assignment of NORs, it was shown that these elements occurred on different chromosome pairs rather than on quadruplets (Fontana 1994; Fontana *et al.* 1998b). Therefore, these authors suggested diploidy for sturgeon with ~120 chromosomes and tetraploidy for sturgeon with ~250 chromosomes. Nonuniform results concerning the ploidy level of acipenseriform species were also found among studies focused on expressed gene products. Birstein *et al.* (1997) observed duplicated protein loci in species with ~120 chromosomes, which they attributed to tetraploidy. By contrast, Kuz'min (1996) assigned diploidy to species with ~120 chromosomes in a densitometric study of serum albumins.

In our study we used highly polymorphic markers (microsatellites) that are randomly distributed in the nuclear genome and inherited in a Mendelian fashion (May *et al.* 1997) to investigate ploidy levels of 20 sturgeon species. Because of the large number of acipenseriform species and the large sample size we wished to examine, traditional methods for assessing ploidy levels and chromosome numbers were not feasible. The assumption of our work was that the number of alleles at a single locus should be a minimal reflection of the ploidy level. We examined a large sample of mostly wild-caught individuals to exclude interpretation difficulties that may result from inbreeding or small numbers of parents.

Hypotheses concerning the evolution of genome formation require testing in a phylogenetic context (Pagel and Harvey 1988). Several recent articles have addressed the phylogeny of Acipenseriformes (Artyukhin 1995; Bemis *et al.* 1997; Birstein and Bemis 1997; Birstein and DeSalle 1998; Krieger *et al.* 2000; Ludwig *et al.* 2000). Cladistic analyses indicate that Acipenser is a sister taxon to the monophyletic lineage formed by Scaphirhynchus and Pseudoscaphirhynchus, and together these three genera comprise the subfamily Acipenserinae with Huso as its sister taxon (Findeis 1997). Mayden and Kuhajda (1996) supported the monophyly of the genus Acipenser with only one autapomorphy. Birstein and DeSalle (1998) and Ludwig *et al.* (2000) called the validity of Huso as a separate taxonomic unit into question. Although both studies dealt with most of the extant sturgeon species (Birstein and DeSalle 1998, 24 species; Ludwig *et al.* 2000, 19 species), they resulted in controversial topologies. Ludwig *et al.* (2000) observed a separation of the Atlantic and the Pacific species, which was not found by Birstein and DeSalle (1998). This separation was supported by other studies that focused only on North American Atlantic and Pacific species (Brown *et al.* 1996; Krieger *et al.* 2000). In this study, we investigated ploidy level in light of molecular phylogeny. To accomplish this, we examined sequences of the entire mitochondrial cytochrome *b* gene. Three additional Asian Pacific species, *A. schrenckii*, *A. sinensis*, and *H. dauricus*, were added to the data of Ludwig *et al.* (2000) with the aim of bringing more light to this postulated Atlantic-Pacific separation.

MATERIALS AND METHODS

Fish samples and DNA extraction: The acipenseriform species examined in this study and their geographical origins are given in Table 1. Sample sizes are listed in Table 2. In addition, data from 96 artificially reproduced juveniles of *A. sturio* resulting from the last successful artificial reproduction of this species in 1995 (Williot *et al.* 1997) were used to examine the inheritance of two microsatellite loci. These fish are reared under aquaculture conditions for future restocking programs in France and Germany. The DNA was isolated from fin or blood samples using QIAamp blood and tissue kits (QIAGEN, Hilden, Germany).

Microsatellite analyses: Allele number and lengths were determined for the loci amplified by the following primer pairs designed from lake sturgeon (*A. fulvescens*): Afu-19, Afu-34, Afu-39, Afu-54, Afu-57, and Afu-68 (May *et al.* 1997). Primers were labeled with 6-FAM, HEX, and TET (ABI, Foster City, CA). Amplifications were performed in a total volume of 25 μl containing 50 ng genomic DNA, 0.25 units *Taq* DNA polymerase (QIAGEN), 5 pmol of each primer, 0.10 mm Tris-HCl (pH 8.8 at 25°), 50 mm KCl, 1.5 mm $MgCl_2$, 0.1 μg/μl bovine serum albumin (BSA), 0.08% (v/v) Nonidet P40, and 100 μm of each dNTP. Amplification was performed with 30 cycles of the following steps: 30 sec at 94°, 30 sec at 52° up to 57° (Table 2), 30 sec at 72°, and a 5-min final extension at 72°. The size of alleles was determined using an ABI 310 DNA sequencer with internal standards. In principle, detection of two electropherogram peaks differing in size from one diploid individual would represent two different alleles (four different peaks would represent four alleles in a tetraploid species, etc.). For those fish exhibiting two or more peaks differing in size, the ratio of peak heights and areas was calculated with ABI GENSCAN 2.0 and these data were used to estimate gene dosages in the same manner as we performed in Jenneckens *et al.* (2001).

Amplification and sequencing of cytochrome *b*: The entire cytochrome *b* gene (cyt-*b*) and some flanking sequences, comprising 1221 bp, were amplified using flanking tRNA primers L-14735 (Wolf *et al.* 1999) and cytb-rev1, as well as four additional primers to permit sequencing of overlapping fragments, as described in Ludwig *et al.* (2000). The following reaction conditions were used: 60 sec at 94°, 30 sec at 55°, 90 sec at 72° for 30 cycles, and a final extension at 72° for 5 min. Amplifications were carried out with 1 unit *Taq* DNA polymerase (QIAGEN), 10 pmol of each primer, 50 ng DNA, 0.10 mm Tris-HCl (pH 8.8 at 25°), 50 mm KCl, 1.5 mm $MgCl_2$, 0.1 μg/μl BSA, 0.08% (v/v) Nonidet P40, and 100 μm of each dNTP. PCR products were run on a 1.5% agarose gel at 150 V for 2 hr. The bands of interest were excised from the gel and extracted using the QIAquick gel extraction kit (QIAGEN). Direct sequencing was performed in both directions using the Big

TABLE 2

Number of analyzed specimens and ranges of allele size

Species	Sample size	Range of allele sizes observed at each microsatellite locus					
		Afu-19 (bp) [52°]	Afu-34 (bp) [57°]	Afu-39 (bp) [57°]	Afu 54 (bp) [52°]	Afu-57 (bp) [57°]	Afu-68 (bp) [57°]
A. baerii	139	118–151 (7)	134–146 (5)	117–147 (8)	177–269 (13)	—	128–236 (15)
A. brevirostrum	6	120–129 (3)	133–145 (5)	124–145 (7)	180–256 (10)		123–127 (2)
A. fulvescens	31	127–130 (2)	—	123–129 (2)	180–220 (9)	155–203 (13)	108–211 (7)
A. gueldenstaedtii	99	118–154 (10)	134–149 (6)	108–156 (16)	173–237 (15)	—	104–248 (28)
A. medirostris	37	121–124 (4)	127–139 (2)	—	164–176 (2)	154–199 (16)	119–175 (13)
A. mikadoi	5	121–127 (3)	136–142 (3)	—	178 (1)	158–202 (11)	131–179 (11)
A. naccarii	20	109–130 (19)	131–155 (7)	114–138 (6)	177–237 (9)	—	134–170 (8)
A. nudiventris	15	121–130 (2)	133 (1)	132 (1)	195–212 (5)	—	128–146 (2)
A. oxyrinchus	90	121–145 (4)	138–145 (6)	120–123 (2)	184–192 (3)	—	132–160 (8)
A. persicus	17	121–163 (12)	130–149 (7)	120–156 (12)	185–246 (12)	—	116–248 (18)
A. ruthenus	156	130–142 (5)	137–155 (7)	108–126 (4)	—	—	176–232 (15)
A. schrenckii	3	117–120 (2)	127–143 (6)	115 (1)	149–185 (6)	—	116–160 (9)
A. sinensis	5	108–123 (5)	128–151 (7)	105–117 (4)	160–164 (2)	—	132–160 (8)
A. stellatus	93	124–142 (7)	131–149 (7)	111 (1)	157–197 (11)	—	116–152 (9)
A. sturio[a]	140	118–145 (6)	140–149 (4)	120–123 (2)	180–188 (3)	—	128–152 (4)
A. transmontanus	12	—	137–140 (2)	114 (1)	148 (1)	132–149 (4)	119–151 (9)
H. huso	74	127–157 (6)	137–140 (2)	114–150 (4)	213–285 (10)	—	136–164 (4)
H. dauricus	3	118–121 (2)	136–139 (2)	115–118 (2)	160–168 (3)	—	124–148 (5)
S. albus	6	123 (1)	140–149 (3)	120–123 (2)	146 (1)	119–131 (3)	115–123 (3)
S. platorynchus	11	120–129 (3)	140–149 (3)	120–123 (2)	146–150 (2)	115–131 (4)	111–135 (6)

Numbers of alleles in analyzed microsatellite loci are indicated in parentheses; annealing temperatures for each locus are indicated in brackets.

[a] Including 96 artificially reproduced specimens.

Dye RR Terminator cycle sequencing kit (ABI); sequencing products were visualized using an ABI 310.

Microsatellite data analysis: The microsatellite-allele-counting approach used for investigation of ploidy levels is limited in that the number of alleles in an individual is an estimate of the ploidy level. The number of alleles observed in a single individual, within a sample of individuals, may not represent the maximum possible number of alleles at that locus for that species. Furthermore, the observation of four alleles in a single individual may indicate tetrasomic inheritance at that locus, or it may indicate the presence of two disomically inherited loci. Theoretically, low levels of polymorphism may be especially prevalent in small populations such that it would be highly unlikely to find an individual with the maximum possible number of alleles. This problem is more acute in 2n species than in 4n species or 8n species because the number of heterozygous individuals is larger in these systems (Allendorf *et al.* 1975; Nadeau and Sankoff 1997). To increase our confidence in the evidence presented here, we examined allelic distributions of artificially produced *A. sturio* (~120 chromosomes), demonstrating the inheritance pattern for the loci examined in this species.

Sequence data analysis: We computed basic sequence statistics with the program DnaSP 2.2 (Rozas and Rozas 1997). Analysis of molecular variance (AMOVA) values were computed with the program ARLEQUIN 2.000 (Schneider *et al.* 2000). Tree calculations in this study were performed on the entire cyt-*b* gene (1141 bp). Phylogenetic relationships among taxa were estimated using maximum parsimony and maximum-likelihood algorithms, implemented by the PAUP* (Swofford 1998) and PUZZLE v. 2.5.1. (Strimmer and von Haeseler 1996) program packages. Bootstrap analyses were performed with 1000 iterations. The consistency index (CI) and retention index (RI) were calculated in PAUP* (Swofford 1998). Maximum-likelihood/quartet-puzzling support values are equivalent to bootstrap values.

Spectral analysis was conducted using the program SPECTRUM 2.0 (Charleston 1996) to measure the amount of support for, and conflict against, a given phylogenetic arrangement. A spectrum is a representation of all inferred or estimated branch lengths of a phylogenetic tree, which is generated without the necessity of actually inferring a tree. For a given set of sequences of aligned characters, the support for a hypothesized partition (split) of the sequences into two parts is the sum of the sequence positions that have a unique character state in that partition. The conflict value for a split is the sum of all other splits that contradict the partitioning of sequences in the first split. A detailed description and application of this method is given in Lento *et al.* (1995) and Pitra and Veits (2000). The main focus of this study is the Atlantic-Pacific separation.

RESULTS

Microsatellite data: Table 2 summarizes the ranges of allele sizes and number of alleles observed at each locus within each species.

Table 3 gives an overview of the ploidy levels inferred from microsatellite analyses. Wherever a ">" precedes the allelic band patterns observed (*e.g.*, 8n), the exact number of alleles was not detectable using the quantitative measurements obtained by GENESCAN, resulting from technical limitations. PCR amplification of microsatellites often generates stutter, that is, products that are one or a couple of motif sizes smaller or larger than the actual intended product size. These bands are less well amplified than the actual microsatellite product, but they create false peaks in an electropherogram, making gene dosage calculations often difficult. This problem is exacerbated if there are eight or more alleles that are similar in size. In addition, the strength of amplification depends not only on the number of DNA copies in the genome but also on the length of the fragment. Shorter fragments sometimes amplify more intensively. Both factors make an exact assessment of the allele number using gene dosage calculations difficult. In these cases, however, the number of single alleles was larger than the number shown. Some microsatellite patterns were not evaluated in some species because they did not amplify (indicated by a dash); amplifications at other loci produced a single allele (indicated by M). With few exceptions (see Table 3), the microsatellite loci examined in this study, Afu-19, Afu-34, Afu-39, Afu-54, Afu-57, and Afu-68, showed disomic allelic patterns in species with ~120 chromosomes and tetrasomic allelic patterns in species with ~250 chromosomes. In one group of species (*A. brevirostrum, A. fulvescens, A. gueldenstaedtii, A. medirostris, A. mikadoi,* and *A. naccarii*), microsatellite patterns indicated octosomic or greater allelic band patterns at a minimum of one locus. Allelic band patterns in an additional four species (*A. baerii, A. persicus, A. sinensis,* and *A. transmontanus*) showed evidence of possible octosomy (or greater than tetrasomy) at a minumum of one locus. Allelic band patterns greater than octosomic were observed in *A. mikadoi* at two loci.

None of the microsatellite loci examined in Acipenseridae amplified in *P. spathula.* Microsatellite data obtained in this study are used for discussion of ploidy levels and not for questions of population genetics. Aspects of population genetics or interspecific distribution of alleles are discussed in previous publications (May *et al.* 1997; Jenneckens 1999; Jenneckens *et al.* 2001; Ludwig *et al.* 2000). Additional discussions of other aspects of population genetics will be published elsewhere.

Inheritance of microsatellites: The sire and the dam used in creation of *A. sturio* showed allelic variability at two loci, Afu-19 (118/142:118/118) and Afu-54 (184/184:184/188) in one of the parents. Gene dosage assessments indicated that the allele ratios for the heterozygotic individuals were 1:1. Therefore, parental genotypes of the heterozygous individual had to be 118:142 if disomic or 118:118:142:142 if tetrasomic at Afu-19 and 184:188 if disomic or 184:184:188:188 if tetrasomic at Afu-54. The offspring were 52% homozygous for allele 118 and 48% heterozygous for alleles 118 and 142 at Afu-19. At Afu-54, 48% were homozygous for allele 184 bp and 52% were heterozygous for alleles 184 and 188. Approximately equal ratios of homozygous and heterozygous offspring confirm disomic inheritance at these loci for this species.

Cyt-*b* characteristics: Sequences are archived in the

TABLE 3

Ploidy level as inferred from microsatellite analysis for the 20 of the 24–25 extant Acipenseriform species examined in this study

Species	Chromosome no.*	Microsatellite loci examined in this study						Inferred ploidy level
		Afu-19	Afu-34	Afu-39	Afu-54	Afu-57	Afu-68	
A. baerii	248 ± 5	4n	4n	4n	4n	—	>4n	4n
A. brevirostrum	360 or 500[a]	4n	4n	4n	8n	—	2n	4n or 8n
A. fulvescens	250[a]	2n	—	4n	4n	8n	4n	4n
A. gueldenstaedtii	250 ± 8	4n	4n	4n	4n	—	8n	4n
A. medirostris	250[a]	4n	2n	—	2n	8n	8n	4n
A. mikadoi	500[a]	4n	4n	—	M	>8n	>8n	8n
A. naccarii	246 ± 8	4n	4n	4n	4n	—	8n	4n
A. nudiventris	118 ± 2	2n	M	M	2n	—	2n	2n
A. oxyrinchus	120[a]	2n	2n	2n	2n	—	2n	2n
A. persicus	>200	4n	4n	4n	4n	—	>4n	4n
A. ruthenus	118 ± 2	2n	2n	2n	—	—	2n	2n
A. schrenckii	240[b]	2n	4n	M	4n	—	4n	4n
A. sinensis	264 ± 3	4n	4n	4n	2n	—	>4n	4n
A. stellatus	118 ± 2	2n	2n	M	2n	—	2n	2n
A. sturio	116 ± 4	2n	2n	2n	2n	—	2n	2n
A. transmontanus	274	—	2n	M	M	4n	>4n	4n
H. huso	118 ± 2	2n	2n	2n	2n	—	2n	2n
H. dauricus	120[b]	2n	2n	2n	2n	—	4n	2n
S. albus	Unknown	M	2n	2n	2n	2n	2n	2n
S. platorynchus	112 ± 2	2n	2n	2n	2n	2n	2n	2n

Chromosome numbers are taken from Birstein *et al.* (1997). Numbers beneath microsatellite loci indicate allelic band patterns observed for each species at each locus; 2n indicates disomic allelic band patterns, 4n indicates tetrasomic allelic band patterns, and so on. M indicates monomorphy or a single allele observed at the locus indicated. — indicates no amplification at the locus indicated for that species.

[a] Chromosome number is assumed on the basis of the DNA content.

[b] Only macrochromosomes were counted precisely; all other species were investigated karyologically.

EMBL GenBank (AJ245825-41, AJ249692-4, AJ251451, and AJ252186-7).

The 1221-bp sequences generated by single or multiple amplifications included the entire cyt-*b* gene and an additional 80 bp of related tRNA genes. No direct stop-codon was detected in any of the sequences obtained. The TAA stop signal of the cyt-*b* gene is completed by the addition of 3′ A residues to the mRNA. The 34 bp (primer sequence included) upstream of the cyt-*b* gene was derived from $tRNA^{Glu}$. In most acipenseriform species, two nucleotides serve as a spacer between the $tRNA^{Glu}$ gene that precedes the cyt-*b* gene and cyt-*b* itself; however, the spacer was composed of three nucleotides in *A. nudiventris*, *A. medirostris*, and *A. mikadoi*. In the acipenseriforms, cyt-*b* continues directly into the $tRNA^{Thr}$ gene without any spacing nucleotides. Downstream of the cyt-*b* gene, inclusive of primer nucleotides, 46 nucleotides in the amplified fragment derived from the $tRNA^{Thr}$ gene were amplified. The 1141 bases comprising the cyt-*b* gene sequences from 22 acipenseriform taxa included 324 variable characters (28.4% of the total cyt-*b* gene sequence), with most of the changes (79.9%) occurring at the third codon position. The majority of the changes (90.7%) were synonymous; those remaining resulted in 18 amino acid substitutions that were informative for parsimony analysis. Most amino acid substitutions involved exchanges between hydrophobic residues. Alignment of the 22 cyt-*b* sequences revealed no nucleotide insertions or deletions within sequences. The cyt-*b* sequences can be fully translated using the bovine mitochondrial code (Anderson *et al.* 1982) without nonsense or intervening stop codons. Finally, the alignment revealed neither an increased abundance of first and second codon position changes nor a shift in the typical mammalian mtDNA transition bias, both of which are known to occur in mtDNA sequences translocated to the nuclear genome (Zhang and Hewitt 1996).

Molecular phylogeny: An identical branching topology was best supported by both maximum parsimony (single-most-parsimonious tree $L = 1101$ steps, CI = 0.639, RI = 0.645) and maximum-likelihood (LnL = −6645.70; transition/transversion ratio, 2.7 ± 0.2) analyses. A representative maximum-likelihood tree is shown in Figure 1. Four robustly supported conclusions can be inferred from the trees: (I) Acipenser and Scaphirhynchus form a monophyletic assemblage, the Acipenserinae (maximum likelihood = 84%, maximum parsimony = 100%); (II) the *A. oxyrinchus-A. sturio* cluster (maximum likelihood = 100%, maximum parsimony =

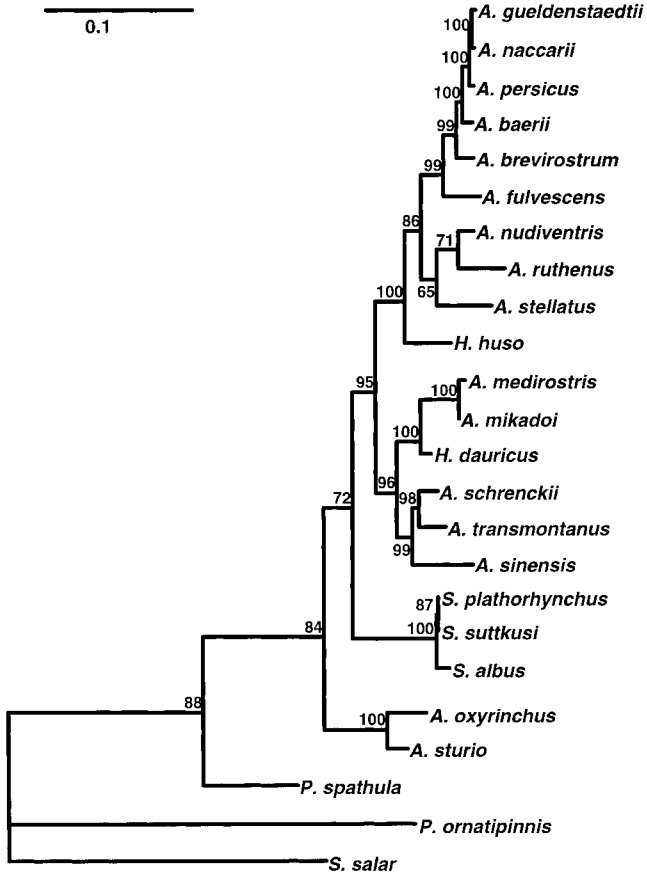

FIGURE 1.—Phylogenetic tree based on cytochrome-*b* gene sequences estimated by the maximum-likelihood algorithm using PUZZLE. The quartet-puzzling support values are shown at each node and were derived from 1000 puzzling steps. The tree was rooted using the corresponding outgroup sequences from *Salmo salar* and *Polypteris ornatipinnis*.

100%) is basal for the clade; (III) the two species of Huso are embedded within the genus Acipenser—it appears that the genus Huso had a polyphyletic origin or that it is an inappropriate taxonomic unit; (IV) there are two monophyletic groups within the Acipenser/Huso assemblage. These include the Atlantic species *A. baerii*, *A. brevirostrum*, *A. fulvescens*, *A. gueldenstaedtii*, *A. naccarii*, *A. nudiventris*, *A. persicus*, *A. ruthenus*, *A. stellatus*, and *H. huso* as one group (maximum likelihood = 100%, maximum parsimony = 94%); and the Pacific species *A. medirostris*, *A. mikadoi*, *A. schrenckii*, *A. sinensis*, *A. transmontanus*, and *H. dauricus* as a second group (maximum likelihood = 96%, maximum parsimony = 71%). To further assess the reliability of this previously uncovered subdivision in Acipenser (LUDWIG *et al.* 2000), we used spectral analysis to measure the definitive phylogenetic signals in our data set, supporting or conflicting with this hypothesis (Figure 2). The resulting spectrum indicates that the second strongest split signal (see split 16,775) in the cyt-*b* data set supports the partition of the Acipenser/Huso complex independently of any conventional tree-construction method. It is interesting to note that 11 of the most frequent partitions also occur in the corresponding maximum-likelihood tree (Figure 1). Therefore, spectral analysis demonstrates both that there is a high level of phylogenetic signal in our cyt-*b* data set and that the partition of the Acipenser/Huso complex is well supported.

Acipenserid phylogeography: The primary phylogenetic subdivision in the Acipenser/Huso complex correlated with the current geographic distribution. The first monophyletic group contained 10 species inhabiting tributaries of the Atlantic Ocean. The second group included 6 species inhabiting tributaries of the Pacific Ocean. The phylogeographic bipartition involved 7.5 ± 1.2% Kimura2 corrected sequence divergence and 80.4 nucleotide differences. At the amino acid level, positions 230 (Phe ↔ Leu) and 371 (Trp ↔ Leu) are diagnostic for the oceanic divergence within the Acipenser/Huso complex. The AMOVA showed that 33.2% ($P < 0.0001$) of the total variance occurs between the two phylogeographic clades, and 23.4% ($P < 0.0001$) was distributed among species within clades.

DISCUSSION

Evolution of ploidy levels in sturgeon: Analyses of karyotypes of 21 of the 24 or 25 extant acipenseriform species revealed that most species possessed either ~120 or ~250 chromosomes (reviewed in BIRSTEIN *et al.* 1997, Table 3). On the basis of genome quantification estimated using flow cytometry calibrated against reference species with previously analyzed chromosome numbers, however, two sturgeon species with higher chromosome numbers were identified. Flow cytometry allows inference of ploidy levels from the ratios of DNA content among species, provided the basal ploidy level is known. It proved to be informative in developing hypotheses regarding genome evolution. For example, *A. medirostris* and *A. mikadoi* are almost indistinguishable morphologically and were considered to be one species by some authors (ARTYUKHIN and ANDRONOV 1990; ARTYUKHIN 1995). A taxonomic separation of the North American species (*A. medirostris*) from the East Asian Pacific coast species (*A. mikadoi*) was evident from measurements of DNA content. In *A. mikadoi*, the DNA content is approximately two times higher (14.4 pg) than in sturgeon species with ~250 chromosomes (7.8–8.3 pg), including *A. medirostris*, and approximately four times higher than in species with ~120 chromosomes (BIRSTEIN 1993b,c). On the basis of DNA content, therefore, BIRSTEIN (1993c) and BIRSTEIN *et al.* (1997) inferred that *A. mikadoi* has ~500 chromosomes. We propose that *A. mikadoi* is octaploid on the basis of our microsatellite data.

BLACKLIDGE and BIDWELL (1993) propose that *A. brevirostrum* is an allopolyploid species, a descendant of ancestral spontaneous triploids (12n = 360 chromosomes). A hybrid origin of *A. brevirostrum* between spe-

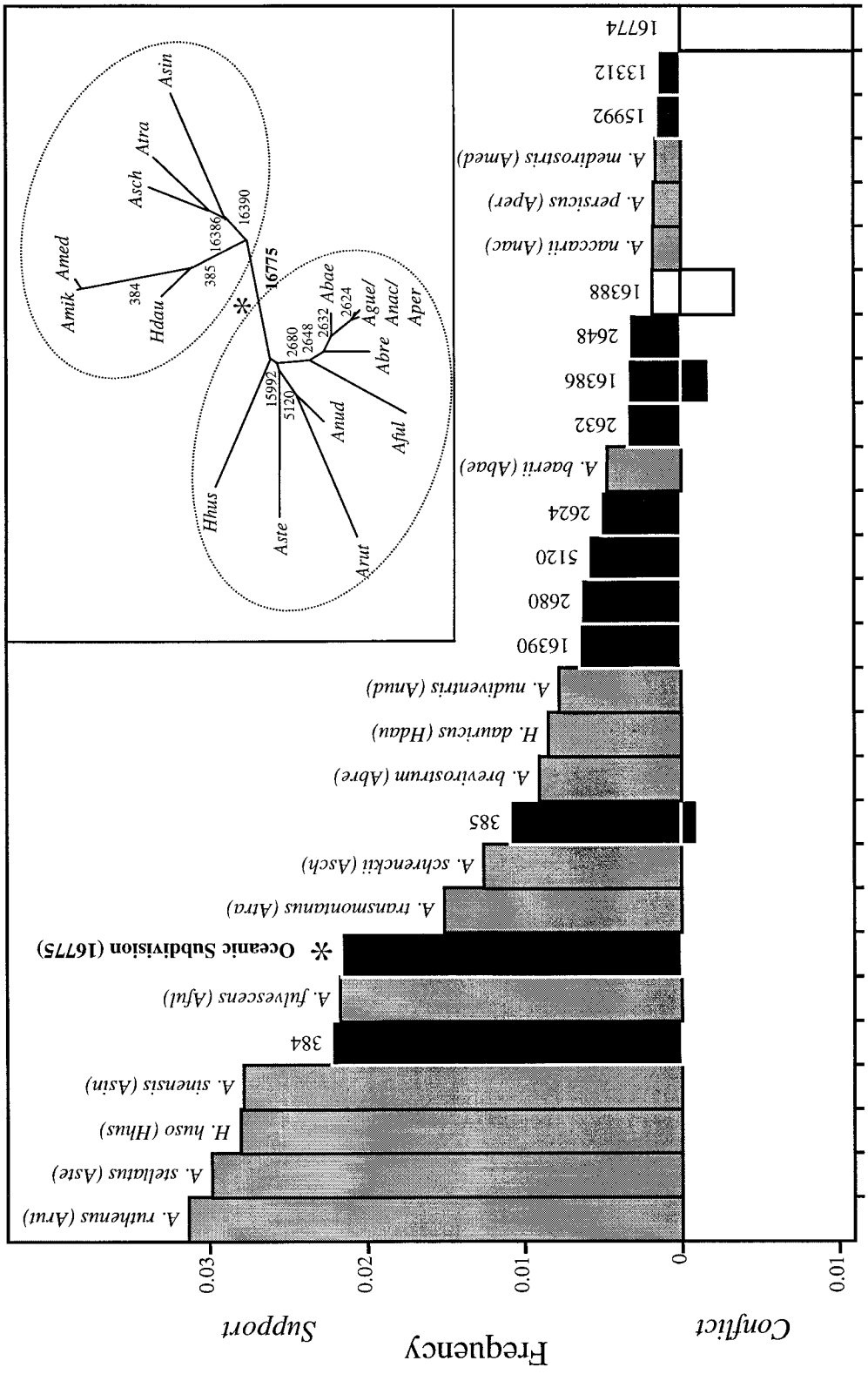

FIGURE 2.—Spectral analysis of the cytochrome-*b* DNA sequences from 16 species of the genera Acipenser and Huso. Bars above the *x*-axis represent frequency of support for each taxonomic grouping (split). Bars below the *x*-axis represent the sum of all conflicts against the corresponding split above the *x*-axis. Solid bars (both support and conflict) indicate the splits that were selected for inclusion in the optimal tree shown in the inset. Terminal taxa (shaded bars) are labeled by the corresponding taxon name, and internal splits (open bars) are labeled by their split number. There are 32,768 possible splits in this data set and the vast majority have little or no support, so only the strongest 27 are shown. The asterisk indicates the second highest-ranking split supporting the oceanic subdivision in the Acipenser/Huso complex.

cies with ~250 and ~500 chromosomes, as they proposed, may be possible, but no species with ~500 chromosomes are known within the Atlantic group, which could be put forth as its likely ancestral form. Allopolyploidy is extremely rare in other acipenserids, and therefore Birstein *et al.* (1997) found it more probable that this is a 16n species; this proposal is in keeping with their inferred chromosome number of ~500. On the basis of evidence at one microsatellite locus, we propose octaploidy in *A. brevirostrum*. *A. brevirostrum* is a U.S. federally endangered (since 1973) species and therefore we were able to obtain only six samples. The low sample size, and a recent decrease in population size that could have increased genetic drift, could reduce the total number of microsatellite alleles observed, which could reduce the ploidy number inferred from these data. Additional analyses of chromosome numbers of *A. brevirostrum* would be necessary to provide conclusive evidence regarding the ploidy level of this species.

The *in vitro* amplification of polymorphic nuclear markers, such as microsatellite loci, permits a direct although partial view of the genome. Observation of the maximum number of alleles per individual at a given locus provides evidence of ploidy level at that locus. For a diploid locus a maximum of two alleles differing in size (base pairs) is expected. For species with ~120 chromosomes, one or two peaks were detected at most microsatellite loci within each individual. For a heterozygous specimen the two peaks observed showed an approximately equal peak area and size. On the basis of the assumption that species with ~120 chromosomes are diploid, the presence of four alleles in one individual at a locus implies tetraploidy. The presence of no more than two alleles may indicate diploidy at that locus but does not contradict tetraploidy (or higher). Allelic band patterns inferred by methods such as these (including also isozyme data) indicate the maximum number of homologous forms a locus may have in a genome. However, evidence of a disomic band pattern at one locus precludes neither tetraploidy at another locus nor that the species in question is tetraploid. Rather, should a mixture of evidence be found in a species, it indicates a transitional ploidy level in which some loci have diverged sufficiently from their genomic duplicates such that they no longer amplify in polymerase chain reaction or are no longer expressed as protein products (Allendorf and Thorgaard 1984). This transition toward diploidy, or diploidization, is expected to occur in polyploid vertebrates until a species may be considered to be functionally diploid. Diploidization is completed when only divalents are formed during meiosis; this behavior, however, is extremely difficult to observe empirically, especially in species with many chromosomes, and thus ploidy levels are more commonly inferred indirectly. Even in fully diploidized species, residual evidence of polyploid ancestry is occasionally observed because of gene homology. Similarly, tandem duplications will create apparent polyploidy when those genes are examined.

Among 11 microsatellite loci studied by Pyatskowit *et al.* (2001) in *A. fulvescens* (~250 chromosomes), octosomic allelic patterns were never observed; all loci showed di- or tetrasomic allelic patterns. In another study, 1 of these loci, Afu-39, showed no more than two alleles in 501 fish sampled from six sturgeon species with ~120 chromosomes and four alleles in 265 samples from four species with ~250 chromosomes (Jenneckens *et al.* 2001). This supported di- and tetraploidy rather than tetra- and octaploidy in species with ~120 and ~250 chromosomes, respectively. However, although microsatellite loci Afu-19, Afu-34, Afu-39, and Afu-54 investigated in the present study support this pattern, loci Afu-57 and Afu-68 appear to be duplicated in species with ~250 chromosomes (Table 3). No more than two alleles were observed in all individuals at all loci for all species with ~120 chromosomes (with the exception of Afu-68 in *H. dauricus*, Table 3). This pattern strongly indicates a functional diploidy for species with ~120 chromosomes. Additional functional diploidy in species with ~120 chromosomes was also supported by the inheritance of two loci in *A. sturio*.

All phylogenetic trees (Figure 1) indicate that *Polyodon spathula* is basal to all other sturgeon examined in this study. This finding is consistent with common understanding of ancient divergence of the families Polyodontidae and Acipenseridae in acipenseriform evolution (*e.g.*, Birstein and DeSalle 1998). At the next level, the three species of Scaphirhynchus and the *A. sturio/A. oxyrinchus* clade are basal within the Acipenseridae. All six of these species have ~120 chromosomes; thus, it is likely that the common ancestor of all members of the family Acipenseridae had this chromosome number.

We agree with Birstein *et al.* (1997) that the common diploid ancestor of all Acipenseriformes had a karyotype of 60 chromosomes. A genome duplication event must have occurred in this ancestor to produce the Acipenserid lineage, whose basal species have ~120 chromosomes. A reestablishment of the diploid condition, as postulated by Fontana (1994), before the radiation of this order seems plausible. The proposal of genome duplication and subsequent reduction in functional ploidy is not rare; even in the evolution of diploid vertebrates, genome quadruplication is discussed (Leipoldt 1983; Spring 1997). Although the chromosome number of some Acipenseriformes, for example, *Psephurus gladius* (family Polyodontidae), is not known at this time, additional genome duplication events seem to have evolved more recently because they are observed in certain species within the genus Acipenser only. We found evidence for locus duplication (Afu-57 and Afu-68) only in species with ~250 or ~500 chromosomes but not in species with ~120 chromosomes (Table 3 and Figure

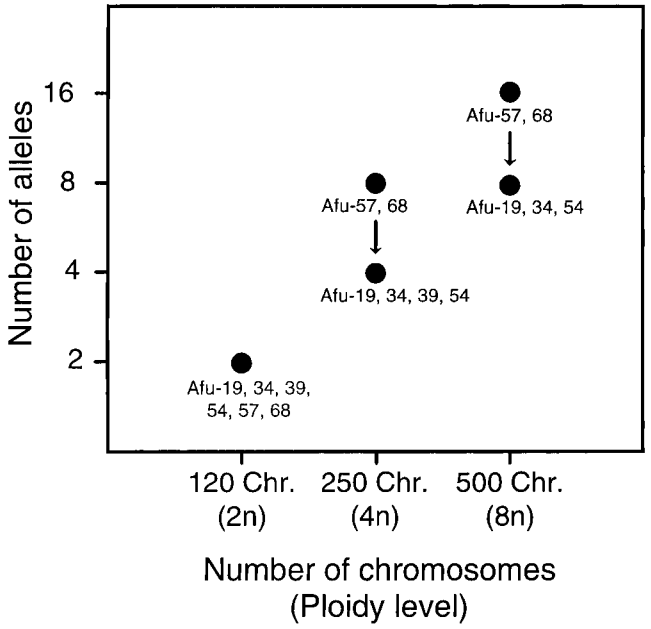

FIGURE 3.—Evidence of single gene duplication events associated with chromosome number and inferred ploidy levels in sturgeon species with ~120, ~250, and ~500 chromosomes (n = 866 individuals from 20 species).

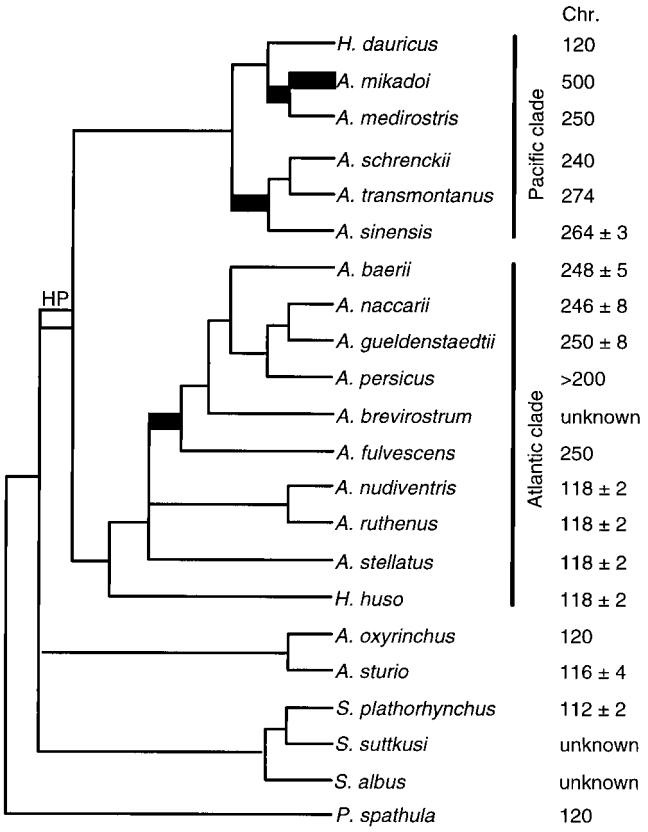

FIGURE 4.—Ploidy evolution in the Acipenseridae, including some related Acipenseriform taxa for comparison. The tree represents the topology, calculated using maximum parsimony, of the entire cytochrome-*b* gene. Polyploidization events are mapped on the tree using black boxes. The existence of heteroplasmy resulting from length variations of the mtD-loop is shown by a white box (data were taken from LUDWIG *et al.* 2000; *A. schrenckii*, *A. sinensis*, and *H. dauricus*; A. LUDWIG, unpublished data; ZHANG 1998). The number of chromosomes is reviewed in BIRSTEIN *et al.* (1997). See also Table 3.

3). This supports our proposal that the nearest ancestor to Acipenserids was functionally diploid with ~120 chromosomes (tetraploid derived, which is reflected in DNA content) and that species with higher chromosome numbers are the result of more recent genome duplication events. In contrast to the proposed single ancient genome duplication in a common ancestor of all salmonid fishes (ALLENDORF and THORGAARD 1984), our studies of sturgeon molecular phylogeny showed that several independent genome duplication events happened during sturgeon evolution (Figure 4).

It is quite possible that the observation of low numbers of duplicated locus expression in the acipenseriforms, in spite of a genome duplication event, may be explained by subsequent gene silencing, as found for Salmonids and Catostomids (FERRIS and WHITT 1978; STONEKING *et al.* 1981) and for paddlefish (CARLSON *et al.* 1982; DANIELSON *et al.* 1999). A good example of gene silencing is the existence of two proopiomelanocortin (POMC) genes in paddlefish (*P. spathula*) and sturgeon (*A. transmontanus*). In both of the sturgeon POMCs, the N-terminal paired-basic proteolytic cleavage site that normally flanks γ-melanocyte-stimulating-hormone (MSH) is missing. In place of the paired-basic residues, the two sturgeon POMC genes have nonbasic residues (ALRUBAIAN *et al.* 1999; DANIELSON *et al.* 1999). A degeneration of the γ-MSH core sequences was reported in one of the duplicated sturgeon POMC sequences (DANIELSON *et al.* 1999). Therefore DANIELSON *et al.* (1999) concluded that the loss of the basic residues at the N-terminal cleavage site occurred prior to duplication of the ancestral POMC gene and that degeneration of γ-MSH core sequence was a subsequent event that occurred after POMC duplication. The loss of a γ-MSH proteolytic cleavage site in the ancestral chondrostean lineage served to functionally eliminate the γ-MSH region by preventing this sequence from being cleaved from the POMC precursor (DANIELSON *et al.* 1999). Like sturgeon and paddlefish, salmonids have two POMC genes (DANIELSON *et al.* 1999). Phylogenetic analysis suggests that duplication of the salmonid POMCs was an evolutionary event that occurred independently and long after the duplication of the chondrostean POMC sequences. Compared to the Chondrosteii, however, the rates of amino acid substitution between paralogously related salmonid POMC sequences are much higher in rainbow trout and sockeye salmon (DANIELSON *et al.* 1999). Furthermore, tetraploid loaches (Cobitidae), although arising more recently than salmonids, show a greater rate of loss of duplicated genes (BAILEY *et al.*

1978). This supports the theory of very slow molecular evolution in Chondrosteii (Rico *et al.* 1996) and demonstrates that gene and/or genome duplication events happened independently in several lineages. It is likely, therefore, that the process of gene silencing has lineage-specific patterns.

Blacklidge and Bidwell (1993) observed great differences in the mean genome sizes observed among isolated populations of *A. transmontanus*. Apart from gene silencing, chromosomal rearrangements may be assumed to play another important role in the acipenseriform genome formation. This is heavily supported for *A. transmontanus* by combining the data we obtained at different microsatellite loci with the outcome of the study of van Eenennaam *et al.* (1998b). These authors showed no evidence of multivalent formation in synaptonemal complex analyses when they examined males of this taxon. This suggests a completion of the diploidization process (van Eenennaam *et al.* 1998b). However, allelic scores at microsatellite loci Afu-57 and Afu-68 indicated tetrasomic allelic band patterns at these loci.

The octosomic allelic band patterns observed at Afu-54 for *A. brevirostrum* and the tetrasomic allelic band patterns observed at Afu-68 for *H. dauricus* may be explained by tandem duplications of single genes rather than polyploidization events. Observation of single gene duplications may be more common in fishes than some other taxa. Analysis of zebrafish showed that these fish often have more multigene families than do mammals (Postlethwait *et al.* 1998, 2000). The additional genes in zebrafish could have arisen from either (I) a greater propensity of tandem gene duplications in the fish lineage, (II) less loss of paralogous gene copies in fish, or (III) additional independent chromosome duplication or tetraploidization in fish. Mapping data gave no evidence for the clustering of additional genes in zebrafish, thus reducing the likelihood that tandem duplication is a general mechanism for gene duplication (Postlethwait *et al.* 1998). A lower rate of molecular evolution in fish is often described (Rico *et al.* 1996), thus providing support for the second proposed explanation. Our data corroborate the proposal of additional independent chromosome duplication or tetraploidization because they show this effect in one of the most primitive fish lineages.

Within all 492 wild-caught specimens of species with ~120 chromosomes, the number of alleles per locus ranged between 2.22 (Afu-39) and 6.71 (Afu-57). This range was lower than the range observed in 363 specimens of species with ~250 chromosomes, between 5.25 (Afu-34) and 12.77 (Afu-68). From this evidence, we propose that higher levels of ploidy permit a greater flexibility in accommodating mutations that modify functions because of complementation among partially divergent gene family members. Mutations that are deleterious when only a single gene is present may be neutral if a gene duplicate compensates for the loss of function (Nadeau and Sankoff 1997).

These data, taken as a whole, indicate strongly an ongoing process of functional reduction of ploidy level in sturgeon. The data are highly consistent in indicating the following functional ploidy levels: for species with ~120 chromosomes, 2n; ~250 chromosomes, 4n; and ~500 chromosomes, 8n.

Molecular phylogeny and acipenserid phylogeography: To investigate the consistency of the chromosomal acipenseriform evolution that we proposed in this study, the chromosome number for each species was superimposed on a reconstructed molecular phylogenetic tree based on the entire cyt-*b* gene sequence (Figure 4). All previous studies of molecular phylogeny of sturgeon resulted in the following agreements: Acipenser and Scaphirhynchus form a monophyletic assemblage, the *A. oxyrinchus/A. sturio* cluster is most distantly related to the other species of the genus Acipenser, and species of the genus Huso do not cluster together, but within separate clusters of the genus Acipenser (Brown *et al.* 1996; Birstein and DeSalle 1998; Krieger *et al.* 2000; Ludwig *et al.* 2000). We address a postulated oceanic subdivision of the order, Acipenseriformes, into Atlantic and Pacific lineages (Ludwig *et al.* 2000). From our phylogenetic tree (Figure 1), a separation into two clades, Atlantic and Pacific, emerged. The spectral analysis indicated that the second strongest subdivision was an internal node (split 16,775; Figure 2), reflecting the same primary oceanic subdivision in Acipenser and Huso observed in the conventional phylogenetic reconstructions, independently corroborating this proposal.

The evolution of chromosomes and distribution of heteroplasmy (Figure 4), the structure of repeat units and general organization of the mtD-loop region (Buroker *et al.* 1990; Brown *et al.* 1992, 1996; Ludwig *et al.* 2000), as well as geographic distribution (discussed in Ludwig *et al.* 2000) strongly supported the molecular phylogeny constructed in this study (Figures 1 and 4).

Concluding remarks and future perspectives: Distribution of allele number within microsatellites shows an ongoing process of functional genome copy reduction in sturgeon. The number of alleles observed at Afu-57 and Afu-68 showed the process of diploidization to be more recent in species with ~250 chromosomes and ~500 chromosomes. Considering the outcomes of previous studies and this study, it appears that gene silencing, chromosomal rearrangements, and transposition events are playing important roles in the acipenseriform genome formation. We believe that the common diploid ancestor of Acipenseriformes had ~60 chromosomes. A genome duplication event must have occurred in this ancestor. A genomic duplication event, followed by a reestablishment of a functional diploid condition before the radiation of this order, seemed to be plausible. Functional ploidy levels as indicated by our microsatellite approach are for species with ~120 chromosomes =

2n, ~250 chromosomes = 4n, and ~500 chromosomes = 8n. In contrast to single genome duplication events in other fish taxa, our results indicated that genome duplication occurred repeatedly during sturgeon evolution, making sturgeon an ideal taxonomic group to address the selective pressures that favored extensive duplication of the genome.

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