



NEW METHODS FOR OLD DNA
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PHYLOGEOGRAPHY OF THE EUROPEAN STURGEON

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STURGEONS

(ACIPENSERIFORMES: ACIPENSERIDAE)

- Acipenseriformes – 25 sturgeon and 2 paddlefishes
- Evolved in the Holarctic and are widely distributed through the whole North America and Eurasia
- They are known for their large size and for caviar
- „Living fossils” – they have undergone little morphological changes since they originated around 400 Mya

SEA STURGEON LINEAGE

- The sea sturgeon lineage – two sister species which separated around 60 Mya

European sturgeon – *A. sturio* L. 1758

Atlantic sturgeon – *A. oxyrinchus* Mitchill, 1815

- Large and long-lived fish with a life span up to 60 years
- Anadromous - spending several years at the sea before maturing and returning to natural born rivers to spawn



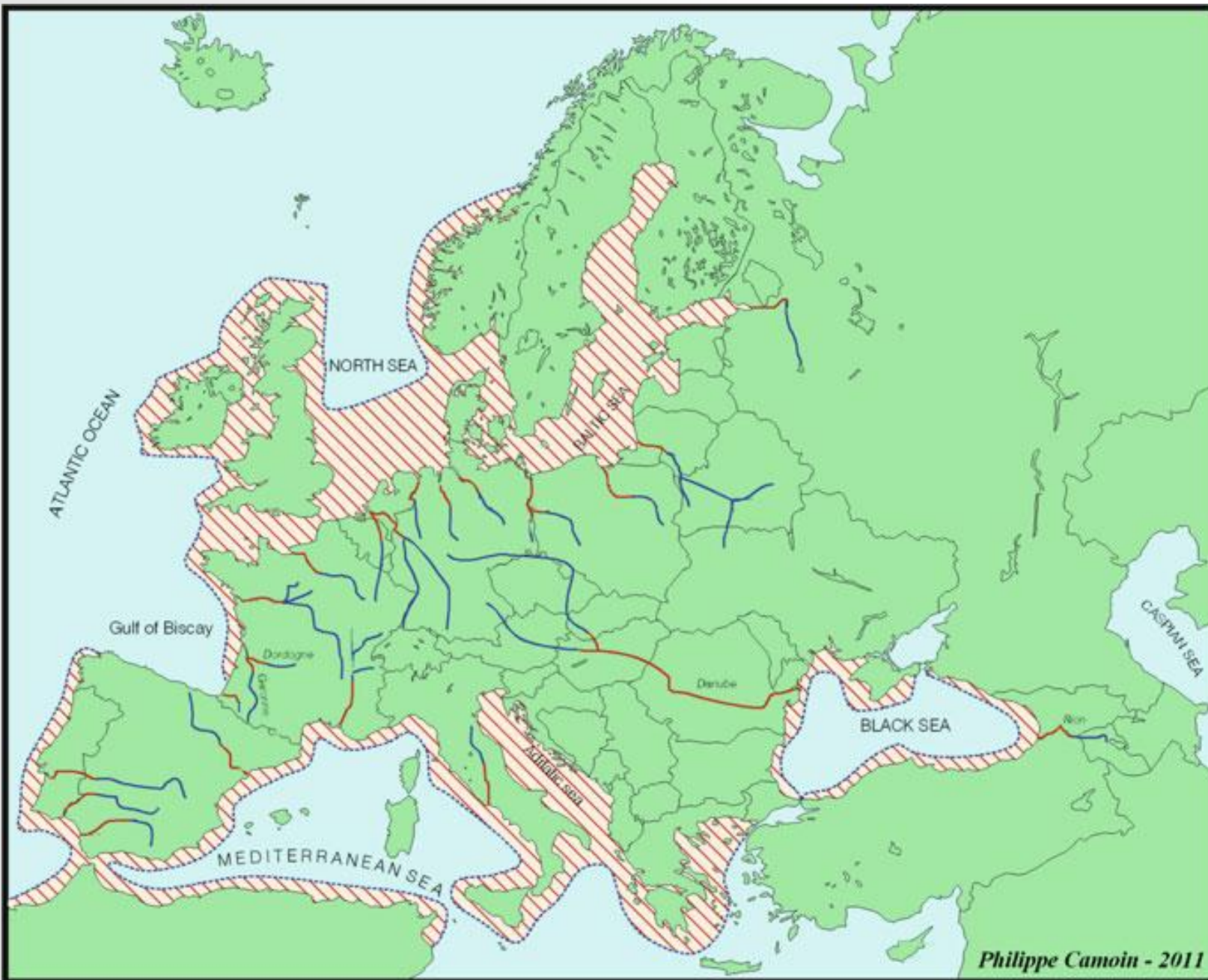
<http://www.livt.net/Clit/Ani/Cho/Ost/Gan/gan008.jpg>



<http://www.tier-fotos.eu/1024/atlantischer-stoer-acipenser-oxyrinchus-2615.jpg>

HISTORICAL *A. STURIO* DISTRIBUTION

- It was present along the whole Atlantic coast in Europe, Mediterranean and Black Sea
- Spawned in all large European rivers
- Economically important

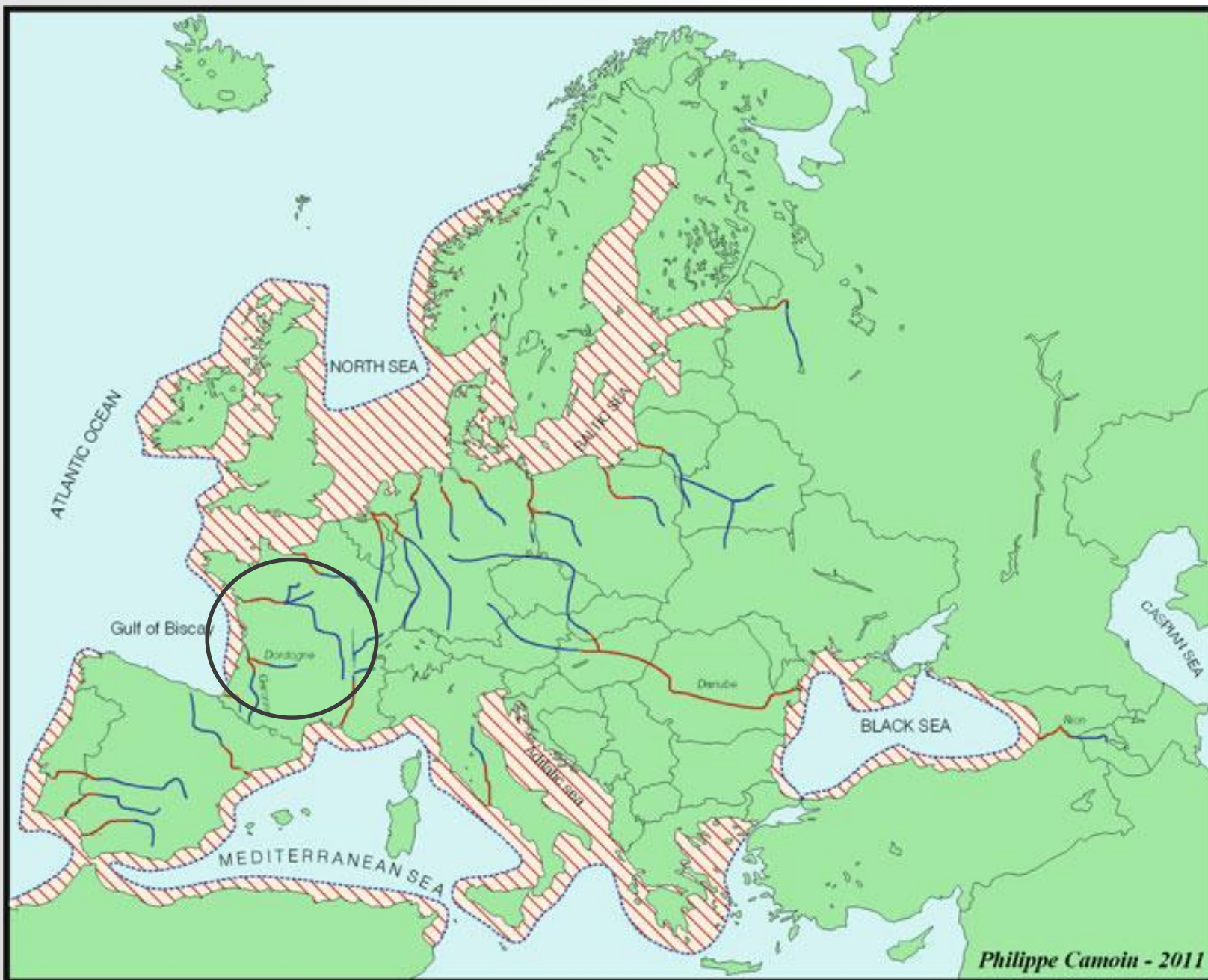


Williot, P et al (2011) Brief Introduction to Sturgeon with a Special Focus on the European Sturgeon, *Acipenser sturio* L. 1758. Biology and conservation of the European sturgeon *Acipenser sturio* L. 1758: the reunion of the European and Atlantic sturgeons (ed. by P. Williot, E. Rochard, N. Desse-Berset, F. Kirschbaum and J. Gessner), pp. 3–11. Springer, Berlin.

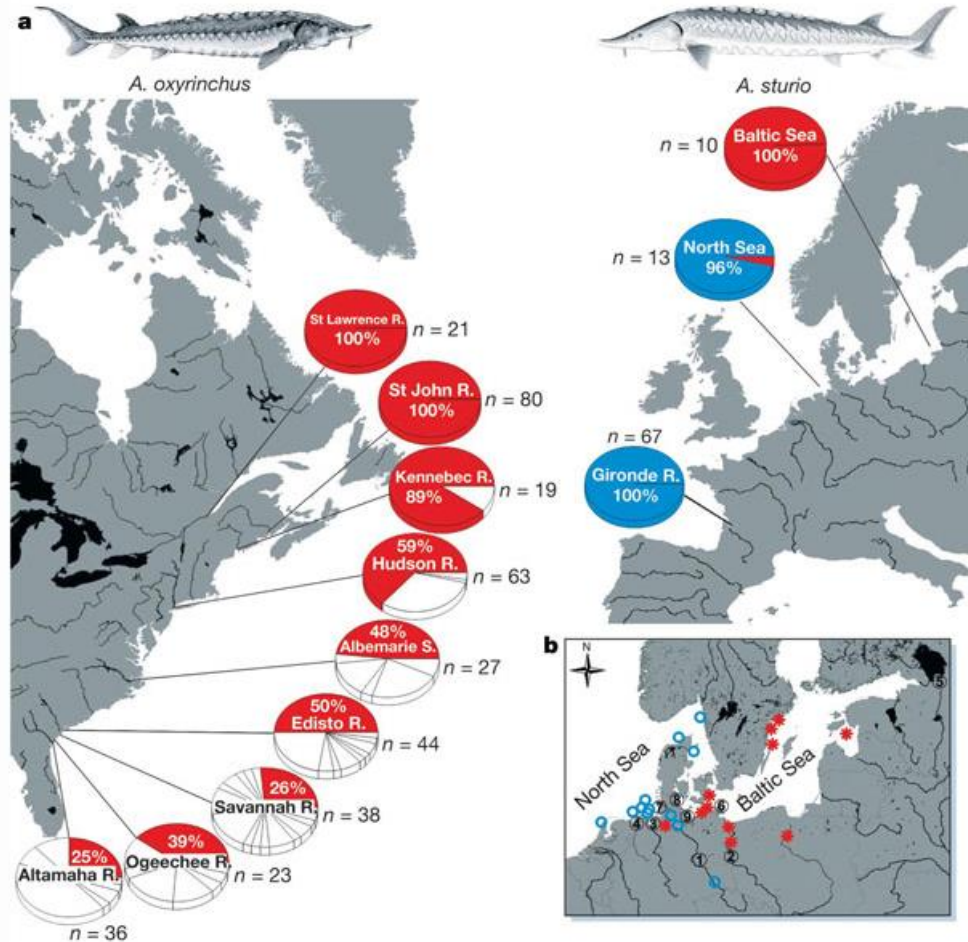
CURRENT *A. STURIO* DISTRIBUTION

- Only one natural population in the Gironde estuary in France (Atlantic coast)

- Critically endangered species at the edge of extinction



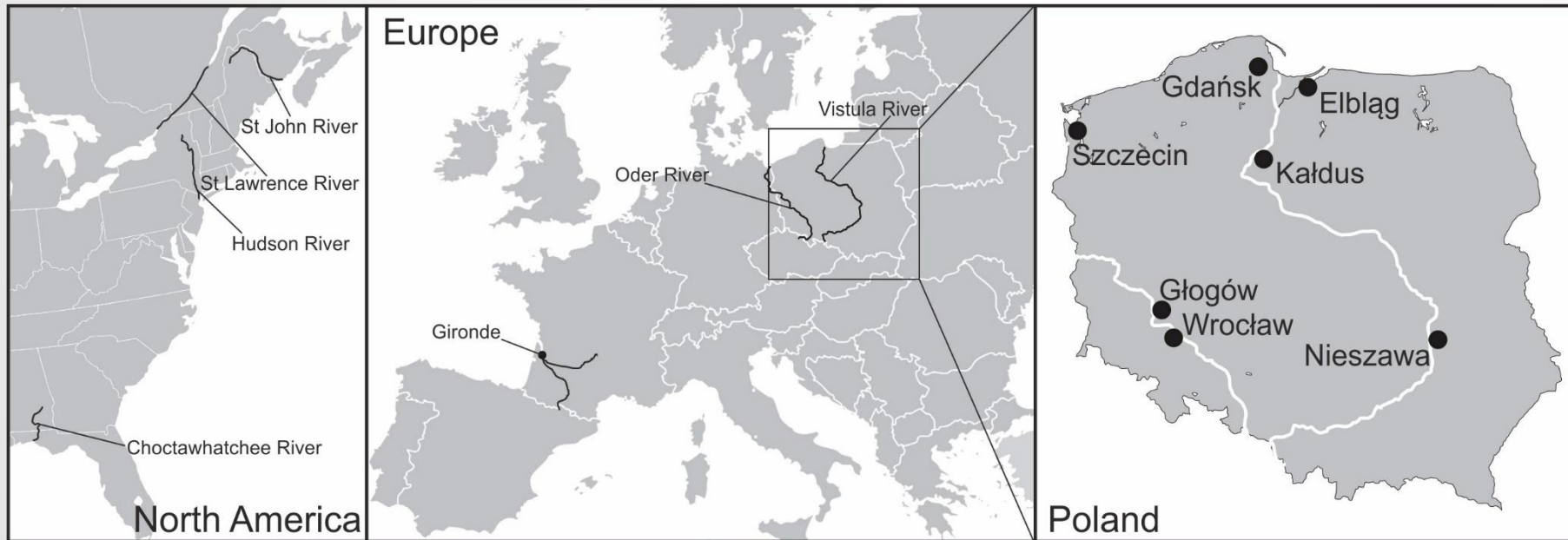
EUROPEAN STURGEON – *A. STURIO* / *A. OXYRINCHUS*?



- Ludwig et al. (2002) - the first evidence that *A. oxyrinchus* existed in Europe
- Genetic analysis of archeological remains of sturgeon excavated in Baltic Sea drainage basin – 200 bp of control region of the mtDNA and 230 bp sequence of nuclear DNA flanking the microsatellite Aox-23
- They have postulated that the Atlantic sturgeons colonized Baltic Sea during Little Ice Age (XIV – XVII century), when it replaced the European sturgeon.

FIGURE 1. Geographical distribution of two lineages of mitochondrial DNA haplotypes found in Atlantic sea sturgeon from North America and Europe.

THE HISTORY OF THE STURGEON IN THE BALTIC SEA

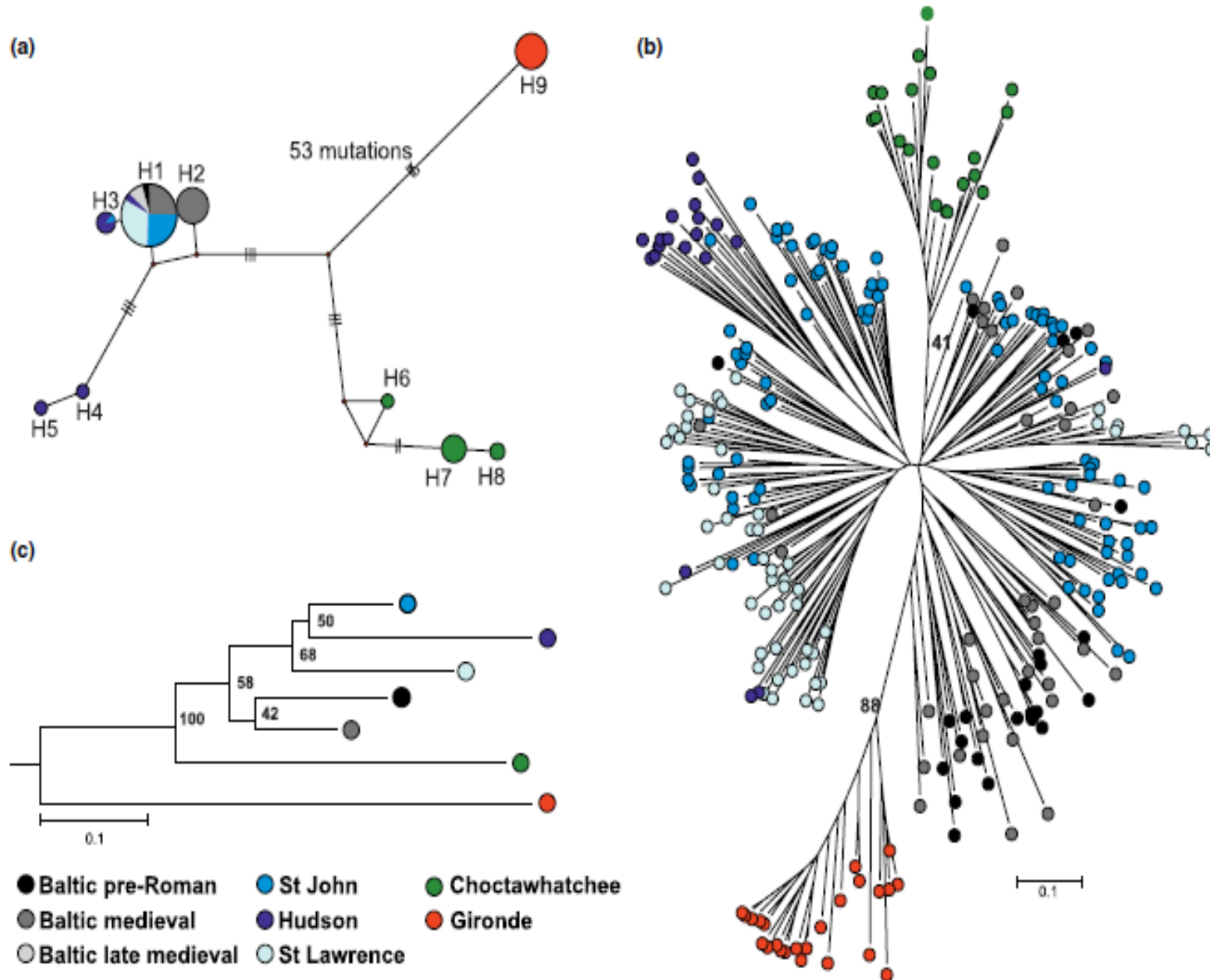


Sampling sites of contemporary North American (225 specimens) and European populations of sturgeon (24 specimens) and archeological sites of the ancient sturgeon remains (188 specimens).

Baltic population –

1. pre - Roman (2,300 – 2,100 ya)
2. medieval (1,700 – 1,600 ya)
3. late medieval (700 – 600 ya)
4. modern (19 - 20th century)

THE BALTIC STURGEON



- 125 samples from the Baltic Sea, only four was found to represent *A. sturio*
- (a) Median - joining network of 633 bp of control region and cytochrome b of 193 individuals
- Unrooted neighbour-joining tree based on msDNA Nei D_A distances between (b) pairs of individuals and (c) pairs of populations

ESTIMATION OF THE COLONIZATION TIME OF THE BALTIC SEA

	<p>ancestral population</p> <p>Baltic population</p> <p>Atlantic population</p> <p>Model with common ancestral population for Baltic and Atlantic populations (scenario 1)</p>			<p>ancestral population</p> <p>Baltic population</p> <p>Atlantic population</p> <p>Model without common ancestral population for Baltic and Atlantic populations (scenario 2)</p>			<p>ancestral population</p> <p>Baltic population</p> <p>Model with ancestral population for Baltic population (scenario 3)</p>
	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]

MATERIAL

- 349 samples representing all past distribution range of the European sturgeon
- different museums (Finland, Denmark, Sweden, Belgium, Ireland, Germany, France, Spain, Italy, Croatia, Bulgaria, Romania)
- archaeological sites (Russia, Poland, Belgium, Netherlands, France, United Kingdom, Serbia)
- tissue fragments preserved in alcohol
- dry skin fragments
- bone fragments

DNA EXTRACTIONS

1. Modified phenol/chloroform method (Baca et al., 2012)
2. Automated extraction with Maxwell® 16 Forensic Instrument (Promega) - uses paramagnetic particles as a purification medium to bind and wash nucleic acids
3. Column based method (Dabney et al., 2013)

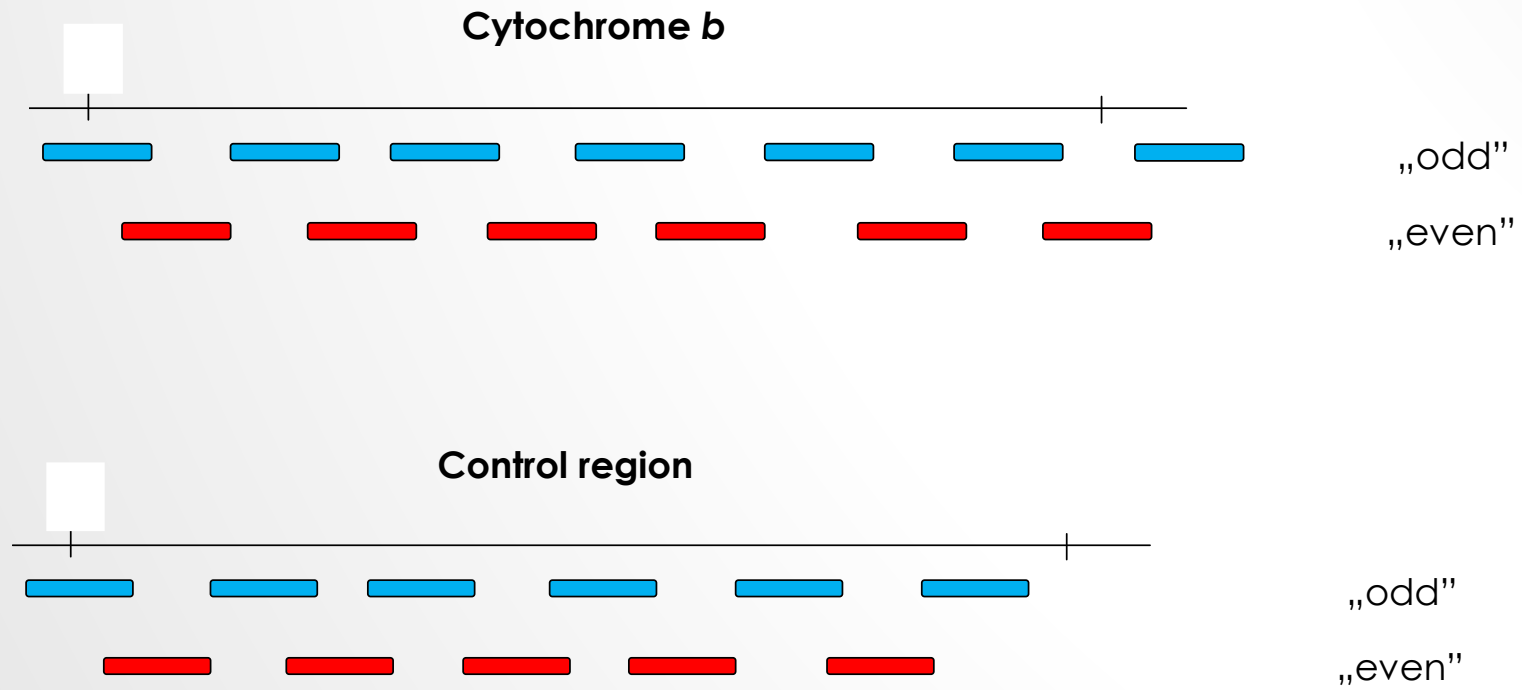
DNA AMPLIFICATIONS

1. Amplification of the short fragment of mtDNA:
 - ~120 bp of cytochrome *b*
 - ~250 bp of the control region
2. Quality of the extracted DNA
3. Taxonomic identification
4. Amplification of the complete cytochrome *b* and control region of the mtDNA

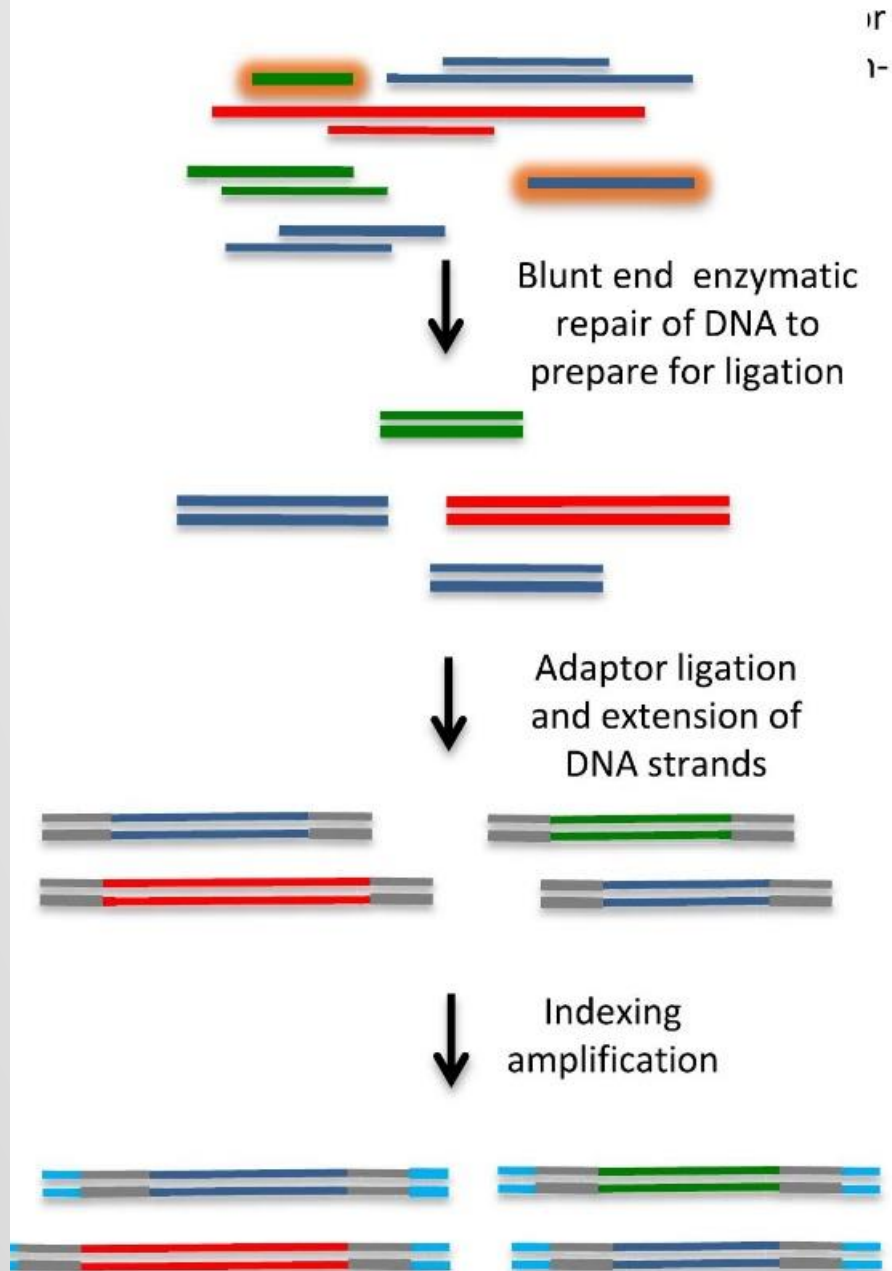
TAXONOMIC IDENTIFICATION

- DNA sequence – 211 specimens
- *A. sturio* – 66 specimens – North Sea, Atlantic coast of Europe, Mediterranean, Adriatic and Black Sea
- *A. oxyrinchus* – 127 specimens – Baltic Sea, Staraya Ladoga, North Sea and Atlantic coast
- other *Acipenser* sp. – 18 specimens

MULTIPLEX PCR



B) Double-stranded Library Preparation



LIBRARY PREPARATION

Double - indexed library – according to protocol dedicated to ancient DNA
Meyer & Kircher (2010) CH

- Green line = Target DNA in sample
- Blue line = Background DNA in sample
- Red line = Exogenous modern contamination
- Grey line = Universal adaptor (short DNA sequence)
- Blue line = Index (acts like a barcode to ID each library)
- Orange line = Single-stranded DNA

SEQUENCING ON MISEQ ILLUMINA PLATFORM

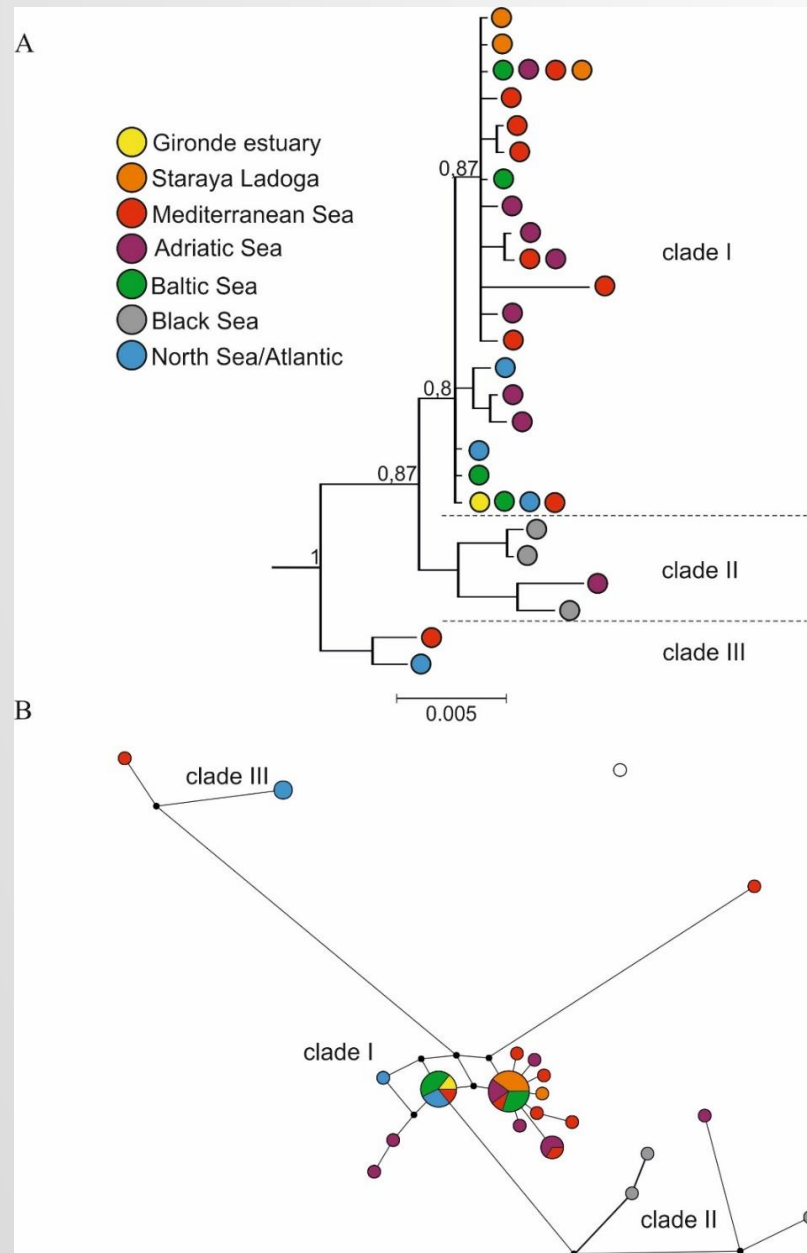
1. Quantification of libraries – fluorometer (Qubit), Real-Time PCR
2. Equimolar libraries were pooled and sequenced using MiSeq Reagent kit v2, 500 cycles
3. Obtained data were assembled using SeqMan NGen (DNA STAR)
4. Consensus sequences were based on the two independent replicates

RESULTS

- ***A. oxyrinchus*** – 1449 bp of cytochrome b and control region for 34 specimens
- 6 haplotypes – five in the North Sea, two in the Baltic Sea and only one in the Ladoga Lake
- Eastward direction of the colonization of the Baltic Sea
- ***A. sturio*** -1902 bp of cytochrome b and control region for 37 specimens
- 25 haplotypes

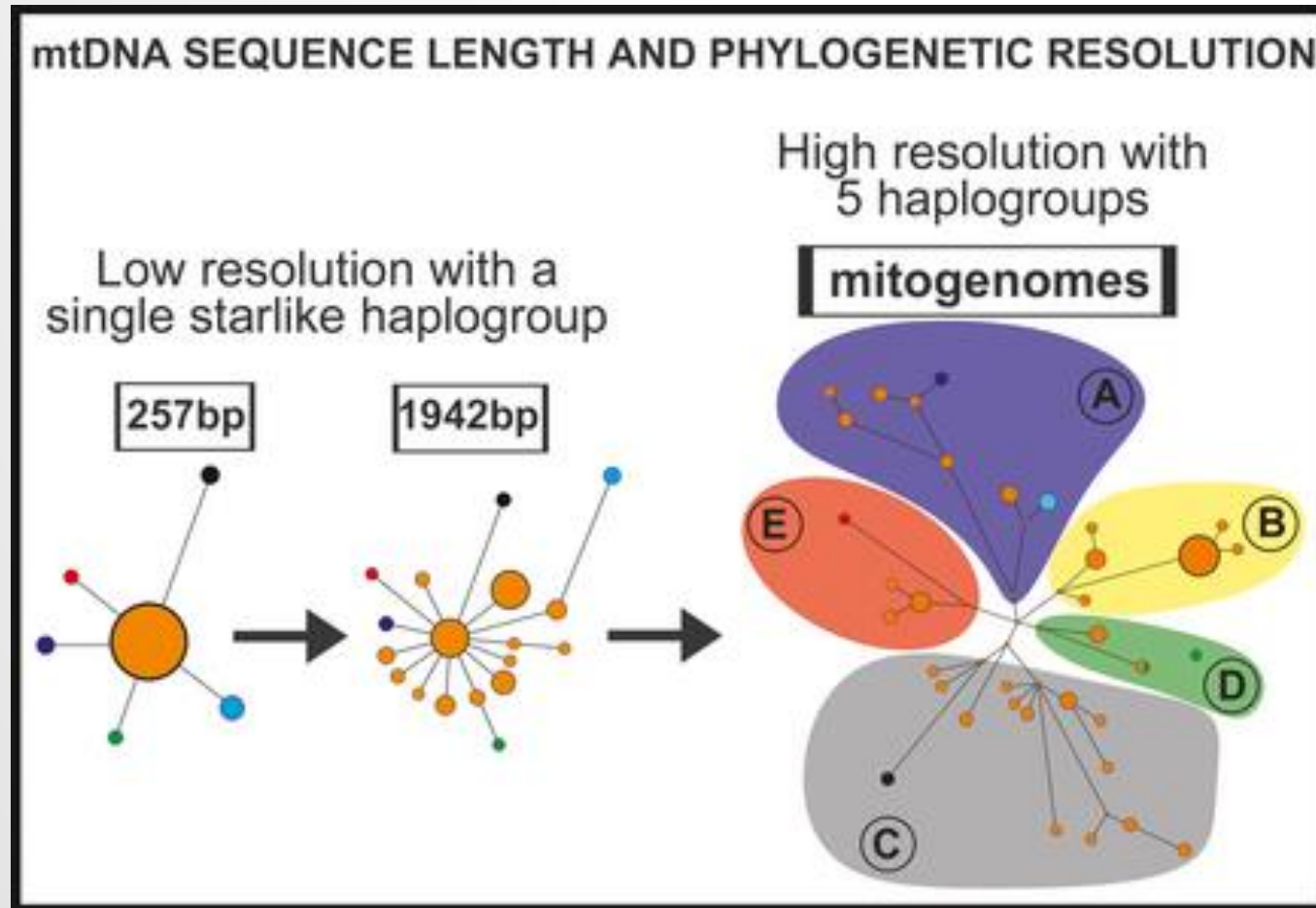
PHYLOGENETIC OF THE EUROPEAN STURGEON

- Three different clades
- Clade I – distributed in all analyzed regions with exception of the Black Sea
- Clade II - could indicate that in the Black sea was separate lineage which mixed with Adriatic sea around 10,000 ya
- Clade III - the oldest lineage
- There is no strong correlation between phylogenetic positions and either geographic locality or age of samples



A MrBayes phylogenetic tree.
B Median-joining haplotype network based on 1902 bp. The area of the circles are proportional to haplotype frequency and length of the connections line corresponds to number of substitutions.

COMPARISON OF PHYLOGENETIC RESOLUTION BETWEEN MTDNA DATASETS



MITOGENOMES SEQUENCING

- 24 specimens belonging to different clades

Extraction of DNA (Dabney et al., 2013)



Double - indexed genomic libraries

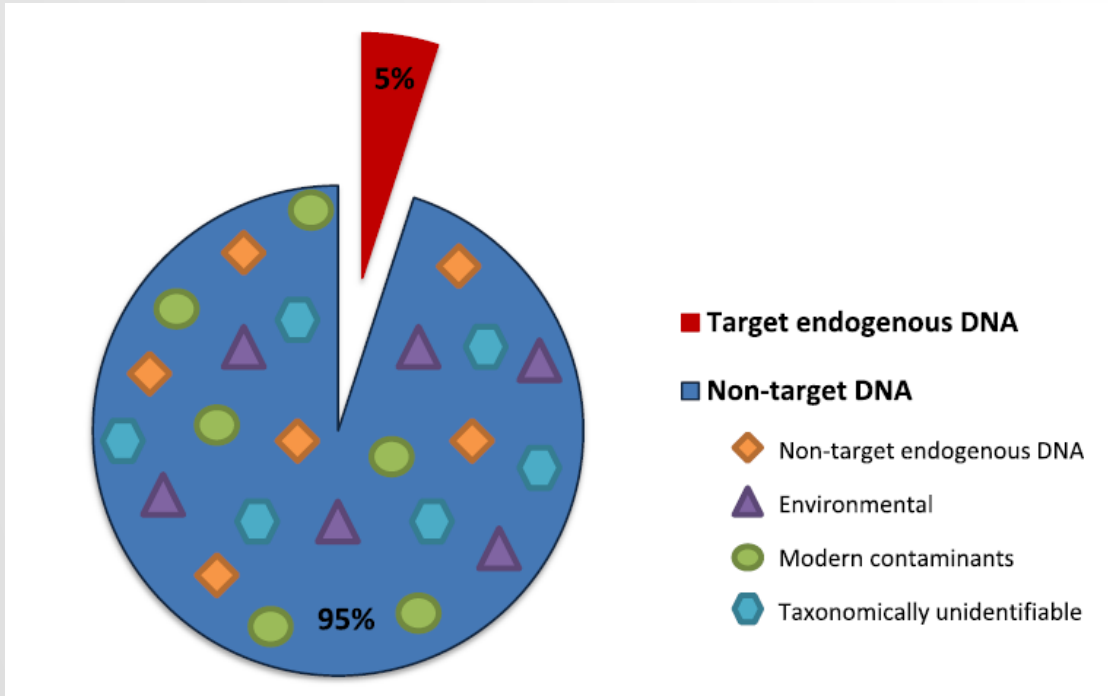


Target enrichment *via* DNA hybridization capture

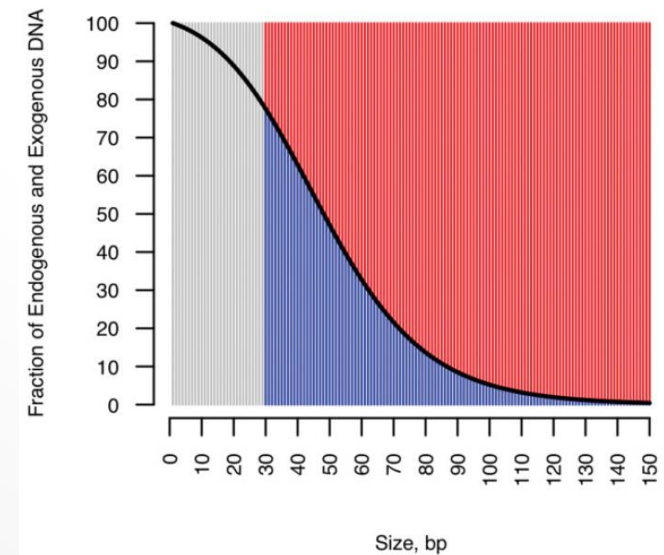
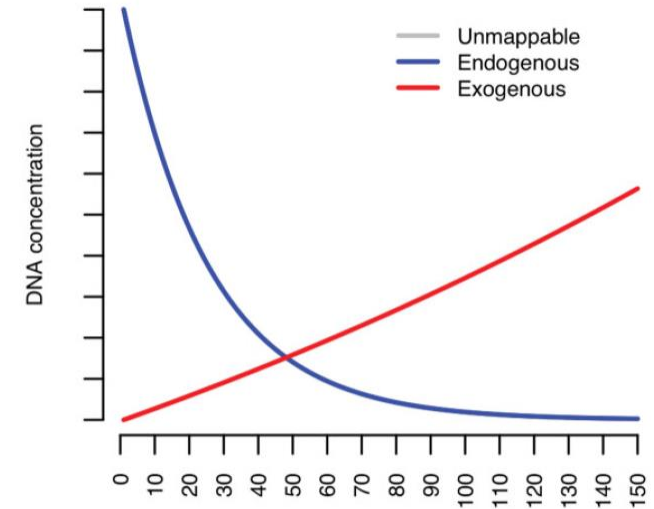


Sequencing on NextSeq Illumina platform

ENDOGENOUS VS. EXOGENOUS DNA

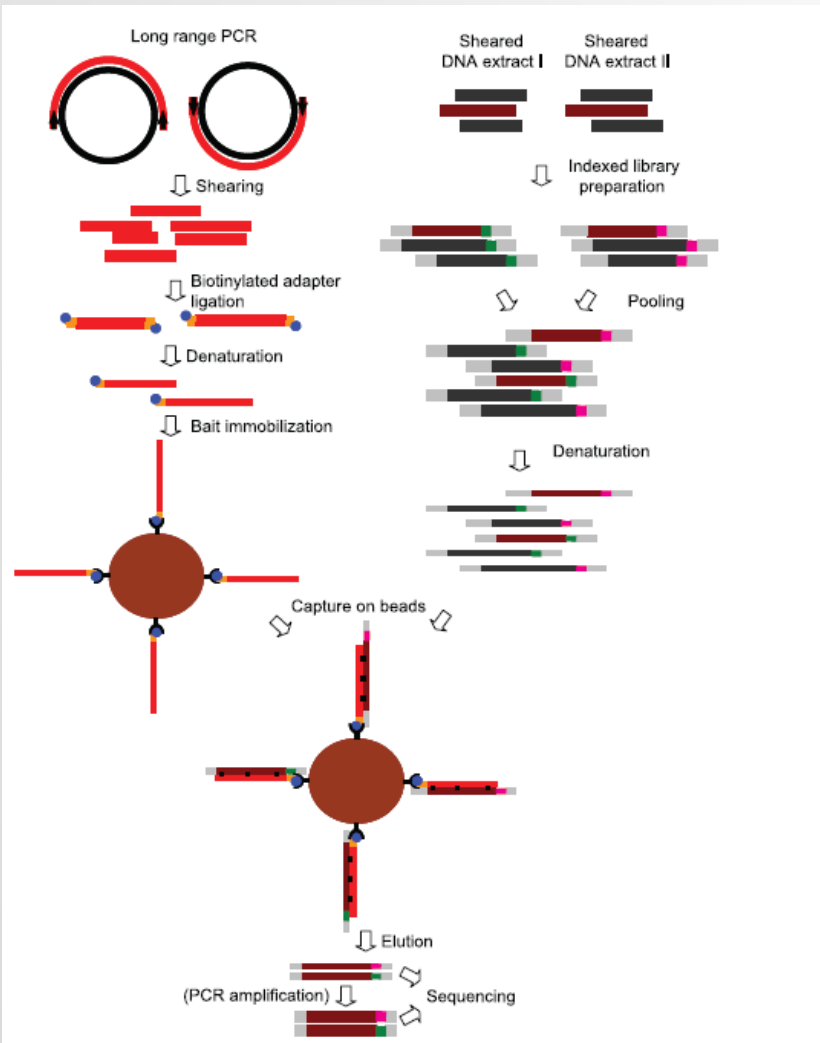


Marciniak et al. (2015) *Journal of Human Evolution*, 79, 21-34.



Orlando (2014) *Bioesseyes* 36:598-605

TARGET ENRICHMENT WITH HYBRIDIZATION CAPTURE



1. Bait

- complete mitogenome of *A. sturio*
- sonication of DNA to fragments ca. 400bp
- enzymatic preparation of the bait

2. Double – indexed genomic libraries

3. Hybridization capture – Horn 2012

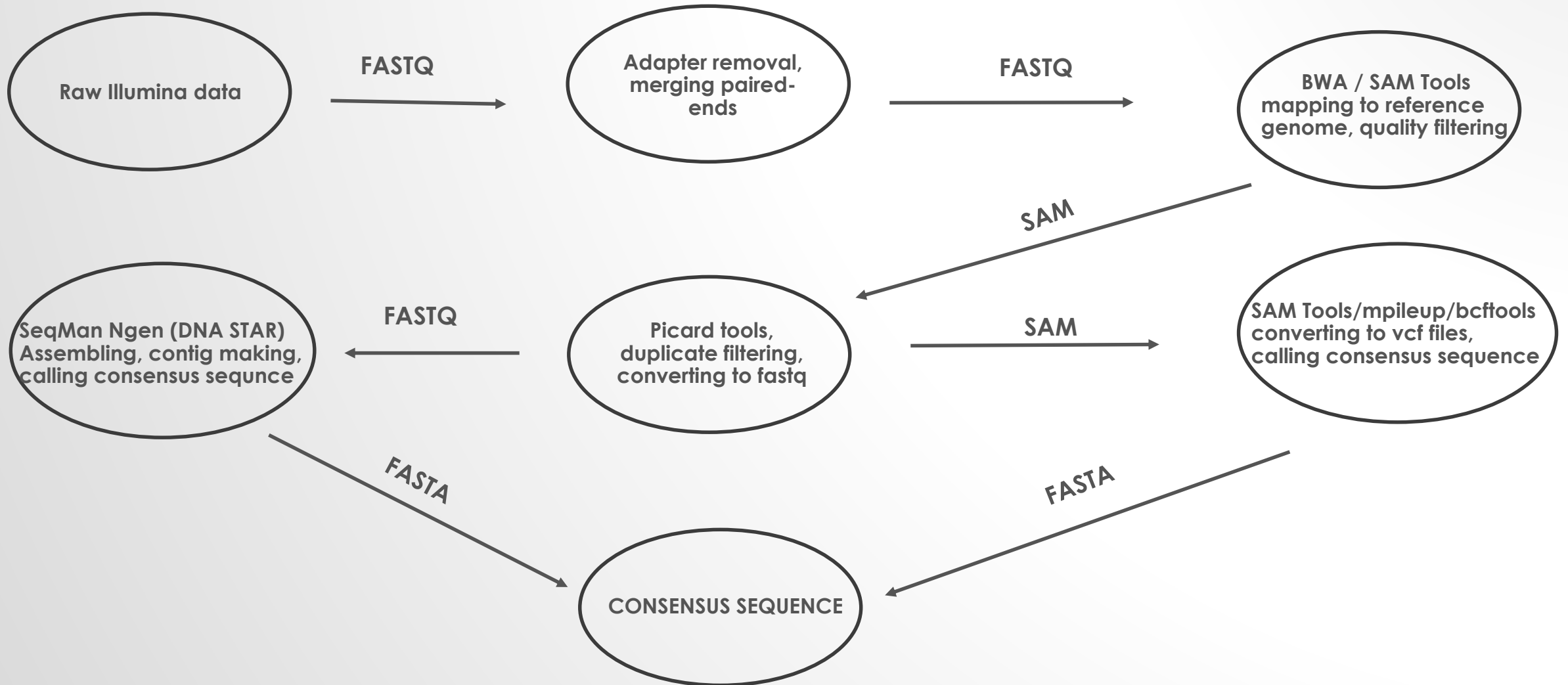
SHOTGUN SEQUENCING VS. TARGET ENRICHMENT AND HYBRIDIZATION CAPTURE

Sample		Reads	mapped to mtDNA		mapped to mtDNA (q>30) without duplicates	
			reads	% reads	reads	% reads
TW002L1	shotgun sequencing	137 710	0	0,000%	0	0%
	hybridization capture	3 208 007	46 857	1,461%	11319	0,35%
TW0020A1L1	shotgun sequencing	137 380	6	0,004%	6	0,004%
	hybridization capture	5 743 275	172 987	3,01%	17935	0,31%
TW021A1L1	shotgun sequencing	266 056	62	0,02%	62	0,02%
	hybridization capture	2 604 025	25 829	0,99%	8444	0,32%

SEQUENCING ON THE NEXTSEQ ILLUMINA PLATFORM

1. Quantification of libraries – fluorometer (Qubit), Real-Time PCR
2. Quality control and size selection - High-sensitivity DNA assay (Bioanalyzer)
3. Equimolar libraries were pooled and sequenced using NextSeq kit v2 150 reads, MidOutput

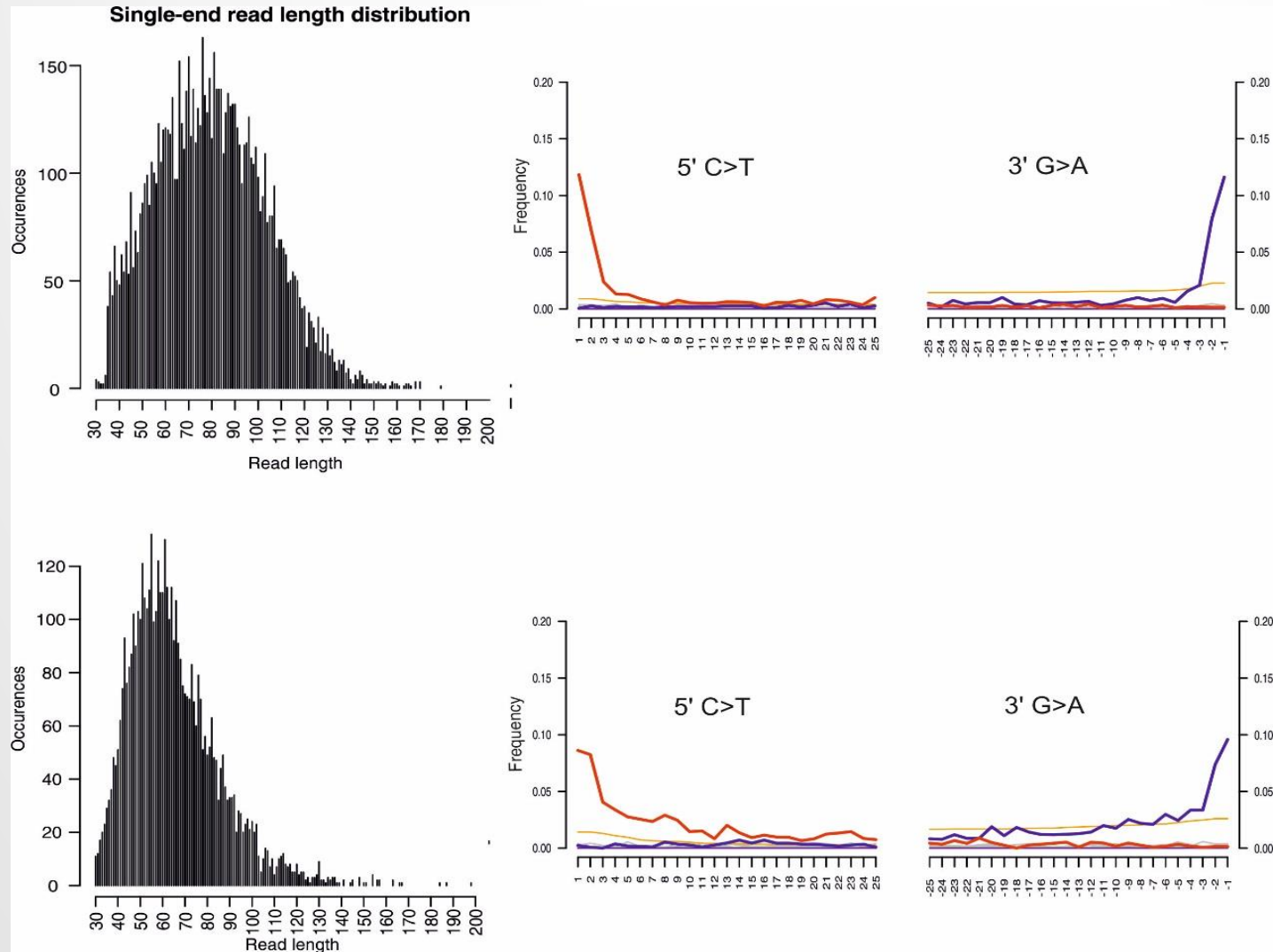
BIOINFORMATICS' ANALYSIS



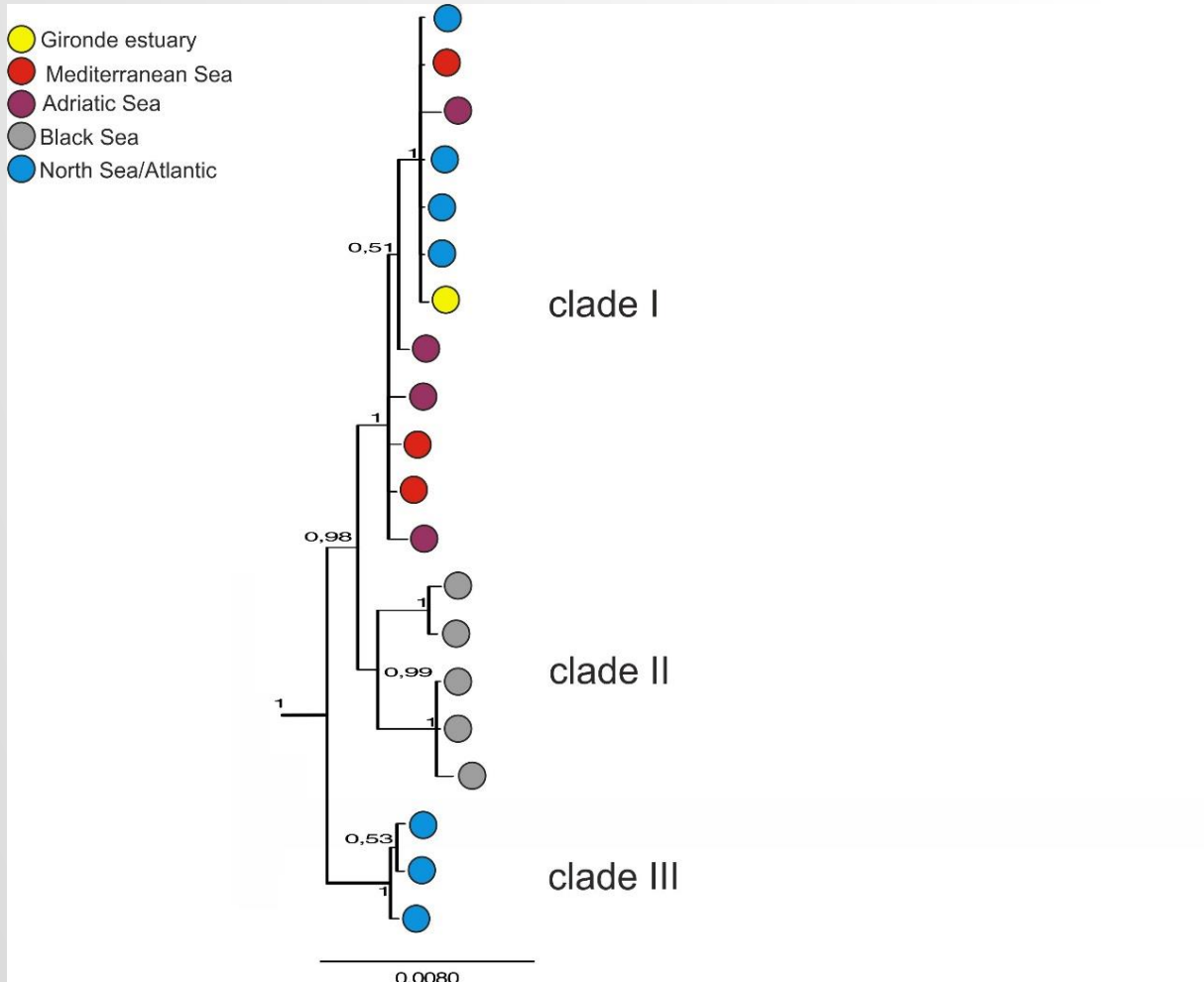
SUMMARY OF SEQUENCE DATA

Samples	Raw reads	mapped to mtDNA		mapped to mtDNA (q>30) without duplicates	
		reads	% reads	reads	% reads
1	1 851 768	55 555	3,00%	16 871	0,91%
2	3 474 709	18 608	0,54%	10 098	0,29%
3	2 379 837	25 770	1,08%	6 542	0,27%
4	3 707 265	6 430	0,17%	4 926	0,13%
5	243 820	3 883	1,59%	3 485	1,43%

DNA DAMAGE PATTERN AND SIZE DISTRIBUTION

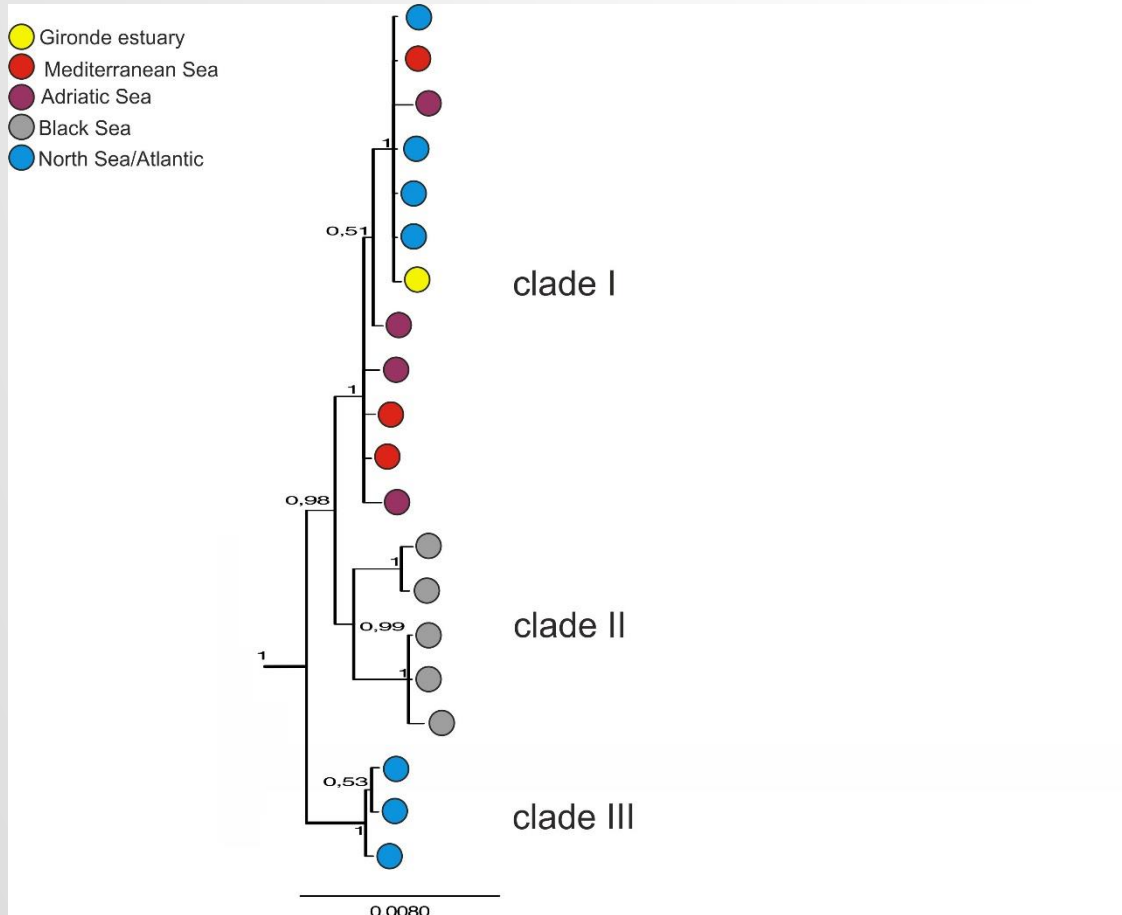


PHYLOGENETIC OF THE EUROPEAN STURGEON

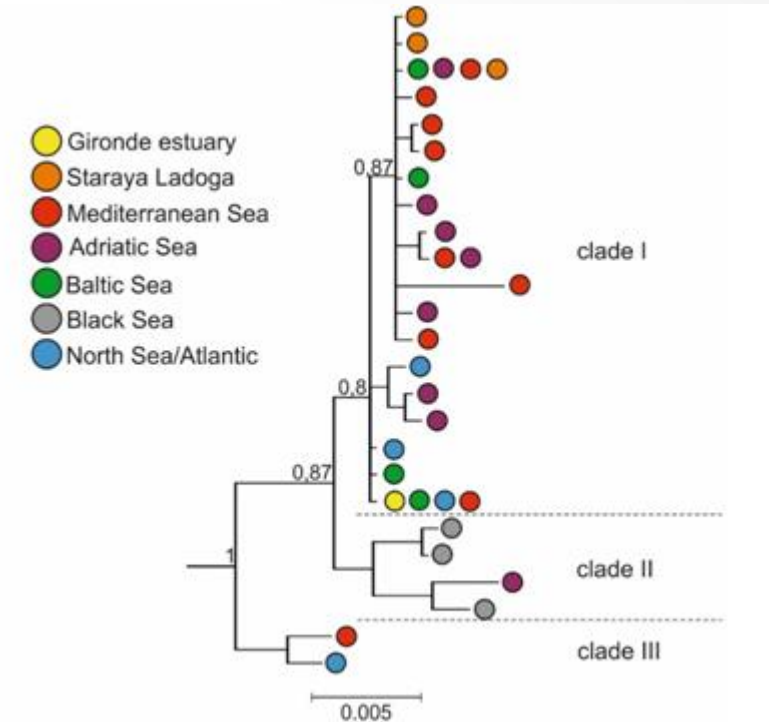


- 19 specimens – 17 haplotypes
- Three different clades
- Clade I – distributed in all analyzed regions excluding the Black Sea
- Clade II - could indicate uniqueness of the Black Sea lineage
- Clade III - the oldest lineage
- Sample bias could not be excluded

PHYLOGENETIC OF THE EUROPEAN STURGEON



complete mtDNA genomes



1902 bp of cytochrome b and control region

SUMMARY

- Museum samples were found to be a very good source of DNA!
- Next generation sequencing has radically changed research of this type of samples

ACKNOWLEDGMENTS

- My colleagues who I had worked on this project - Mateusz Baca and Hanna Panagiotopoulou.
- All people who provided the samples and cooperate in this project.
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THANKS FOR YOUR ATTENTION