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Continuous mitochondrial diversity of Danube sturgeon species over millennia: insights from ancient DNA

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Sturgeons, an iconic group of large fishes inhabiting marine and freshwater ecosystems, have historically had significant economic and cultural value, particularly prized for their meat and roe (caviar). Furthermore, sturgeons play a vital ecological role as mesopredators of prey fish and invertebrates. In the Danube basin, the European (*Acipenser sturio*) and fringebarbel or ship sturgeon (*Acipenser nudiventris*) are locally extinct, while beluga (*Huso huso*), Russian (*Acipenser gueldenstaedtii*), stellate (*Acipenser stellatus*) and sterlet (*Acipenser ruthenus*) sturgeon have significantly declined since the nineteenth century owing to overfishing, habitat loss and pollution. Archaeological evidence suggests that sturgeon exploitation along the Danube began as early as 11.6 thousand years before the present. This study explores the genetic landscape of Danube sturgeons over the past approximately 10 000 years using ancient DNA (aDNA) from archaeological specimens. Despite challenges posed by limited sample size, phylogenetic analyses of mitochondrial genomes and the D-loop reveal high genetic diversity within beluga, Russian and ship sturgeon populations. In addition, shared haplotypes between modern and historical specimens of both beluga and Russian sturgeons suggest genetic continuity within each species over time. This study provides, to our knowledge, the first high-coverage sequencing of ancient sturgeon mitogenomes establishing the foundation for future aDNA research.
This article is part of the theme issue 'Shifting seas: understanding deep-time human impacts on marine ecosystems'.

1. Introduction

Sturgeon populations of the Danube (collectively known as 'Danube sturgeons') belong to the family Acipenseridae and comprise five anadromous species—beluga (*Huso huso*), Russian (*Acipenser gueldenstaedtii),* stellate (*Acipenser stellatus),* European (*Acipenser sturio*), fringebarbel or ship sturgeon

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(*Acipenser nudiventris*)—which live in the Black Sea and spawn in the Danube River, and one potamodromous species—sterlet sturgeon (*Acipenser ruthenus*)—which completes its migrations entirely within the river system[1]. Note that a recent publication [1] proposed reclassifying the *Acipenser* species included in this study into the genus *Huso*. However, as this nomenclature has not yet been widely adopted, we have retained the traditional genus names throughout this work. Currently, the Russian, stellate and beluga sturgeon are considered *critically endangered* [2] and their migratory routes in the Danube have been confined to its lower section and the delta. The European and ship sturgeon are listed as *locally extinct* [2,3], even though for the latter some relict populations still survive in the eastern Black Sea [4,5]. The sterlet sturgeon has undergone a dramatic population decline over the past few centuries [6–8] and it is currently classified as an *endangered* species [2]. The first signs of sturgeon stock collapse were documented in the Hungarian stretches of the Danube from the sixteenth century [9–11], and by the eighteenth century excessive trade in European markets and the use of wooden weirs to trap beluga sturgeon led to a rapid decline in the amount of fish caught upstream of the Danube Gorges. Most detrimentally, the canalizations and alterations of the Danube bed that began in the nineteenth century [6,12], as well as the massive hydropower plant construction that commenced in the mid-twentieth century in the Danube Gorges [6,9,13] resulted in a loss of habitat and the sturgeon's natural spawning ground [9,14–16].

Human interactions with the Danube and its ecosystem extend further back than historical documentation with the first archaeological evidence of sturgeon fishing dating to the Epipaleolithic (15.0–11.5 thousand years before the present (kyr BP)) [17]. Archaeological remains of sturgeons in the middle section of the Danube during the Mesolithic and Neolithic (approx. 11.6–6.7 kyr BP) [11,18,19] suggest the continuity of fishing practises. In particular, during the Late Mesolithic (approx. 9.4–8.2 kyr BP) sturgeons were consumed in the Danube Gorges (Padina, Lepenski Vir, Vlasac, Kula, Schela Cladovei and Icoana) [18,20–22] and continued to be a part of the diet of the first agricultural communities along the Danube River during the Neolithic (approx. 8.0–6.7 kyr BP) as confirmed by sturgeon remains as well as δ^{13}C-depleted and δ^{15}N-enriched values in human bone collagen [18,19,23–26].

During the Roman and Late Roman periods (approx. 2.0–1.5 kyr BP), sturgeon represented a symbol of prestige for the elite, and specialized fishery activities are attested in the Danubian provinces [27,28]. From the Medieval period (approx. 1.1–0.4 kyr BP), a significant intensification of catches was recorded by historical sources, which described trade in the middle Danube region from fisheries located in the Danube Gorges [29] and in the Hungarian stretch of the Danube [30]. For instance, the fish assemblage at the medieval monastery of Studenica (Raška district, central Serbia) suggests that sturgeon was probably transported salted and in large barrels 200 km away, from the fishing spots of the Gorges [29].

Recent studies on mitochondrial DNA (mtDNA) markers and nuclear markers have investigated the genetic diversity of contemporary Danube sturgeon species in the Caspian, Azov and Black Seas [31–36] and, in some cases, detected hybridization events [7,32,37–39]. Given the long-term impact on the Danube's fish stock, the analysis of genetic variation through time would assist in evaluating the status of ancient populations under diverse fishing pressures, and contribute to a more effective management and conservation strategies of contemporary populations [40]. Shotgun sequencing methods of ancient DNA (aDNA) have enabled the detection of short DNA molecules from degraded fishbone samples [41–43], which combined with modern datasets can provide a better understanding of the genetic fluctuations caused by human exploitation [44,45]. However, because of their poor fossil preservation, only a few studies have used palaeogenomics to investigate ancient sturgeon assemblages. Phylogenetic analyses using mitochondrial and nuclear markers have focused on Atlantic (*Acipenser oxyrhynchus*) and European sturgeon (*A. sturio*) [46–51], white (*Acipenser transmontanus*) and green sturgeon (*Acipenser medirostris*) [52] and Adriatic populations of beluga sturgeon [53], while studies on ancient specimens of Russian, stellate, ship and sterlet sturgeon are still lacking.

Here, we apply aDNA shotgun sequencing on 25 sturgeon bone samples using a recently developed single stranded library preparation protocol (Santa Cruz) [41] to investigate the genetic diversity of five sturgeon species: the anadromous beluga (*H. huso*), stellate (*A. stellatus),* Russian (*A. gueldenstaedtii*), fringebarbel or ship (*A. nudiventris*) sturgeon and the freshwater sterlet (*A. ruthenus*) sturgeon. The samples were obtained from archaeological collections spanning the Mesolithic to the Early Neolithic (approx. 11.6–7.5 kyr BP), the Roman and Late Roman periods (approx. 2.0–1.5 kyr BP) and the Medieval period (approx. 1.1–0.4 kyr BP). We analyse the mitochondrial genome to resolve undetermined taxa and explore the relationships among all sturgeon specimens within the modern phylogenetic landscape. In addition, we investigate the haplotype identity of the historical samples of stellate, Russian and beluga sturgeon compared with extant populations from the Black, Azov and Caspian Seas to infer mtDNA lineage continuity and variability over a temporal and geographical scale.

2. Methods

(a) Archaeozoological analysis and sample collection

A total of 52 sturgeon bone specimens were selected from fish faunal assemblages from several archaeological sites in Serbia (figure 1a): Padina, Lepenski Vir and Kula (the Danube Gorges, eastern Serbia), spanning from the Mesolithic to the Early Neolithic (approx. 11.6–7.5 kyr BP); the Early Neolithic site of Donja Branjevina (approx. 8-7.6 kyr BP, West Bačka district, Vojvodina province, northern Serbia) the Roman-Late Roman (approx. 2.0–1.5 kyr BP) fortress of Diana-Karataš (Danube Gorges, eastern Serbia) and the Roman site of Viminacium (Braničevo district, eastern Serbia); the medieval sites (approx. 1.1–

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0.4 kyr BP) of Studenica (Raška district, central Serbia) and Dupljaja-Grad (South Banat district, Vojvodina province, northern Serbia). All sites are located within the Danube basin, however, Studenica is found at a considerable distance from the river, in the vicinity of the Ibar, a tributary of the West Morava, approximately 200 km south of the Danube.

The fish faunal assemblages, including the selected specimens, have previously been subjected to archaeozoological analysis (e.g. [18,19,27,29,54]). Their taxonomic identification (to the species, genus and family level) and the determination of the skeletal element have been undertaken by using the reference collection of the Laboratory for Bioarchaeology, Department of Archaeology, Faculty of Philosophy, University of Belgrade, as well as relevant atlases and publications (e.g. [55–58]).

The 52 specimens were subsampled at the Laboratory for Bioarchaeology, Department of Archaeology, Faculty of Philosophy, University of Belgrade, using a Dremel hand tool (Micro 8050) and following strict aDNA precautions to avoid cross-contamination [59,60]. When possible, the selection criteria included well-preserved, robust skeletal elements, both those that could be attributed to particular taxa with more certainty on the basis of morphology (e.g. maxillare, dentale) and those identified to the genus or family level only (e.g. fragmented cranial elements, bony scutes, pectoral spines). The criteria were also to ensure the representation of distinct individuals and diverse archaeological contexts from all of the aforementioned sites, including samples morphologically identified as *H. huso, A. gueldenstaedtii, A. stellatus, A. nudiventris* and specimens classified to the genus or family level (table 1; electronic supplementary material, table S5).

The age of the samples, ranging from the Mesolithic to the Medieval period, was estimated based on the absolute or relative dating of each archaeological context from which they originated (figure 1a; table 1).

(b) DNA extraction and library preparation

DNA extraction and library preparation were performed in the dedicated aDNA laboratories at the University of Copenhagen. First the bone surface was removed and cut into a chunk of about 600 mg, using a Dremel hand tool (Micro 8050) . This was then exposed to UV light for 5 min on each side before pulverizing it with a hammer.

All 52 samples were hereafter extracted for DNA, including two negative controls, using a double digestion protocol [43] and for samples with the presence of dirt surface or soil remnants in the chunks, we applied a double digestion with the addition of a mild bleach pre-treatment (electronic supplementary material, table S6) [42].

A total of 150 mg of bone material was pre-digested under gentle three-dimensional shaking for 1 h at 37°C. Following centrifugation and removal of the supernatant, the residual chunks were incubated for 48 hours at 37°C in a revolving mixer with a freshly produced digestion buffer and adding 25 µl of proteinase K after the first 24 h. The amount of material used for the mild bleach pre-treatment (150 mg × 2) was then combined after the second digestion. DNA was extracted using the silica-based High Pure Viral Nucleic Acid kit (Roche Diagnostics) and purified with MinElute columns (QIAGEN) according to manufacturer's instructions. DNA was then eluted in a 60 µl preheated Elution buffer and quantified in a Qubit 2.0 Fluorometer (Thermo Fisher).

Prior to library preparation, we selected a total of 25 extracts based on their DNA quantification (as measured by Qubit values; electronic supplementary material, table S6) while accounting for species representation. Single-stranded libraries were built using the Santa Cruz reaction following the procedure described in Kapp *et al*. [41]. Amplification cycles were determined using a quantitative polymerase chain reaction (qPCR), following Kapp *et al*. [41], modified to 30 cycles to reduce over-amplification. The chosen number of cycles given to each library were cutting threshold value +2 cycles. Unique indexes were added to each library in a 50 µl volume PCR containing 25 µl KAPA HiFi Uracil + mastermix (Roche) with 0.8 µM of each forward and reverse primer added to 21 µl of the library. An initial denaturation step at 98°C for 45 s was followed by a variable number of cycles of 98°C for 15 s, 65°C for 30 s and 72°C for 30 s. We then purified the libraries with magnetic beads (MagBio HighPrep PCR, MagBio Genomics Inc., USA) at a 1 : 1.6 ratio. Library concentration was assessed with a high sensitivity DNA assay on the Bioanalyzer 2100 (Agilent). Finally, all libraries were equimolarly pooled and sequenced on a NovaSeq 6000 150 bp paired end at the GeoGenetics Sequencing Core, University of Copenhagen.

(c) Mitochondrial DNA analyses

The sequences were processed following the Paleomix workflow [61], with a few modifications. We first trimmed the raw reads with AdapterRemoval (v2.3.0) [62], discarding reads shorter than 30 bp and bases with quality scores below 30, followed by collapsing of the remaining reads (*--mm 3 --minlength 30 --trimns --trimqualities --minquality 30 --collapse*) (electronic supplementary material, table S7). For the purpose of identifying the unresolved taxa at species level, we initially performed a competitive mapping of all collapsed reads per sample against the mitochondrial reference genome of the five sturgeon taxa historically present in the Danube River, including two Adriatic species (electronic supplementary material, table S8), using Bowtie2 [63]. We hereafter selected the best fit species for each sample based on the reference genome with the highest number of covered bases and depth of coverage (electronic supplementary material, table S9). Prior to mapping we edited the reference genomes in Geneious Prime 2023.0.1 (https://www.geneious.com) adding 50 bp to the ends of the sequence to improve the mapping coverage of the strand termini. The reference genomes detected as the best fit, were then realigned separately for each library using both BWA with *aln* algorithm [64] and Bowtie2 in—*very-sensitive* mode to compare their performance (electronic supplementary material, figure S1.2). Duplicates were removed with PicardTools (http://broadinstitute.github.io/picard/) and mapping statistics were visualized with BAMCOV (https://github.com/fbreitwieser/bamcov) (electronic supplementary material, figure S1.1). Deamination patterns were assessed with metaDMG [65] using *global* mode and *forward only* parameters (electronic supplementary material, figure S2.1).

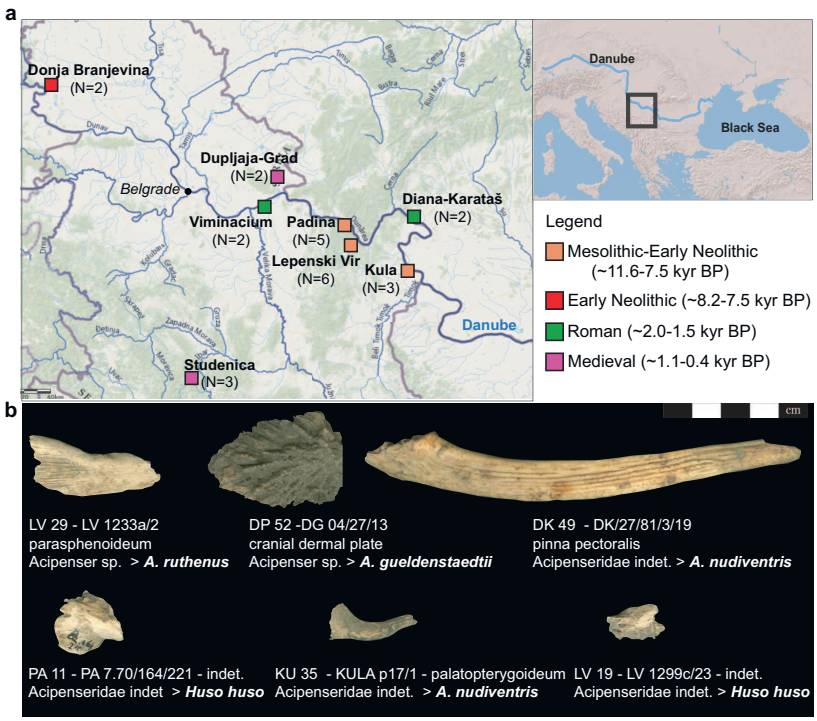

Figure 1. The provenance of samples and their aDNA identification. (a) The locations and number of samples used in this study for each archaeological site. (left map: created in ArcGIS using USGS, Esri Romania, HERE, Garmin, FAO, NOAA, USGS and World Hydro Reference Overlay; right map: created in QGIS v. 3.34.11 using Esri World Shaded Relief and river data from Natural Earth (1 : 10 m scale, 2024)). (b) The six Acipenserid bone fragments initially classified at the genus and family levels, with their species-level identification refined through mtDNA analysis.

We first investigated the phylogenetic relationships of the ancient sturgeon complete mitochondrial sequences with modern reference genomes from GenBank belonging to Adriatic and Pacific populations (figure 2; see the electronic supplementary material, table S10 for accessions). The consensus mitochondrial sequences were generated using ANGSD (v. 0.940) [66] with the parameters *-doFasta 2 -doCounts 1 -minQ 30 -minMapQ 30* to ensure high-confidence base calls and minimize sequencing errors. The alignment used consisted of the concatenated 13 protein-coding genes of the mitochondrial genome edited with Geneious Prime 2023.0.1 (ND1, ND2, COI, COII, ATP8, ATP6, COIII, ND3, ND4L, ND4, ND5, Cytb, ND6) for samples with a breadth of coverage of at least 90% and a depth of coverage above threefold (electronic supplementary material, figure S1.3). We excluded from this analysis the most divergent D-loop region and the transfer RNAs/12S ribosomal RNA owing to the variable number of tandem repeats within the species *H. huso* that can reduce the resolution of the phylogenetic reconstruction across different species [67,68]. Thereafter, we defined the optimal site model for the different partitions of our alignment using PartitionFinder (v. 2.1.1) with the greedy algorithm [69–71]. We then inferred a Bayesian phylogenetic tree using the Yule model in BEAST2 (v. 2.7.3) [72] with 50 million iterations and a strict clock. To further evaluate genetic diversity within the beluga sturgeon phylogeny (figure 3), we applied the same Bayesian approach, given also the larger number of samples available (eight). Finally, we performed a maximum likelihood analysis using IQ-Tree (v 1.6.12) [73] with 1000 replicates to compare the consistency of the results (electronic supplementary material, figures S3.1 and S3.2).

For the three key caviar-producing species—beluga, Russian and stellate sturgeon—we assessed the survival of ancient haplotypes in modern species by extracting the control region (D-loop) from our sequenced mitochondrial genomes, and using modern D-loop data from GenBank. In addition to the sequences generated in this study, we included a total of 118 sequences of beluga (*H. huso*) [32,33,74], 67 sequences of Russian (*A. gueldenstaedtii*) [32,33,75] and 214 sequences of stellate sturgeon (*A. stellatus*) [32–34,76] with a range of geographical provenances including species from the Caspian Sea, Sea of Azov, Black Sea and the Danube River (figure 4*a*; electronic supplementary material, table S11). We truncated the D-loop sequences of beluga, Russian and stellate sturgeon to 471 bp, 648 bp and 562 bp, respectively to ensure alignment consistency with the shorter sequences retrieved from GenBank. In addition, the undetermined bases in the ancient samples were masked across all sequences after performing the alignment with MAFFT (v. 7.490) to prevent the introduction of biases. We excluded the ship and sterlet sturgeon in this analysis owing to limited publicly available data [7,68,77]. A neighbour-joining tree was generated with Geneious using the Tamura-Nei model and 1000 replicates. The haplotypes were also organized in a network with Popart [78] using the median-joining network algorithm [79] (figures 3b and 4b-d; electronic supplementary material, figures S4.1 and S4.2).

Finally, we computed the number of haplotypes (h), nucleotide diversity (π), haplotype diversity (Hd) and number of polymorphic sites (S) with DnaSp v.6 [80] to investigate the genetic diversity in the control region between modern and ancient sequences of beluga, Russian and sterlet sturgeon.

Table 1. The specimens analysed in this study and the dating of their context. (Samples highlighted in bold indicate the mtDNA identification of specimens that were previously determined at the genus or family level, or those with different genetic identification from their morphological classification.)

sample ID	site	taxon (morphological ID)	taxon (mtDNA ID)	context
PA-1	Padina	*H. huso*	*H. huso*	Mesolithic
PA-4	Padina	*A. gueldenstaedtii*	***A. ruthenus***	Late Mesolithic
PA-6	Padina	*A. stellatus*	*A. stellatus*	Late Mesolithic
PA-11	Padina	Acipenseridae indet.	***H. huso***	Mesolithic–Neolithic Transition/Neolithic
PA-16	Padina	*A. gueldenstaedtii*	***A. nudiventris***	Early-Middle Neolithic
LV-17	Lepenski Vir	*H. huso*	*H. huso*	Early Mesolithic
LV-19	Lepenski Vir	Acipenseridae indet.	***H. huso***	Middle Mesolithic
LV-22	Lepenski Vir	*H. huso*	*H. huso*	Middle Mesolithic
LV-24	Lepenski Vir	*H. huso*	*H. huso*	Mesolithic–Neolithic Transition
LV-28	Lepenski Vir	*H. huso*	***A. gueldenstaedtii***	Mesolithic–Neolithic Transition
LV-29	Lepenski Vir	*Acipenser* sp.	***A. ruthenus***	Early Neolithic
KU-32	Kula	*H. huso*	*H. huso*	Late Mesolithic/Mesolithic-Neolithic Transition
KU-34	Kula	*A. stellatus*	*A. stellatus*	Late Mesolithic/Mesolithic-Neolithic Transition
KU-35	Kula	Acipenseridae indet.	***A. nudiventris***	Late Mesolithic/Mesolithic–Neolithic Transition
DB-38	Donja Branjevina	*A. gueldenstaedtii*	***A. nudiventris***	Early Neolithic
DB-39	Donja Branjevina	*A. stellatus*	*A. stellatus*	Early Neolithic
VM-43	Viminacium	*H. huso*	*H. huso*	Roman/Late Roman[a]
VM-44	Viminacium	*H. huso*	*H. huso*	Roman/Late Roman[a]
DK-47	Diana-Karataš	*H. huso*	*H. huso*	Late Roman
DK-49	Diana-Karataš	Acipenseridae indet.	***A. nudiventris***	Late Roman
DP-52	Dupljaja-Grad	*Acipenser* sp.	***A. gueldenstaedtii***	High Medieval
DP-53	Dupljaja-Grad	*A. gueldenstaedtii*	*A. gueldenstaedtii*	High Medieval
ST-54	Studenica	*A. gueldenstaedtii*	***H. huso***	Late Medieval
ST-55	Studenica	*H. huso*	*H. huso*	Late Medieval
ST-56	Studenica	*H. huso*	*H. huso*	Late Medieval

[a] The exact dating of the context is under process.

3. Results

(a) Genetic identification of sturgeon species

A total of 3 339 502 159 reads were trimmed for adapters and filtered for low-quality sequences, resulting in a total of 2 563 087 350 reads, of which 46 578 aligned to the various sturgeon mitogenomes (electronic supplementary material, table S9). The competitive mapping of each read to the mitochondrial genomes of the five Danubian species shows that BWA aligner enhances alignment efficiency compared with Bowtie2 (electronic supplementary material, figure S1.2), making it the preferred choice for the following alignment of mitochondrial genomes by species.

Our species identification of the archaeological specimens aligns with the morphological determination at the species level in 14 out of the 25 samples (table 1). In addition, we refine the classifications from genus and family levels to species level in a further six of the samples (figure 1b; table 1), while identifying a different species than originally recorded in five samples (table 1).

We subsequently performed a separate mapping of the ancient specimens to the mitochondrial genome reporting the highest coverage, resulting in a general (17 out of 25) breadth of coverage above 75% and a threefold to 30-fold average coverage (electronic supplementary material, figure S1.3 and table S9). Exceptionally, the majority of the samples (four out of six) from the site of Lepenski Vir present a breadth of coverage below 50% and a depth of coverage below 0.3-fold, suggesting poor preservation at this site (electronic supplementary material, figure S1.3 and table S9). However, an in-depth examination of the damage estimation and mean fragment length of samples with a mapping read count of greater than 100, reveals that the read lengths from Lepenski Vir resemble those from the site of Kula, while DNA degradation seems to be comparable with other Mesolithic and Neolithic samples from the same period (electronic supplementary material, figures S2.1 and S2.2).

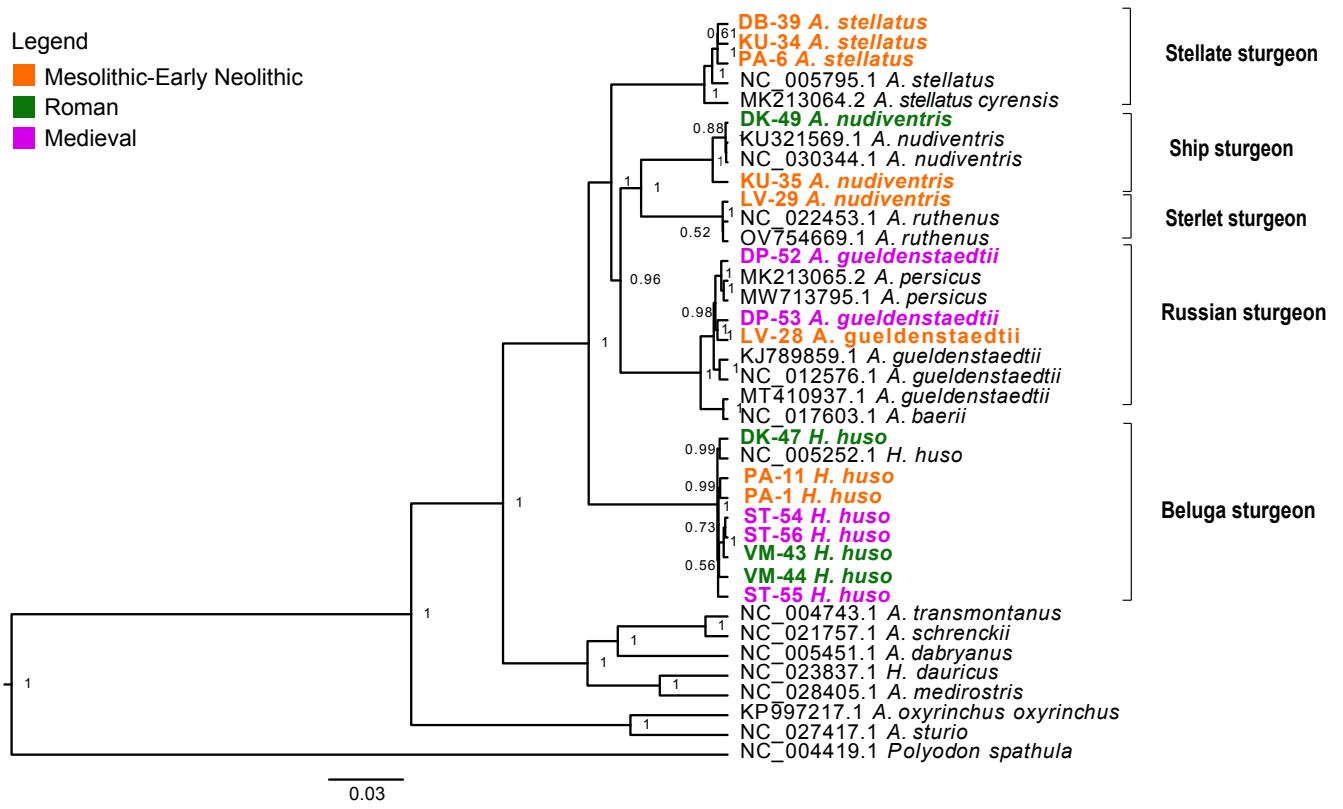

Figure 2. Phylogenetic placement of ancient and modern sturgeon species. Phylogenetic placement generated using Bayesian inference (Yule model) with 50 million iterations. Samples from this study are outlined in colours distinguishing different time periods. Abbreviations define archaeological sites (DK = Diana-Karataš, VM = Viminacium, DB = Donja Branjevina, PA = Padina, KU = Kula, LV = Lepenski Vir, ST = Studenica, DP = Dupljaja Grad).

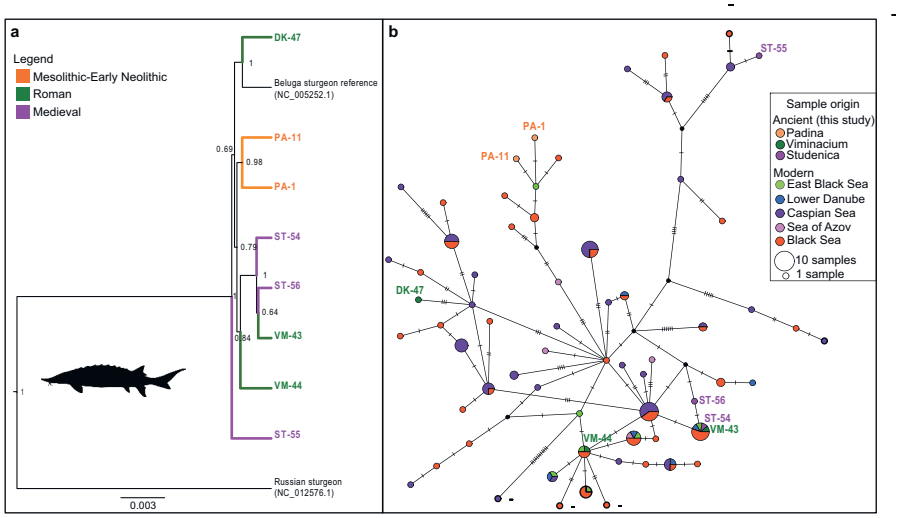

Figure 3. Phylogeny (a) and haplotype network (b) of the beluga sturgeon. (a) Phylogenetic relationship between the archaeological specimens of beluga sturgeon generated using Bayesian inference; using the Russian sturgeon as an outgroup. Ancient samples are coloured by period. (b) Haplotype network of beluga sturgeon generated in PopArt using the median-joining network algorithm. Abbreviations refer to archaeological sites (DK = Diana-Karataš, VM = Viminacium, PA = Padina, ST = Studenica).

(b) Phylogenetic placement

The phylogenetic analysis of ancient sturgeon specimens and modern populations revealed consistent results across both Bayesian and maximum likelihood methods (figure 2; electronic supplementary material, figure S3.1 and table S1 for excluded samples). Our tree topology (figure 2) places the beluga sturgeon as basal to all Danubian sequences, with monophyletic groups of the stellate sturgeon (*A. stellatus*) and the Russian sturgeon (*A. gueldenstaedtii*) forming two different clades. Sterlet sturgeon (*A. ruthenus*) clusters with ship sturgeon (*A. nudiventris*), both showing a common ancestor with the Russian sturgeon.

At the temporal scale, we observe differences between the clustering of the sturgeons associated with Mesolithic/Neolithic periods compared with the sturgeons from Roman-Late Roman and Medieval periods. While the Russian sturgeon phylogeny suggests complex relationships within the clade, in particular an individual from Lepenski Vir (LV-28), found in a Mesolithic–Neolithic Transition context clusters together with an individual from the medieval Dupljaja-Grad (DP-53), while another

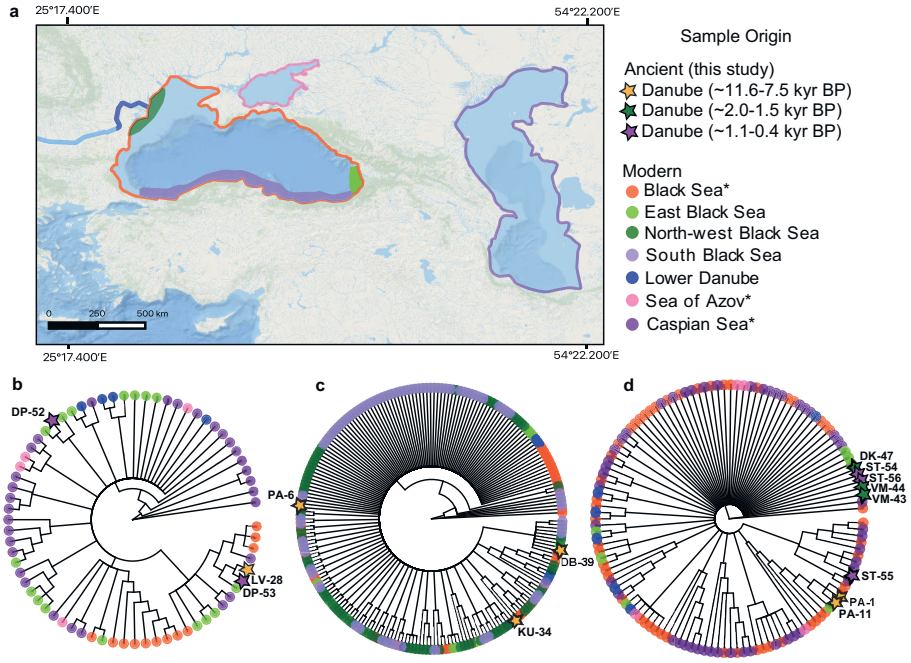

Figure 4. Spatial haplotype distribution of the Russian (b), stellate (c) and beluga (d) sturgeon. (a) The location of the modern sequences used to infer the neighbour-joining analysis (QGIS v. 3.34.11, Esri World Terrain Base Map. River data: Natural Earth, 1 : 10 m scale, 2024). An asterisk (*) indicates unavailable specific sample locations; in such cases, the general provenance from the basin is highlighted in colour. (b–d). Neighbour-joining trees showing phylogenetic relationships based on the D-loop of ancient specimens from the middle Danube settlements (DK = Diana-Karataš, VM = Viminacium, DB = Donja Branjevina, PA = Padina, KU = Kula, LV = Lepenski Vir, ST = Studenica, DP = Dupljaja Grad) within the modern genetic landscape [32–34,74–76].

specimen from the same site (DP-52) is placed on a sister branch clustering together with the Persian sturgeon (*Acipenser persicus*—MW713795.1, MK213065.2), a mitochondrial lineage of *A. gueldenstaedtii* present in the southern Caspian Sea and in the Black Sea [75]. The ship sturgeon from Kula (KU-35) found in a context dated to the Late Mesolithic/Mesolithic–Neolithic Transition forms a separate branch, while the Late Roman specimen from Diana-Karataš (DK-49) clusters with modern species from the Caspian and Azov Sea. The phylogeny of the beluga sturgeon exhibits similar placement by period. Considering the sample size (eight), we conducted a separate analysis, comparing the Bayesian with the maximum likelihood approach, however, both demonstrated consistency in our placements (figure 3*a*; electronic supplementary material, figures S3.1 and S3.2).

We observe that the samples from Padina (PA-1, PA-11) corresponding to broadly Mesolithic and Mesolithic–Neolithic transitional contexts respectively, cluster together. By contrast, the Roman/Late Roman specimens from Viminacium (VM-43, VM-44) and the medieval specimens from Studenica (ST-54, ST-55) form a separate clade as a sister branch to the Mesolithic–Neolithic samples with posterior support values of 0.79/1 (figure 3*a*). Additionally, an individual from the Roman site of Diana-Karataš (DK-47) forms a separate clade with the reference genome (NC_005252.1), situated at the basal node of the previously mentioned specimens with a support of 0.69/1. The single individual, ST-55, from Studenica occupies the basal node of the phylogeny, with full support (1/1), suggesting an earlier divergence within the clade.

(c) Haplotype identification

We next investigated the genetic variability of the three most exploited caviar-producing species—beluga, Russian and stellate sturgeon—by analysing haplotype diversity over a temporal and geographical scale. The phylogenetic analysis of the control region (D-loop) (figures 3b and 4); electronic supplementary material, figures S4.1 and S4.2) enabled direct comparison with recent studies from the Black Sea basin [32–34,74–76], revealing the genetic relationships between ancient and modern sturgeon populations. Two archaeological samples of Russian sturgeon (figure 4*b*) from the Mesolithic–Neolithic settlement of Lepenski Vir (LV-28) and the Medieval Dupljaja-Grad (DP-53) cluster together in a clade with individuals from the eastern Black Sea, a sister branch of other Caspian and Black Sea variants. This placement is supported by the haplotype network (electronic supplementary material, figure S4.1), which indicates that the two specimens show a limited genetic variability over time, differing by only three mutations (electronic supplementary material, figure S4.1). Another sample from the same site (DP-52) exhibits an identical haplotype to that of a specimen from the eastern Black Sea (MZ665971, haplotype 10) and also shows only three mutations when compared to modern populations in the eastern Black Sea and Caspian Sea.

The three samples of stellate sturgeon (figure 4*c*) seem to represent unique haplotypes within contemporary populations of the Black Sea basin. The Mesolithic and Neolithic specimens from Padina, Kula and Donja Branjevina (PA-6, KU-34, DB-39) do not cluster together in the tree, or in the haplotype network (electronic supplementary material, figure S4.2). This suggests a continuous high level of haplotype diversification and a lack of clear geographical differentiation within the Black Sea, consistent with previous findings regarding modern populations [33,53]. An exception is observed in the haplotypes from the southern part of the Black Sea, which is consistent with the newest findings [34]. However, none of our ancient sequences align with this cluster.

Table 2. mtDNA diversity of ancient and modern populations of Russian, stellate and beluga sturgeon. (Abbreviations: N = numbers of individuals, h = number of haplotypes, Hd = haplotype diversity; K = average number of differences, S = number of polymorphic sites, π = nucleotide diversity.)

species	samples	*N*	*h*	Hd	*K*	*S*	*π*
A. gueldenstaedtii	modern	67	45	0.98236	16.62958	102	0.03269
	ancient	3	3	1	10.66667	16	0.02164
	total	70	48	0.98385	16.6795	104	0.03272
A. stellatus	modern	214	148	0.99452	11.04124	220	0.01989
	ancient	3	3	1	11.33333	17	0.02042
	total	217	151	0.99467	11.03076	222	0.01988
H. huso	modern	118	83	0.99044	8.6038	101	0.01654
	ancient	8	5	0.96429	8.96429	25	0.02094
	total	126	88	0.98971	8.65168	103	0.01683

We also identify five new haplotypes (out of eight) among the archaeological specimens of beluga sturgeon (figures 3b and 4d) that were not detected in modern populations. These include the Mesolithic–Neolithic specimens from Padina (PA-1, PA-11), a Roman sample from Diana-Karataš (DK-47) and two from Medieval Studenica (ST-55, ST-56). By contrast, the phylogeny of the beluga sturgeon suggests the survival of ancient lineages within modern populations. A Roman specimen from Viminacium (VM-44) clusters with haplotypes identified in the Black Sea region (HUS24-MW183700, HUS73-MW183749) and specifically in individuals from its eastern area (haplotype 1-MZ665990). In addition, another Roman specimen (VM-43) and a medieval specimen (ST-54) exhibit identical variations with populations from the Lower Danube (Dan280-KF431844) and the Black Sea (HUS19-MW183695), including individuals from the eastern Black Sea (haplotype 5-MZ665994).

Lastly, the genetic differentiation analysis on the control region (table 2) conducted with DnaSp [80], indicates a high level of haplotype diversity in both ancient and modern populations for each of the three species (Hd = 0.96–1), alongside a low nucleotide diversity (π = 0.033–0.017). A high average number of differences (K) is detected in Russian (*A. gueldenstaedtii*: K = 16.7) and stellate sturgeon (*A. stellatus*: K = 11), indicating a marked genetic diversity. In comparison, we observe a slightly lower value (K = 8) for beluga sturgeon (*H. huso*), which may reflect a reduced genetic variability within this species.

4. Discussion

This work presents, to our knowledge, the first attempt to outline the spatiotemporal genetic landscape of the Danube sturgeon populations using the mitochondrial genetic diversity through aDNA. Our mitochondrial genome analysis successfully identifies acipenserid bone fragments of sterlet (*A. ruthenus*) and ship sturgeon (*A. nudiventris*), while also refining previous morphological determinations (table 1). Although based on a limited sample set, such an approach could greatly enhance our understanding of historical fish biodiversity and fishing strategies when applied to larger datasets [81]. Furthermore, these findings highlight the importance of integrating molecular techniques with morphological evaluations to address the primary challenges in archaeozoological studies. These include the difficulties in the determination of closely related species that can often arise from highly degraded or fragmented bones that exhibit a limited number of diagnostic markers, as was the case with our samples. In addition, the morphological identification is further hindered by the similarity of skeletal elements in members of the same genus or family (i.e. *Acipenser* or Acipenseridae), the occurrences of hybridization, the features of dermal bones covering of the head which exhibit a great degree of irregularity and asymmetry even within the same individual, poor survival rate of sturgeon remains owing to their largely cartilaginous skeleton [11,30,46,50–52,82,83], and the absence of adequate osteological reference collections [84] owing to the extirpation and global decline of these species of fish [7,68,77].

Our phylogenetic analysis, which used 13 protein-coding genes of mtDNA from the three most exploited caviar-producing species, aligns with the findings of Mugue *et al*. [68], which also places the beluga basal to all Danube sturgeons. The phylogenetic relationships within each clade are also consistent with the results obtained from the hypervariable control region of the D-loop. The Russian sturgeon specimens (figure 4*b*; electronic supplementary material, figure S4.1) from the Mesolithic–Neolithic Transitional settlement of Lepenski Vir (LV-28) and the Medieval Dupljaja-Grad (DP-53) share a common ancestry, even though they exhibit two distinct haplotypes. Their close genetic relationship implies a degree of continuity in the genetic makeup of these populations from the Mesolithic–Neolithic to the Medieval period, suggesting relative genetic stability over time. Additionally, another medieval sample from Dupljaja-Grad (DP-52) exhibits the same haplotype as modern individuals from the eastern Black Sea (electronic supplementary material, figure S4.1) and differs by only three mutations from Caspian Sea specimens (electronic supplementary material, figure S4.1 and table S4). Our whole mitogenome phylogenetic analysis groups this sample with the Persian sturgeon (*A. persicus*), a subspecies found in the southern Caspian Sea. Despite the inability to detect the full genetic diversity of the Persian sturgeon population through mtDNA analysis [33,70,71,78], our findings indicate a close genetic relationship between the ancient specimens and contemporary populations in the Caspian Sea. This points to historical interactions and potential gene flow, probably dating back to pre-Holocene periods when the Black Sea and Caspian Sea were connected (see further details in the following paragraphs).

The beluga sturgeon's phylogeny (figures 3 and 4d) deveals a high degree of similarity among samples from the Roman site of Viminacium (VM-43) and the Medieval Studenica (ST-54), both clustering with modern individuals from the Black Sea and

lower Danube regions. Notably, most samples exhibit unique haplotypes (five) with a Roman specimen (DK-47) and a medieval specimen (ST-55) showing a significant number of mutations (7–16) compared with the other historical samples. This may suggest long-term genetic divergence of distinct lineages persisting through the Roman and Medieval periods. In particular, ST-55 is part of the assemblage from the monastery of Studenica, located around 200 km from the Danube. There is neither historical nor ecological evidence to indicate that sturgeons were migrating to the area of the Ibar or its tributaries. Instead, written records explicitly indicate that sturgeon consumed during religious celebrations were imported from the Danube [29]. The basal position of ST-55 in the phylogenetic tree (figure 3*a*), along with the high number of mutations in the D-loop (figure 3*b*), suggests that it may represent an ancestral lineage contributing to the high genetic variability observed in modern beluga populations inhabiting the Black Sea.

Conversely, we found no evidence of common ancestry between modern populations of stellate sturgeons from the Mesolithic and Neolithic periods. Instead, our analysis identifies unique haplotypes that are closely related to individuals from the northwestern Black Sea and southern Black Sea, indicating a high level of variation in these populations (figure 4*c*; electronic supplementary material, figure S4.2).

Overall, the analysis of the D-loop does not reveal clear geographical differentiation within the three species of beluga, stellate and Russian sturgeon. This finding is consistent with previous studies on contemporary populations, which have identified a complex structure characterized by hundreds of unique haplotypes present among the Caspian, Black and Azov Seas. These results suggest multiple radiation events probably resulting from past reiterated admixture processes [33,34,53,85]. The high genetic variability observed and a lack of geographical differentiation may be influenced by multiple introgression events driven by palaeo-geographical changes in the last 5 million years, during which the Black Sea, Sea of Azov and the Caspian Sea were interconnected. The most recent contact occurred during the end of the Pleistocene period (approx. 15.0–11.0 kyr BP) [53,86].

Our results on genetic indices of variation (table 2) confirm a high genetic diversity within ancient and modern sturgeon populations (Hd = 0.96–1), although there are minimal differences at the nucleotide level (π = 0.033–0.017). This could indicate either a recent divergence between different sturgeon populations, or a population bottleneck [33]. Despite the small sample size, our analysis of the control region supports this trend in the ancient specimens of stellate sturgeon (three) which exhibit unique genetic lineages, as well as to some extent for the Russian (two) and beluga (five) sturgeons. The identification of unique lineages and potential evidence of genetic bottlenecks in ancient sturgeon populations may also reflect anthropogenic factors such as fishing pressure and habitat modification. Overfishing and targeted fishing practises, especially those aimed at larger, migratory species like the beluga and Russian sturgeons, probably have accelerated stock decline. For example, it is recorded that the beluga sturgeon population had a dramatic reduction upstream of the Danube Gorges owing to the intensification of catches starting from the sixteenth century as well as the use of massive weirs and the expansion of the caviar trade in European markets, well attested from the eighteenth century [9,11,87,88]. In addition to fishing pressure, riverbed alterations and dam construction have significantly impacted sturgeon migratory routes [6,11,13,16], further contributing to the isolation of gene pools and limited gene flow between populations. This geographical isolation could have reinforced local adaptation and genetic differentiation while also reducing genetic exchange across populations. Alternatively, the limited number of common haplotypes observed may also reflect undetected lineages rather than a decline in genetic diversity, and could potentially increase with the inclusion of additional reference genomes. Furthermore, incorporating a larger number of archaeological sturgeon samples may be necessary to evaluate the homogeneity of the results and to mitigate biases related to the sample selection. Alternative approaches, such as the use of nuclear markers [36,56] could provide valuable insights into past hybridization events and offer a more detailed understanding of the demographic history of sturgeons in the Black Sea. Lastly, for species like *A. nudiventris*, which has been extirpated from the Black Sea, archaeological specimens may represent a unique source of genetic data, offering valuable insights into the species' past distribution and population structure.

Recent advancements in single-stranded DNA preparation protocols, combined with high-throughput sequencing, have effectively enhanced the detection and analysis of highly fragmented aDNA molecules [41,89]. These advances hold great promise for future research on archaeological sturgeon specimens, facilitating a more precise reconstruction of past population structures. Our findings highlight the importance of integrating archaeological and genetic data to enhance our understanding of the historical dynamics of aquatic species, particularly their ecological roles and the implications of human exploitation.

Ethics. All fossil specimens in this study are part of the permanent zooarchaeological collection at the Laboratory of Bioarchaeology, in the Department of Archaeology, Faculty of Philosophy, University of Belgrade, which also issued the relevant permits. Their inventory numbers are provided in the electronic supplementary material, table S5.

Data accessibility. The sequencing data analysed in this article (fastq files) and the associated sample metadata are deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB81886. The mitochondrial genome sequences used in the phylogenetic analyses have been submitted to GenBank under accession numbers PV485394-PV485410. The codes, metadata and scripts used in this study are available on Zenodo, with a permanent doi [90].

Supplementary material is available online [91].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. G.Z.: formal analysis, investigation, validation, visualization, writing—original draft, writing—review and editing; M.L.M.: investigation, validation, writing—review and editing; IŽ.: resources, validation, writing—review and editing; K.P.: resources, writing—review and editing; S.V.: resources, writing—review and editing; T.M.: resources, writing—review and editing; D.M.: resources, writing—review and editing; N.M.: resources, writing—review and editing; D.R.: resources, writing—review and editing; D.O.: validation, writing—review and editing; M.T.O.: conceptualization, funding acquisition, methodology, project administration, supervision, writing—original draft; M.W.P.: conceptualization, funding acquisition, methodology, project administration, supervision, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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References

1. Brownstein CD, Near TJ. 2025 Toward a Phylogenetic Taxonomy of Sturgeons (Acipenseriformes: Acipenseridae). *Bulletin of the Peabody Museum of Natural History* **66**, 3–23. (doi:10.3374/014.066.0101)
2. IUCN. 2024 *The IUCN Red List of Threatened Species*. See https://www.iucnredlist.org (accessed February 2024).
3. Congiu L, Gessner J, Ludwig A. 2023 IUCN Red List reassessment reveals further decline of sturgeons and paddlefishes. *Oryx* **57**, 9–10. (doi:10.1017/s0030605322001260)
4. Beridze T, Edisherashvili T, Anderson C, Scheele F. 2021 Ship sturgeon rediscovered in the Rioni River in Georgia. *Oryx* **55**, 9–9. (doi:10.1017/s0030605320001003)
5. Jarić I, Lenhardt M, Cvijanović G, Ebenhard T. 2009 *Acipenser sturio* and *Acipenser nudiventris* in the Danube - extant or extinct? *J. Appl. Ichthyol*. **25**, 137–141. (doi:10.1111/j.1439-0426.2009.01227.x)
6. Schmid M, Haidvogl G, Friedrich T, Funk A, Schmalfuss L, Schmidt-Kloiber A, Hein T. 2023 The Danube: on the environmental history, present, and future of a great European river. In *River culture: life as a dance to the rhythm of the waters* (ed. KM Wantzen), pp. 637–671. Paris, France: UNESCO Publishing, xiii + 901. (doi:10.54677/INTF8577)
7. Ludwig A, Lippold S, Debus L, Reinartz R. 2009 First evidence of hybridization between endangered sterlets (*Acipenser ruthenus*) and exotic Siberian sturgeons (*Acipenser baerii*) in the Danube River. *Biol. Invasions* **11**, 753–760. (doi:10.1007/s10530-008-9289-z)
8. Friedrich T. 2018 Danube sturgeons: past and future. In *Riverine ecosystem management. science for governing towards a sustainable future, vol. 8. aquatic ecology series* (eds S Schmutz, J Sendzimir), pp. 507–518. Cham, Switzerland: Springer. (doi:10.1007/978-3-319-73250-3_26)
9. Schletterer M, Kuzovlev VV, Zhenikov YN, Tuhtan JA, Haidvogl G, Friedrich T, Górski K, Füreder L. 2018 Fish fauna and fisheries of large European rivers: examples from the Volga and the Danube. *Hydrobiologia* **814**, 45–60. (doi:10.1007/s10750-017-3370-5)
10. Reinartz R. 2002 *Sturgeons in the Danube river. Biology, status, conservation literature study*. Bezirk Oberpfalz: Landesfischereiverband Bayern, Austria: International Asociation for Danube Research (IAD).
11. Bartosiewicz L, Bonsall C, Şişu V. 2008 Sturgeon fishing in the middle and lower Danube region. In *The Iron Gates in prehistory: new perspectives,* vol. 1893. *BAR International series* (eds C Bonsall, V Boroneanț, I Radovanović), pp. 39–54. Oxford, UK: Archaeopress.
12. Schiemer F, Guti G, Keckeis H, Staras M. 2004 Ecological status and problems of the Danube River and its fish fauna: a review (eds RL Welcomme, T Petr). In *RAP, Int. Symp. on the Management of Large Rivers for Fisheries, 11-14 Feb, vol. I*, *Phnom Penh, Cambodia*, pp. 273–300. Bangkok, Thailand: FAO; MRC.
13. Fürst H. 2006 The Hungarian-Slovakian conflict over the Gabčíkovo-Nagymaros Dams: an analysis. *Intermarium* **6**, 1–15. https://ciaotest.cc.columbia.edu/olj/int/int_0602b.pdf
14. Wiesner C, Schotzko N, Cerny J, Guti G, Davideanu G, Jepsen N. 2008 *Technical report with results from the fish sampling and analyses from the Joint Danube Survey 2007*. (eds I Liška, F Wagner, J Slobodník), ICPDR – International Commission for the Protection of the Danube River, Vienna, Austria.
15. Liška I, Wagner F, Sengl M, Deutsch K, Slobodník J. 2015 Joint Danube Survey 3: a comprehensive analysis of Danube water quality. Final report, International commission for the protection of the Danube river (ICPDR), Vienna, Austria.
16. Friedrich T. 2013 *Sturgeons in Austrian rivers: historic distribution, current status and potential for their restoration,* vol. 5, *World Sturgeon Conservation Society: special publication*, p. 80. Norderstedt, Germany: BoD–Books on Demand.
17. Borić D. 2011 Adaptations and transformations of the Danube Gorges foragers (c. 13,000–5500 cal. BC): an overview. In *Beginnings – new research in the appearance of the Neolithic between northwest Anatolia and the Carpathian Basin. Papers of the International workshop 8th - 9th April 2009, Istanbul, Turkey* (eds R Krauß, D Ciobotaru, B Horejs), p. 231. Rahden/Westf, Germany.: Verlag Marie Leidorf GmbH.
18. Živaljević I. 2017 Ribolov na Đerdapu u ranom *holocenu* :(10.-6. milenijum pre n.e.). Универзитет у Београду, Филозофски факултет. https://nardus.mpn.gov.rs/handle/123456789/8257.
19. Živaljević I, Dimitrijević V, Stefanović S. 2017 Faunal remains from Kula, a Mesolithic-Neolithic site at the exit of the Danube Gorges (Serbia). In *From hunter-gatherers to farmers: human adaptations at the end of the Pleistocene and the first part of the Holocene* (eds M Margarit, A Boroneant), pp. 113–133. Targoviște, Romania: Editura Cetatea de Scaun. (Papers in Honour of Clive Bonsall).
20. Bălăşescu A, Radu V, Boroneanţ A, Bonsall C. 2021 Mesolithic Icoana revisited (II) – a reappraisal of the faunal remains. In *Scripta praehistorica. miscellanea in honorem mariae bitiri dicata. târgoviște: supplementum i to materiale și cercetări arheologice,* vol. 1 (eds R Dobrescu, A Boroneanț, A Doboş), pp. 373–412. Târgoviște, Romania: Editura Cetatea de Scaun. (doi:10.3406/mcarh.2021.2218)
21. Bartosiewicz L, Boroneant V, Bonsall V, Stallibrass S. 2001 New data on the prehistoric fauna of the Iron Gates: a case study from Schela Cladovei, Romania. In *From the Mesolithic to the Neolithic. Proceedings of the International Archaeological Conference held in the Damjanich museum of Szolnok, 22-27 September 1996* (eds R Kertesz, J Makkay), pp. 15–21. Budapest, Hungary: Archaeolingua.
22. Bartosiewicz L, Bonsall C, Boroneanţ V, Stallibrass S. 1995 Schela Cladovei: a preliminary review of the prehistoric fauna. In *Mesolithic miscellany,* vol. 16(2) (ed. C Bonsall), pp. 2–19. Edinburgh, UK: University of Edinburgh: Department of Archaeology.
23. Borić D, Price TD. 2013 Strontium isotopes document greater human mobility at the start of the Balkan neolithic. *Proc. Natl Acad. Sci. USA* **110**, 3298–3303. (doi:10.1073/pnas.1211474110)
24. Nehlich O, Borić D, Stefanović S, Richards MP. 2010 Sulphur isotope evidence for freshwater fish consumption: a case study from the Danube Gorges, SE Europe. *J. Archaeol. Sci*. **37**, 1131–1139. (doi:10.1016/j.jas.2009.12.013)
25. Grupe G, Mikic Z, Peters J, Manhart H. 2003 Vertebrate food webs and subsistence strategies of Meso-and Neolithic populations of central Europe. In *Documenta archaeobiologiae, vol. 1* (eds G Grupe, J Peters). Munich, Germany: Bavarian State Collection for Anthropology (SNSB-SAM).
26. Jovanović J, de Becdelièvre C, Stefanović S, Živaljević I, Dimitrijević V, Goude G. 2019 Last hunters–first farmers: new insight into subsistence strategies in the Central Balkans through multi-isotopic analysis. *Archaeol. Anthropol. Sci*. **11**, 3279–3298. (doi:10.1007/s12520-018-0744-1)
27. Zivaljevic I, Vukovic-Bogdanovic S, Bogdanovic I. 2019 Ad palatinas acipensem mittite mensas: fish remains from viminacium. In *Starinar 69* pp. 183–202 (doi:10.2298/sta1969183z)
28. Long HS, Scholfield AF. 1959 *Aelian. on the characteristics of animals. volume ii: books 6-11*, p. 89, vol. **53**. Cambridge: Loeb Classical Library 448, MA: Harvard University Press.

29. Živaljević I, Marković N, Maksimović M. 2019 Food worthy of kings and saints: fish consumption in the medieval monastery Studenica (Serbia). *Anthropozoologica* **54**, 201. (doi:10.5252/anthropozoologica2019v54a16)
30. Bartosiewicz L, Bonsall C. 2008 Complementary taphonomies: medieval sturgeons from Hungary. In *Archéologie du poisson, 30 ans d'archéo-ichtyologie au cnrs. hommage aux travaux de jean desse et de nathalie desse-berset, xxviiie rencontres internationales d'archéologie et d'histoire d'Antibes* (eds P Béarez, S Grouard, B Clavel), pp. 35–45. Antibes, France: APDCA.
31. Kohlmann K, Kersten P, Geßner J, Eroglu O, Firidin S, Ciorpac M, Taflan E, Suciu R. 2018 Validation of 12 species-specific, tetrasomic microsatellite loci from the Russian sturgeon, *Acipenser gueldenstaedtii*, for genetic broodstock management. *Aquac. Int*. **26**, 1365–1376. (doi:10.1007/s10499-018-0290-y)
32. Beridze T, Boscari E, Scheele F, Edisherashvili T, Anderson C, Congiu L. 2022 Interspecific hybridization in natural sturgeon populations of the Eastern Black Sea: the consequence of drastic population decline? *Conserv. Genet*. **23**, 211–216. (doi:10.1007/s10592-021-01413-7)
33. Dudu A, Georgescu SE, Costache M. 2014 Molecular analysis of phylogeographic subspecies in three ponto-caspian sturgeon species. *Genet. Mol. Biol*. **37**, 587–597. (doi:10.1590/s1415-47572014000400016)
34. Holostenco DN *et al*. 2022 Genetic population structure of the critically endangered stellate sturgeon (*Acipenser stellatus*) in the Black Sea basin: implications for conservation. *Aquat. Conserv.* **32**, 1926–1939. (doi:10.1002/aqc.3892)
35. Jenneckens I, Meyer JN, Debus L, Pitra C, Ludwig A1461-0248. 2000 Evidence of mitochondrial DNA clones of Siberian sturgeon, *Acipenser baerii*, within Russian sturgeon, *Acipenser gueldenstaedtii*, caught in the River Volga. *Ecol. Lett*. **3**, 00179.x. (doi:10.1046/j.1461-0248.2000.00179.x)
36. Luo D, Li Y, Zhao Q, Zhao L, Ludwig A, Peng Z. 2019 Highly resolved phylogenetic relationships within Order Acipenseriformes according to novel nuclear markers. *Genes* **10**, 38. (doi:10.3390/genes10010038)
37. Havelka M, Boscari E, Sergeev A, Mugue N, Congiu L, Arai K. 2019 A new marker, isolated by dd[scp]RAD[/scp] sequencing, detects Siberian and Russian sturgeon in hybrids. *Anim. Genet*. **50**, 115–116. (doi:10.1111/age.12733)
38. Boscari E, Barmintseva A, Pujolar JM, Doukakis P, Mugue N, Congiu L. 2014 Species and hybrid identification of sturgeon caviar: a new molecular approach to detect illegal trade. *Mol. Ecol. Resour*. **14**, 489–498. (doi:10.1111/1755-0998.12203)
39. Boscari E *et al*. 2017 Fast genetic identification of the Beluga sturgeon and its sought-after caviar to stem illegal trade. *Food Control* **75**, 145–152. (doi:10.1016/j.foodcont.2016.11.039)
40. Barrett JH. 2019 An environmental (pre)history of European fishing: past and future archaeological contributions to sustainable fisheries. *J. Fish Biol*. **94**, 1033–1044. (doi:10.1111/jfb.13929)
41. Kapp JD, Green RE, Shapiro B. 2021 A fast and efficient single-stranded genomic library preparation method optimized for ancient DNA. *J. Hered*. **112**, 241–249. (doi:10.1093/jhered/esab012)
42. Boessenkool S, Hanghøj K, Nistelberger HM, Der Sarkissian C, Gondek AT, Orlando L, Barrett JH, Star B. 2017 Combining bleach and mild predigestion improves ancient [scp]DNA[/scp] recovery from bones. *Mol. Ecol. Resour*. **17**, 742–751. (doi:10.1111/1755-0998.12623)
43. Damgaard PB, Margaryan A, Schroeder H, Orlando L, Willerslev E, Allentoft ME. 2015 Improving access to endogenous DNA in ancient bones and teeth. *Sci. Rep*. **5**, 11184. (doi:10.1038/srep11184)
44. Hofman CA, Rick TC, Fleischer RC, Maldonado JE. 2015 Conservation archaeogenomics: ancient DNA and biodiversity in the Anthropocene. *Trends Ecol. Evol*. **30**, 540–549. (doi:10.1016/j.tree.2015.06.008)
45. Martínez-García L *et al*. 2021 Historical demographic processes dominate genetic variation in ancient Atlantic cod mitogenomes. *Front. Ecol. Evol*. **9**. (doi:10.3389/fevo.2021.671281)
46. Ludwig A, Arndt U, Debus L, Rosello E, Morales A. 2009 Ancient mitochondrial DNA analyses of Iberian sturgeons. *J. Appl. Ichthyol*. **25**, 5–9. (doi:10.1111/j.1439-0426.2009.01184.x)
47. Ludwig A, Arndt U, Lippold S, Benecke N, Debus L, King TL, Matsumura S. 2008 Tracing the first steps of American sturgeon pioneers in Europe. *BMC Evol. Biol*. **8**, 221. (doi:10.1186/1471-2148-8-221)
48. Pagès M, Desse-Berset N, Tougard C, Brosse L, Hänni C, Berrebi P. 2009 Historical presence of the sturgeon *Acipenser sturio* in the Rhône basin determined by the analysis of ancient DNA cytochrome b sequences. *Conserv. Genet*. **10**, 217–224. (doi:10.1007/s10592-008-9549-6)
49. Tiedemann R, Moll K, Paulus K, Scheer M, Williot P, Bartel R, Gessner J, Kirschbaum F. 2007 Atlantic sturgeons (*Acipenser sturio*, *Acipenser oxyrinchus*): American females successful in Europe. *Naturwissenschaften* **94**, 213–217. (doi:10.1007/s00114-006-0175-1)
50. Chassaing O, Desse-Berset N, Duffraisse M, Hughes S, Hänni C, Berrebi P. 2013 Palaeogenetics of western French sturgeons spotlights the relationships between *Acipenser sturio* and *Acipenser oxyrinchus*. *J. Biogeogr*. **40**, 382–393. (doi:10.1111/j.1365-2699.2012.02785.x)
51. Nikulina EA, Schmölcke U. 2016 Reconstruction of the historical distribution of sturgeons (Acipenseridae) in the eastern North Atlantic based on ancient DNA and bone morphology of archaeological remains: implications for conservation and restoration programmes. *Divers. Distrib*. **22**, 1036–1044. (doi:10.1111/ddi.12461)
52. Grindle ED, Rick TC, Dagtas ND, Austin RM, Wellman HP, Gobalet K, Hofman CA. 2021 Green or white? Morphology, ancient DNA, and the identification of archaeological North American Pacific Coast sturgeon. *J. Archaeol. Sci*. **36**, 102887. (doi:10.1016/j.jasrep.2021.102887)
53. Boscari E *et al*. 2021 Defining criteria for the reintroduction of locally extinct populations based on contemporary and ancient genetic diversity: the case of the Adriatic beluga sturgeon (*Huso huso*). *Divers. Distrib.* **27**, 816–827. (doi:10.1111/ddi.13230)
54. Mladenović T. 2022 Ekonomija naselja od 11. do sredine 13. veka u jugozapadnom Banatu: arheozoološki pristup. PhD thesis, Faculty Philosophy, University of Belgrade, Belgrade, Serbia.
55. Bartosiewicz L, Takács I. 1997 Osteomorphological studies on the great sturgeon (*Huso huso* Brandt). *Archaeofauna 6* 9–16. (doi:10.15366/archaeofauna1997.6.001)
56. Radu V. 2005 *Atlas for the identification of bony fish bones from archaeological sites*. Bucureşti, Romania: Editura Contrast.
57. Bakhshalizadeh S, Bani A, Abdolmalaki S. 2013 Morphometric features in the pectoral fin spine of the great sturgeon (*Huso huso*) and the ship sturgeon (*Acipenser nudiventris*, Lovetsky, 1828). *J. Appl. Ichthyol*. **29**, 730–732. (doi:10.1111/jai.12223)
58. Hilton EJ, Dillman CB, Paraschiv M, Suciu R. 2022 Cranial morphology of the stellate sturgeon, *Acipenser stellatus* Pallas 1771 (Acipenseriformes, Acipenseridae), with notes on the skulls of other sturgeons. *Acta Zool*. **103**, 57–77. (doi:10.1111/azo.12355)
59. Llamas B, Valverde G, Fehren-Schmitz L, Weyrich LS, Cooper A, Haak W. 2017 From the field to the laboratory: controlling DNA contamination in human ancient DNA research in the high-throughput sequencing era. *STAR: Sci. Technol. Archaeol. Res.* **3**, 1–14. (doi:10.1080/20548923.2016.1258824)
60. Gilbert MTP, Bandelt HJ, Hofreiter M, Barnes I. 2005 Assessing ancient DNA studies. *Trends Ecol. Evol*. **20**, 541–544. (doi:10.1016/j.tree.2005.07.005)
61. Schubert M *et al*. 2014 Characterization of ancient and modern genomes by SNP detection and phylogenomic and metagenomic analysis using PALEOMIX. *Nat. Protoc*. **9**, 1056–1082. (doi:10.1038/nprot.2014.063)
62. Schubert M, Lindgreen S, Orlando L. 2016 AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Res. Notes* **9**, 88. (doi:10.1186/s13104-016-1900-2)

63. Langmead B, Salzberg SL. 2012 Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359. (doi:10.1038/nmeth.1923)
64. Li H, Durbin R. 2009 Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**, 1754–1760. (doi:10.1093/bioinformatics/btp324)
65. Michelsen C, Pedersen MW, Fernandez-Guerra A, Zhao L, Petersen TC, Korneliussen TS. 2022 metaDMG – a fast and accurate ancient DNA damage toolkit for metagenomic data. *BioRxiv* (doi:10.1101/2022.12.06.519264)
66. Korneliussen TS, Albrechtsen A, Nielsen R. 2014 ANGSD: analysis of next generation sequencing data. *BMC Bioinform*. **15**, 356. (doi:10.1186/s12859-014-0356-4)
67. Dadkhah K, Mianji GR, Barzegar A, Farhadi A. 2021 Characterization of the mitochondrial *Huso huso* genome with new aspects into its organization with presence of tandem repeat in the 12srRNA and tRNA-glu. *BMC Ecol. Evol.* **23**, 55. (doi:10.21203/rs.3.rs-894057/v1)
68. Mugue N, Barmintseva A, Schepetov D, Shalgimbayeva G, Isbekov K. 2016 Complete mitochondrial genomes of the critically endangered ship sturgeon *Acipenser nudiventris* from two seas. *Mitochondrial DNA B Resour.* **1**, 195–197. (doi:10.1080/23802359.2016.1144103)
69. Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. 2017 PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol*. **34**, 772–773. msw260. (doi:10.1093/molbev/msw260)
70. Lanfear R, Calcott B, Ho SYW, Guindon S. 2012 PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol. Biol. Evol*. **29**, 1695–1701. (doi:10.1093/molbev/mss020)
71. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010 New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol*. **59**, 307–321. (doi:10.1093/sysbio/syq010)
72. Drummond A, Bouckaert R. 2015 *Bayesian evolutionary analysis with BEAST*. Cambridge, UK: Cambridge University Press. (doi:10.1017/CBO9781139095112)
73. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ. 2016 W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res*. **44**, W232–W235. (doi:10.1093/nar/gkw256)
74. Doukakis P, Birstein V, Salle R. 2005 Intraspecific structure within three caviar-producing sturgeons (*Acipenser gueldenstaedtii*, *A. stellatus*, and *Huso huso*) based on mitochondrial DNA analysis. *J. Appl. Ichthyol.* **21**, 457–460. (doi:10.1111/j.1439-0426.2005.00652.x)
75. Birstein VJ, Doukakis P, DeSalle R. 2000 Polyphyly of mtDNA lineages in the Russian sturgeon, *Acipenser gueldenstaedtii*: forensic and evolutionary implications. *Conserv. Genet.* **1**, 81–88. (doi:10.1023/A:1010141906100)
76. Doukakis P, Birstein VJ, Ruban GI, DeSalle R. 1999 Molecular genetic analysis among subspecies of two Eurasian sturgeon species, *Acipenser baerii* and *A. stellatus*. *Mol. Ecol*. **8**, S117–27. (doi:10.1046/j.1365-294x.1999.00816.x)
77. Beridze T, Scheele F, Edisherashvili T, Anderson C. 2022 Rare but not gone: a relict population of the Black Sea ship sturgeon *Acipenser nudiventris* persists in the Rioni River, Georgia. *Diversity* **14**, 1102. (doi:10.3390/d14121102)
78. Leigh JW, Bryant D. 2015 popart: full-feature software for haplotype network construction. *Methods Ecol. Evol*. **6**, 1110–1116. (doi:10.1111/2041-210x.12410)
79. Bandelt H, Forster P, Rohl A. 1999 Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol*. **16**, 3748.
80. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A. 2017 DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol*. **34**, 3299–3302. (doi:10.1093/molbev/msx248)
81. Živaljević I, Askeyev V, Galimova DN, Askeyev OV. 2021 Size estimations of sturgeons (Acipenseridae) from the Mesolithic-Neolithic Danube Gorges (eds D Borić, D Antonović, B Mihailović). In *Foraging Assemblages, Proc. of the 9th Conf. on the Mesolithic in Europe*, Belgrade, Serbia, pp. 422–427. Serbian Archaeological Society, Belgrade, Republic of Serbia & The Italian Academy for Advanced Studies in America, Columbia University, New York, USA.
82. Brinkhuizen DC, Clason AT. 1986 Features observed on the skeletons of some recent European Acipenseridae: their importance for the study of excavated remains of sturgeon. In *Fish and archaeology. studies in osteometry, taphonomy seasonality and fishing methods. bar international series 294* (eds DC Brinkhuizen, AT Clason), pp. 18–32. Oxford, UK: BAR Publishing.
83. Desse-Berset N. 1994 Sturgeons of the Rhône during Protohistory in Arles (6th-2nd century BC) (ed. W Van Neer). In *Fish Exploitation In The Past. Proceedings of the 7th Meeting of the ICAZ Fish Remains Working Group, 274*, pp. 81–90. Tervuren, Belgium: Royal Museum for Central Africa.
84. Volynskyi T, Kovalchuk O. 2024 Sturgeons in materials from archaeological sites of Ukraine: a review. *Int. J. Osteoarchaeol*. **34**, e3310. (doi:10.1002/oa.3310)
85. Doukakis P, Babcock EA, Pikitch EK, Sharov AR, Baimukhanov M, Erbulekov S, Bokova Y, Nimatov A. 2010 Management and recovery options for Ural River beluga sturgeon. *Conserv. Biol*. **24**, 769–777. (doi:10.1111/j.1523-1739.2010.01458.x)
86. Tomilova AA, Lyubas AA, Kondakov AV, Vikhrev IV, Gofarov MY, Kolosova YS, Vinarski MV, Palatov DM, Bolotov IN. 2020 Evidence for Plio-Pleistocene duck mussel refugia in the Azov Sea river basins. *Diversity* **12**, 118. (doi:10.3390/d12030118)
87. Szilágyi M. 1995 *A tiszai halászat. az eszközök és fogási módok történeti változásai. (Fishing in the Tisza. Historical changes in equipment and methods)*. Budapest, Hungary: Akadémiai Kiadó. See http://real-eod.mtak.hu/15926/.
88. Marsigli L. 1726 *Danubius pannonico-mysicus, observationibus geographicis, astronomicis, hydrographicis historicis, physicis perlustratus*. Amsterdam, The Netherlands: The Hague.
89. Orlando L *et al*. 2021 Ancient DNA analysis. *Nat. Rev. Methods Prim.* **1**, 1–26. (doi:10.1038/s43586-020-00011-0)
90. Zampirolo G. 2024 Continuous-mitochondrial-diversity-of-Danube-sturgeons (v0.1). *Zenodo.* (doi:10.5281/zenodo.14051975)
91. Zampirolo G, McCarthy ML, Živaljević I, Penezić K, Vuković S, Mladenović T *et al*. 2025 Supplementary material from: Continuous mitochondrial diversity of Danube sturgeon species over millennia: insights from ancient DNA. Figshare. (doi:10.6084/m9.figshare.c.7834300)