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Biard, Vincent, Gol'din, Pavel, Vishnyakova, Karina et al. (6 more authors) (2017) Genomic and proteomic identification of Late Holocene remains: Setting baselines for Black Sea odontocetes. *Journal of Archaeological Science Reports*. ISSN 2352-409X

<https://doi.org/10.1016/j.jasrep.2017.07.008>

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TITLE

Genomic and proteomic identification of Late Holocene remains: setting baselines for Black Sea odontocetes

AUTHORS

Vincent Biard^{1*}, Pavel Gol'din², Elena Gladilina^{2,3}, Karina Vishnyakova^{2,3}, Krista McGrath⁴, Filipe G. Vieira¹, Nathan Wales¹, Michael C. Fontaine⁵, Camilla Speller⁴, and Morten Tange Olsen^{1*}

AFFILIATIONS

¹Section for Evolutionary Genomics, Natural History Museum of Denmark, University of Copenhagen, Øster Voldgade 5-7, 1350 Copenhagen K, Denmark

²Schmalhausen Institute of Zoology, National Academy of Sciences of Ukraine, 15 Bogdan Khmelnytskyi Street, Kiev, 01601, Ukraine

³Ukrainian Scientific Centre of Ecology of the Sea, 89 Frantsuzsky Blvd., Odessa, 65009, Ukraine

⁴BioArCh, Department of Archaeology, University of York, Environment Building, York, North Yorkshire YO10 5DD, UK

⁵Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, PO Box 11103 CC, Groningen, The Netherlands

CORRESPONDING AUTHORS

*Vincent Biard

4 Rue Roger Senturenne, D103

33140 Villenave d'Ornon, France

E-mail: vincentbiard33@gmail.com

*Morten Tange Olsen

Section for Evolutionary Genomics

Natural History Museum of Denmark

University of Copenhagen

Øster Voldgade 5-7

1350 Copenhagen K, Denmark

Fax: +45 353210102

E-mail: morten.olsen@snm.ku.dk

ABSTRACT

A critical challenge of the 21st century is to understand and minimise the effects of human activities on biodiversity. Cetaceans are a prime concern in biodiversity research, as many species still suffer from human impacts despite decades of management and conservation efforts. Zooarchaeology constitutes a valuable approach for informing conservation and management decisions by providing baseline information on the past distribution and human uses of species. However, traditional morphological species identification of mixed assemblage bones can be challenging, particularly in the case of cetaceans. To address this issue, we applied and evaluated the performance of three biomolecular approaches – Sanger sequencing, shotgun sequencing and collagen peptide fingerprinting (ZooMS) – for species identification in a mixed assemblage of 800 to 1600 years old odontocete (toothed whale) samples from the site of Chersonesus in Crimea, Ukraine. We found that ZooMS allowed for identification to the taxonomic level for 28 of our 30 samples (>90%), identifying them as either “porpoise” or “dolphin”, and approximately half of those samples could be further identified to species level with the shotgun sequencing approach. In addition, shotgun sequencing produced several complete ancient odontocete mitogenomes and auxiliary nuclear genomic data for further exploration in a population genetic context. In contrast, both morphological identification and Sanger sequencing lacked taxonomic resolution and/or resulted in misclassification of samples. We found that the combination of ZooMS and shotgun sequencing provides a powerful tool in zooarchaeology, and here allowed for a deeper understanding of past marine resource use and its implication for current management and conservation of Black Sea odontocetes.

KEYWORDS

67

68 Species identification; mixed assemblage; odontocete; ZooMS; shotgun sequencing; Sanger
69 sequencing; mitogenome.

70

INTRODUCTION

One of the critical challenges of the 21st century is to investigate and reduce the negative effects of human activities on the Earth's biodiversity. To address these issues, detailed baseline information on the ecology and evolution of plant and animal populations and species is required, including information on past abundance, distribution, species life history or phenology, as well as historic and prehistoric human resource use, context and impacts. While this is true for much of Earth's biota, cetaceans constitute an iconic group where many species and populations, despite decades of management and conservation efforts, still suffer from past and present human impacts, such as overexploitation, habitat alterations, bycatch, and ocean noise (Schipper *et al.* 2008; Pimm *et al.* 2014).

Zooarchaeology is particularly suited to provide such baseline information, as this approach documents a broad range of direct material evidences of animals, including their abundance, distribution, diversity, population structure and individual traits, as well as long-term changes of these parameters (Steadman 1995; Dietl *et al.* 2015; Hofman *et al.* 2015). However, in the case of cetaceans, accurate identification of species constitutes a special problem. On many archaeological sites, cetacean assemblages contain multiple species (Mulville 2002), which are represented by only incomplete fragments of postcranial bones and often are considered to be non-diagnosable by morphological methods (Mulville 2002; Amundsen *et al.* 2013). For example, isolated vertebrae of *Delphinus* sp. are hardly discriminated from *Stenella* sp., small *Tursiops* sp. or *Steno* sp. Furthermore, in an assemblage containing kitchen refuse of *Delphinus* sp., *Stenella* sp. and *Tursiops* sp., most of postcranial fragments were unidentified, unlike well informative cranial and, in particular, dental remains (Cooke *et al.* 2016). In

95 cetaceans, caudal vertebrae (comprising 35–50% of all vertebrae, depending on the species)
96 and vertebral centra with missing processes are especially difficult for diagnostics. This may
97 also be true for other mammalian groups, but many terrestrial mammalian taxa are often
98 represented by a single species in each assemblage or a few species of different size
99 categories and, thus, are more easily discriminated (Dunnell 1971; Grayson 1984).

100
101 The ability to extract and analyse ancient DNA (aDNA) from subfossil material have greatly
102 contributed to the resolution of this problem (Willerslev & Cooper 2005), allowing for
103 determining the species identity of e.g. historical whale remains from whaling stations at
104 South Georgia (Lindqvist *et al.* 2009; Sremba *et al.* 2010). Moreover, although driven
105 primarily by research in human (Briggs *et al.* 2009; Rasmussen *et al.* 2010; Allentoft *et al.*
106 2015) and terrestrial megafauna evolution (Gilbert *et al.* 2008; Lorenzen *et al.* 2011; Dabney
107 *et al.* 2013a), several studies have utilized aDNA to assess the evolutionary and demographic
108 history of cetaceans (see Foote *et al.* 2012a). For instance, McLeod *et al.* (2012) have
109 examined the demographic history of bowhead whales (*Balaena mysticetus*) in the Canadian
110 Arctic using mitochondrial DNA fragment, and have revealed a population expansion over
111 the past 30,000 years BP. Similar historic inference have been made from Eastern Atlantic
112 bowhead whales (Foote *et al.* 2013), as well as common bottlenose dolphin (*Tursiops*
113 *truncatus*) (Nichols *et al.* 2007), killer whales (*Orcinus orca*) (Foote *et al.* 2009) and North
114 Atlantic right whales (*Eubalaena glacialis*) (McLeod *et al.* 2010).

115
116 To date, most molecular approaches for ancient cetacean species determination have been
117 based on Sanger sequencing of short fragments of mitochondrial genes such as cytochrome-b
118 (CytB), ribosomal 12S or cytochrome oxidase-I (COI). However, for highly degraded

material, sufficient DNA template may not be preserved, or this approach might not yield sufficient coverage and resolution for species distinction – in particular if these are from closely related species (Goldstein & DeSalle 2011). Moreover, the focus on a single short fragment often limits additional population genetic inference of evolutionary and demographic trajectories, as well as associated historical anthropogenic and climatic impacts. The recent development of peptide mass fingerprinting of bone collagen (ZooMS), where species identification can be accomplished by comparing ancient peptide fingerprints to modern reference databases, allows for analysis of degraded ancient material otherwise suboptimal for DNA analysis (Buckley *et al.* 2009; Welker *et al.* 2015). However, due to the relatively slow rate of evolution within the collagen chains, taxonomic resolution is often limited to the genus or even family level. Studies combining analysis of aDNA Sanger sequencing and collagen targeting have successfully been applied to a few diverse assemblages of baleen whales (Buckley *et al.* 2014; Evans *et al.* 2016; Speller *et al.* 2016), however for some species and materials, even the combined use of these methods may not provide sufficient resolution for species identification. By generating massive amounts of data across the genome, short-read DNA “shotgun” sequencing techniques may circumvent the obstacles of aDNA fragmentation and taxonomic resolution, as well as provide additional data for population genetic inference of demography and phylogeography (Leonardi *et al.* 2016). Thus, the approach has a great potential to become the gold standard for species identification of ancient material. However to date the applicability of shotgun sequencing relative to Sanger sequencing and collagen profiling have not been evaluated..

All three odontocete populations inhabiting the Black Sea, the common bottlenose dolphin (*Tursiops truncatus*), the short-beaked common dolphin (*Delphinus delphis*) and the harbour

porpoise (*Phocoena phocoena*), though of widespread species, are recognized as separate subspecies and of special management and conservation concern. The IUCN has classified the Black Sea harbour porpoise (*P. p. relicta*) and the Black Sea bottlenose dolphin (*T. t. ponticus*) as endangered (Birkun Jr & Frantzis 2008; Birkun Jr 2012) and the Black Sea common dolphin (*D. d. ponticus*) as vulnerable (Birkun Jr 2008). These have all been severely depleted by decades of extensive hunting (Kleinenberg 1956; Birkun Jr 2002a), and are now affected by fisheries bycatch and consequences of ctenophore *Mnemiopsis leidyi* invasion, undermining their trophic base (Bushuev 2000; Vishnyakova & Gol'din 2015b). The common bottlenose dolphin (Viaud-Martinez *et al.* 2008; Moura *et al.* 2013) and short-beaked common dolphin (Amaral *et al.* 2007) both appear to have colonised the Black Sea a few thousand years ago. Similarly, it has been suggested that a relict harbour porpoise population in the Eastern Mediterranean founded the Black Sea population a few thousand years after the reconnection of the two basins, probably tracking suitable habitats (Fontaine *et al.* 2012; Fontaine *et al.* 2014; Fontaine 2016). Moreover, in contrast to the two other odontocete species present in the basin, the Black Sea harbour porpoise is unique in its isolation to other populations situated in the Atlantic Ocean, around 4,000 km apart. Its absence from the Mediterranean Sea, already noted by Aristotle (350 BC) (Frantzis *et al.* 2001), might be the consequence of changing oceanic conditions, notably warming temperatures and low productivity of Mediterranean ecosystems (Thunell *et al.* 1977; Thunell 1979; Fontaine 2016). Thus isolated, the Black Sea harbour porpoise has evolved distinct morphological and genetic characteristics (Viaud-Martínez *et al.* 2007; Galatius & Gol'din 2011), and additional sub-structuring might even exist among the different water bodies of the Black Sea region (Gol'din 2004; Gol'din & Vishnyakova 2016), although this question requires further investigations.

167

168 Here we tested the applicability of different species identification methods on a mixed
169 assemblage of odontocete (toothed whale) zooarchaeological remains excavated at the site of
170 Chersonesus on the Black Sea coast of Crimea, Ukraine. Specifically, we i) compared and
171 evaluated the performance of four approaches: morphology, Sanger sequencing, collagen
172 peptide mass fingerprinting (ZooMS), and shotgun sequencing of short-read DNA; and ii)
173 discuss our findings in the context of historic marine resource use, and in relation to setting
174 baselines for contemporary conservation and management schemes in the region. To our
175 knowledge this is one of the first studies to utilise the full extent of recent genomic and
176 proteomic techniques to identify and analyse highly degraded ancient odontocete material.
177 Importantly, although the present focus is on odontocetes, our findings should apply to other
178 organisms and study systems.

179

MATERIALS & METHODS

Study site

Chersonesus (also known as Chersonesus Taurica or Tauric Chersonesus = Chersonese) is located on the Black Sea coast in the southern Crimea (Supplementary Figure S1). It was the greatest city, trade and cultural centre of the northern Black Sea region during the Hellenistic, Roman and Byzantine ages between 2400–600 years BP (Strabo 1929; Porphyrogenitus 1993; Carter & Mack 2003). Founded by Greeks from Asia Minor, it was populated by colonists and visited by traders from various Black Sea and Mediterranean localities, and thus, the archaeology of the city shows a great variety of regional economic and cultural practices (Kadeev 1970; Kadeev & Sorochan 1989). In particular, marine fisheries and seafood played an important role in economy and diet of the Chersonesus population, and diverse marine fauna was reported from zooarchaeological evidence, as well as from art representations and some descriptive sources (Semenov-Zuser 1947; Kadeev 1970; Højte 2005; Morales *et al.* 2007).

Zooarchaeological material

The zooarchaeological material comprised 259 samples of small toothed whales excavated in 2011-2013 from the Kruze basilica and adjoining area in Chersonesus (Ushakov 2011), consisting of partial skulls and skull fragments, mandibles, ear bones, isolated teeth, sternum bones, humeri, partial vertebrae, epiphyses of vertebral bodies, ribs and rib fragments and pelvic bones. Of these, 30 samples of vertebrae, teeth, mandibles, epiphyses and skull

fragments were used for comparison of species identification methods, as representing individuals that were identified as different specimens in terms of morphology, taphonomy and archaeological context (Table 1, Figure 1A). The samples dates ranged from 1600 to 800 years BP based on the archaeological context, as presented by age-specific ceramics and coins (Ushakov 2011, 2013; Ushakov *et al.* 2013); but most of specimens came from the layers dated as 1600-1500 years BP (400-530 CE).

Morphological species identification

Based on morphological characteristics, the majority of the samples were assigned to species level (Supplementary Table S1). Identification was conducted by comparing the samples with museum collection specimens (Supplementary Figure S2). Specifically, all three species are well distinguished from their teeth, skull vertices, facial skulls, ear bones, sternum, humerus and pelvic bones (Supplementary Table S2). In addition, bottlenose dolphins are significantly larger than harbour porpoises in all dimensions, independent of ontogenetic age. In contrast to the above distinctions, vertebrae and ribs are less indicative than other bones. Generally, vertebrae of bottlenose dolphins can be identified from their relative large size, and those of common dolphins by their narrow bodies and long spines. However, incomplete vertebrae can generally only be provisionally identified. Here, as for ribs, they were identified to species only with other associated bones.

Genetic species identification

DNA extraction

All DNA extractions were conducted in a designated clean laboratory for ancient DNA analysis at the Centre for GeoGenetics, Natural History Museum of Denmark. First, the outer layer of the bone was removed by drilling and bleaching to limit contamination from soil composition. Then, 72 to 420mg of cleaned bone powder was obtained by drilling the bones inner part or, when drilling was impossible, by grinding of the full samples. Drilling was performed using a Dremel with a rounded drill head, running at <1000rpm to minimise heating of the sample. DNA was extracted using a silica-in-solution method (Rohland & Hofreiter 2007; Dabney *et al.* 2013a; Allentoft *et al.* 2015). Extraction blanks were included in each batch to monitor potential contamination. DNA quality and quantity was assessed using High Sensitivity D1000 ScreenTape for 2200 TapeStation (Agilent Technologies) and a Qubit[®] 2.0 Fluorometer. The former estimates peak molarities of the short-fragment component of the DNA extracts, and is hence expected to provide the most reliable approximation of DNA concentration in our subfossil samples.

Endogenous DNA content

The DNA extracts were tested for the presence and amount of endogenous (odontocete) DNA by real time qPCR using previously designed primers specifically developed for odontocete species identification by targeting a 43bp region of the CytB gene (Foote *et al.* 2012b). qPCRs were performed on a Stratagene Mx3000P (Agilent Technologies) and each 25µL reaction contained 2µL of DNA, 1 x PCR buffer, 2.5mM MgCl₂, 0.8µg/µL BSA, 0.4µM of each primer, 0.25µM mixed dNTPs, 1µL SYBR Green and 0.25µL AmpliTaq Gold[®] enzyme (Applied Biosystems). Thermocycling was performed at 95°C for 10 min, 55 cycles of 95°C

for 30sec, 55°C for 30sec and 72° for 30sec, and concluded by a dissociation step at 95°C for 1min, 50°C for 30sec and 95°C for 30sec. DNA extracts were used at 1:5, 1:10 and 1:20 dilution. Furthermore, for each qPCR reaction, one PCR blank (containing ddH₂O instead of sample) was run in triplicate to control eventual contamination during the qPCR set up. The qPCRs also included a standard, composed of 1:1, 1:10, 1:100 and 1:1000 dilution of modern DNA from a harbour porpoise, which was run in triplicate to monitor the specificity of the amplification. The Ct values (cycle threshold) and amplification efficiency was determined using the Pfaffl method (Pfaffl 2001). The Ct value refers to the numbers of cycles needed to observe the amplification of the DNA template, as the fluorescence level is correlated to the amount of double stranded DNA that is synthesized. Such approach is therefore more specific targeting, theoretically, only endogenous DNA. The Ct values were determined in the exponential phase of the qPCR amplification and normalized to the standard for comparison between the different qPCR assays.

Furthermore, to investigate the eventual impact of the quantity of starting material in the DNA retrieval from the archaeological material, we compared the amounts of bone powder with extract peak molarities and the normalized Ct values from the qPCR assays.

Species identification by Sanger sequencing

To illustrate the traditional approach to species determination, the qPCR products were used as a template for PCR amplification and Sanger sequencing using the CytB primers mentioned above (Figure 1B). Each 25µL reaction contained 2µL of qPCR product, 1 x PCR buffer, 2.5mM MgCl₂, 0.4µg/µL BSA, 0.4µM of each primer, 0.25µM mixed dNTPs and

0.2µL AmpliTaq Gold[®] enzyme (Applied Biosystems) under thermocycling 95°C for 5min, 25 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 30sec, finishing by 72°C for 7min. The resulting PCR products were visualised by electrophoresis on 2% agarose gel and positive PCRs were purified and sent for Sanger sequencing at Macrogen[®], Korea.

Species identification by shotgun sequencing

In order to test the applicability of shotgun sequencing of short-read sequence data for species determination (Figure 1C), DNA extracts were converted to Illumina indexed libraries using the NEBNext[®] DNA Library Prep Master Mix Set for 454[™] (New England BioLabs[®]). The resulting libraries were tested by real time qPCR to determine the optimal number of cycle for the library PCR amplification prior to sequencing. qPCRs were performed on a Stratagene Mx3000P (Agilent Technologies) and each 100µL reaction contained 20µL of 1:20 diluted library, 1 x PCR buffer, 2.5mM MgCl₂, 0.6µg/µL BSA, 0.2µM of each primer (PE1.0 and Index), 0.15µM mixed dNTPs, 1µL SYBR Green and 2µL AmpliTaq Gold[®] enzyme (Applied Biosystems) under thermocycling 95°C for 12min, 40 cycles of 95°C for 20sec, 60°C for 30sec and 72° for 40sec, finishing by 72°C for 5min.

Libraries were amplified by PCR under the above-described conditions (excluding SYBR green). Amplified libraries were purified using the QIAquick[®] PCR Purification Kit following the manufacturer's instructions, and then tested for quality and quantity using High Sensitivity D1000 ScreenTape for 2200 TapeStation (Agilent Technologies). The final libraries were pooled into two groups (BS_01 to BS_17 and BS_18 to BS_30) to equimolarity based on the 2200 TapeStation profiles and single read sequenced on respectively one lane

and a partial lane of the Illumina HiSeq 2000 platform. Moreover, five samples (BS_07, BS_10, BS_11, BS_12 and BS_15) were re-sequenced on a partial lane. The library of the sample BS_16 did not reveal any insert based on the DNA profile and was consequently not sequenced.

Given the lack of nuclear reference genomes for most cetaceans and general low yield of endogenous nuclear DNA from zooarchaeological material, the genetic species determination was done by use of mitochondrial genomic data (mitogenomes). First, sequencing adapters were removed from the de-multiplexed reads and reads shorter than 30bp were discarded using the program AdapterRemoval 1.5.4 (Lindgreen 2012). Then, all reads were mapped to reference mitogenomes of the harbour porpoise (Accession number NC005280) and bottlenose dolphin (Accession number KF570325). Full mitogenomes are not available for short-beaked common dolphin so reference data for this species was created by compiling available mtDNA sequence fragments in GenBank (Accession numbers KC312628, AB481388, and EU685091), as well as by mapping the short-read data to the mitogenome of a sister species, the long-beaked common dolphin (*Delphinus capensis*) (Accession number NC012061). Mapping was done using the program BWA 0.7.5a-r405 (Li & Durbin 2009), reads were quality filtered ($Q>30$) using the program samtools 1.2 (Li *et al.* 2009), and PCR duplicates were removed using the program Picard (<http://picard.sourceforge.net>) before being imported into Geneious (Kearse *et al.* 2012). Also, to aid in species identification by phylogenetic matching, we constructed a phylogenetic tree, including all mitogenome references and samples presenting high coverage mitogenomes, using the MrBayes 3.2.2. (Huelsenbeck & Ronquist 2001) plugging in Geneious. The mitogenome reference of the Ginkgo-toothed beaked whale (*Mesoplodon ginkgodens*) (Accession number NC027593) was

used as an out-group. Four independent chains for 1,000,000 generations under the General-time-reversible (GTR) model substitution were performed, with a burn-in length of 100,000 generations, and a sampling frequency of 5,000 generations.

Taxonomic identification by collagen peptide mass fingerprinting (ZooMS)

The identification of zooarchaeological remains through peptide mass fingerprinting of bone collagen (also known as ZooMS) has been applied in several contexts, including the identification of marine mammals (Buckley *et al.* 2014; Evans *et al.* 2016; Speller *et al.* 2016). We applied ZooMS to 29 of the odontocete samples after they had been processed for ancient DNA analysis (BS_16 was destroyed entirely during DNA analysis). The samples were processed in BioArCh, University of York, using protocols for ancient bone (Buckley *et al.* 2009). We immersed *ca.* 10-30mg of bone in 250µL of 0.6M hydrochloric acid at 4°C until the samples were demineralised. The samples were centrifuged and the supernatant discarded, before being rinsed in 200µL of 0.1M NaOH to remove humics and other chromophoric compounds. The NaOH was removed, and the samples were rinsed three times in 200µL of 50mM ammonium bicarbonate solution (NH₄HCO₃) pH 8.0 (AmBic). A final 100µL of AmBic was then added to the samples, followed by incubation for one hour at 65°C to gelatinise the collagen; 50µL of this supernatant was incubated overnight at 37°C with 0.4µg of trypsin, then acidified to < pH 4 with 0.1% trifluoroacetic acid (TFA), and purified using a 100µl C₁₈ resin ZipTip® pipette tip (EMD Millipore).

For mass spectrometry, we combined 1µL of extract with 1µL of matrix (α -cyano-hydroxycinnamic acid) and spotted them in triplicate along with calibration standards onto a

Bruker ground steel target plate and ran them on a Bruker ultraflex III MALDI TOF/TOF mass spectrometer, resulting in 90 individual spectra. Spectra were analyzed using the mMass software (Strohalm *et al.* 2008); spectra with low signal to noise ratios, or few to no discrete peaks were eliminated from the dataset. We averaged spectra from replicates of the same sample and compared them to the list of m/z markers for both marine and non-marine mammals (Buckley & Kansa 2011; Kirby *et al.* 2013; Buckley *et al.* 2014). We assigned taxonomic identifications at the most conservative level of identification (genus, or family level) based on the presence of unambiguous m/z markers (Figure 1D).

Population genetic analyses

Finally, we illustrate the additional use of short-read DNA sequence data from ancient odontocetes in a population genetic context by assembling and comparing it with publically available modern sequences from studies on same species and geographical regions. As an example, the ancient Black Sea harbour porpoise sequences were aligned with a 706bp fragment of the mitochondrial region of 64 modern harbour porpoise from the Black Sea and the Aegean Sea from Fontaine *et al.* (2012) and a parsimony-based TCS haplotype network was created using PopART ver. 1.7 (Leigh & Bryant 2015). This species was chosen based on the availability of modern reference data.

RESULTS

Morphological species determination

A total of 30 odontocete samples were analysed, comprising 16 vertebrae, four mandibles, four individual teeth, three epiphyses, two skulls and one occipital condyle (Table 1). Of these, 14 were morphologically identified as bottlenose dolphin remains, 12 were identified as harbour porpoises, one was identified as a short-beaked common dolphin, and three were unidentified.

Genetic species identification

Endogenous DNA content

The DNA concentrations were between 0.42ng/μL and 68.0ng/μL with an average of 4.96ng/μL, and peak molarities were ranging from 0.15nmol/L to 42.5nmol/L, with an average concentration of 10.12nmol/L (Supplementary Table S3). The average amplification efficiency of the qPCR assay was of 98.1±1.7%, and the melting curves were composed of single peaks for most of the samples, indicating the successful amplification of DNA from subfossil odontocetes. In a few samples, we also observed amplification in both extraction and qPCR blanks. However, these amplifications started after 40 cycles, more than 10 cycles after the amplification start of the latest amplified sample, and melting curves revealed qPCR products that were different from the standard (Supplementary Figure S3). We therefore attributed these amplifications to the formation of primer dimers and to the high number of

cycles performed compare to modern DNA template qPCR assays. A Pearson's correlation was run to determine the relationship between the amounts of bone powder and the extract peak molarities and no direct correlation was observed ($r(28) = -0.042$, $P > 0.5$) (Figure 2 A). Rather there seemed to be a weak but non-significant correlation with Ct values ($r(28) = -0.250$, $P > 0.1$) (Figure 2 B), indicating that amount of starting material determines endogenous DNA yield, although it is not statistically significant.

Species identification by Sanger sequencing

In the Sanger sequencing approach, CytB sequence chromatograms were obtained from 28 of the 30 samples. However, the quality of the data was poor and chromatograms difficult to score, leaving uncertainties in the base calls. Moreover, even in the cases where quality was sufficient for reliable base calling, the amplified CytB sequence fragment was found to contain few diagnostic sites to reliably distinguish between bottlenose dolphin and short-beaked common dolphin. Thus, Sanger sequencing data only allowed for species identification of eight harbour porpoises, whereas the remaining 20 samples were classified as *Dolphin sp.* (Table 1).

Species identification by shotgun sequencing

Fourteen of the 29 samples that were shotgun sequenced yielded sufficient short read data for assembling partial and high coverage mitogenomes and species determination (Supplementary Table S4). The identified species included seven harbour porpoises, four short-beaked common dolphins and three bottlenose dolphins (Table 1, Supplementary Figure

S4). For these samples, the proportion of mitogenome recovery and depth of coverage ranged from 72% to 100% (average = 90.2%) and 1.6 to 78.5 (average = 19.4) (Figure 3), respectively, whereas the remaining 15 samples yielded a very low number of reads (<120) or insufficient depth of coverage for species identification (Supplementary Table S4).

Species identification by collagen peptide mass fingerprinting (ZooMS)

We used ZooMS to assign broad level taxonomic identifications for all 29 samples tested for both Sanger and shotgun sequencing. Twenty-eight of the samples were confirmed as odontocetes, falling into two broad categories: 21 samples were identified as ‘dolphins’, which includes bottlenose dolphin, striped dolphin (*Stenella coeruleoalba*), short-beaked common dolphin, white-beaked dolphin (*Lagenorhynchus albirostris*); and 7 samples were identified as ‘porpoises’, which includes Dall’s porpoise (*Phocoenoides dalli*) and harbour porpoise, but also white-sided dolphin (*Lagenorhynchus acutus/ obliquidens*) and killer whale (*Orcinus orca*) (Table 1; Figure 1D; Supplementary Table S5). One sample was identified as a carnivore based on a lack of peptide markers common to cetaceans (e.g. m/z value 1,079) (Buckley *et al.* 2014), and the presence of peptides common to the order Carnivora (e.g. m/z values 1,105; 1,453; 2853; 2869) (Buckley *et al.* 2009; Kirby *et al.* 2013). The spectra was compared against available peptide markers for both terrestrial and marine carnivores (e.g. seals) (Kirby *et al.* 2013; Buckley *et al.* 2014; Buckley *et al.* 2017); due to the presence of peptide markers 2,131 (marker (D)) and particularly 1,576 (2t76), the spectra for BS_03 appears to be most consistent with the genus *Canis* (Buckley *et al.* 2017) (Supplementary Figure S5).

Comparison of species identification methods

The three biomolecular methods for species identification – Sanger sequencing, shotgun sequencing and ZooMS – only disagreed for two (BS_03 and BS_10) of the 28 samples where data was obtained from at least two of the molecular methods (Table 1). Specifically, the sample BS_10 – an occipital condyle, and morphologically determined to be Cetacean species – was determined to be a dolphin species by Sanger sequencing, a harbour porpoise by the shotgun sequencing method, and ZooMS identified this as a member of the ‘porpoise’ group, thus agreeing with the shotgun species identification. In the case of BS_03, this sample was morphologically identified as short-beaked common dolphin, but as a harbour porpoise using the Cytb sequencing, while the ZooMS identification pointed to a carnivore. Shotgun sequencing did not produce exploitable data for this sample, leaving its identification uncertain.

For the remaining 26 samples, the three molecular approaches agreed, although only shotgun sequencing provided identification at species level. The molecular approaches also revealed multiple errors in identifications from morphology. For instance, species identification was confirmed only for 6 of 11 specimens morphologically identified as harbour porpoises, whereas the others were re-identified as dolphins (BS_08 was re-identified to species level as a short-beaked common dolphin). Moreover, 2 of 13 specimens morphologically identified as bottlenose dolphins, were re-identified as short-beaked common dolphins using biomolecular approaches. Therefore, all the errors in morphology, which were tracked to the species level by genetic analysis involved short-beaked common dolphins and were likely due to

misidentification or omission of this species, and there was no confusion in discrimination between harbour porpoises and bottlenose dolphins.

Phylogenetic analyses

Four ancient Black Sea harbour porpoises yielded sufficient data for comparison with the mitochondrial control region fragment available for modern data. One transition was observed between the four ancient sequences, segregating them into two distinct haplotypes: BS_09 and BS_14 to one, and BS_11 and BS_15 to the other. Comparison with modern Black Sea sequences reveals that both haplotypes are still present in the modern population, including the southern Crimean waters, with one being the most abundant and central in the star-like topology of network (Figure 4), indicating a recent population expansion. .

DISCUSSION

Species identification in zooarchaeology

Species identification of archaeological materials can be challenging. Morphological identifications can be subject to human error and is sometimes impossible, being limited by reference to a higher taxon. This is especially common in the case of cetaceans, which are often found in multispecies assemblages, consisting of numerous closely related taxa of similar size. Biomolecular approaches may provide an excellent alternative for confirming a morphological label or resolving uncertainties. Here, we applied and evaluated four approaches to identify material from a mixed assemblage of odontocetes; one morphology-based, one protein-based and two DNA-based. We found that the proteomic ZooMS approach and Sanger sequencing could assign taxonomic grouping (“porpoise” or “dolphin”) to the majority of samples. In contrast, shotgun sequencing only yielded sufficient genetic information for half of the samples, but for those samples working well, it allowed for identification to species level and provided the first ancient mitogenomes from Black Sea odontocetes, allowing for species level identification and additional population genetic inference.

There are several advantages to a protein-based method such as ZooMS for species identification of subfossil material. First, collagen in particular, is a robust protein, and can generally be recovered from tropical or subtropical archaeological sites where DNA may not be preserved (Welker *et al.* 2015). Second, ZooMS can identify samples without prior taxonomic knowledge potentially identifying ‘non-target’ species that might fail PCR based

approaches such as Sanger sequencing due to a lack of primer specificity. The main limitation of ZooMS is its level of taxonomic resolution: although all odontocete samples were identified, they could only be assigned to broad taxonomic groups due to the lack of diversity within the collagen sequences of odontocetes and poor resolution within certain areas of the spectrum (Buckley *et al.* 2014). For instance, species such as killer whales and white-sided dolphin within the Delphinidae family have a peak at mass 1652 grouping them with porpoises, but differ from porpoises and matches other dolphins at masses in the 1500s area of the spectrum. However this area is often poorly resolved, making conclusive identification difficult. Thus, ZooMS classifications may not be correct in systems unlike ours, where we can rule out certain species based on their known taxonomic distributions, habitat preference and absence from the Black Sea. For example, we could quite confidently rule out killer whale, Dall's porpoise, white-beaked dolphin, and white-sided dolphin from the Black Sea, leaving the 'dolphin' group to include the genera *Tursiops*, *Delphinus* and, hypothetically, *Stenella*, and the 'porpoise' group to include only *Phocoena*. More precise taxonomic identifications can occasionally be obtained from the mass spectra, but this requires the successful recovery of multiple diagnostic peptides.

Sanger sequencing has previously been successfully applied for species identification in cetacean mixed assemblages (Lindqvist *et al.* 2009; Sremba *et al.* 2010; Evans *et al.* 2016). Although the primers used here have been successful in a previous study for identifying odontocetes (Foote *et al.* 2012b), we generally found the chromatograms difficult to interpret, the approach failed to identify samples beyond taxonomic level, and two samples (BS_03 and BS_10) were misclassified. The CytB fragment that we targeted contains 12 and 15 variations sites that differentiate between harbour porpoise and bottlenose dolphin or harbour porpoise

and common short-beaked dolphin respectively. The two dolphin species differ at 3 sites, which in our case was insufficient for clear species identification. These obstacles may be overcome by designing new primers and/or targeting larger mitochondrial fragments, as reported for some baleen whale samples (e.g. Yang & Speller 2006; Evans *et al.* 2016). However, for highly fragmented ancient DNA, amplification of large PCR products may be problematic, so targeting of short regions are recommended. If such short fragment primers can be designed and highly degraded ancient material can be avoided, Sanger sequencing may provide species identification, but the obtained sequences will likely not provide much data for population genetic inference.

In contrast, having previously been limited by costs and laborious methodology, shotgun sequencing has emerged as an efficient and simple method for generating a large number of short-read sequence data for species identification and population genetic inference from degraded material (Blow *et al.* 2008). In particular, this method can by-pass difficulties occurring using Sanger sequencing, including significant DNA fragmentation, which was an issue in our study. In addition, the ability to recover larger regions of the mitochondrial genome rather than just a short fragment can often provide a more robust taxonomic identification. Still, shotgun sequencing is not without limitations. Specifically, although all of the samples seemed to contain well-preserved collagen, only half of the samples yielded sufficient amounts of sequence data for species identification. The geographical region from which our samples originate is characterised by temperate to arid subtropical climate, and thus typically not considered optimal for preservation of aDNA (Willerslev & Cooper 2005; Dabney *et al.* 2013b). Moreover, the samples are likely kitchen refuse and some have certainly been boiled or cooked, probably resulting in some DNA degradation already before

burying. As observed here, sequencing success could potentially be improved by increasing the amount of starting material (e.g. bone powder), and for small or valuable samples where material is limited, issues of poor sequence recovery may be solved by re-sequencing troublesome samples, applying capture-enrichment methods (Ávila-Arcos *et al.* 2011; Ávila-Arcos *et al.* 2015) or using bioinformatics software capable of handling low depth sequencing data (Korneliussen *et al.* 2014).

ZooMS has been suggested as a pre-screening method ahead of more intensive or destructive bimolecular approaches, including PCR-based and shotgun DNA analyses (Von Holstein *et al.* 2014; Brown *et al.* 2016; Speller *et al.* 2016), shotgun proteomics (Welker *et al.* 2016) or radiocarbon dating (Harvey *et al.* 2016). As ZooMS is typically less expensive and requires less bone powder than other biomolecular techniques, pre-screening with ZooMS can provide both taxonomic information, as well as an indication of overall biomolecular preservation. In this study, the ZooMS technique provided comparable taxonomic resolution as the Sanger sequencing approach, and could therefore act as a cost-effective pre-screening method ahead of shotgun sequencing. Thus, we recommend using a combination of ZooMS and shotgun sequencing for more effectively resolving uncertainties in species determination. Under such an approach, ZooMS will likely allow for the assignation to a taxonomic level of most or all species, as well as a pre-screen of suitable material that can be shotgun sequenced for further identification to species level and generation of mitogenomic and nuclear data for population genetic inference.

Marine resource use in ancient Chersonesus

574 The overall species composition of wild fauna on archaeological sites, and particularly in
575 ancient kitchen refuse, provides numerous insights for the reconstruction of ancient
576 environments, paleoclimatic conditions, catch techniques and seasonality of different hunting
577 and fisheries activities. For example, numerous anchovy (*Engraulis encrasicolus*) remains at
578 the Chersonesus sites are indicative of set net fisheries during the autumn and spring seasonal
579 migration; turbot (*Scophthalmus maximus*) is indicative for bottom gill net fisheries, which
580 are the most productive in spring; and tuna (*Thunnus sp*) is indicative for longline and
581 intensive boat tending activities in warm season. From these perspectives, the identify
582 odontocetes can be either a product of incidental catches in bottom gill nets set for turbot, as it
583 is in present times (Vishnyakova & Gol'din 2015a), specimens recovered from strandings, or
584 an object of direct hunt from boats in coastal waters, as it appeared in the early 20th century
585 (Kleinenberg 1956). Of these alternatives, the former idea is well supported by abundant
586 turbot remains in the Kruze basilica. Meanwhile, strandings of common dolphins are
587 relatively rare in Crimea, as well as bycatches in any modern fishery practices in the Black
588 Sea (Birkun Jr 2002b). On a global scale, bycatch is generally associated with modern types
589 of fisheries, such as pelagic trawling and big purse seine fisheries (Tregenza *et al.* 1997;
590 Bilgmann *et al.* 2008), rather than artisanal fisheries. Therefore, stable catches of common
591 odontocetes might have implied boat-based direct hunting. For instance, Black Sea fishermen
592 hunted common dolphins in early 20th century, using a few small boats setting a single purse
593 seine (*alaman*) (Kleinenberg 1956). Aelian described a similar net for tuna fisheries used in
594 the Roman-era southern Black Sea and mentioned dolphin catches in this context. In an
595 earlier evidence, Strabo (1929) mentioned ancient dolphin catches in the same area in
596 association with tuna and bonito fisheries. Also, Brito & Vieira (2009) found evidence for
597 catches of common dolphins and tuna in medieval Portugal with the same type of trap net

(*almadrava*). Additionally, Kadeev (1970) suggested that dolphins could be hunted by Chersonesus fishermen with a harpoon. Our study showed a stable presence of common dolphins in archaeological kitchen refuse assemblages, comprising more than half of the specimens that were identified to “dolphin” by ZooMS and could be further identified to species by shotgun sequencing. These were greatly underestimated by the morphological analyses and, thus, support the idea of diverse odontocete catch practices in the ancient Chersonesus. These practices seem to have affected all the local odontocete species and possibly included, not only common fisheries bycatch products, but also, direct cetacean-focused hunting.

Implications for management and conservation of Black Sea odontocetes

The geographically isolated Black Sea harbour porpoise, as well as other Black Sea odontocetes, seem to have undergone dramatic population size reductions during the past century as a result of substantial hunting and bycatch peaking between the 1930s and 1950s and killing hundreds of thousands, if not a million, animals (Kleinenberg 1956; Birkun Jr 2002a; Fontaine *et al.* 2012; Fontaine *et al.* 2014). The extensive fisheries and other anthropogenic disturbances also impacted fish abundance and resulted in an ecosystem regime shift, intrusion of invasive species and economic collapse in the 1970s (Bushuev 2000; Daskalov 2002, 2003; Daskalov *et al.* 2007; Llope *et al.* 2011). Consequently, the three odontocete species inhabiting the Black Sea have been classified as species under special management and conservation concern.

621 To supplement management policies, detailed baseline knowledge on past occurrence and
622 human impacts is needed. Although human activities have clearly escalated in recent decades,
623 our species identification of Late Holocene zooarchaeological material indicate that Black Sea
624 odontocetes, similarly to other marine resources, have been impacted by hunting and fisheries
625 bycatch for millennia. For instance, our population genetic analyses of ancient harbour
626 porpoise samples supported previous studies based on modern material (Fontaine *et al.* 2012),
627 suggesting a long term presence of harbour porpoise in the Black Sea. A more complete
628 understanding of the relative roles of past and present human activities on odontocete
629 demography will require additional zooarchaeological investigations, and upscaling current
630 genetic efforts to include genomic data from both contemporary and ancient samples. In
631 demonstrating the use of biomolecular approaches for species identification, and obtaining the
632 first full mitogenomic and auxiliary nuclear data from ancient Black Sea odontocetes, we
633 have taken the first step.

ACKNOWLEDGEMENTS

The authors wish to thank the Dr Bøje Benzon Foundation and the Section for Evolutionary Genomics at the Natural History Museum of Denmark for their financial support. The authors also thank Sergey Ushakov, Alexey Ivanov and Larisa Sedikova for providing access to the Black Sea samples and the Danish National High-Throughput DNA Sequencing Centre for sequencing the samples.

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Authors Contribution

V.B., P.G. and M.T.O. designed the research; E.G., K.V. and P.G. collected zooarchaeological samples and processed the morphological analysis; V.B. performed aDNA based laboratory work advised by N.W. and M.T.O; K.MG. and C.S. performed protein based laboratory work; V.B., K.MG., F.G.V., N.W., M.C.F., C.S. and M.T.O. analysed the molecular data; V.B., P.G., C.S. and M.T.O. wrote the manuscript; V.B. and M.T.O. provides funding; all authors commented on previous version of the manuscript and approved the final version.

Data Accessibility

DNA sequences: Genbank accession XXXXX (to be added upon accept of the manuscript)

Supporting Information

Table S1. Morphology of cetacean remains from the Kruze basilica in Chersonesus (400–530 CE), excavations 2011-13.

Table S2. Description of the morphological characteristics of bones structures used for the species identification.

Table S3. Description of the DNA extract characterisation per sample.

Table S4. Description of the sequencing results per samples. NS=Not Sequenced.

Table S5. Designated peptide markers used for taxonomic identification of the Black Sea samples.

Figure S1. Map showing the sampling locations of the Black Sea samples. *Inset* shows area of the main map.

Figure S2. Picture of caudal vertebrae of extant *Tursiops truncatus* (A) and *Delphinus delphis* (B) typically used for morphological species determination of subfossil material.

Figure S3. Melting curves for BS_29 qPCR amplifications. The gray line with dots, the green line with squares and the blue line with upside down triangles represent respectively the results for the 1:5, 1:10 and 1:20 dilutions. The other four yellow curves represent negative controls. Amplification can be observe for two of the negative controls, however the dissociated products are different form those obtained for the samples dilutions.

Figure S4. Bayesian phylogenetic tree of ancient mitogenomes infer with MrBayes algorithm. *Mesoplodon ginkgodens* was used as an out-group and numbers on nodes represent posterior probabilities.

Figure S5. Averaged MALDI-ToF mass spectra from sample BS_03. Rectangles indicate the peptides used to identify the sample as a probable carnivore.

FIGURES & TABLES

Figure 1. Summary of the different techniques for identification of subfossil odontocete remains. (A) Odontocete samples from Chersonesus showing the state of preservation and degree of fragmentation. (B) Chromatograms resulting from the Sanger sequencing of CytB gene fragments from odontocete samples. (C) Reads alignment to the *Phocoena phocoena* reference mitogenome resulting from the Shotgun sequencing of the sample BS_14. (D) MALDI-ToF mass spectra displaying peptide marker (P2) distinguishing the porpoise from the dolphin group.

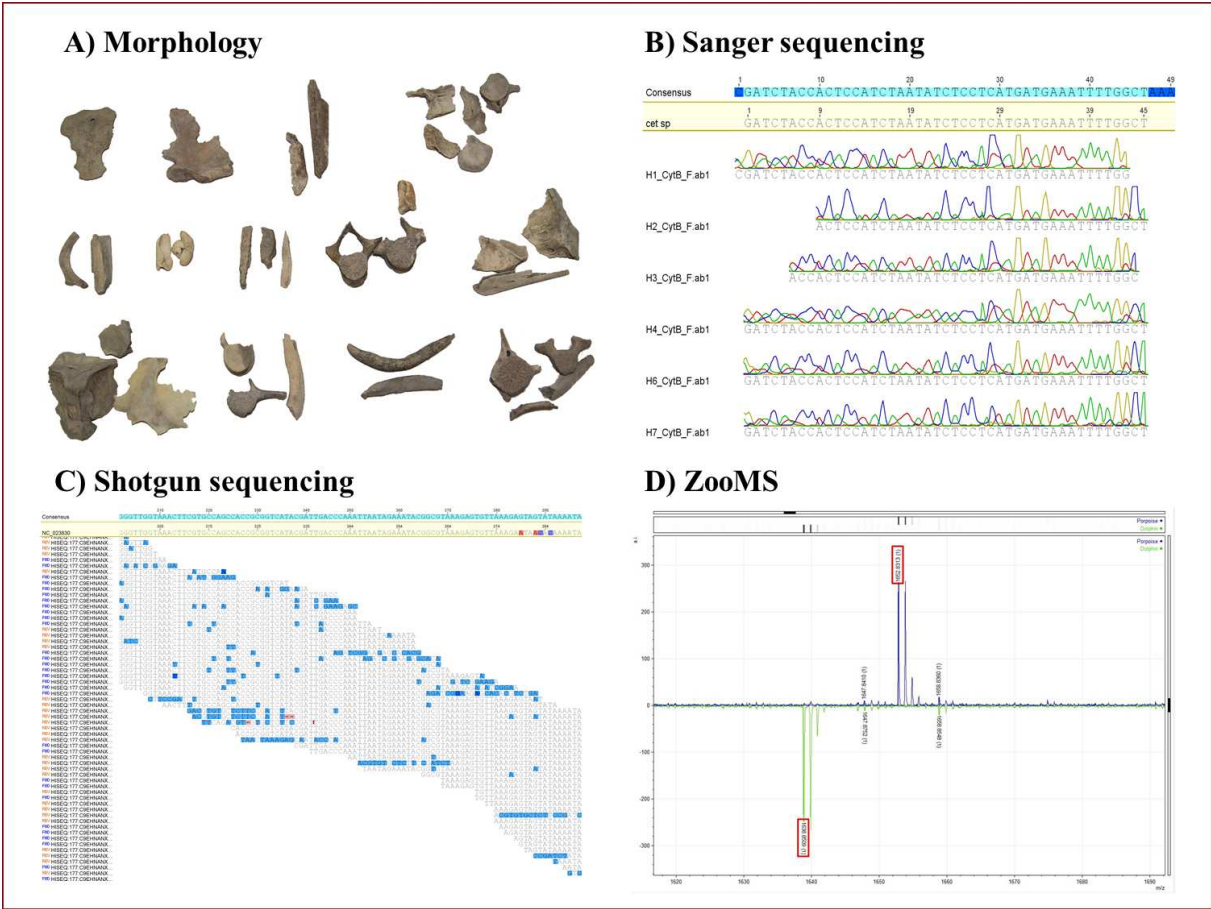


Figure 2. The correlation between amount of starting material (bone powder weight) and (A) peak molarity and (B) normalized qPCR Ct-values. Note that higher peak molarity and lower Ct-values typically signifies higher DNA content.

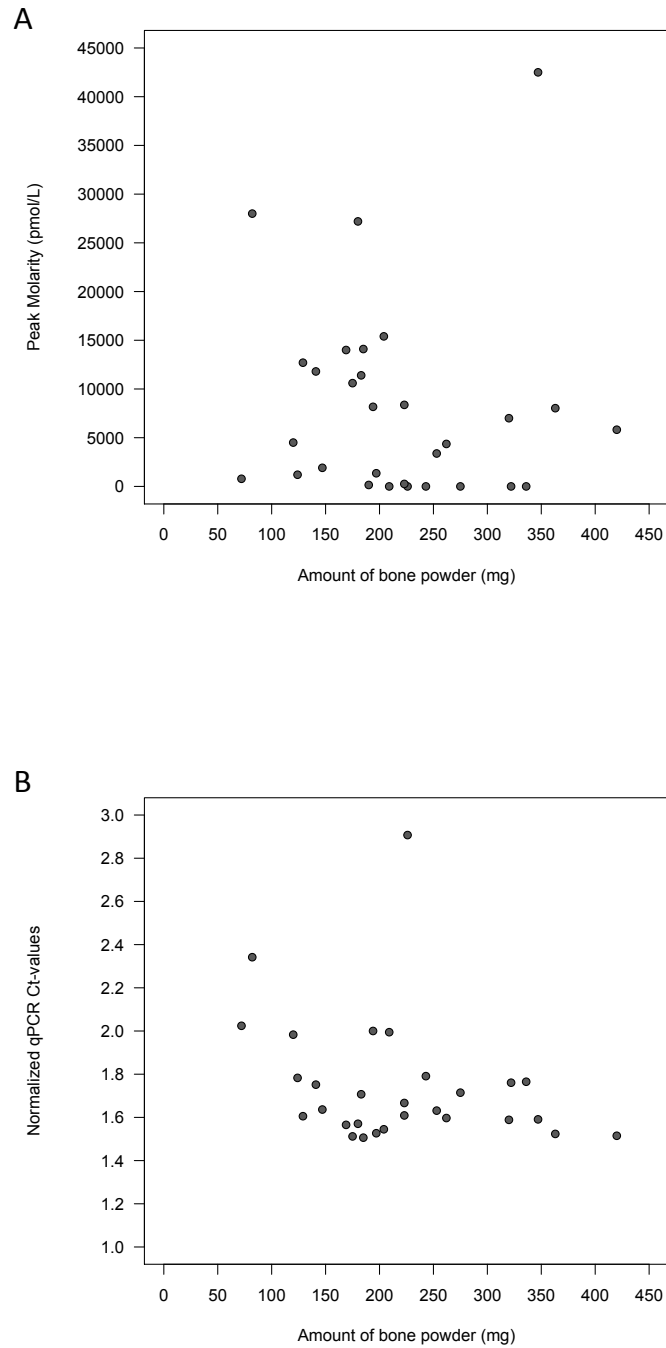


Figure 3. Mitogenome recovery and sequencing depth of coverage resulting from the shotgun sequencing.

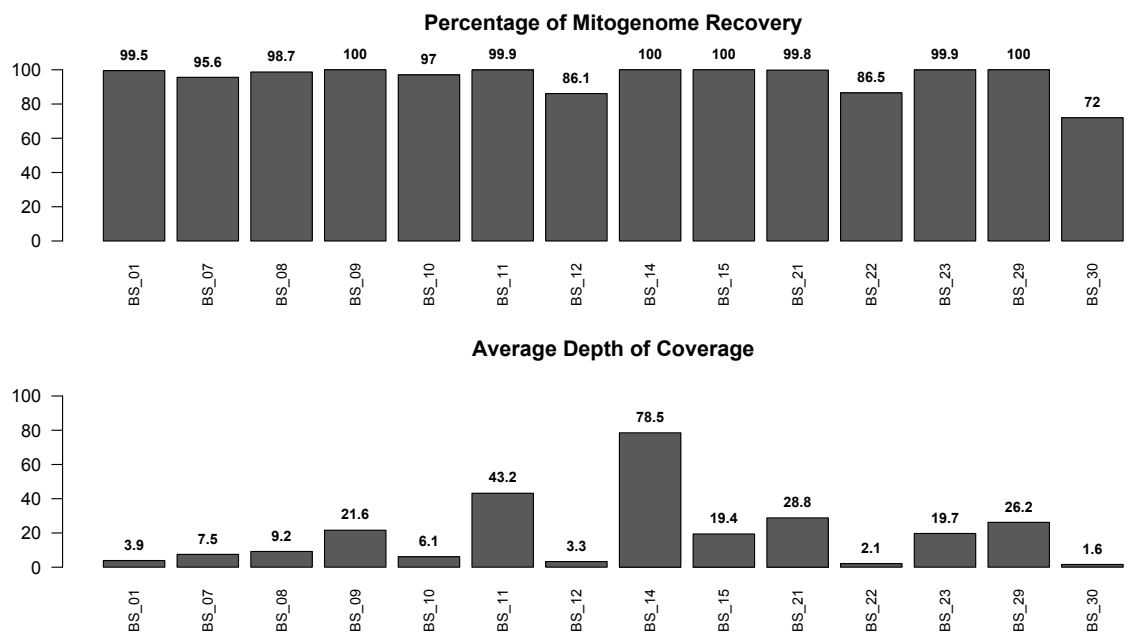


Figure 4. Haplotype network among Black Sea harbour porpoise based on the 706bp mitochondrial sequence.

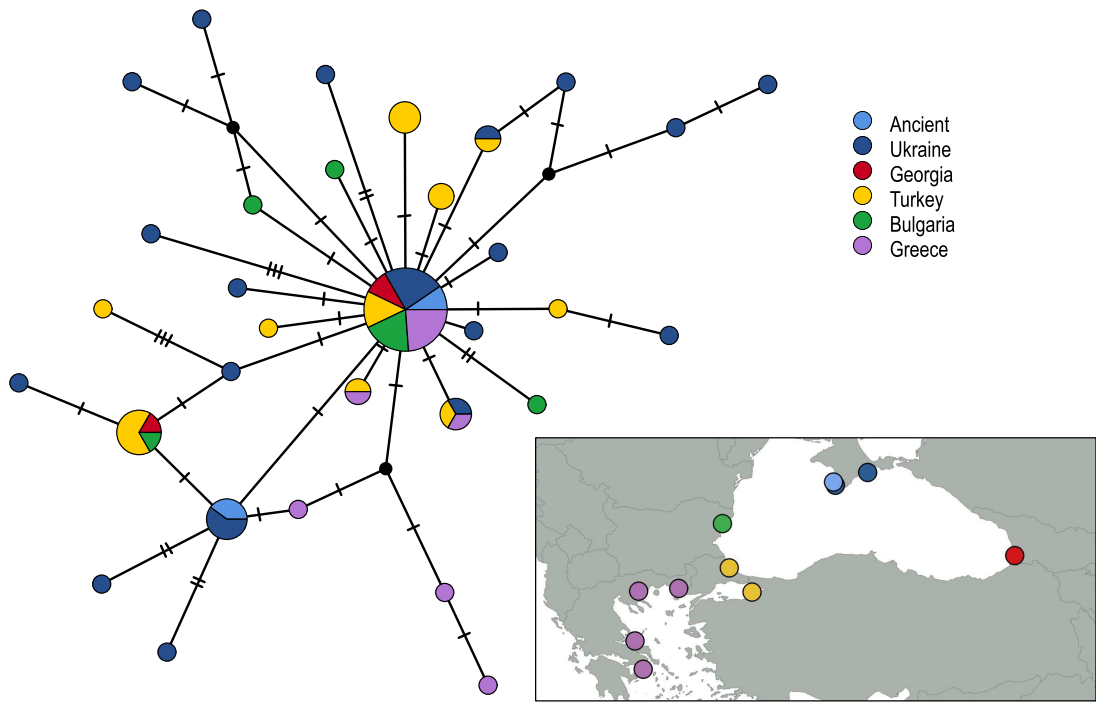


Table 1. Odontocete samples and results of the species determination by morphology, CytB Sanger sequencing, shotgun sequencing of mitogenomes and ZooMS. Notice that the three molecular approaches gave overall very similar results, with the exception of samples BS_3 and BS_10.

ID	Year (CE)	Bone	Morphology	Sanger	Shotgun	ZooMS ^a
BS_01	400-530	Mandible	Cetacea sp.	Dolphin sp.	<i>D. delphis</i>	'Dolphin'
BS_02	400-530	Mandible	Cetacea sp.	Dolphin sp.	Failed	'Dolphin'
BS_03	275-300	Mandible	<i>D. delphis</i>	<i>P. phocoena</i>	Failed	Carnivora
BS_04	400-530	Mandible	<i>P. phocoena</i>	Dolphin sp.	Failed	'Dolphin'
BS_05	400-530	Tooth	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_06	400-530	Vertebra	<i>P. phocoena</i>	Dolphin sp.	Failed	'Dolphin'
BS_07	400-530	Vertebra	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_08	400-530	Epiphysis	<i>P. phocoena</i>	Dolphin sp.	<i>D. delphis</i>	'Dolphin'
BS_09	400-530	Epiphysis	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_10	400-530	Occip. condyle	Cetacea sp.	Dolphin sp.	<i>P. phocoena</i>	'Porpoise'
BS_11	400-530	Vertebra	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_12	400-530	Vertebra	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_13	400-530	Vertebra	<i>P. phocoena</i>	Dolphin sp.	Failed	'Dolphin'
BS_14	400	Teeth	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_15	400	Skull	<i>P. phocoena</i>	<i>P. phocoena</i>	<i>P. phocoena</i>	'Porpoise'
BS_16	400	Skull	<i>P. phocoena</i>	<i>P. phocoena</i>	N/A	N/A
BS_17	1000-1200	Vertebra	<i>P. phocoena</i>	Dolphin sp.	Failed	'Dolphin'
BS_18	470-500	Tooth	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_19	470-500	Tooth	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_20	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_21	470-500	Epiphysis	<i>T. truncatus</i>	Dolphin sp.	<i>T. truncatus</i>	'Dolphin'
BS_22	470-500	Vertebra	<i>T. truncatus</i>	Failed	<i>D. delphis</i>	'Dolphin'
BS_23	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	<i>D. delphis</i>	'Dolphin'
BS_24	1000-1100	Vertebra	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_25	1000-1100	Vertebra	<i>T. truncatus</i>	Failed	Failed	'Dolphin'
BS_26	1000-1100	Vertebra	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_27	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_28	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	Failed	'Dolphin'
BS_29	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	<i>T. truncatus</i>	'Dolphin'
BS_30	470-500	Vertebra	<i>T. truncatus</i>	Dolphin sp.	<i>T. truncatus</i>	'Dolphin'

^a 'Dolphin' includes '*Tursiops truncatus*, *Stenella coeruleoalba*, *Delphinus delphis*, *Lagenorhynchus albirostris*'; 'Porpoise' includes '*Orcinus orca*, *Phocoenoides dalli*, *Phocoena phocoena*, *Lagenorhynchus obliquidens*'; Note that sample BS_16 was not shotgun sequenced or tested using ZooMS.