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ORIGINAL ARTICLE

The history of sturgeon in the Baltic Sea

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ABSTRACT

Aim Migrants of the Atlantic sturgeon, *Acipenser oxyrinchus*, from North America are thought to have founded the Baltic sturgeon population during the Little Ice Age around 1200 years ago, replacing the European sturgeon, *Acipenser sturio*. To test this hypothesis and to further elucidate the colonization of the Baltic Sea by *A. oxyrinchus*, we carried out DNA analyses of ancient and contemporary populations of both species.

Location We analysed DNA from 188 specimens of sturgeons collected from archaeological sites and museums in Poland and of 225 contemporary specimens from North American and European populations.

Methods Several mitochondrial DNA fragments were sequenced and eight microsatellite loci were genotyped for species identification, polymorphism and population structure analyses. Approximate Bayesian computation was used to estimate when the Baltic Sea was colonized.

Results Of 125 ancient sturgeon specimens from the Baltic Sea, only four were classified as *A. sturio*, the remainder being *A. oxyrinchus oxyrinchus*. The ancient *A. o. oxyrinchus* population over two different time periods was highly polymorphic and genetically distant from contemporary populations of this taxon. The time of entry into the Baltic Sea was estimated to be 4000–5000 years ago. We also detected introgression of *A. sturio* into the *A. o. oxyrinchus* gene pool, caused by a prior hybridization event.

Main conclusions For the past 2000 years at least, *A. o. oxyrinchus* has been the dominant sturgeon in the Baltic Sea, indicating a much earlier origin than previously suggested. The most similar extant sturgeon populations to the extinct Baltic stock are those from the St John and St Lawrence rivers in Canada. These populations should be considered the best source of breeding material for the ongoing sturgeon restitution programmes in Poland and Germany.

Keywords

***Acipenser oxyrinchus*, *Acipenser sturio*, ancient DNA, Baltic sturgeon, colonization, founding population, hybridization, introgression, marine biogeography.**

INTRODUCTION

Sturgeons (Acipenseriformes: Acipenseridae) are one of the oldest osteichthyan fish families, dating from at least the Early Jurassic, approximately 200 Ma. Based on analyses of mitochondrial DNA sequence variation, the origin of Acipenseriformes was dated to 389.7 Ma (Peng *et al.*, 2007). Since that time, sturgeons have undergone little morphological change, and the group are considered to be 'living fossils'. All the extant species – 25 sturgeons and two paddlefish – evolved in the Holarctic and the family is now distributed throughout North America and Eurasia (Birstein & Doukakis, 2000). Within the Acipenseridae, the divergence time between the Pacific and Atlantic clades was estimated to be 121 Ma. The split between the Atlantic sturgeon (*Acipenser oxyrinchus* Mitchill, 1815), inhabiting rivers draining the Atlantic coastline of North America, and the European sturgeon (*Acipenser sturio* Linnaeus, 1758), found in rivers

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around the Atlantic coastline of Europe, took place about 58 Ma (Peng *et al.*, 2007), probably as a result of vicariance caused by continental drift and the opening of the Atlantic Ocean, followed later by the Pliocene cooling (Peng *et al.*, 2007). The two species are considered sister species and form a separate clade from other sturgeons within the Acipenseridae (Peng *et al.*, 2007). *Acipenser oxyrinchus*, the American species, is further divided into two subspecies, the Atlantic sturgeon proper (*A. o. oxyrinchus*) and the Gulf sturgeon (*A. o. desotoi* Vladykov, 1955), which is found in rivers draining the Gulf of Mexico (Vladykov, 1955).

Atlantic and European sturgeons are large and long-lived fishes. Both species are anadromous, spending several years at sea before maturing and returning to their riverine spawning grounds (Smith & Clugston, 1997; Acolas *et al.*, 2011). During the marine phase, they may undertake long coastal migrations, up to thousands of kilometres (Rochard *et al.*, 1997; Erickson *et al.*, 2011), but both species exhibit strong homing behaviour and usually return to their natal rivers for spawning (King *et al.*, 2001; Williot *et al.*, 2011). Straying is, however, also possible and they can colonize other river systems if new habitats become available (Wirgin *et al.*, 2007; Chassaing *et al.*, 2011).

The Atlantic sturgeon, *A. o. oxyrinchus*, historically inhabited most major rivers on the eastern coast of North America from Labrador to Florida. Nowadays, only 14–17 spawning populations persist in the USA, with between two and five in Canada (Wirgin *et al.*, 2007). The European sturgeon, *A. sturio*, inhabited most of the European coastline from the White Sea through the Norwegian Sea, North Sea, Baltic Sea (including Lake Ladoga), the Atlantic Ocean and Mediterranean Sea to the Black Sea (Holčík, 2000). Currently, however, only one relict population of this species survives, spawning in the Gironde watershed (Atlantic basin, France) (Williot & Castelnaud, 2011).

Recently, it was discovered that the range of *A. o. oxyrinchus* was not originally limited to the North American coast (Ludwig *et al.*, 2002), but that up to the beginning of the 20th century, this species was also abundant in Europe. Subsequently, as a result of overexploitation, this species disappeared from the East Atlantic and the Baltic Sea, and the last catch of *A. o. oxyrinchus* in the Vistula River was reported in 1965 (Kolman *et al.*, 2011). Based on DNA studies of sturgeons from museums and archaeological excavation sites in the Western Baltic region, Ludwig *et al.* (2002, 2008) claimed that *A. o. oxyrinchus* colonized the Baltic Sea directly from North America as recently as the Little Ice Age (*c.* 1200 years ago), and replaced *A. sturio*, a species requiring higher temperatures for egg development. The range of *A. o. oxyrinchus* in Europe then extended to other regions, including Great Britain (Ludwig *et al.*, 2009) and the French Atlantic coast (Chassaing *et al.*, 2013).

According to Chassaing *et al.* (2013) on the other hand, *A. o. oxyrinchus* first colonized Western Europe at least 5000 yr BP and then migrated north-east to reach the Baltic Sea. It seems certain that after this colonization event, *A. o. oxyrinchus* occupied the same breeding rivers as *A. sturio*, enabling introgression, a common phenomenon in sturgeons (Birstein *et al.*, 1997). This was probably reciprocal, leading to fertile offspring and back-crosses (Tiedemann *et al.*, 2007; Ludwig *et al.*, 2008; Chassaing *et al.*, 2013).

To test these hypotheses concerning the history of *A. o. oxyrinchus* in Europe and in the Baltic Sea, we undertook DNA analyses of sturgeon specimens collected from various excavation sites and from museum collections in Poland, dated from 2300–2100 years ago until the present. We sequenced five fragments of mitochondrial DNA (mtDNA) for four ancient sample collections and obtained profiles of eight microsatellite loci for two of them. The genetic diversity and structure of the ancient sturgeon populations was then compared with those of contemporary populations from North America and Western Europe. The time of Baltic Sea colonization, as well as the size and source of the founding population were estimated.

MATERIALS AND METHODS

Nine sturgeon populations, four ancient and five contemporary, were studied. Tissue fragments of 225 contemporary and 188 ancient sturgeons were used for DNA extraction and amplification (Table 1). The Baltic collection was composed of four populations – pre-Roman (1), medieval (2), late medieval (3) and modern (4) – and included specimens obtained from various archaeological sites and museums (Fig. 1, Table 1). The oldest samples from Baltic pre-Roman populations were dated by the ^{14}C method at the Poznań Radiocarbon Laboratory, Poland (Poz-29920; 2165 ± 30 uncal. yr BP). The remaining samples were dated by archaeological context. The specimens from archaeological sites were represented by bony scutes, whereas the 19th and 20th century archival samples were fragments of skin and fins. DNA from contemporary specimens was extracted from fragments of fins preserved in 70% ethanol.

The sampled contemporary populations were: *A. o. oxyrinchus* from the St Lawrence (5) and St John (6) rivers in Canada, and the Hudson River, USA (7); *A. o. desotoi* from the Choctawhatchee River, USA (8); and *A. sturio* from the Gironde, France (9) (Fig. 1, Table 1).

DNA extraction and amplification

To avoid contamination, the extraction of DNA from ancient samples and all pre-PCR manipulations were performed in a laminar flow cabinet in a sterile room dedicated to ancient DNA analyses at the Institute of Genetics and Biotechnology of the University of Warsaw. This room was over-pressurized and equipped with a system of airlocks. It was never used for work with contemporary DNA samples or PCR products. The room was UV-irradiated when not in use. The work with ancient and contemporary samples was carried out by different people. Researchers wore lab coats, masks and two pairs of gloves. Fish bones, skin and fin fragments for DNA

Table 1 The number and origin of samples of ancient Baltic and contemporary North American and European populations of sturgeon (*Acipenser oxyrinchus* and *A. sturio*) analysed in this work. *n*, number of analysed individuals; —, no data obtained. Numbers in parentheses represent the number of haplotypes in corresponding populations. Samples were dated by archaeological context and site stratigraphy.

Origin of samples	Population	Dating	*n*	Number of obtained sequences				Number of combined CR+*cytb*123 sequences*	Number of obtained sequences†				Number of obtained msDNA profiles
				CR	*cytb*1	*cytb*2	*cytb*3		16S	12S	*ND5*_1	*ND5*_2	
Archaeological site Nieszawa 5	Baltic pre-Roman (1)	2300–2100 years ago	45	17	5	22	5	5(H1)	8(2)	—	12(2)	13(2)	23
Natural History Museum, Wrocław	Baltic medieval (2)	1700–1600 years ago	5	1	—	—	—	—	—	—	—	—	—
Archaeological sites: Gdańsk, Elbląg, Kałdus, Ląd, Szczecin, Głogów		1100–700 years ago	84	65	66	53	52	52(H1, H2)	10(1)	6(1)	—	—	38
Natural History Museum, Wrocław	Baltic late medieval (3)	700–600 years ago	16	1	6	—	—	—	—	—	—	—	—
		300–200 years ago	33	11	11	14	11	11(H1)	—	—	13(2)	14(2)	—
Natural History Museum, Lviv	Baltic modern (4)	100–50 years ago	1	1	1	—	—	—	—	—	—	—	—
University of Agriculture, Szczecin		1965	1	1	1	—	—	—	—	—	—	—	—
Museum of Central Pomerania, Słupsk		1965	1	—	—	—	—	—	—	—	—	—	—
Central Maritime Museum in Gdynia		1972	1	1	1	1	1	1(H1)	—	—	—	—	—
Estonian coast of Baltic Sea		1996	1	1	1	1	1	1(H1)	—	—	—	—	—
St Lawrence River, Canada	St Lawrence (5)	Contemporary samples	54	40	40	40	40	40(H1)	3(1)	3(1)	1(1)	1(1)	54
St John River, Canada	St John (6)		107	101	33	33	33	32(H1, H3)	5(1)	3(1)	1(1)	1(1)	94
Hudson River, USA	Hudson (7)		20	15	17	17	17	15(H1, H3, H4, H5)	3(1)	3(1)	1(1)	1(1)	20
Choctawhatchee River, USA	Choctawhatchee (8)		20	14	19	19	19	14(H6, H7, H8)	2(1)	3(2)	3(1)	3(1)	20
Gironde, France	Gironde (9)		24	22	22	22	22	22(H9)	3(1)	3(1)	3(1)	3(1)	24
						Total		**193(9)**	**34(4)**	**21(4)**	**34(2)**	**36(3)**	**273**

*Combined CR + *cytb*123 sequences were used to construct the haplotype network. Haplotypes H1–H9 corresponds to those in Fig. 2.
†All obtained sequences are presented in Appendix S2.

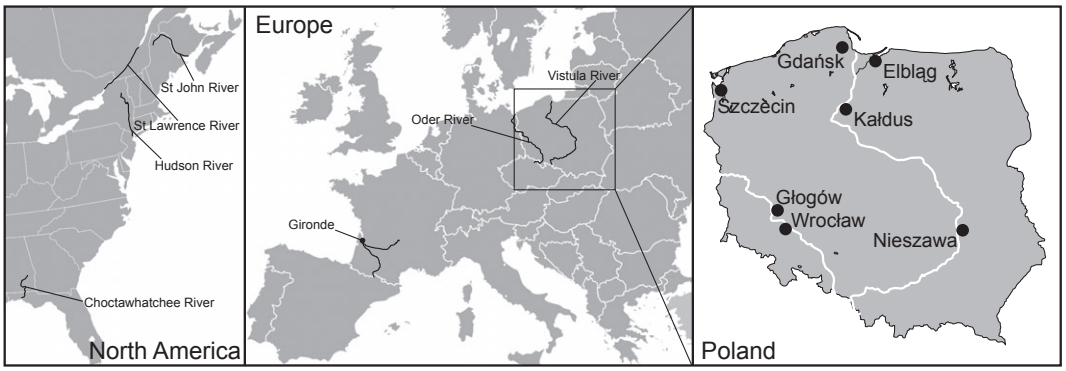


Figure 1 Sampling sites of contemporary North American and European populations of sturgeon (*Acipenser oxyrinchus* and *A. sturio*) and archaeological sites of the ancient sturgeon remains.

isolation were cleaned with a scalpel and sandpaper, and then washed with bleach, alcohol and double-distilled water to remove surface contaminations and impurities. Tissues were pulverized in a 6750 Freezer/Mill (SPEX CertiPrep, Metuchen, NJ, USA) and 300–500 mg was suspended in 1.6 mL of extraction buffer: 0.1 M EDTA, pH 8.0, 20 mM dithiothreitol (DTT), 0.5% N-lauroylsarcosine, 5 mM N-phenacylthiazolium bromide (PTB), 30 μL proteinase K (20 μg mL^{-1}). DNA was isolated using a phenol–chloroform procedure and precipitated in 95% isopropanol containing Pink Co-Precipitant (Bioline, London, UK). DNA pellets were resuspended in 30 μL TE buffer. Negative controls were included in every batch of DNA extractions. For DNA isolation from contemporary samples, the protocols of the Sherlock AX kit (A&A Biotechnology, Gdynia, Poland), Genomic Mini (A&A Biotechnology) or Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) were applied. DNA was resuspended or eluted with sterile double-distilled water to a final volume of 100 μL and stored at −20 °C.

Mitochondrial DNA and microsatellite analysis

Eight fragments of the mitochondrial genome (mtDNA): control region (CR), cytochrome *b* (*cytb* 1–3), NADH dehydrogenase 5 (*ND5* 1–2), 12S rDNA and 16S rDNA, were amplified using primers from the literature or designed using PRIMER3 (Untergrasser *et al.*, 2012) (see Table S1 in Appendix S1 of the Supporting Information). These mtDNA regions were chosen to maximize the possibility of identifying population-specific polymorphisms that could be diagnostic for Baltic sturgeons.

In the case of *cytb*, for which more than one haplotype was found in ancient populations, amplification products were cloned using the PCR Cloning Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. A minimum of six clones per PCR product were sequenced using M13 universal primers and checked for consistency. The PCR-RFLP method described by Panagiotopoulou *et al.* (2014) was used to screen the contemporary samples for this polymorphism.

Eight microsatellite loci were analysed: *Aox*45 (King *et al.*, 2001), *Aox*C45, *Aox*D54, *Aox*D161, *Aox*D297, *Aox*D170, *Aox*D188 and *Aox*D234 (Henderson-Arzapalo & King, 2002) (see Appendix S1 for PCR reaction mixtures and amplification conditions). At least two independent amplifications were conducted for each ancient sample, alongside negative controls. For ambiguous alleles, some individuals were genotyped up to six times. Alleles were accepted if they appeared at least twice.

Statistical analysis

Population diversity indices, differentiation and structure

The obtained DNA sequences were aligned using BIOEDIT 7.0.9.0 (Hall, 1999). The median-joining network (Bandelt *et al.*, 1999) based on the 633-bp alignment of 193 sturgeon sequences was constructed using NETWORK 4.6.1 (Fluxus Engineering, Clare, UK; available at: http://www.fluxus-engineering.com/).

PEAK SCANNER 1.0 (Applied Biosystems, Carlsbad, CA, USA) was used to bin, score and output microsatellite data. MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004) was applied to each predefined population separately to test for genotyping errors caused by stuttering, null alleles and/or large-allele dropout using 1000 iterations and 95% confidence intervals. The number of alleles per locus (A), allelic richness (R) and inbreeding coefficients (F_{IS}) were obtained using FSTAT 2.9.3.2 (Goudet, 2002). The effective number of alleles (N_e) and number of private alleles (N_p) for each group were calculated with GENALEX 6.41 (Peakall & Smouse, 2006). Observed and expected heterozygosities (H_O and H_E, respectively) were estimated with ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). Deviations from Hardy–Weinberg equilibrium (HWE) were tested using the Markov chain Monte Carlo (MCMC) method (1000 dememorization steps, 1000 batches, 1000 iterations per batch) to estimate exact *P*-values (Guo & Thompson, 1992) as implemented in GENEPOP 4.0.10 (available at http://genepop.curtin.edu.au/; Raymond & Rousset, 1995). The statistical significance of

heterogeneity in allele frequency distribution between samples was tested using the genotypic differentiation test in GENEPOP with 1000 iterations of the MCMC and *G* exact test.

The allele-size randomization test (Hardy *et al.*, 2003) of SPAGEDI 1.1.b (Hardy & Vekemans, 2002), applying 10,000 permutations, was used to test if stepwise-like mutations have contributed significantly to the genetic differentiation among populations. Measures of genetic differentiation between populations were quantified by calculating pairwise Θ in FSTAT. To reduce the likelihood of type I errors over multiple tests, a sequential Bonferroni correction was applied. The allele-size-based measure of differentiation rho (an unbiased estimator of Slatkin's R_{ST}) was determined using R_{ST} CALC 2.2 (Goodman, 1997), implementing 1000 bootstrap pseudoreplicates and 1000 permutations. Factorial correspondence analysis (FCA) was performed in GENETIX 4.05.2 using the '3D-sur populations' command (Belkhir *et al.*, 2004) to visualize the spread of the variance for each population in genetic distance between individuals. Individual and population pairwise Nei's genetic distances (D_A) with 1000 bootstrap pseudoreplicates over loci were obtained with MICROSATELLITE ANALYSER (MSA) 4.05 (Dieringer & Schlötterer, 2003). A consensus neighbour-joining tree was constructed using PHYLIP 3.68 (Felsenstein, 1989) and drawn with MEGA 5.2.2 (Tamura *et al.*, 2011).

In order to identify the number of genetically different clusters (*K*) and examine how the predefined sturgeon populations corresponded to the clustering solution based on the microsatellite (msDNA) data, we used the Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) (see Appendix S1 for details of parameters). The most probable number of clusters (*K*) was determined using the ΔK method in STRUCTURE HARVESTER 0.6.8 (Earl & vonHoldt, 2012). The average coefficients of membership (*Q*) across the 20 runs for the optimal ΔK were computed using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007). DISTRUCT 1.1 (Rosenberg, 2004) was used to graphically display the genetic membership coefficient of individuals to each of the predefined clusters.

Inference of colonization time and population sizes by ABC approach

The estimation of colonization times and the sizes of founding populations was carried out on the microsatellite data by approximate Bayesian computation (ABC) (Beaumont *et al.*, 2002) as implemented in DIYABC 1.0.4.46 (Cornuet *et al.*, 2008; see Appendix S1 for parameters and detailed description).

RESULTS

We obtained mitochondrial sequences for 125 ancient and 199 contemporary sturgeon samples (out of 188 and 225 samples, respectively). Obtained sequences were deposited in GenBank (JX669880–JX669882; see Appendix S2). MsDNA analysis was performed for two ancient Baltic (pre-Roman and medieval) and five contemporary populations. For the other ancient samples, the quality of DNA did not allow us to obtain complete msDNA profiles. Detailed information regarding the number of haplotypes and individuals analysed for each of the selected markers is given in Table 1.

Haplotype attribution and mtDNA diversity

The mtDNA fragments were analysed to determine species-specific haplotypes and to estimate the genetic diversity of sturgeon populations. The sequences of all analysed fragments allowed for clear species distinction (see Appendix S2). In the Baltic population, four individuals, one dated to 2300–2100 years ago, one to 1100–700 years ago, and two to 300–200 years ago, carried the diagnostic *A. sturio* haplotype. The remaining individuals had characteristic *A. o. oxyrinchus* haplotypes.

Overall, the polymorphism of the mtDNA in the ancient Baltic population appeared to be very low. For the 12S rDNA, 16S rDNA and *ND5* mtDNA fragments, we recorded no variation within either *A. oxyrinchus* or *A. sturio* (see Appendix S2).

Within the control region, all Baltic sturgeons carried haplotype A (GenBank AF162716), typical for contemporary sturgeons from the St Lawrence and St John rivers. Only one contemporary specimen from the St John River had haplotype B (GenBank AF162721), characteristic of the Hudson River population (Wirgin *et al.*, 2000). Two different *cytb*2 haplotypes (H1 and H2) were found in the ancient Baltic population. Most individuals shared the same haplotype as contemporary *A. o. oxyrinchus*, but 24 out of 89 specimens bore a single G → A synonymous substitution (haplotypes H1 and H2; Fig. 2a), characteristic of *A. sturio* (GenBank AF006145). Haplotype H2 was not found among 167 specimens of *A. o. oxyrinchus* and *A. o. desotoi* from contemporary North American populations (Fig. 2a).

Genetic polymorphism of sturgeon populations based on the msDNA analysis

Altogether, 61 ancient and 212 contemporary specimens (out of 134 and 225, respectively) were analysed for variation at eight msDNA loci (Tables 1 & 2). In the case of the ancient samples, profiles with more than two missing loci were excluded from the data set. No evidence of allele dropouts or scoring errors were found in any of the eight loci. Null alleles with frequencies higher than 0.15 were observed only in the locus *Aox*D188 in the two ancient Baltic populations. The rate of allele amplification was also low in this case, and it was therefore excluded from further analysis.

All loci, with the exception of *Aox*C45 in the Hudson River population of *A. o. oxyrinchus*, were polymorphic in the studied populations (Table 2). The overall mean number of alleles per locus was 19.7, varying from 6 (*Aox*C45) to 36

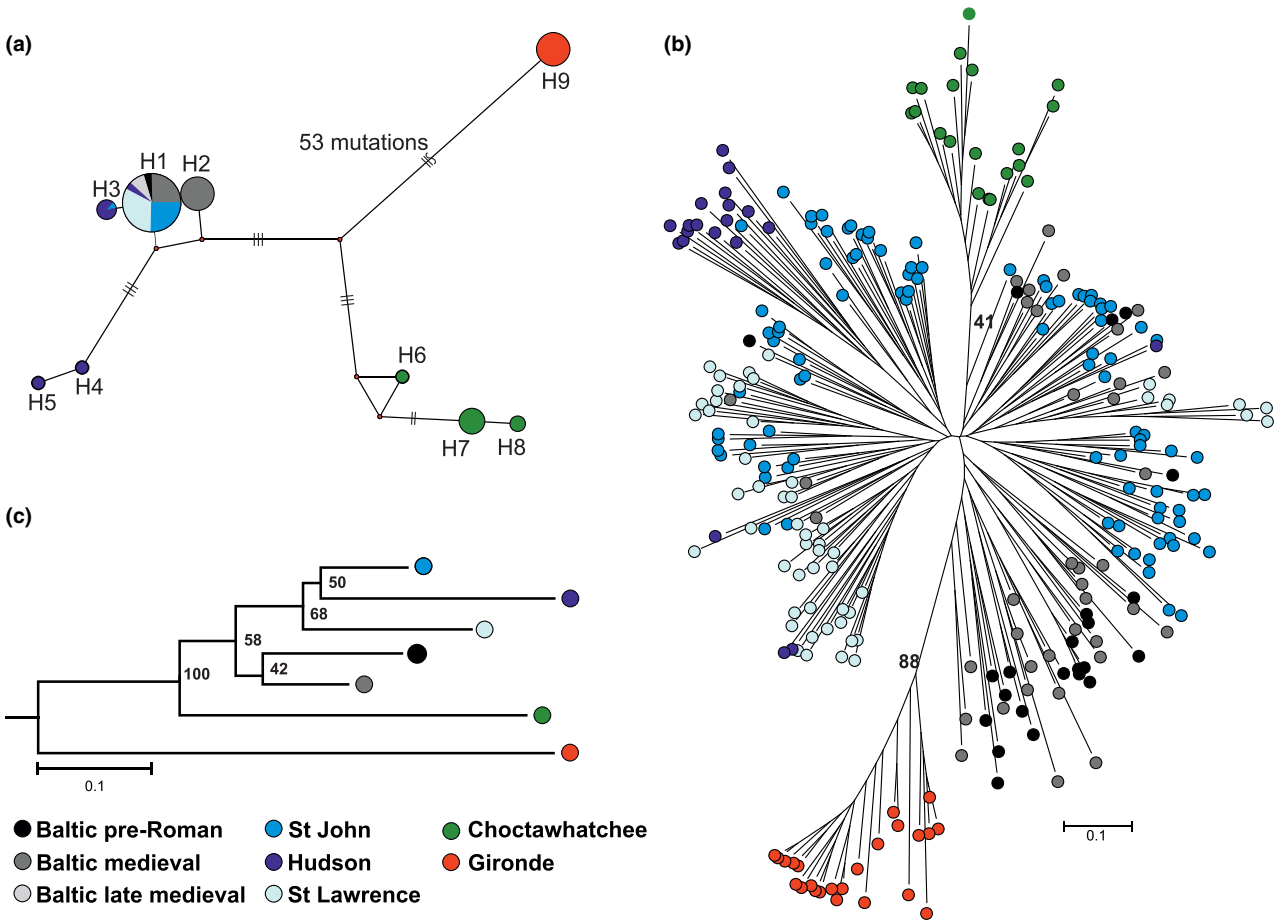


Figure 2 (a) Median-joining network based on 633 bp of control region and cytochrome *b* mtDNA sequences from 193 individuals of sturgeon (*Acipenser oxyrinchus* and *A. sturio*). Inferred missing haplotypes are presented as red dots. The area of the circles is proportional to the haplotype frequency, and the length of the connecting lines corresponds to the number of substitutions. Unrooted neighbour-joining tree based on msDNA Nei D_A distances between (b) pairs of individuals and (c) pairs of populations. Numbers at nodes indicate bootstrap support values. Individuals representing different populations as well as haplotypes are coloured according to their population of origin in a consistent way.

Table 2 Allelic variation and genetic diversity within ancient Baltic and contemporary North American and European populations of sturgeon (*Acipenser oxyrinchus* and *A. sturio*). *A*, mean number of alleles; *R*, allelic richness; N_p, number of private alleles; N_e, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; and F_{IS}, inbreeding coefficient. Significant deviations from Hardy–Weinberg equilibrium (HWE) are indicated by * for $P < 0.05$; n.s., not significant.

Population	*n*	*A*	*R*	N_p	N_e	H_O	H_E	F_{IS}	HWE
Baltic pre-Roman (1)	23	7.86	7.51	1.00	4.32	0.68	0.73	0.070	†
Baltic medieval (2)	38	9.29	7.84	1.43	4.35	0.66	0.70	0.064	†
St Lawrence (5)	54	7.29	5.53	1.00	3.23	0.63	0.63	0.004	*
St John (6)	94	11.00	7.07	1.43	4.32	0.62	0.65	0.037	n.s.
Hudson (7)	20	8.14	8.00	0.86	4.55	0.80	0.81	0.015	*
Choctawhatchee (8)	20	4.43	4.35	0.71	2.60	0.46	0.49	0.045	n.s.
Gironde (9)	24	3.43	3.38	1.43	2.46	0.62	0.58	−0.063	*

†Calculations not performed as samples constituted of individuals separated in time, not belonging to one generation.

(*Aox*D297) (data not shown). The lowest mean allelic richness, effective number of alleles and heterozygosity values were found in the *A. sturio* and *A. o. desotoi* collections. The most polymorphic populations were the two ancient Baltic collections and the contemporary Hudson River. The highest numbers of private alleles was observed in the St John River, Baltic medieval and *A. sturio* populations. The significant and positive inbreeding coefficients (F_{IS}) observed in the contemporary American populations could result either from inbreeding or from the Wahlund effect, a reduction in

heterozygosity caused by an existence of different breeding subunits and therefore substructure in the studied samples (Wahlund, 1928).

The negative and statistically significant F_{IS} value in the Gironde population indicates an excess of heterozygotes, most probably caused by bottleneck and/or sibling occurrence.

Structure and differentiation of sturgeon populations

The allelic distribution differed among all pairs of populations across all loci with highly significant *P*-values obtained using the *G* exact test ($P \approx 0$). Population pairwise Θ (Table 3) and the distribution of variance within and between populations as depicted by FCA (Fig. S1 in Appendix S3), indicated that the genetic distances within and between *A. o. oxyrinchus* populations (including two ancient Baltic populations) are much smaller than the distances between them and the populations of *A. sturio* and *A. o. desotoi*. The smallest genetic distance was observed between the two Baltic populations. The differentiation between *A. sturio* and the ancient Baltic populations was smaller than that among *A. sturio* and the contemporary *A. o. oxyrinchus* populations (Table 3, Fig. 2b–c). The highest divergence was observed between *A. sturio* and *A. o. desotoi*, and reached very high values ($\Theta = 0.44$). A similar pattern was obtained when the rho values were calculated, although the estimated genetic differentiation of *A. sturio* from other populations was twice as high as the Θ comparisons (Table 3). The results of allele-size randomization tests (data not shown) revealed that R_{ST} (based on the allele size differences and stepwise mutation model) was a better measure of genetic distance than F_{ST} (based on the allele identity information, assuming an infinite-allele mutation model) only in the case of *A. sturio* pairwise comparisons. This implies that mutations that arose during the long period of separation between the two species have contributed more than migration to the observed genetic differentiation (Hardy *et al.*, 2003).

STRUCTURE analysis of all the studied populations identified $K = 6$ as the uppermost hierarchical level of structure with the same subdivision and similar values of membership coefficients (*Q*) for both assumed models (Fig. 3; Fig. S2 and Table S2 in Appendix S3). Specimens from the Baltic population represented by collections at two different time points were assigned to a separate cluster with *Q*-values ranging from 75% to 90% depending on the allele frequency dependency model (Table S2a,b). The remaining 10–25% of ancestry belonged mainly to Canadian populations (St John and St Lawrence rivers). It is worth noting that, only in the case of the Baltic population, 1% of *Q* was found in the *A. sturio* cluster (Table S2). When the ancient Baltic populations were analysed separately from the contemporary ones, no apparent substructuring was visible (data not shown).

Past hybridization between *A. sturio* and *A. o. oxyrinchus*

The comparison of allelic distribution between populations of *A. o. oxyrinchus* (Baltic and North America) and *A. sturio*

Table 3 Pairwise estimates of Θ (F_{ST} analogue) below the diagonal and rho (R_{ST} analogue) above the diagonal among seven ancient Baltic and contemporary North American and European populations of sturgeon (*Acipenser oxyrinchus* and *A. sturio*). All values were significant ($P < 0.05$).

Θ / rho	Baltic pre-Roman	Baltic medieval	*A. o. oxyrinchus* St Lawrence	*A. o. oxyrinchus* St. John	*A. o. oxyrinchus* Hudson	*A. o. desotoi* Choctawhatchee	*A. sturio* Gironde
Baltic pre-Roman (1)		0.05	0.13	0.20	0.09	0.22	0.56
Baltic medieval (2)	0.04		0.08	0.10	0.04	0.16	0.57
St Lawrence (5)	0.14	0.11		0.10	0.09	0.18	0.66
St John (6)	0.08	0.07	0.11		0.08	0.25	0.65
Hudson (7)	0.14	0.14	0.17	0.12		0.30	0.60
Choctawhatchee (8)	0.28	0.25	0.31	0.28	0.32		0.72
Gironde (9)	0.27	0.29	0.37	0.34	0.32	0.44	

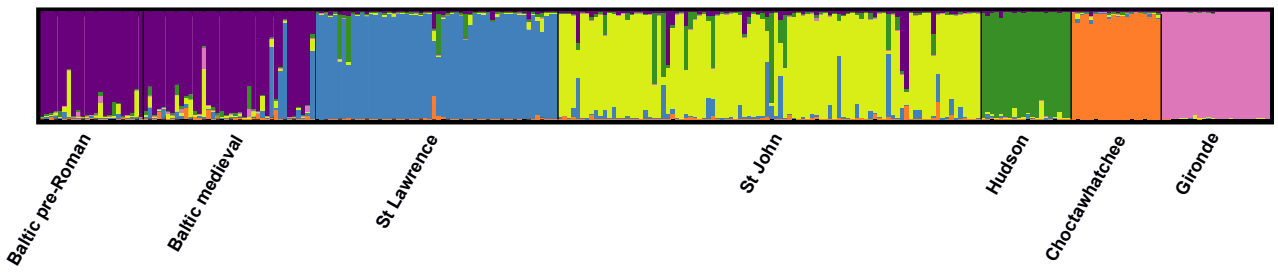


Figure 3 Summary plot of *Q*-values (membership coefficients) in admixture analysis of ancient and contemporary sturgeons (*Acipenser oxyrinchus* and *A. sturio*) using admixture model and correlated allele frequencies for $K = 6$. Each individual is represented by a vertical bar.

revealed that 50% of alleles were unique to each species (Fig. 4), and only 4% of alleles were common to all three sturgeon groups. The contemporary *A. o. oxyrinchus* and Baltic populations, on the other hand, shared 40% of observed alleles. The Baltic population had 5% of alleles in common with *A. sturio*, whereas the North American *A. o. oxyrinchus* populations shared only 1% with this species.

Allele ranges at the loci *Aox*D188, *Aox*D297 and *Aox*D161 were species-specific (Fig. 5). Baltic sturgeons exhibited a very broad range of allele sizes, exceeding the ranges observed in all remaining *A. o. oxyrinchus* and *A. sturio* populations. The calculated percentage of species-specific *A. sturio* alleles across the three diagnostic loci in the ancient Baltic populations treated jointly was 11.3% (Baltic pre-Roman, 14.7%; Baltic medieval, 9.4%).

In 52% of Baltic pre-Roman ($n = 12$) and 34% of Baltic medieval ($n = 13$) specimens, at least one allele specific to *A. sturio* was scored. This could be interpreted as evidence of past hybridization. Out of four Baltic specimens that had the *A. sturio* mtDNA haplotype, we obtained an msDNA profile for only one individual from the Baltic pre-Roman population. This individual revealed no *A. sturio* species-specific alleles and its membership coefficient (Q) to the *A. sturio* cluster equalled 0.4%, strongly suggesting that this individual was of hybrid origin.

Colonization of the Baltic Sea and North American rivers by *A. o. oxyrinchus*

The ABC method was applied to calculate the approximate time of the Baltic Sea colonization by *A. o. oxyrinchus*. Three different scenarios were considered, assuming that the Baltic Sea population had: (1) originated from the same ancestral population as the North American population; (2) diverged from the North American population; or (3) had its own ancestral population. The calculated time of colonization was 3140–3940 years ago for the first scenario, 5000–6600 years ago for the second, and 4280 years ago for the third (Fig. 6). The North American population included populations from the St John, St Lawrence and Hudson rivers, whereas the Baltic population was composed of two collections, the Baltic pre-Roman and Baltic medieval, separated by 1000 years. We have also performed calculations separately for the two Baltic populations, for the Hudson River, and for the St John and St Lawrence rivers, obtaining similar results as for the combined populations (Fig. 6). Whichever scenario was adopted, the calculations

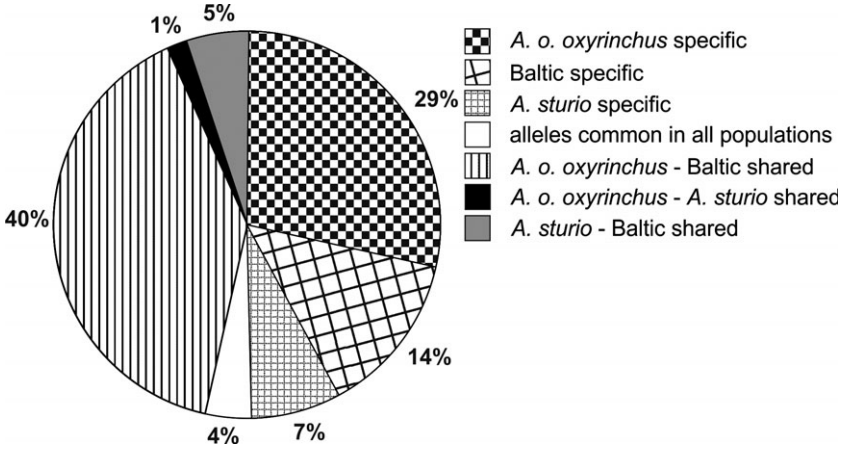


Figure 4 Comparison of the allelic distribution between the ancient Baltic population, *Acipenser oxyrinchus oxyrinchus*, treated as one group and *A. sturio* population in the seven analysed microsatellite loci.

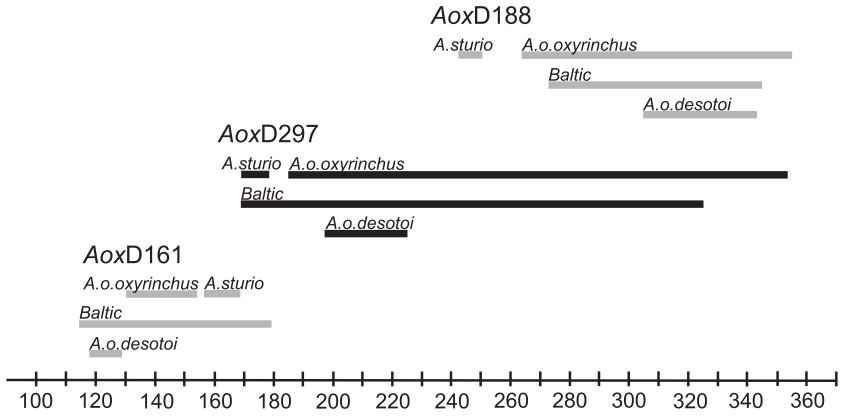
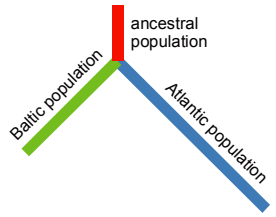
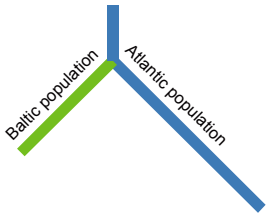


Figure 5 Observed allele size ranges in the three analysed groups of sturgeons at three species-specific microsatellite loci distinguishing *Acipenser o. oxyrinchus* and *A. sturio*.





	Model with common ancestral population for Baltic and Atlantic populations (scenario 1)			Model without common ancestral population for Baltic and Atlantic populations (scenario 2)			Model with ancestral population for Baltic population (scenario 3)
	Atlantic population:			Atlantic population:			
	Canada+Hudson	Canada	Hudson	Canada+Hudson	Canada	Hudson	
Estimated colonization time of the Baltic Sea	3140 [2500-5740]	3220 [2500-6000]	3940 [2540-7200]	5000 [2640-7700]	5440 [2760-7820]	6600 [3240-7960]	4280 [2560-7580]
Founder size	56 [7-98]	68 [14-100]	59 [8-99]	58 [9-99]	67 [13-99]	59 [8-99]	50 [6-98]

Figure 6 Estimated mean colonization time (years ago) and effective size of the founding population of sturgeon (*Acipenser oxyrinchus*) of the Baltic Sea for different colonization scenarios. Group 'Canada' represent both (5) St Lawrence River and (6) St John River populations; 'Atlantic' includes the (5) St Lawrence River, (6) St John River and (7) Hudson River populations. The 95% highest probability density (HPD) interval is shown in square brackets.

gave comparable results with respect to the estimated effective population size of the Baltic founder population (N_{ef}), varying from 50 to 68 individuals (Fig. 6).

To check whether sturgeons from Canadian rivers (St John and St Lawrence) could have served as founder populations for the Baltic, the ABC method was used to calculate the time of their colonization by *A. o. oxyrinchus*. Two different scenarios were tested. Under the first, the Canadian populations originated from the Hudson River population, whereas the second scenario assumes an unknown ancestral population. Under both scenarios, the obtained values of colonization times were around 9000 years ago for the St John River and 7400 years ago for the St Lawrence River, respectively. The size of the St John River founding population varied between 40 and 60 individuals for both scenarios tested (Fig. S3 in Appendix S3).

DISCUSSION

The notion that *A. sturio* was the only sturgeon species which historically inhabited Europe (Holčík, 2000) was overthrown by Ludwig *et al.* (2002, 2008), who analysed the mtDNA sequences from sturgeon remains dated to 1200–1000 yr BP from Germany's Baltic coast, and found that they belonged to *A. o. oxyrinchus*. Our results support these findings: out of 125 Baltic sturgeon specimens dating from at least 2000 years ago to the present day, only four possessed *A. sturio* mitochondrial haplotypes with all the remainder bearing haplotypes of *A. o. oxyrinchus*, implying its dominance in the Baltic through this period of time.

Acipenser o. oxyrinchus was extirpated in the Baltic in the 20th century, but populations still exist in North America. According to Ludwig *et al.* (2002), these populations were the founders of the *A. o. oxyrinchus* populations in Europe. We have performed analysis of genetic polymorphism and structure of the two Baltic collections dated to 2300–2100 years ago (Baltic pre-Roman) and 1100–700 years ago (Baltic medieval) and compared them with the North American populations. Pairwise estimates of genetic differentiation both among sturgeon populations and between individuals have shown that the genetic distances between *A. sturio*, *A. o. desotoi* and *A. o. oxyrinchus* are – as expected – two to three times greater than the distances between populations of *A. o. oxyrinchus*. The genetic divergence between populations of *A. sturio* and *A. oxyrinchus* was even more pronounced when values of rho, a more reliable index, were calculated, judging by the results of the randomization test. It is worth noting that the distances between the Baltic, St John River and St Lawrence River populations were smaller than those between St John River and Hudson River populations. This observation was supported by the results of the mtDNA analyses. Both Baltic and Canadian (St John and St Lawrence) specimens had the mtDNA control region haplotype A. Among Canadian sturgeons, we found only one individual with haplotype B, characteristic of the Hudson River and populations located further south (Wirgin *et al.*, 2002).

Our results show that *A. o. oxyrinchus* formed a highly polymorphic population in the Baltic at least 2000 years ago. All indices of genetic diversity for the species at this date are similar to or higher than those obtained for contemporary sturgeon populations. Both ancient Baltic populations shared a very similar level of polymorphism implying no drastic changes in their demography during the more than 1000 years between samples. We assume that, in order to have built up an independent and stable genetic structure by this time, the Baltic population must have been founded

much earlier. Our calculations, based on coalescence analysis of microsatellite variation, suggests that *A. o. oxyrinchus* was present in the Baltic at least 3000 and probably around 4000–5000 years ago. Additionally, 26% of specimens belonging to the ancient Baltic population bore the unique mtDNA haplotype H2, which, given its absence from contemporary North American populations, may have originated in Europe. This relatively frequent haplotype, assuming a very low mutation rate in Acipenseridae, supports a much earlier colonization time of the Baltic Sea by *A. o. oxyrinchus* than that suggested by Ludwig *et al.* (2002, 2008). This is in agreement with recent results of archaeological excavations in western and southern France, which showed that *A. o. oxyrinchus* was present in Western Europe at least 5000 years ago (Chassaing *et al.*, 2013).

The Baltic Sea opened for migrants *c.* 8000 yr BP, when the brackish Littorina Sea was formed and the English Channel and the Danish Straits circulations were established (Mörner, 1995). Conditions suitable for sturgeon colonization, however, only developed around 7500 years ago (Makowiecki, 2008). Our data suggest that the Canadian St John and St Lawrence rivers were colonized by *A. o. oxyrinchus* prior to this time, and therefore could have served as a source of the Baltic population.

It is difficult to tell whether the Baltic population emerged as a result of a single immigration event, or whether gene flow extended over a long period. Recently, a two-step colonization of the Baltic Sea via Western Europe was proposed (Chassaing *et al.*, 2013). Our results show that the effective size of the Baltic sturgeon founding population was around 50 individuals. Given the life-history traits of this species, the real number of sturgeons entering the Baltic Sea must have been much larger, and the colonization was probably a long-lasting process allowing the establishment of new spawning sites. The evidence for at least two stages in the colonization of the Baltic comes from studies on the level of introgression between *A. sturio* and *A. o. oxyrinchus* in the Baltic region. Hybridization between these two species has already been reported (Tiedemann *et al.*, 2007; Ludwig *et al.*, 2008; Chassaing *et al.*, 2013). Our results show that 5% of the scored alleles of the Baltic population are shared with *A. sturio*, whereas North American populations of *A. o. oxyrinchus* share only 1% with *A. sturio*. As North American and European sturgeon populations had no possibility to interbreed, alleles shared between them should be treated either as traces of ancestral polymorphism or as an effect of homoplasy. A total of 5% of *A. sturio* alleles in the Baltic population may therefore suggest introgression between these populations. In two of the three species-specific loci, *Aox*D161 and *Aox*D297, the Baltic sturgeons had alleles considered unique to *A. sturio*. At the third species-specific locus (*Aox*D188), all Baltic specimens possessed only alleles specific to *A. o. oxyrinchus*. We have evaluated introgression as the percentage of alleles unique to *A. sturio* across the three species-specific loci. In the ancient Baltic population, a value of 11% was obtained and, taking into account all three species-specific loci, we have found that *c.* 40% of specimens belonging to the ancient Baltic population (52% Baltic pre-Roman and 34% Baltic medieval), bore at least one allele of *A. sturio*. This suggests that hybridization was more intense in the ancient Baltic population than in the younger populations studied by Ludwig *et al.* (2008) and Chassaing *et al.* (2013), who observed 14% and 11% of introgressed specimens, respectively.

The absence of specimens that could be assigned to pure *A. sturio* implies that this species has never occurred in Baltic as a sustainable population. The high level of introgression of the Baltic population by *A. sturio* alleles and the lack of first-generation hybrids suggest that the Baltic was colonized by *A. o. oxyrinchus* specimens which had already hybridized with *A. sturio*. We agree with the scenario of Baltic Sea colonization proposed by Chassaing *et al.* (2013), in the first step of which, *A. o. oxyrinchus* appeared along the Atlantic coast of Western Europe, where it cohabitated with *A. sturio*. In the second step. it then entered the Baltic to found a population which began to evolve independently from *A. sturio* and the North American *A. o. oxyrinchus*.

CONCLUSIONS

Acipenser o. oxyrinchus colonized the Baltic Sea at least 2000 years ago according to dating of the samples and most probably 3000–5000 years ago as revealed by ABC analysis, and should be considered a native species in the region. The sturgeons from the St John and St Lawrence rivers in Canada are genetically closest to the extinct Baltic population. This is important for sturgeon restitution programmes in Poland and Germany. The introgression of *A. sturio* into the ancient Baltic *A. o. oxyrinchus* occurred before the Baltic Sea was colonized by the latter.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary materials and methods.
Appendix S2 Sequences obtained in this study.
Appendix S3 Supplementary results.

BIOSKETCHES

This work is part of the PhD thesis of **Danijela Popović,** which concerned genetic polymorphism of populations of four anadromous fish species, their conservation and restitution in Poland. One of the aims in this thesis was focused on ancient sturgeon population in the Baltic Sea. It is also part of the PhD study of **Hanna Panagiotopoulou**, who is involved in genetic analyses of the Atlantic sturgeon populations in terms of its restitution in Polish waters. Additionally, Hanna is interested in other aspects of sturgeon biology and biogeography, including past distribution ranges, polymorphism and hybridization of Atlantic and European sturgeon.

Author contributions: D.P, H.P., A.S. and P.W. conceptualized the project; S.K., D.M. and T.K. collected the samples; D.P., H.P., J.G. and A.S. performed experimental works; D.P., H.P., A.S., M.B. and P.M. analysed the data; and D.P., H.P., A.S., M.B., P.M. and P.W. wrote the manuscript.

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