SHORT COMMUNICATION

Atlantic sturgeons (*Acipenser sturio*, *Acipenser oxyrinchus*): American females successful in Europe

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Abstract Recent molecular data on the maternally inherited mitochondrial (mt) DNA have challenged the traditional view that the now extinct Baltic sturgeon population belonged to the European sturgeon *Acipenser sturio*. Instead, there is evidence that American sea sturgeon *Acipenser oxyrinchus* historically immigrated into the Baltic Sea. In this study, we test the hypothesis that *A. oxyrinchus* introgressed into, rather than replaced, the *A. sturio* population in the Baltic. We established four single nucleotide polymorphisms (SNPs) in the nuclear MHC II antigen gene with a species-specific SNP pattern.

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Using an ancient DNA approach and two independent lines of molecular evidence (sequencing of allele-specific clones, SNaPshot), we detected both *A. sturio* and *A. oxyrinchus* alleles in the available museum material of the now extinct Baltic sturgeon population. The hybrid nature of the Baltic population was further confirmed by very high levels of heterozygosity. It had been previously postulated that the immigration of the cold-adapted *A. oxyrinchus* into the Baltic occurred during the Medieval Little Ice Age, when temperature likely dropped below the degree inducing spawning in *A. sturio*. Under this scenario, our new findings suggest that the genetic mosaic pattern in the Baltic sturgeon population (*oxyrinchus* mtDNA, *sturio* and *oxyrinchus* MHC alleles) is possibly caused by sex-biased introgression where spawning was largely restricted to immigrating American females, while fertilization was predominantly achieved by abundant local European males. The hybrid nature of the former Baltic sturgeon population should be taken into account in the current reintroduction measures.

Introduction

The European sturgeon (*Acipenser sturio*) was historically widely distributed in Europe, but is extirpated in its historic range, apart from a relict population in the Gironde, France (Lepage and Rochard 1995). Traditionally, the now extinct Baltic sturgeon population had been classified as "European sturgeon" (Billard and Lecointre 2001). This view has been challenged recently in the framework of the German sturgeon restoration program (Kirschbaum et al. 2006), as

Ludwig et al. (2002) hypothesized a complete replacement of European sturgeon by American sea sturgeon *Acipenser oxyrinchus* in the Baltic Sea. These authors investigated maternally inherited mtDNA, morphology, and one nuclear microsatellite. This spectacular scenario postulates two steps of population expansion in American sturgeon, i.e., (1) eastbound dispersal from Eastern North America to Europe and (2) replacement of European by American sturgeon in the Baltic about 800 years ago, associated with the Medieval Little Ice Age. The molecular evidence for step 1 is overwhelming, as all analyzed Baltic sturgeon specimens exhibit an mt genotype presently only found in American sea sturgeon (Ludwig et al. 2002). The postulated complete replacement of European sturgeon is more difficult to validate. Given that (a) the two sturgeon species are closely related (Birstein and DeSalle 1998), and (b) natural hybrids regularly occur even among more distantly related sturgeon species (Birstein et al. 1997; Congiu et al. 2001), we forward in this study an alternative hypothesis, i.e., that American sturgeons introgressed into, rather than replaced, the Baltic population. Under our new hypothesis, we predict ancient hybridization in the Baltic. In fact, Acipenseriformes are characterized by a high capacity for hybridization, as—in sympatry—nearly all species are able to hybridize (Billard and Lecointre 2001).

Due to uniparental maternal inheritance, hybrid status of an individual will not be reflected in mtDNA. For the genetic detection of sturgeon hybrids, amplified fragment length polymorphism (AFLP) at multiple nuclear loci had been successfully applied (Congiu et al. 2001). This approach was however precluded in this study, as our analysis of the Baltic Sea sturgeon population had to rely entirely on ancient museum material, which does not yield DNA of a quality suitable for reliable AFLP screening. We hence established a new species-specific nuclear marker in *A. sturio* and *A. oxyrinchus* in the MHC II antigen gene (Venkatesh et al. 1999). We also argue that any sign of an ancient hybridization is more likely to be preserved in markers potentially subject to balancing selection (e.g., MHC genes; cf. Sommer 2005) than in neutral markers, evolving through random genetic drift (e.g., microsatellites).

Materials and methods

As reference samples, we obtained tissue samples (preserved in ethanol) from wild caught specimens of both *A. sturio* (Gironde River, France, $n=17$) and *A. oxyrinchus* (St. Lawrence, Canada, $n=10$; and St. John River, Canada, $n=17$). The latter two samples comprise the geographic region where *A. oxyrinchus* is fixed for the mt haplotype detected in Baltic sturgeons (Ludwig et al. 2002). DNA was extracted with the DNeasy kit (Qiagen) according to the manufacturer's protocols. Our analysis of the extinct Baltic sturgeon population was based on ancient museum material (15 samples from eighteenth to twentieth century, four of which also included in Ludwig et al. 2002; bones or old tissue, either dry or ethanol-preserved; see electronic supplementary material S1 for details). For the analysis of the museum samples, we followed the established criteria for ancient DNA analysis (Hofreiter et al. 2001). In particular, ancient DNA samples were processed in a separate dedicated, ancient DNA laboratory never used for work with the recent DNA. Bone samples were pulverized in a freezer mill (Spex Certiprep 6750) under liquid nitrogen. DNA isolation was performed with DNeasy (Qiagen) according to the manufacturer's bone DNA isolation protocol.

We had previously established four single nucleotide polymorphisms (SNPs) with a species-specific SNP pattern (cf. American sea sturgeon *A. oxyrinchus* GenBank accession no. AJ515709; European sturgeon *A. sturio* GenBank accession no. AJ515708). We genotyped wild caught reference samples with standard PCR [1 cycle at 94°C for 5 min, 40 cycles at 94°C for 1 min, at 53°C (primer specific Tm) for 30 s, at 72°C for 45 s, and a final extension at 72°C for 2 min; see Tiedemann et al. 2005 for experimental details] using the primers AcMHC-1 5′-TGCAGCGCGTAC GAGTTCTACCC-3′ and AcMHC-2 5′-CTCCACCAAG CAGCTGTAGGAGT-3′, followed by sequence analysis, i.e., cycle sequencing with the BigDye v1.1 Terminator Cycle Sequencing Kit and stratification on an AB 3100 automatic sequencer (Applied Biosystems). PCR products of heterozygous specimens were cloned (TA cloning kit, Invitrogen) before sequencing. PCR on ancient samples was performed using the same conditions as for the reference samples, except

Table 1 Allele distribution at MHC II antigen gene in extant (Canadian and French) and extinct (Baltic) sturgeons

Origin	$2n$	"oxy"	"stur"	I1–I4	H_E	H_O
American sea sturgeon (*A. oxyrinchus*) St. Lawrence/St. Johns (Canada)	54	52	1	1	0.07	0.04
European sturgeon (*A. sturio*) Gironde (France)	34	–	33	1	0.06	0.06
Baltic sturgeon (*A. oxyrinchus* × *sturio*) Baltic Sea (eighteenth to twentieth century)	30	6	10	14	0.79	0.73

See electronic supplementary material for the exact genotype information.
2n Number of alleles studied, "oxy"*A. oxyrinchus* reference allele (GenBank accession no. AJ515709), "stur"*A. sturio* reference allele (GenBank accession no. AJ515708), *I1–I4* other intermediate alleles, H_E expected heterozygosity, H_O observed heterozygosity
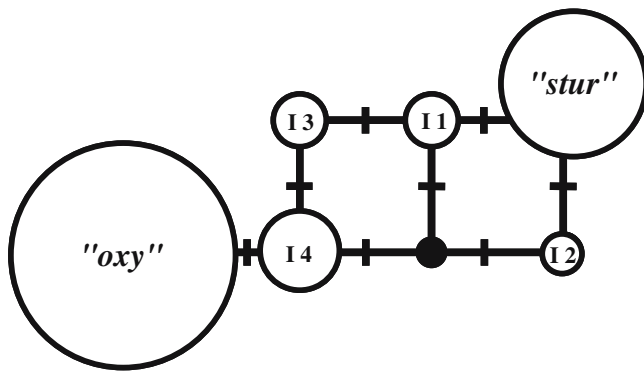
Fig. 1 Phylogenetic network of MHC alleles in recent and ancient sturgeons. *"oxy"* Dominant allele (96%) in *A. oxyrinchus* reference samples (GenBank accession no. AJ515709), *"stur"* dominant allele (97%) in *A. sturio* reference sample (GenBank accession no. AJ515708). *Circle area* represents absolute allele frequency; *bars between circles* represent single nucleotide polymorphisms (SNPs). *Black circle* represents a hypothetical intermediate type not found in our sample

for: (1) a separate PCR machine dedicated to ancient samples was used and (2) a nested PCR was performed with specific internal primers (AcMHC-1b 5′-GAGATAAAGGT GACCTGGCTGAG-3′ and AcMHC-2b 5′-GCAGGTAC (AG)AGTGGATCTGATAGG-3′). PCR equipment was UV-irradiated before use to minimize cross-contamination. All PCR products from ancient samples were cloned and subsequently sequenced to determine allelic states using the same methods as for the reference samples.

With low amounts and potential degradation of DNA—as typical for ancient samples—allelic dropout could erroneously indicate homozygote state for heterozygous individuals. To circumvent this difficulty, we confirmed the allelic state in a separate SNP analysis: After a first PCR as above, character state at each SNP was detected by the SNaPshot™ (Applied Biosystems) technology, i.e., an

adjacent oligonucleotide was designed for each SNP (sequences available on request) such that heterozygous state could be detected by specific addition of a single fluorescent-labeled didesoxynucleotide, followed by detection on an automatic sequencer (Quintans et al. 2004).

Observed (H_O) and expected (H_E) heterozygosities were calculated (refer to, e.g., Hedrick 2000, formulae 2.18b and 2.18c, respectively). We checked for Hardy–Weinberg equilibrium (HWE) by statistically comparing H_O and H_E. A phylogenetic network of alleles was determined according to Bandelt (1992).

Results

Our analysis of the reference samples confirmed the previously established species-specific SNP pattern: In American sea sturgeon *A. oxyrinchus* from two Canadian rivers, 96% of the alleles (52 out of 54) comprised the MHC reference allele for this species ("oxy"; Table 1, Fig. 1). In the extant relict population of European sturgeon *A. sturio* in the Gironde, 97% of the alleles (33 out of 34) were the respective reference alleles for this species ("stur"; Table 1, Fig. 1). Note that the "stur" allele was also detected once in *A. oxyrinchus* (corresponding to about 2% frequency) (Fig. 2). This single shared allele is potentially a remnant of the common ancestry of both species.

It is interesting to note that in the 15 Baltic specimens, we found both the "stur" (*A. sturio*) and the "oxy" (*A. oxyrinchus*) MHC alleles in frequencies of 33 and 20%, respectively (Table 1). Moreover, a high percentage (47%) in the Baltic sturgeons comprised alleles intermediate between the "oxy" type and the "stur" type (I1 to I4; Table 1, Fig. 1). These intermediate alleles were rare or absent in the extant reference populations. Whereas observed (H_O) and expected (H_E) heterozygosities were

Fig. 2 Relative frequency of *A. oxyrinchus* (*black*), *A. sturio* (*white*), and intermediate other (*gray*) genotypes at the maternally inherited mtDNA (data from Ludwig et al. 2002) and the biparentally inherited MHC locus in the extant reference populations as well as in the extinct Baltic population. Note that mtDNA and MHC analyses only partially refer to the same samples (see electronic supplementary information)
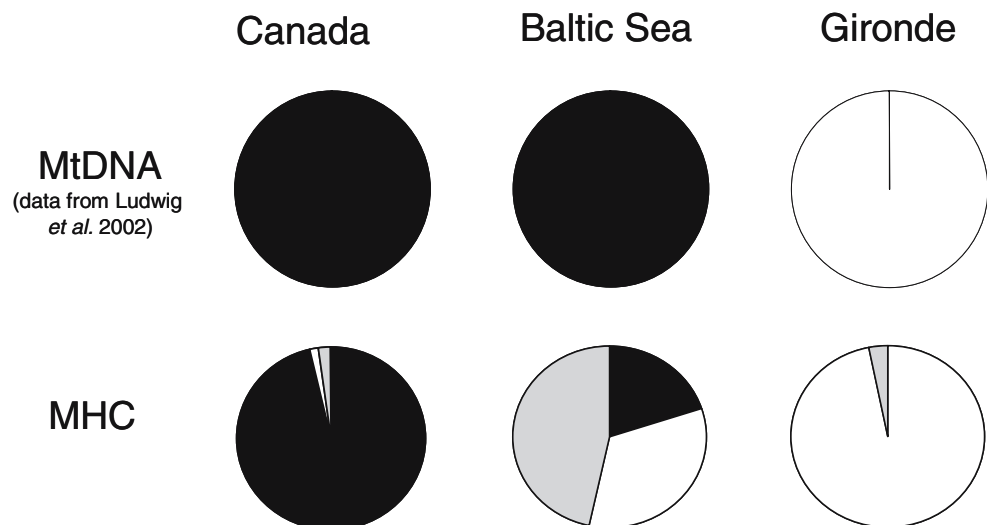
very low in the extant populations (0.04–0.06 and 0.06–0.07, respectively), these values were high in the extinct Baltic population (H_O=0.73, H_E=0.79). Regarding their MHC allele frequencies, all populations were in HWE, i.e., there was no significant difference between H_O and H_E. HWE indicates that a population consists of a single randomly interbreeding group.

Discussion

Given that Baltic sturgeons carry "oxy" and "stur" alleles as well as their intermediates, our data suggest that the Baltic was inhabited by a hybrid population, i.e., *A. oxyrinchus × sturio*. Together with the mtDNA data from Ludwig et al. (2002), our findings support our new hypothesis that *A. oxyrinchus* has hybridized with, rather than replaced, the former autochthonous Baltic sturgeon population.

An alternative explanation would be that both species have lived together in the Baltic—without intermixing. Under this scenario, we should detect alleles of both species, but single specimens should be mostly homozygous for one of the types, i.e., either "oxy" or "stur", such that a heterozygote deficit should be expected, i.e., expected heterozygosity should be significantly larger than observed heterozygosity. This prediction was—however—not met, as all populations including the Baltic one were in HWE, i.e., there was no significant difference between expected and observed heterozygosities. HWE is indeed indicative of a single interbreeding population. Several Baltic specimens carried both the "oxy" and the "stur" MHC allele, at least one of which carried the *oxyrinchus* mtDNA genotype (Ludwig et al. 2002, see supplementary information). Moreover, the high percentage (over 70%) of heterozygous specimens in the Baltic is a further indication of its hybrid nature when compared to the very low observed heterozygosity in the Canadian and French reference samples. Genetic variability (H_E) was also considerably higher in the now extinct Baltic populations than in the extant reference groups.

Given that all Baltic specimens shared an American genotype of the maternally inherited mtDNA (Ludwig et al. 2002), but both American ("oxy") and European ("stur") MHC alleles, our data suggest a sex-biased introgression: American female sturgeons had apparently more success in contributing to the Baltic gene pool than their male conspecifics. While there is no evidence for sex-biased dispersal in sturgeon, a possible explanation for a superior success of American females could lie in the climatic conditions during the assumed period of immigration (Ludwig et al. 2002): During the Little Ice Age (about 800 years ago), water temperature presumably dropped below 20°C, the degree inducing spawning in *A. sturio*,

possibly leaving the field for female immigrants of the cold-adapted American *A. oxyrinchus*, which are reported to spawn between 13.3 and 17.8°C (Ludwig et al. 2002). In males, however, there is no evidence for such temperature preferences during sperm deposit. The genetic mosaic pattern in the Baltic sturgeon population (*oxyrinchus* mtDNA, *sturio* and *oxyrinchus* MHC alleles) is possibly caused by a sex-biased introgression where spawning was largely restricted to immigrating American females, while fertilization was predominantly achieved by abundant local *A. sturio* males. The hybrid nature of the former Baltic sturgeon population should be taken into account in the current reintroduction measures (Gessner et al. 2006). From a conservation point of view, it is not straightforward to develop an appropriate strategy for the reestablishment of an extinct natural hybrid population. One might argue that—given this hybrid nature of the extinct population—both *A. sturio* and *A. oxyrinchus* should be released in the Baltic Sea. It is however unlikely that any such man-made hybrid population released under the present environmental conditions would reestablish the specific preextinction hybrid population. Any restocking involving *A. oxyrinchus*—if successful in the Baltic Sea—implies the possibility that these sturgeons would spread further in Europe. If so, this would constitute a potential threat for the last remaining "pure" *A. sturio* population in the Gironde, as it might put this relict population at the risk of being either introgressed or replaced.

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