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# Evolution of Polyploidy and Functional Diploidization in Sturgeons: Microsatellite Analysis in 10 Sturgeon Species

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## Abstract

Sturgeons (family Acipenseridae) are one of the most endangered groups of animals. Two hundred million years of evolution and multiple ploidy levels make this group a unique subject for studying the evolution of polyploidy in animals. As most sturgeon species have gone through significant functional diploidization, 2 scales of ploidy levels can be distinguished: the "evolutionary scale," which indicates the maximum ploidy level achieved and the "recent scale," which indicates the current functional ploidy level. This study analyzes published and new microsatellites to check the ploidy level and to determine the degrees of functional diploidization in 10 sturgeon species from Europe and Asia. We screened 50 primer pairs newly developed for *Acipenser gueldenstaedtii* and 40 primer pairs previously developed in other studies for other sturgeon species. The maximal number of alleles per individual of a given species was assessed at 20 microsatellite loci, which showed consistent amplification in most of the 10 analyzed species. Taken together, our data on the percentage of disomic loci in different species suggest that functional diploidization is an ongoing process in sturgeons. We observed lower levels of diploidization in tetraploid species from the Atlantic clade than in the species from the Pacific clade, which can be explained by the more recent genome duplication in tetraploid species from the Atlantic clade. Based on the recent findings and results of this study, we propose that the evolution of sturgeons has been affected by at least 3 different polyploidization events.

**Subject areas:** *Molecular systematics and phylogenetics*

**Key words:** Acipenser, *disomic, microsatellites, polyploidy, sturgeon, tetrasomic*

Sturgeons (family Acipenseridae, order Acipenseriformes, infraclass Chondrostei) are among the most ancient fish still present on Earth, dating back to Upper Cretaceous times (Bemis et al. 1997) and believed to have diverged from an ancient pre-Jurassic teleost lineage approximately 200 million years ago (Patterson 1982). They are considered "living fossils" because they do not seem to have changed much morphologically since the origin of the group (Krieger and Fuerst 2002). In addition, they have one of the lowest rates of molecular evolution of all recent vertebrates (Krieger and Fuerst 2002). Sturgeons provide a unique model for studying genome duplication events as the species exhibit multiple ploidy levels (Ludwig et al. 2001). Many questions, such as the chromosomal evolution or the phylogeny of these fishes, are still not completely resolved, and the threatened or endangered status of many species indicates that the time left to study these fishes may be limited.

Sturgeons nowadays comprise 25 species that are distributed exclusively in the northern hemisphere, the Ponto-Caspian region currently having the greatest species diversity (Bemis et al. 1997). Most sturgeons live at sea and some in freshwater; however, all species spawn in freshwater (Bemis and Kynard 1997).

The 25 species of family Acipenseridae have been divided into 4 genera defined in the 19th century based on morphological characters: genus *Pseudoscaphirynchus* (3 species), genus *Scaphirynchus* (3 species), genus *Huso* (2 species), and genus *Acipenser* (17 species) (Bemis and Kynard 1997). Evolutionary relationships within the order Acipenseriformes are still partially unresolved (Krieger et al. 2008). For example, current molecular evidence suggests that the genus *Huso* is not monophyletic, with the 2 species of *Huso* found embedded separately within the genus *Acipenser* (Birstein and DeSalle 1998; Ludwig et al. 2001; Krieger et al. 2008) (Figure 1).

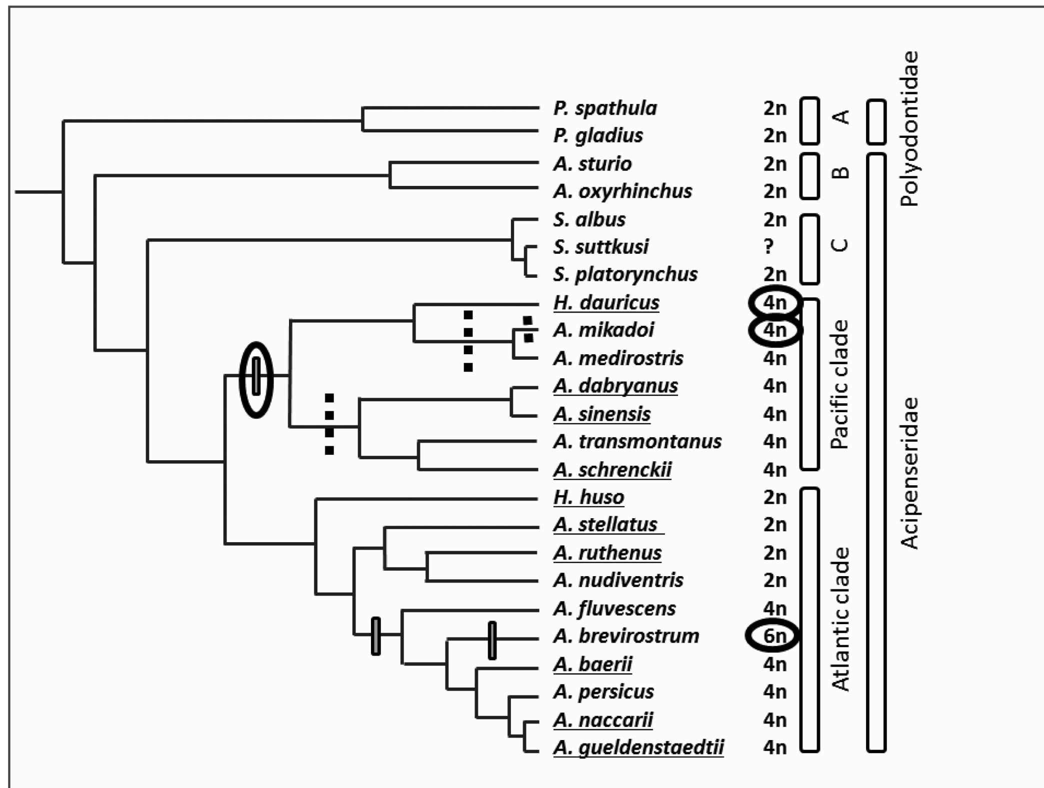


**Figure 1.** Phylogenetic tree modified from Peng et al. (2007): the underlined taxa have been investigated in this study, the ellipses indicate new information, the vertical bars are deduced duplication events, and the dotted vertical lines indicate duplication events that are no longer necessary to invoke. A = genus *Pseudoscaphirynchus*, B = sea sturgeons clade, and C = genus *Scaphirynchus*. Recent data are ploidy levels in *Huso dauricus* (4n), *Acipenser mikadoi* (4n) (Vasil'ev et al. 2009), and *Acipenser brevirostrum* (6n) (Fontana et al. 2008) with presumable duplication events, proposed by Vasil'ev et al. (2010) and supported by this study, mapped on the branches. However, evolution of polyploidy in *A. brevirostrum* is probably more complex, as proposed by Fontana et al. (2008) and likely also involves allopolyploidy.

Recent cytological evidence (Vasil'ev et al. 2009; Zhou et al. 2011) confirms the polyphyletic origin of genus *Huso* and Vasil'eva et al. (2009) has proposed that the 2 species should be considered as members of genus *Acipenser*.

According to the most recent sturgeon phylogenetic studies (Peng et al. 2007; Krieger et al. 2008), Acipenseridae can be divided into 4 major monophyletic groups: 1) a clade composed of the members of the genus *Scaphirynchus*, 2) sea sturgeon clade composed of north Atlantic *Acipenser oxyrinchus* and the European *Acipenser sturio*, 3) Pacific clade constituting a geographic part of the *Acipenser*/*Huso* complex, and 4) Atlantic clade comprising the other part of the complex. The Pacific clade includes 7 species: *Acipenser schrenckii*, *Acipenser transmontanus*, *Acipenser sinensis*, *Acipenser dabryanus*, *Acipenser medirostris*, *Acipenser mikadoi*, and *Huso dauricus*. The Atlantic clade contains 10 species: *Acipenser gueldenstaedtii*, *Acipenser naccarii*, *Acipenser persicus*, *Acipenser baerii*, *Acipenser brevirostrum*, *Acipenser fulvescens*, *Acipenser nudiventris*, *Acipenser ruthenus*, *Acipenser stellatus*, and *Huso huso*.

The notion that whole-genome duplication (polyploidy) is an important evolutionary mechanism was first proposed by Susumu Ohno almost half a century ago (Ohno 1970). In plants, the role of polyploidy in evolution and diversification has long been recognized. The possibility that genome duplication has played an important role in the evolution of vertebrates has only recently received more attention (Berrebi et al. 1996; Van de Peer et al. 2003; Dehal and Boore 2005).

Polyploids may arise through chromosome duplication within a species (autopolyploidy) or in association with interspecific hybridization (allopolyploidy). In sturgeons, several evolutionary scenarios have been proposed to explain the origin of polyploidy: from several independent whole-genome duplication events in different lineages (Birstein and DeSalle 1998; Ludwig et al. 2001) and multiple hybridization events (Vasil'ev 1999, 2009; Fontana 2002) to both auto- and allopolyploidy combinations (Fontana et al. 2008).

According to Vasil'ev (2009), all investigated Acipenseriformes can be divided into 3 discrete groups depending on the number of chromosomes: 1) species with 112–146 chromosomes (e.g., *H. huso*, *A. stellatus*, and *A. ruthenus*), 2) species with 240–270 chromosomes (e.g., *A. gueldenstaedtii*, *A. baerii*, *A. naccarii*, *A. transmontanus*, *A. fulvescens*, *A. medirostris*, *A. sinensis*, and *A. schrenckii*), and 3) species with 360–370 chromosomes (*A. brevirostrum*).



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There is evidence that ~120 chromosome species are of tetraploid origin (Birstein and Vasil'ev 1987) with their diploid ancestor being extinct (Dingerkus and Howell 1976). The karyotype with 120 chromosomes is relatively large, which is not typical of diploid fish species and its DNA content is about twice as large compared with most fish species (Vasil'ev et al. 2009). Data on allozyme variability also confirm the tetraploid status of the ~120 chromosomal species. For example, *A. stellatus*, with 146 chromosomes, has 31% duplicated allozyme loci (Ryabova and Kutergina 1990). Consequently, the ~250 and ~370 chromosome species are of octoploid and dodecaploid origins, respectively. However, because ~120 chromosome species have reached a significant level of functional genome diploidization, 2 scales of Acipenseriformes ploidy have been proposed: 1) the "evolutionary scale": diploid (extinct), tetraploid (~120 chromosomes), octoploid (~250 chromosomes), and dodecaploid (~370 chromosomes) species and 2) the "contemporary scale," which is the most frequently considered: diploid (~120 chromosomes), tetraploid (~250 chromosomes), and hexaploid (~370 chromosomes) species (Vasil'ev et al. 2009).

Functional diploidization is one of the most intriguing yet still not entirely explained aspects of genome evolution. It is the evolutionary process whereby the gene content of a tetraploid species (after whole-genome duplication) degenerates to become functionally diploid but maintains twice as many distinct chromosomes (Wolfe 2001). This procedure enables the correct pairing of homologous chromosomes during meiosis/mitosis (diploid mode of inheritance) (Panopoulou and Poustka 2005). The key event is the switch from 4 chromosomes, which form a quadrivalent at meiosis, to 2 pairs of chromosomes each of which forms a bivalent (Wolfe 2001). In population genetic terms, functional diploidization is the switch from 4 alleles at a single locus (tetrasomic inheritance) to 2 alleles at each of 2 distinct loci (disomic inheritance) (Wolfe 2001). In both plants and animals, a mixture of tetraploid and diploidized loci can be found in a single species, which suggests that diploidization does not necessarily happen at the same time for all chromosomes (Berrebi et al. 1993; Wolfe 2001; Havelka et al. 2013).

It is often assumed that polyploids that form bivalents during meiosis are allopolyploids, whereas those that form multivalents during meiosis are autopolyploids (Otto and Whitton 2000). However, completely homolog chromosome tetrads formed after an autotetraploidization event can undergo secondary differentiation in 2 homolog pairs (Stift et al. 2008; Boscari et al. 2011). They tend to rediploidize over time as mutations accumulate and chromosomes diverge (Otto and Whitton 2000). In theory, during the functional diploidization process in autopolyploids, the genomes of an autopolyploid will diverge through drift with the evolution of disomic inheritance. This results from the lack of recombination between all combinations of homologs (Le Comber et al. 2010).

On the other hand, an allopolyploid inherits some divergence between chromosome pairs from the 2 parental species. However, unless the genomes are sufficiently diverged, homoeologous pairing may still occur (Le Comber et al. 2010) and crossing over between homoeologous chromosomes can homogenize the genome (Stift et al. 2008). Thus, it cannot be assumed that a paleopolyploid is necessarily allopolyploid solely because it shows disomic inheritance (Otto 2007).

Allozymes have been used to study functional diploidization in salmonids (Allendorf and Utter 1973; Allendorf and Thorgaard 1984), catostomids (Ferris and Whitt 1975; Buth 1981), and cyprinids (Berrebi et al. 1990). With allozyme data, functional diploidization corresponds to the loss of an enzymatic function. Nowadays, DNA-based markers such as microsatellites can be used directly to provide evidence of duplicate genes rather than the duplication of expressed products (Mable et al. 2011). Furthermore, analyzing DNA allows the sample to be taken without killing protected animals such as the sturgeon species, all of which are protected. However, using DNA, there are some difficulties that may blur the estimation of disomy for a locus based on codominant markers such as microsatellites. Two recently diploidized loci, still sharing the same alleles but constituting independent loci, cannot be easily distinguished from 1 tetrasomic locus. This bias can be limited by choosing highly polymorphic microsatellite loci and scoring numerous individuals.

There is evidence that with sturgeons microsatellites, functional diploidization rarely corresponds to flanking region mutations (as null alleles), but rather to chromosome transitions from tetrasomy to disomy allowing drift and the differentiation of homolog loci. This assumption can be deduced from the observations of crosspriming among species. The very high level of crosspriming among sturgeons (May et al. 1997; McQuown et al. 2000; King et al. 2001; Moghim et al. 2012) coincides with the stability of the flanking regions (see Shao et al. 2011, Table 1), among species sometimes separated by more than 1 million years (Peng et al. 2007). This stability has been already observed in other taxa (Rico et al. 1996). The relatively high frequency of diploidization observed at least in American sturgeons (Welsh et al. 2003) can be explained by the classical release of selection on duplicated genes and not by null alleles.

In sturgeon species from the ~250 chromosome species group, it is possible to detect a trend toward functional diploidization from a tetraploid karyotype (Fontana et al. 2001). This trend is indicated in the tetraploid species *A. gueldenstaedtii* by the presence of chromosomal rearrangements, shown by telomeric sequences scattered along 2 chromosomes (Fontana et al. 1998). It is also indicated in the tetraploid species *A. naccarii* by heterogeneity within the larger acrocentrics, which cannot be grouped into quadruplets but rather into homologous pairs (Fontana et al. 1999, 2001). Inheritance studies on North American tetraploid sturgeon species showed a combination of locus-specific disomic and tetrasomic inheritance for *A. fulvescens* (lake sturgeon: Pyatskowit et al. 2001; McQuown et al. 2002), *A. transmontanus* (white sturgeon: Rodzen and May 2002), and *A. medirostris* (green sturgeon: Welsh et al. 2003). This suggests that these species are still in the process of diploidization. There is no known case of complete diploidization in fish, except perhaps in the salmonids (Allendorf and Thorgaard 1984).

The prospects of using microsatellite markers have induced a search for microsatellite loci with disomic



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**Table 1** Specimen information for 10 sturgeon species examined in this study

| Species | Common name | Geographic origin of specimens | Number of individuals | Provider of samples[a] |
|---|---|---|---|---|
| Pacific lineage | | | | |
| *Huso dauricus* | Kaluga | Beijing fish farm, China | 10 | Ma GuoJun |
| *Acipenser dabryanus* | Yangtze sturgeon | Yibin fish farm, China | 9 | Zhou ShiWu |
| *Acipenser sinensis* | Chinese sturgeon | Yangtze river, China | 16 | Zhu Bin, Xiao Hui |
| *Acipenser schrenckii* | Amur sturgeon | Beijing fish farm, China | 12 | Ma GuoJun |
| Atlantic lineage | | | | |
| *Huso huso* | Great sturgeon/beluga | Danube river, Romania | 5 | Radu Suciu |
| *Acipenser stellatus* | Stellate sturgeon/sevruga | Danube river, Borcea branch, Romania | 5 | Radu Suciu |
| *Acipenser ruthenus* | Sterlet | Borcea branch of Danube river, Romania | 5 | Radu Suciu |
| *Acipenser naccarii* | Adriatic sturgeon | Giovanni fish farm, Italy | 25 | Leonardo Congiu |
| *Acipenser baerii* | Siberian sturgeon | Ianca fish farm, Romania | 21 | Patrick Berrebi |
| | | Irstea fish farm, France | 20 | Patrick Chèvre |
| *Acipenser gueldenstaedtii* | Russian sturgeon/osetra | Danube river, Borcea branch, Romania | 12 | Radu Suciu |
| | | Ianca and Tamadau fish farms, Romania | 12 | Radu Suciu, Patrick Berrebi |

[a]Ma GuoJun—Institute of Amur River Fisheries, People's Republic of China; Zhou ShiWu—Institute of Rare Aquatic Animals; Xiao Hui—Institute of Chinese Sturgeon, YiChang, People's Republic of China; Radu Siciu—Danube Delta National Institute, Romania; Leonado Congiu—University of Ferrara, Italy; Patrick Berrebi—University of Montpellier II, France; Patrick Chèvre—Irstea, France.

inheritance in polyploid sturgeon species. Sturgeon studies targeting the development of disomic microsatellites were initiated in the North American sturgeon species and paddlefish (May et al. 1997; McQuown et al. 2000; King et al. 2001; Pyatskowit et al. 2001; Heist et al. 2002; Henderson-Arzapalo and King 2002; Welsh et al. 2003; Welsh and May 2006). In the case of lake sturgeon and green sturgeon in North America, disomic markers have been developed, so traditional population genetic metrics could be used. In a study by Welsh et al. (2003), out of the primer pairs that successfully amplified the DNA of tetraploid green sturgeon (*A. medirostris*) and lake sturgeon (*A. fulvescens*), 35.5% and 20.9%, respectively, appeared disomic.

In this study, we used 20 polymorphic microsatellite markers to investigate the ploidy levels and the degree of functional diploidization in 10 sturgeon species—6 species that belong to the Atlantic lineage: *A. gueldenstaedtii*, *A. naccarii*, *A. baerii*, *A. ruthenus*, *A. stellatus*, and *H. huso* and 4 species that belong to the Pacific lineage: *A. schrenckii*, *A. sinensis*, *A. dabryanus*, and *H. dauricus*. Knowledge of ploidy in different sturgeon species is important for several reasons. It can help explain the origin of polyploidy in sturgeon and clarify phylogenetic relationships. The detection of disomic loci in polyploid species makes it possible to apply the rich population genetic statistics developed for diploid species only. The goal of this study is first to detect and count the disomic microsatellite loci in polyploid species.

## Materials and Methods

Biological samples (fin tissue) were obtained from 152 sturgeon individuals. Fin samples were collected in the wild, in sturgeon farms, and in sturgeon research institutes in different countries before being preserved in 95% ethanol. The geographical origin, sample size, and providers for each sturgeon species are listed in Table 1. The total genomic DNA was extracted from small pieces of fin tissue using the Chelex extraction method (Estoup et al. 1996).

A library of 200 microsatellite markers for *A. gueldenstaedtii* was produced by a private company (Genoscreen, Lille, France), coupling multiplex microsatellite enrichment isolation with the 454 GS-FLX Titanium pyrosequencing platforms (Malausa et al. 2011), and 50 most promising ones were selected for their large number of repeat units and their absence of compound repeat for polymerase chain reaction (PCR) amplification effectiveness. These 50 unlabeled primer pairs were tested, as were 40 sets of unlabeled published microsatellite primers developed for other sturgeon species (Supplementary Table S1 online), including Adriatic sturgeon *A. naccarii* (Zane et al. 2002; Forlani et al. 2008), Atlantic sturgeon *A. oxyrinchus oxyrinchus* (King et al. 2001; Henderson-Arzapalo and King 2002), green and white sturgeon *A. medirostris* and *A. transmontanus* (Börk et al. 2008), Chinese sturgeon *A. sinensis* (Zhu et al. 2005), paddlefish *Polyodon spathula* (Heist et al. 2002), lake sturgeon *A. fulvescens* (May et al. 1997; Welsh et al. 2003), and shovelnose sturgeon *Scaphirhynchus platorynchus* (McQuown et al. 2000). This was done by amplifying DNA of several (4–5) wild individuals of *A. stellatus*, *A. gueldenstaedtii*, *A. ruthenus*, *H. huso*, and the farmed fish *A. baerii* (called a 5 × 5 test for 5 individuals and 5 species). The amplifications were performed in a total volume of 20 µL. Each reaction mixture consisted of template DNA, 1X GoTaq Reaction Buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.4 U of GoTaq Polymerase (Promega), 0.5 µM of each primer, and 2 mM of each dNTP. Amplifications were carried out using an Eppendorf Mastercycler gradient thermal



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cycler (Eppendorf-Netheler-Hinz, Hamburg, Germany) with the following temperature profile: initial denaturation at 95 °C for 3 min followed by 35 amplification cycles of 45 s at 95 °C, 45 s at locus-specific annealing temperature (Supplementary Table S1 online), 45 s at 72 °C, and a final elongation at 72 °C for 5 min. PCR products were mixed with 1 µL of loading dye and separated by electrophoresis on 2% agarose gels containing ethidium bromide, in 1× TBE (Tris, Borate, EDTA) buffer. Products were visualized using an ultraviolet illuminator (Fisher Bioblock Scientific, Illkirch, France).

Concerning the newly developed primer pairs for *A. gueldenstaedtii* that showed amplification, PCR reactions were optimized by gradually increasing the annealing temperature using the gradient function of the Eppendorf Mastercycler Gradient in order to reduce unspecific amplification. PCR products obtained with 3 different temperatures were mixed with 2.5 µL of loading buffer and loaded on an appropriately chosen 8.7 cm Spreadex® gels (Elchrom Scientific AG, Switzerland) in order to determine the optimal annealing temperature for each primer set. Electrophoresis was performed using the Origins by Elchrom™ Scientific advanced electrophoresis apparatus (Elchrom Scientific AG). The gels were stained with ethidium bromide for 30 min, destained with distilled water for 30 min, and visualized using an ultraviolet illuminator (Fisher Bioblock Scientific).

Forward primers from the primer pairs that showed good amplification in analyzed species were labeled with 6-FAM or Cy3 fluorescent dye (Eurofins MWG Operon, Ebersberg, Germany) and screened through the 5 × 5 test (see unlabeled primers Elchrome method) where the PCR products were visualized on high-resolution polyacrylamide gel to evaluate polymorphism and the quality of amplification. The resolution of loci was classified as good if alleles were clear and sharp with no stutter bands, fair if alleles were clear but stutter bands existed, or poor if product amplification was apparent, but it was difficult to distinguish individual alleles. Primer pairs with good amplification results in these species were screened in the same way in the other 5 species. Amplifications with labeled primers were performed in a final volume of 10 µL. Following amplification, PCR products were mixed 1:4 v/v with bromophenol blue and denatured at 95 °C for 3 min before gel loading. Electrophoresis was performed at 1200 V for 50 min using 6% denaturing polyacrylamide gel and visualized using FMBIO II Fluorescence Imaging System (Hitachi, Tokyo, Japan).

Primer pairs that yielded polymorphic products showing limited or no spurious bands, such as stutter bands and nonspecific amplification in most of the species, were included in this study. In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with GenBank at the NCBI.

The banding patterns (maximal number of alleles per individual) were recorded for each locus and each species. A locus was provisionally considered disomic when individuals displayed single band (indicating homozygosity) or 2 bands of even intensity (indicating heterozygosity). Aspect of scanned bands on acrylamide gels is of great help to attribute them to the good locus: each locus has similar bands in terms of intensity (light gray up to dark black), width, and even shape of density curve. Analyzing several individuals, the species considered should express more than 2 alleles to be suitable for disomy detection. The same rule has been applied for tetrasomy determination: a given individual genotype expressing more than 2 alleles and the species (5–25 individuals analyzed) showing more than 4, thus suggesting polysomy.

There is no perfect method to determine the transmission pattern of a given locus (disomy and polysomy). There are several confusing situations, mainly low or no polymorphism and presence of spurious band showing an aspect close to the true alleles. The ways to overcome these difficulties are multiple: 1) the status of a locus is determined by analyzing several individuals, which generally allow us to determine the pattern; 2) in the cases where it was not possible to determine the banding pattern using 4–5 analyzed individuals, additional individuals (from 5 to 37 individuals, in some cases from different populations) were used to confirm the ploidy status; 3) the use of scanned polyacrylamide gels gives a photograph of the band showing different aspects: density, sharp/fuzzy, width, and thickness, thus bands with similar characteristics were assumed to be homologous. Other rules such as the lower density of the larger alleles are applied.

The percentage of disomic loci (among the loci for which the ploidy level could be determined) was calculated for each species. Although the deduced locus status can include some mistakes, the difference of expected diploidization among species is more informative than the real level of diploidization, which is difficult to establish.

## Results

After the initial screening of the 90 unlabeled primer pairs on agarose gel, 38 primer pairs (42%) resulted in poor or no amplification and were abandoned. For the other 52 primer pairs (25 developed for *A. gueldenstaedtii* and 27 developed for other sturgeon species from the literature), crosspriming with samples of 4 other sturgeon species showed very high levels of cross-species utility.

In the next step among a total of 52 labeled microsatellite primer pairs examined on polyacrylamide gel, we obtained reproducible amplification in most of the analyzed sturgeon species for 20 loci (Table 2). The remaining 32 loci were discarded because they displayed difficulty in amplification, showed nonspecific products, stutter bands (in which case it proves very difficult to read the allelic profile), or were monomorphic among species. Amplification results for the 52 examined loci are listed in Supplementary Table S2 online.

Allelic band patterns indicating the ploidy level for the 10 analyzed species at 20 microsatellite loci are shown in Table 2. In some cases, question marks indicate that it was not possible to definitely determine the ploidy level at a particular locus due to the presence of nonspecific artifact bands (nonspecific amplification) or stutter bands (these loci have been retained because informative in other species). The ploidy level at these loci was marked as ambiguous (with a question mark). If amplification in a particular species produced

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**Table 2** Disomic (2n) and tetrasomic (4n) transmissions of the 20 microsatellite loci analyzed in 10 sturgeon species

| Microsatellite locus | Species examined in this study | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Huso huso* | *Acipenser ruthenus* | *Acipenser stellatus* | *Acipenser gueldenstaedtii* | *Acipenser baerii* | *Acipenser naccarii* | *Acipenser dabryanus* | *Acipenser sinensis* | *Acipenser schrenckii* | *Huso dauricus* |
| *Afu19* | 2n | 2n | 2n | 4n | 4n | 4n | 4n | 4n | 4n | 4n |
| *Afu34* | 2n | 2n | M | 4n | 4n | 4n | 2n | 2n | 4n | 2n |
| *Afu39* | 2n | 2n | M | 4n | 4n | 4n | 4n | 4n | 4n | 2n |
| *Afu54* | 2n | 2n | 2n | 4n | 4n | 4n | 4n | 2n | 4n | 4n |
| *Ag01* | 2n | 2n | 2n | >4n | 4n | 4n | 4n | 4n | M | 4n? |
| *Ag09* | 2n | 2n | 2n | 4n | 2n | 4n? | 4n | 4n | 4n | 4n? |
| *Ag12* | 4n | 2n | 2n | >4n | >4n | 4n | >4n | >4n | >4n | >4n |
| *Ag14* | 2n | 4n | 2n | >4n | 4n | 4n | 4n | 4n | 4n | 4n |
| *Ag18* | 2n | 2n | 2n | 2n | 4n | 2n? | 4n | 4n | 4n | 4n |
| *Ag22* | 2n | 2n | 2n | >4n | 4n | 4n | 2n | 4n | 4n | 2n |
| *Ag28* | 2n | 2n | 2n | 4n | 4n | 4n | 4n? | 4n | 4n | 2n? |
| *Ag49* | 2n | M | 2n | 4n | 4n | 4n | 4n | 4n | >4n | 4n? |
| *AnacC11* | 2n | 2n | 2n | 4n | 4n | 4n | M | 4n | 4n | 4n |
| *Aox27* | 4n | M | 4n | 4n | 4n | 4n | 4n | 4n | 4n | 4n |
| *AoxB28* | 2n | 2n | 2n | 4n | 4n | 4n | ? | ? | ? | ? |
| *AoxD234* | 2n | 2n | 2n | 4n | 4n | 4n | 4n | 4n | 4n | 4n? |
| *Spl101* | 2n | 2n | 2n | 4n | 4n | 4n | 4n | 4n | 4n | 4n |
| *Spl113* | 2n | 2n | 2n | 4n | 4n | 4n | 2n | 4n? | 4n | 2n? |
| *Spl123* | 4n | 4n | 4n | 4n | 4n | 4n | ? | ? | 4n | 4n |
| *Spl170a* | 2n | 2n | 2n | 4n | 4n | 4n | 4n | 4n | 4n | 4n |

Numbers indicate allelic band patterns observed for each species at each locus—2n: disomic allelic band patterns; 4n: tetrasomic allelic band patterns; >4n: more than 4 alleles; M: monomorphy or a single allele observed at the locus indicated; ?: ambiguous.

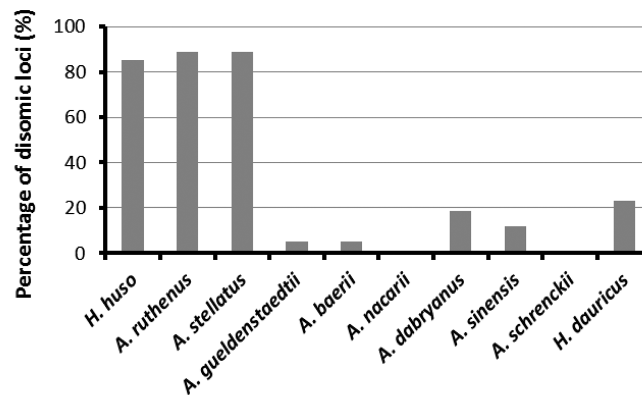


**Figure 2.** Percentage of disomic microsatellite loci detected in the 10 sturgeon species analyzed in this study.

a single allele in all analyzed individuals, it was not possible to determine the ploidy level in that species; such loci are marked as monomorphic (M) in the table.

Locus Spl123 displayed tetrasomic banding patterns in all the species analyzed, including recent diploids. Locus Ag12 appeared highly duplicated and displayed more than 4 bands in *A. gueldenstaedtii*, *A. baerii*, *A. dabryanus*, *A. sinensis*, *A. schrenckii*, and *H. dauricus*. In *A. gueldenstaedtii*, 4 loci designed for this species showed more than 4 bands (Ag01, Ag12, Ag14, and Ag22).

The percentage of disomic loci per species among the loci for which the ploidy level could be definitely determined is shown in Figure 2. The 3 species (*H. huso*, *A. stellatus*, and *A. ruthenus*) that are considered as recent diploids showed 80% or more of disomic loci. The other 7 species that are considered as recent tetraploids all showed less than 23% of disomic loci. No disomic loci were detected in *A. naccarii* and *A. schrenckii*. In *A. gueldenstaedtii* and *A. baerii*, only 5% of loci were identified as disomic (one in each species). The other 3 species from the Pacific clade (*A. sinensis*, *A. dabryanus*, and *H. dauricus*) showed 12, 19, and 23% of disomic loci, respectively.

One out of the 5 individuals of *H. huso* showed a tetrasomic pattern at most of the loci analyzed: 3 alleles (Afu39, Ag09, Ag18, Ag22, Ag28, Ag49, and AoxB28), 4 alleles (Afu19, Afu34, Afu54, Ag01, Aox27, Spl101, Spl113, Spl123, and Spl170a), and more than 4 alleles (Ag12 and Ag14). It was excluded from the banding pattern analysis. This pattern was consistent with the pattern found in *A. gueldenstaedtii*, which could be considered as an indication that this



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individual was incorrectly labeled as *H. huso* and it may actually be *A. gueldenstaedtii*. Another individual that was labeled as *H. huso* (ind. 4 - ST278) displayed 3 bands at loci Ag01 and Ag22. This may be an indication that this individual was incorrectly labeled as pure species and, in fact, it may be a hybrid between *A. gueldenstaedtii* and *H. huso* as has already been described by Dudu et al. (2011).

## Discussion

Sturgeons (Acipenseridae) have long been used as model fish because of their favorable characteristics such as their wide distribution in the north hemisphere, their variable ploidy level according to the taxon, and numerous hybridization events that still occur. Disentangling the main events of duplication and simplification (functional diploidization) of their genome is a step toward understanding genome evolution.

The main structuring feature is the ploidy state, which can be investigated through the chromosome number, the DNA amount, or the genetic expression of nuclear alleles. Most of the published studies that have developed and analyzed nuclear markers in sturgeon were performed on North American species. Table 3 shows the chromosome numbers, ploidy levels according to the evolutionary and recent scale, and the percentage of loci of different ploidy levels for 19 sturgeon species according to this study and previous studies.

**Table 3** Chromosome number, ploidy level, and percentage of disomic, tetrasomic, and octasomic microsatellite loci found in this study and other published studies for 19 sturgeon species

| | | Ploidy level[b] | | Microsatellite loci (%) | | | |
|---|---|---|---|---|---|---|---|
| Species | Chromosome number[a] | Evolutionary scale | Recent scale | Disomic | Tetrasomic | >Tetrasomic | Reference[c] |
| *Acipenser sturio* | 116 ± 4 | 4 | 2 | 100 | 0 | 0 | Ludwig et al. (2001) |
| *Acipenser oxyrinchus* | 121 ± 3 | 4 | 2 | 100 | 0 | 0 | Ludwig et al. (2001) |
| | | | | 75 | 25 | 0 | Havelka et al. (2013) |
| Pacific clade | | | | | | | |
| *Huso dauricus* | 268 ± 4 | 8 | 4 | 23 | 69 | 8 | This study |
| *Acipenser mikadoi* | 262 ± 4/249 ± 8 | 8 | 4 | 0 | 50 | 50 | Ludwig et al. (2001) |
| | | | | 30 | 50 | 20 | Havelka et al. (2013) |
| *Acipenser medirostris* | 249 ± 8 | 8 | 4 | 35.5 | 64.5 | 0 | Welsh et al. (2003) |
| *Acipenser dabryanus* | ? | 8 | 4 | 19 | 75 | 6 | This study |
| *Acipenser sinensis* | 264 ± 4 | 8 | 4 | 12 | 82 | 6 | This study |
| *Acipenser transmonatus* | 248 ± 8/~271 | 8 | 4 | 0 | 41 | 59 | Drauch-Schreier et al. (2011) |
| *Acipenser schrenckii* | 238 ± 8/266 ± 4 | 8 | 4 | 0 | 89 | 11 | This study |
| Atlantic clade | | | | | | | |
| *Huso huso* | 116 ± 4 | 4 | 2 | 100 | 0 | 0 | Moghim et al. (2012) |
| | | | | 82 | 18 | 0 | This study |
| | | | | 73 | 27 | 0 | Havelka et al. (2013) |
| *Acipenser stellatus* | 118 ± 4/118 ± 2 | 4 | 2 | 100 | 0 | 0 | Moghim et al. (2012) |
| | | | | 89 | 11 | 0 | This study |
| | | | | 80 | 20 | 0 | Havelka et al. (2013) |
| *Acipenser ruthenus* | 118 ± 2 | 4 | 2 | 89 | 11 | 0 | This study |
| | | | | 64 | 36 | 0 | Havelka et al. (2013) |
| *Acipenser nudiventris* | 118 ± 2 | 4 | 2 | 100 | 0 | 0 | Moghim et al. (2012) |
| | | | | 80 | 20 | 0 | Havelka et al. (2013) |
| *Acipenser fulvescens* | 262 ± 6 | 8 | 4 | 21 | 79 | 0 | Welsh et al. (2003) |
| *Acipenser brevirostrum* | ~372/372 ± 6 | 12 | 6 | 20 | 60 | 20 | Ludwig et al. (2001) |
| | | | | 0 | 18 | 82 | Havelka et al. (2013) |
| *Acipenser baerii* | 249 ± 5 | 8 | 4 | 43 | 43 | 14 | Fopp-Bayat (2008) |
| | | | | 5 | 90 | 5 | This study |
| | | | | 0 | 73 | 27 | Havelka et al. (2013) |
| *Acipenser persicus* | ~258 | 8 | 4 | 0 | 72 | 28 | Moghim et al. (2012) |
| | | | | 18 | 55 | 27 | Havelka et al. (2013) |
| *Acipenser naccarii* | 239 ± 7 | 8 | 4 | 0 | 100 | 0 | Boscari et al. (2011) |
| | | | | 0 | 100 | 0 | This study |
| | | | | 9 | 64 | 27 | Havelka et al. (2013) |
| *Acipenser gueldenstaedtii* | 250 ± 8 | 8 | 4 | 0 | 54 | 46 | Moghim et al. (2012) |
| | | | | 5 | 75 | 20 | This study |
| | | | | 0 | 64 | 36 | Havelka et al. (2013) |

[a]Chromosome numbers are after Vasil'ev et al. (2010) and references therein.

[b]Established according to the chromosome numbers, except for *A. dabryanus* (estimated according to microsatellites inheritance in this study).

[c]Microsatellite studies establishing loci inheritance: disomic, tetrasomic, and more.

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When no indication is given in this text, we have used the "recent scale" terminology, which means that the diploid species have around 120 chromosomes (evolutionary tetraploids) and the tetraploid ones around 240 (evolutionary octoploids).Within the Pacific clade, *A. mikadoi* was until recently considered as octaploid species, which was supported by nuclear DNA content measurements (Birstein et al. 1993) and microsatellite analysis (Ludwig et al. 2001). *Huso dauricus* was considered as diploid species, which was probably influenced by morphological similarity to the diploid *H. huso*, but genus *Huso* was shown to be of polyphyletic origin (Vasil'eva et al. 2009). However, recent karyological evidence (Vasil'ev et al. 2009; Zhou et al. 2013) and the measurement of DNA content (Vishnyakova et al. 2009; Zhou et al. 2011) have shown that both species are tetraploids. The observation that a majority (69%) of the loci in this study of *H. dauricus* is in agreement with the new karyological evidence showing that this species has 268 ± 4 chromosomes and that it should be considered tetraploid (Vasil'ev et al. 2009). Ludwig et al. (2001) classified *H. dauricus* as diploid based on disomic allelic band patterns observed at 4 of the 5 loci investigated, even though a tetrasomic pattern was observed at 1 locus (Afu68). However, only 3 individuals were analyzed in their study.

Among other loci, we analyzed the same 4 loci (Afu19, Afu34, Afu39, and Afu54) that were among the 6 loci analyzed by Ludwig et al. (2001). Unlike the authors of studies who found all of these 4 loci to show a disomic pattern in *H. dauricus*, we detected a tetrasomic pattern for 2 of them (Afu19 and Afu54). The same is true for other species for which the sample size used by Ludwig et al. (2001) was very small as in *A. schrenckii* where a disomic pattern was observed at locus Afu19, and locus Afu39 was designated as monomorphic in this species. In this study, however, we found a tetrasomic pattern at both loci. In *A. sinensis*, Zhao et al. (2005) and Zhao and Chang (2006) also found a tetrasomic pattern at Afu54, the only locus that was identified as disomic in this species by Ludwig et al. (2001). Our larger sample size for *H. dauricus* (10 individuals), *A. schrenckii* (12 individuals), and *A. sinensis* (16 individuals) and the larger number of loci investigated give a better estimate of the ploidy level.

All other species from the Pacific clade (*A. medirostris, A. dabryanus*, *A. sinensis*, *A. schrenckii*, and *A. transmontanus*) are considered as tetraploids based on the chromosome numbers and DNA content (reviewed in Birstein et al. 1997; Vasil'ev et al. 2010). In *A. medirostris*, Welsh et al. (2003) found 35.5% of disomic microsatellite loci and all other loci (64.5%) were identified as tetrasomic. In *A. sinensis*, Ludwig et al. (2001) found 1 locus (Afu68) in more than 4 copies, and all other loci analyzed in this species were identified as tetrasomic (Ludwig et al. 2001; Zhao et al. 2005; Zhao and Chang 2006). To the best of our knowledge, this study is the first to investigate microsatellite patterns in *A. dabryanus*. The percentage of disomic loci found in this species (12%) is similar to the level found in its sister species, *A. sinensis* (19%) in this study.

The study by Ludwig et al. (2001) is the only one to have examined microsatellite patterns in *A. schrenckii*. However, the results of this study for *A. schrenckii*, *H. dauricus*, and *A. sinensis* must be interpreted with caution due to a very small sample size for these species. In *A. transmontanus*, Rodzen and May (2002) found only 1 possibly disomic locus in males, and Drauch-Schreier et al. (2011) did not identify any disomic locus. Nonetheless, these 2 studies found a very high percentage (59%) of loci with more than 4 alleles. No disomic loci were identified in this study in *A. schrenckii*, and no disomic locus was identified previously in its sister species, *A. transmontanus*. In this study, the 11% of loci presenting more than 4 copies in *A. schrenckii* and 59% of such loci in *A. transmontanus* (Drauch-Schreier et al. 2011) indicate that the level of diploidization in this clade is lower than in its sister clade composed of *A. sinensis* and *A. dabryanus*. More than 4 alleles present at 1 locus (Ag12) in all 4 Pacific species analyzed in this study indicate that some loci still did not go through diploidization.

Within the Atlantic clade, the lineage composed of *A. fulvescens*, *A. brevirostrum*, *A. baerii*, *A. persicus*, *A. naccarii*, and *A. gueldenstaedtii* includes 5 tetraploid species and 1 hexaploid species. *Acipenser brevirostrum* is considered as hexaploid because Kim et al. (2005) and Fontana et al. (2008) found its chromosome number to be ~370. This was confirmed by fluorescence in situ hybridization (FISH), a molecular cytogenetic technique (Fontana et al. 2008). Havelka et al. (2013) found hexaploid allelic band pattern at 5 loci in this species, octaploid allelic band pattern at 1, and 12 distinct alleles were observed at a single locus. Welsh et al. (2003) analyzed microsatellite loci developed for North American *A. fulvescens* and found 20.9% of disomic loci in this species. The rest of the analyzed loci was identified as tetrasomic. Pyatskowit et al. (2001), who examined inheritance patterns in the same species using 4 primer pairs, found that 2 of them amplified disomic loci, 1 pair amplified 2 disomic loci, and the last one amplified a tetrasomic locus.

A study by Fopp-Bayat (2008) that examined inheritance patterns in *A. baerii* found that among the 7 microsatellite loci studied, 3 segregate disomically, 3 tetrasomically, and 1 octasomically. In this study, however, we found only 5% of disomic loci in *A. baerii*. In the study of inheritance patterns in *A. naccarii*, all of the 24 loci examined showed tetrasomic inheritance patterns (Boscari et al. 2011), which is in concordance with our results that showed only tetrasomic pattern in this species. Moghim et al. (2012) who analyzed loci developed for *A. persicus* found 72% of tetrasomic loci in *A. gueldenstaedtii* and 54% in *A. persicus*. The rest of the polymorphic loci analyzed in these 2 species was identified as octasomic (28% and 46%, respectively). Taken together with our results for *A. gueldenstaedtii* (only 5% of disomic loci), the level of diploidization in this species seems very low. We can conclude that in the Atlantic clade, a high level of diploidization is present only in the North American species *A. fulvescens*, whereas the diploidization level in the 4 Eurasian tetraploid species from the *gueldenstaedtii* complex seems to be much lower.

The other 4 species from the Atlantic clade (*H. huso*, *A. stellatus*, *A. ruthenus*, and *A. nudiventris*) are all considered as diploid species based on the chromosome numbers and DNA content (reviewed in Birstein et al. 1997; Vasil'ev et al. 2010). In this group of species, some of the previous microsatellite

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studies identified only disomic patterns (Ludwig et al. 2001; Moghim et al. 2012). In a study by King et al. (2001) that examined amplification of 6 pairs of microsatellite primers in *H. huso* and *A. stellatus*, one locus was nonetheless found to be polysomic in *A. stellatus* (Aox27) and one presumably polysomic in *H. huso* (Aox45). Polysomic patterns in *H. huso*, *A. ruthenus*, and *A. stellatus* have also been detected in 1 of the 7 analyzed microsatellite loci (Afu57) (Ferguson et al. 2000). Havelka et al. (2013) found tetraploid allelic banding pattern at 2–4 loci in these 4 species.

In the 3 diploid species from the Atlantic clade analyzed in this study (*H. huso*, *A. stellatus*, and *A. ruthenus*), we identified between 11% (*A. stellatus* and *A. ruthenus*) and 15% (*H. huso*) of tetrasomic loci. The presence of tetrasomic loci in the diploid species can be explained by their tetraploid origin. This is an indication that the diploidization process in these species is not completed. Likewise, in the group of species which are considered tetraploids, more than 4 alleles detected in a same individual at 4 loci (20%) in *A. gueldenstaedtii* and at 1 locus (5%) in *A. baerii* indicate the octaploid origin of this group of species.

Taken together, our data on the percentage of disomic loci in different species suggests that functional diploidization is an ongoing process in sturgeon. As all species in the Pacific clade are tetraploids according to recent findings, only 1 polyploidization event in the ancestor of the Pacific clade is the most likely scenario (Figure 1), unlike what was proposed by Ludwig et al. (2001) and Peng et al. (2007).

We observed a lower level of diploidization in the tetraploid species from the Atlantic clade than in the tetraploid species from the Pacific clade. This can be explained by the more recent genome duplication in the tetraploid species from the Atlantic clade, which likely happened after the separation from other diploid species that belong to the Atlantic clade (Figure 1). Our findings support the polyploidization scenario proposed by Vasil'ev et al. (2010), according to which 2 polyploidization events occurred in the Atlantic species group: 1) in the common ancestor of the tetraploid Atlantic species and 2) in the origin of hexaploid *A. brevirostrum*. However, the evolution of polyploidy in *A. brevirostrum* is probably more complex, as proposed by Fontana et al. (2008) and also likely to involve allopolyploidy. In conclusion, at least 3 polyploidization events occurred in the evolution of the family Acipenseridae (Figure 1), but additional hybridization events cannot be refuted.

The other groups of fish in which functional diploidization has been investigated are salmonids, catostomids, and cyprinids (mainly barbins). The common ancestor of salmonids experienced a whole-genome duplication event between 25 and 100 million years ago, and modern species are in the process of reverting to a stable diploid state (Allendorf and Thorgaard 1984). Segregation ratios consistent with tetrasomic inheritance or partial tetrasomic ratios are observed following male meioses (Allendorf and Danzmann 1997), indicating that the genomes of salmonids have not completely returned to disomy (Danzmann et al. 2008). Allozyme studies found that catostomid and salmonid fish retain about 50% of the duplicate gene expression, despite up to 100 million years of divergence as polyploids (Ferris and Whitt 1977). In these 2 groups, many duplicate genes have been silenced and many of the remainders have diverged in their gene expression and specificity to different tissues (Ferris and Whitt 1979; Allendorf and Thorgaard 1984). The taxonomy of barbins and especially of the genus *Barbus* has been reorganized in the light of their successive polyploidizations (Berrebi et al. 1996; Tsigenopoulos et al. 2002). In this group, any successful polyploidization generally induces a new radiation as evidenced in African hexaploid barbels (Tsigenopoulos et al. 2010) and in the birth of genus *Capoeta* (Levin et al. 2012). Functional diploidization is still evolving in this group, which renders the use of classical population genetic parameters difficult (Berrebi et al. 1990).

A low percentage of the duplicated locus expression in the diploid sturgeons (contemporary scale) is expected if we assume that only 1 genome duplication event occurred in these species in the ancestor of all Acipenseriformes around 200 million years ago. However, we would expect a higher percentage of duplicated locus expression in species that diverged after presumably more recent duplication events (tetraploids). Ludwig et al. (2001) proposed that low numbers of duplicated locus expressions in sturgeon may be explained by subsequent gene silencing, as found for salmonids and catostomids and for paddlefish.

As a whole, screening 20 microsatellite loci transmission in 10 sturgeon species allowed to suggest a new evolution history of Acipenseridae, reducing the expected polyploidizations from 3 to 1 (Figure 1). Being more parsimonious, it is supposed to be more likely.

## Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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