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Genetic characterization, age and reproductive status of dwarf sperm whales (*Kogia sima*) stranded along the Italian coastlines

Nicola MAIO[1], Tatiana FIORAVANTI[2], Lucrezia LATINI[2], Agnese PETRACCIOLI[1], Marcello MEZZASALMA[3], Gianni INSACCO[4], Andrea SPLENDIANI[2], Francesco POLLARO[5], Fabio Maria GUARINO[1] and Vincenzo CAPUTO BARUCCHI[2]

[1] Dipartimento di Biologia, Università degli Studi di Napoli Federico II, Via Cinthia 26, 80126 Napoli, Italy
[2] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
[3] Dipartimento di Biologia, Ecologia e Scienze della Terra, Università della Calabria, Via P. Bucci 4/B, 87036 Rende, Cosenza, Italy
[4] Museo Civico di Storia Naturale di Comiso, via degli Studi 9, 97013 Ragusa, Italy
[5] Centro Studi Ecosistemi Mediterranei, Via Caracciolo, 84068, Pollica, Salerno, Italy

Corresponding authors: Fabio Maria GUARINO, fabio.guarino@unina.it; Marcello MEZZASALMA, marcello.mezzasalma@unical.it

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Abstract

The knowledge of the life history traits of cetaceans, especially in the case of species that are difficult to observe in the wild, often benefits from the study of stranded individuals. The dwarf sperm whale (*Kogia sima*) is a vagrant cetacean species in the Mediterranean Sea, which is rarely sighted in water and occasionally is found stranded along the Italian coasts. In this work, we investigated the genetics, age, and reproductive status of three dead individuals (1 male, 1 female, 1 unsexed), stranded in three different sites along Italian coastlines. We used mitochondrial DNA markers for tracing the geographical origin of the animals, tooth sectioning as a proxy for their age estimation and gonadal histology for evaluating their reproductive status. Genetic analyses revealed that the male and the female are *K. sima* belonging to the Atlantic clade (A clade). The female, with a total body length (TBL) of 1.95 m, had an estimated age of 12 years, and ovaries with one corpus luteum. The male, with a TBL of 2.07 m, had testicles in late spermatogenesis and an estimated age of 22-24 years. From the unsexed individual, which had an estimated age of at least 20 years, no gonadal samples were obtained as it was stranded as a skeleton. Our findings will contribute to increase the knowledge of some unexplored provenance and life-history traits of *K. sima* visiting the Mediterranean.

Keywords: Cetacea; Kogiidae; Mediterranean Sea; mitochondrial DNA; age estimation; gonadal histology.

Introduction

The Kogiidae family includes only two species, *Kogia breviceps* (de Blainville, 1838) and *K. sima* (Owen, 1866), which, despite being globally distributed, are still among the less known species of marine mammals. In fact, most of our knowledge on their anatomy and life history comes from isolated and rare observations on stranded individuals. The dwarf sperm whale, *K. sima* (Cetartiodactyla, Cetacea, Kogiidae) is a small sized cetacean that inhabits warm, temperate and tropical oceans of both hemispheres. It reaches a maximum length of about 2.7 m and a weight of 280 kg, with females being possibly larger than males (Plön & Baird, 2021). Its range covers the western Atlantic from southeastern U.S.A. to Brazil, including the Antilles, the eastern Atlantic from Portugal to Cape Province, the Indian Ocean from Cape Province to India and South Australia, the western Pacific from Japan to New Zealand, the eastern Pacific from southern Canada to Chile; the species is also found in the Sea of Japan and in the Persian Gulf (Kiszka & Braulik, 2020). In the Mediterranean Sea, *K. sima* is regarded as a "vagrant" species (Plön & Baird, 2021; Maio *et al.*, 2022). Historically, the records of this species in the Mediterranean Sea are limited to four individuals stranded in different localities: in 1988, at the Foce Chiarone located on the border between Lazio and Tuscany (Baccetti *et al.*, 1991); in 2002, at Eraclea Minoa near Agrigento, in western Sicily (Bortolotto *et al.*, 2003); in 2015, at Mnar, Tanger, in the Strait of Gibraltar (Benchoucha *et al.,* 2018) and in 2017 at Baia di Trentova, Agropoli, Salerno (Maio *et al.*, 2017). Although no stable population of *Kogia* spp. has been identified in the Mediterranean so far (Notabartolo di Sciara, 2002; Loy *et al.*, 2019), kogiids are known to exist in the Mediterranean region due to the findings of a few late Neogene fossil specimens (Collareta *et al.*, 2019).

Multidisciplinary approaches have the power to link different kind of data together (e.g., Mezzasalma *et al*., 2017, 2019; Petraccioli *et al*., 2019; Maio *et al*., 2023) in a unifying perspective. This work was performed on biological samples of dwarf sperm whales stranded along the Italian coastlines using a combination of molecular and morphological analyses and was aimed at: i) confirming that the samples genetically analysed come from individuals of *K. sima*, ii) tracing their possible source populations using mitochondrial DNA (mtDNA) markers, iii) estimating the individual age by means of tooth histological section analysis, and iiii) evaluating their reproductive status on the basis of a gonadal histological analysis.

Materials and Methods

Samples

We analysed three individuals (1 male, 1 female, 1 unsexed) of *K. sima* stranded in 1988, 2002 and 2017, respectively, in different localities along Italian coastlines (Table 1). A detailed description of the three individuals and their stranding locality can be found in Baccetti *et al.* (1991), Bortolotto *et al.* (2003), and Maio *et al.* (2017, 2022).

Genetic Analysis

Samples of muscle tissue stored in 70% ethanol (KSa and KSb) and small tooth fragments (KSc) were used as sources of DNA for genetic analysis. Total DNA was extracted using the standard phenol-chloroform method (Sambrook *et al*., 1989), and the quantity and purity of extracted DNA were checked using a Nanodrop-ND1000 spectrophotometer (NanoDrop Technologies) (Fioravanti *et al*., 2022). A fragment of 498 bp of the 5’ end of the mtDNA control region (CR) and 496 bp of the 5’ end of the mtDNA cytochrome b gene (CytB) were amplified using two specific primer pairs designed with Primer3Plus software (Untergasser *et al*., 2007). Primers KsCR_F (5’-CATCAACACCCAAAGCTGAG-3’) and KsCR_R (5’-AGATGAAAATGGCCCTGAAG-3’) were used for the CR sequence, and primers KsCytb_F (5’-TGGACTCAAACCATGACCAA-3’) and KsCytb_R (5’-CGGTTGCTCCTCAGAATGAT-3’) for the CytB sequence. PCR amplifications were performed in 25 µL solutions containing 5 µL of 5X MyTaq™ Reaction Buffer (BioLine), 2.5 µL of F+R primer solution [5 µM], 0.3 µL of MyTaq™ DNA Polymerase (BioLine), 3 µL of extracted DNA [20-40 ng/µL] and 14.2 µL of ddH2O. An amplification profile consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 sec., 55°C for 45 sec. and 72°C for 90 sec., with a final extension step at 72°C for 7 min. PCR products were visualized on a 2% agarose gel stained with GelRed™ (Biotium) and then sent to BMR Genomics (Padova, Italy) for purification by exoSAP-IT™ (USB Corp.) and Sanger sequencing in both directions using an ABIPRISM 3730XL automated sequencer (Applied Biosystems). The match of obtained sequences with those of *K. sima* available on GenBank database was checked using BLAST (Altschul *et al*., 1990). Sequences of CR and CytB were combined, as done by Chivers *et al.* (2005), and aligned with both *K. sima* and *K. breviceps* sequences from their paper using CLUSTALW (Larkin *et al*., 2007). A Maximum Parsimony (MP) tree was inferred from the alignment using MEGA11 v11.0.13 (Tamura *et al*., 2021). The tree was rooted using a sperm whale (*Physeter macrocephalus*) sequence and 1,000 bootstrap replicates were set to assess the reliability of each node. The resulting MP tree was visualized and edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Since more CR sequences are available in GenBank than those of CytB, to have a larger dataset of dwarf sperm whales from different geographical areas, a second alignment was created including the two CR sequences obtained from this study and all those described so far in the literature (Chivers *et al*., 2005; Viricel, 2012; Piboon *et al*., 2022; Nishida *et al*., 2023; Plön *et al*., 2023). The sequences were downloaded from GenBank (accession numbers in Table S1), trimmed manually to make them comparable (about 400 bp in length) and then aligned on CLUSTALW (Larkin *et al*., 2007). The alignment file was used to construct a Median-Joining (MJ) network (Bandelt *et al*., 1999) on Network 10.2.0.0 (http://www.fluxus-engineering.com) to highlight relationships among haplotypes and their geographical origin. The ε parameter was set to 0 and gaps in the alignment were considered.

Age determination

For age estimation, we used tooth sections by applying the standard procedure of paraffin embedding according to Guarino *et al.* (2021). For each individual one man-

Table 1. Specimens of *Kogia sima* used in this study. F: female, M: male; ND: not determined; TBL: total body length.

ID	Stranding location	Stranding date	Sex	TBL (m)	References
KSa	Baia di Trentova (Agropoli, Salerno)	04/02/2017	F	1.95	Maio *et al.* (2017)
KSb	Eraclea Minoa (Agrigento)	11/09/2002	M	2.07	Bortolotto *et al.* (2003)
KSc	Foce Chiarone (Grosseto)	24/05/1988	ND	2.20	Baccetti *et al.* (1991)

dibular tooth was decalcified in RDO (Apex Engineering Products Corporation, Illinois, USA) for 18-24 hours according to the size of the sample. The tooth was subsequently washed in running tap water for 12 h, dehydrated through a series of graded ethanol baths, cleaned with a terpene of natural origin (Bioclear, Bio Optica, Milan, Italy), and embedded with molten paraffin (60 °C). Tooth longitudinal sections (15 µm thick) were obtained using a standard rotative microtome (Reichert–Jung/Leica 2045, Germany) and stained with Mayer's hematoxylin for 30 min. The tooth sections were examined using a Leica EZ4 stereo microscope to check for the best-stained sections closest to the midline of the tooth and a Motic BA340 compound light microscope. The tooth sections observed under light microscope at high magnification were successively photographed with a digital camera and stitched together into a single image. The images obtained of the tooth sections were contrasted using Adobe Photoshop 6.0 in order to enhance the distinctiveness of the growth layer groups (GLGs) (Read *et al.*, 2018). The number of GLGs was assessed independently by two authors (FMG, NM). In the case of discrepancies in the GLG count, the sections were read again until a final consensus was reached. Although no GLG calibration for *K. sima* is available so far, we assumed that the deposition rate of dentinal GLG is annual similar to that found in all other odontocetes for which calibration was carried out (Read *et al.*, 2018). Consequently, the estimation of the individual age is based on the number of GLGs counted.

Reproductive status

Samples from the testes and ovaries were fixed in neutral buffered formalin and embedded in paraffin following standard protocols (Guarino *et al.*, 2020; Maio *et al.*, 2023). They were then sectioned using a semi-automated rotary microtome and the 7 µm thick sections were stained with Mallory Trichrome (Bioptica, Milano, Italy) and observed under a Motic BA340 light microscope equipped with a digital camera.

Results

Genetics

DNA was successfully obtained from two out of the three samples analysed (KSa and KSb). Only a small tooth fragment was collected from the KSc specimen, and the available material did not allow to obtain a sufficient amount of DNA for PCR amplification. The BLAST search highlighted that the CR and CytB sequences of both KSa and KSb samples correspond to *K. sima* sequences already described and available in the GenBank database. The CR and CytB sequences of the KSa individual are identical to the sequences registered with accession numbers AY943735 and AY943796, respectively. The CR and CytB sequences of the KSb individual instead show a 100% correspondence with the sequences registered with accession numbers JX403781 and AY943802, respectively. All the CR and CytB sequences obtained in this study were submitted to GenBank (accession numbers PP541710-PP541713). The phylogenetic analysis carried out combining CR and CytB sequences generated a MP tree in which the two distinct *K. sima* clades (A and B clades, see Chivers *et al.*, 2005) are clearly distinguishable. Both KSa and KSb sequences are included in the *K. sima*-A clade, which exclusively contains sequences of dwarf sperm whales from the Atlantic Ocean (Fig. 1A). The clear separation of *K. sima* in two different clades on the basis of the geographical origin of haplotypes is also evident from the MJ network constructed using only CR sequences (Fig. 1B). Specifically, the CR sequence resulting from the KSa sample corresponds to a sequence previously described by Chivers *et al.* (2005) in dwarf sperm whales from the Atlantic Ocean (Table S1; Fig. 1B). The lack of information about the exact origin of all the individuals analysed in this previous work prevents us from narrowing down the distribution area of this haplotype. The KSb sample instead showed a sequence corresponding to a haplotype already described by Viricel *et al.* (2012) in the Western North Atlantic (Table S1, Fig. 1).

Age determination

Distinct dentinal GLGs were observed starting from the neonatal line (NL) in the teeth of all three individuals studied (Fig. 2). In general, GLGs were composed of a light boundary layer and a thick darkly stained layer but the alternating of these layers stained with varying intensity was not always clear within each GLG. Accessory layers (sensu Evans *et al.*, 2007) were also observed.

The most consistent ages for both the female KSa and the adult male KSc were respectively estimated as 12 and 22-24 years on the basis of the GLGs counted. The age of the unsexed individual KSb was less clear but was estimated as more than 20 yrs.

Gonadal histology

Both the testes and ovaries were in a variable state of autolysis with structural alterations due to poor state of preservation, but they still showed histologically interpretable shape-based features necessary for evaluating the reproductive status. The testes were in late spermatogenesis; showing large seminiferous tubules with an open lumen and spermatids (Fig. 3A). The ovaries had several partially unaffected primary follicles and a structure recognizable as the corpus luteum (Fig. 3B).

Discussion

So far, only four stranding events have been recorded in the Mediterranean area (Baccetti *et al.*, 1991; Bortolotto *et al.*, 2003; Benchoucha *et al.*, 2018; Maio *et al.*, 2017) and all individuals have been identified as *K. sima*

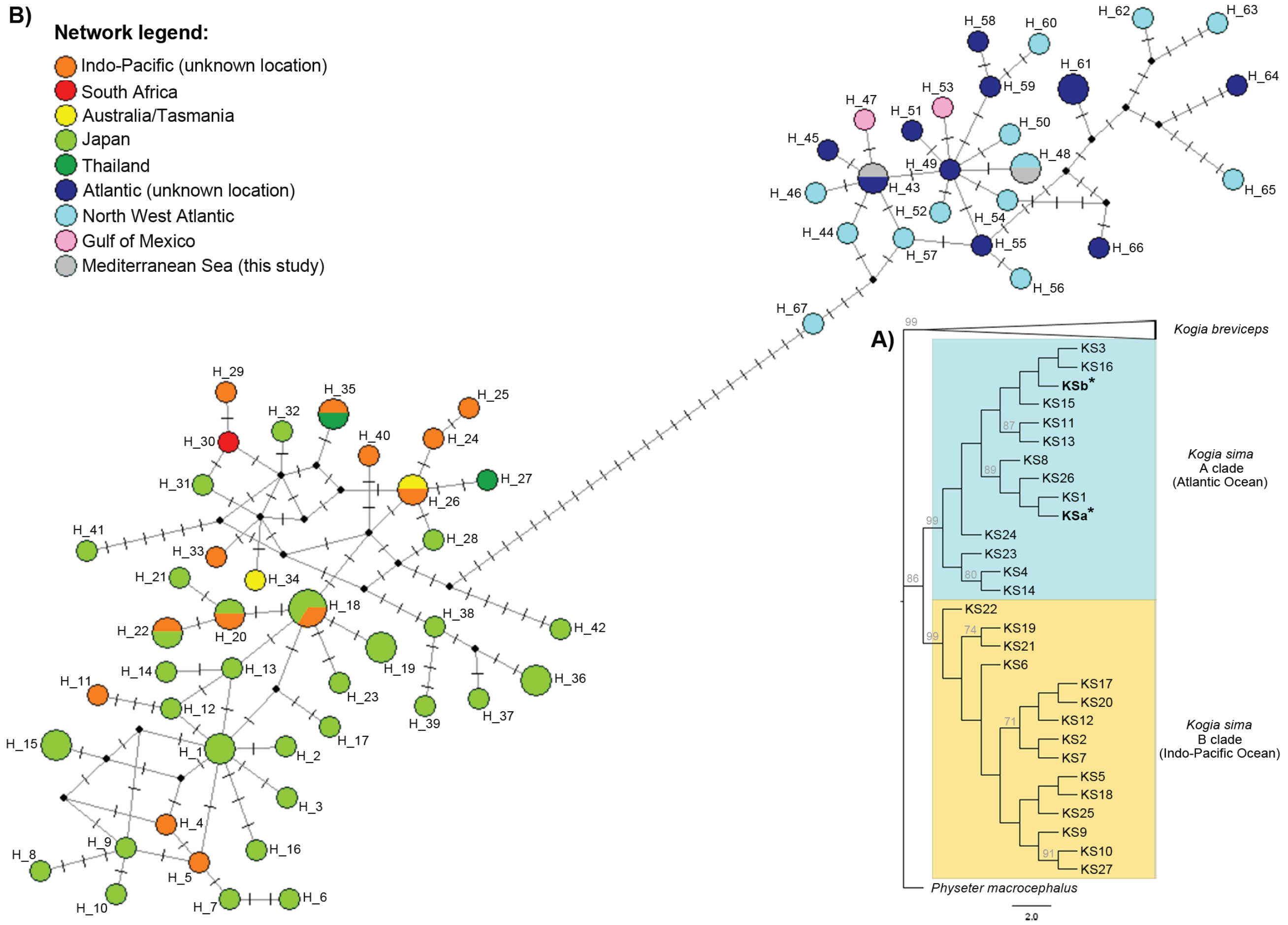

Fig. 1: A) Maximum Parsimony tree showing phylogenetic relationships among *Kogia* spp. sequences (CR + CytB). The sequences resulting from this study are indicated by an asterisk. Bootstrap values (in grey) are indicated on the branches as percentage, only ≥70 values are displayed. The clade including all the *K. breviceps* sequences is collapsed to reduce the tree size, and the two *K. sima* clades are highlighted in light blue (*K. sima*-A) and orange (*K. sima*-B). B) Median-Joining network displaying evolutionary relationships among CR haplotypes of *K. sima*. The size of the nodes is proportional to the number of sequences collapsed in each haplotype after trimming (see details in Table S1) and the colours identify the sampling area.

Fig. 2: Decalcified and stained longitudinal tooth section of *K. sima* KSa (above) and KSc (below). White arrows indicate GLGs. AL: probable accessory layers; NL: neonatal line; ?: GLG is doubtful.

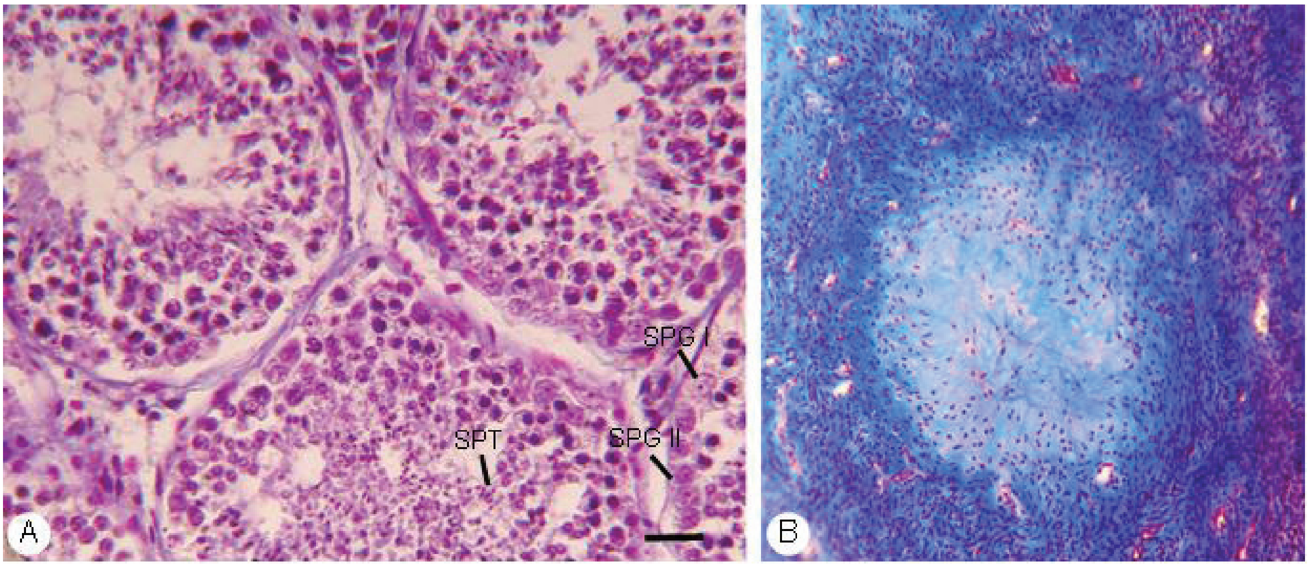

Fig. 3: Histological sections of gonads of *K. sima* stained with Mallory's trichrome. A) testes of KSb (TBL = 2.07m). SPG I: primary spermatogonia; SPG II: secondary spermatogonia; SPT: spermatids. B) Ovary of KSa (TBL = 1.95m) with corpus luteum. Bar corresponds to 30 µm in A and 75 µm in B.

on the basis of morphological characteristics. For individuals stranded in 2002 near Agrigento (Sicily, Italy) and in 2017 near Agropoli (Campania, Italy), the identification was also genetically supported by the analysis of a mtDNA 16S rRNA gene fragment (Maio *et al.*, 2017). Present work clearly confirms that at least the two samples genetically analysed (KSa and KSb) come from individuals of *K. sima*. In order to reveal the geographic origin of dwarf sperm whales sporadically found in the Mediterranean area, stranded specimens were genetically analysed using the more polymorphic mitochondrial CR and CytB sequence markers. An Atlantic origin was previously suggested for *K. sima* individuals found in the Mediterranean after the stranding of a dwarf sperm whale at Mnar (Tanger, Morocco), a locality situated in the proximity of the Strait of Gibraltar (Benchoucha *et al.*, 2018). It also seems unlikely that dwarf sperm whales enter the Mediterranean Sea via the Canal of Suez, as only a single stranding event was recorded for the species near the strait of Bab al Mandab, which connects the Red Sea to the Gulf of Aden (Notarbartolo di Sciara *et al.*, 2017), and also because among cetaceans, only the Indian Ocean humpback dolphin *Sousa plumbea* is regarded as a Lessepsian migrant (Kerem *et al.*, 2001; Ozbilgin *et al.*, 2018; Frantzis, 2018). Chivers *et al.* (2005) reconstructed the phylogeny of the Kogiidae family, using a combination of CR and CytB sequences, and revealed the presence of three monophyletic clades: all *K. breviceps* haplotypes group together in a single clade, while Atlantic and Indo/Pacific haplotypes of *K. sima* are clustered in two separate clades (*K. sima*-A and *K. sima*-B, respectively). These two clades probably evolved during glacial maxima events in the Pleistocene period when cool water temperatures limited the movement of individuals through the Cape of Good Hope (South Africa) and promoted the isolation of Atlantic and Indo-Pacific dwarf sperm whales. This separation is currently maintained by the presence of the phylogeographic break resulted by the meeting of Agulhas and Benguela currents (Chivers *et al.*, 2005). However, a recent research focused on the mtDNA characterization of the species in the Southern Hemisphere highlighted that, contrary to what was observed by Chivers *et al.* (2005), samples from St Helena Bay (South Africa, Atlantic Ocean) fell into the Indo-Pacific clade. This result suggested that South Africa does not represent a strong barrier to gene flow and that the Atlantic clade has a narrower distribution than previously proposed (Plön *et al.*, 2023). The sequences obtained from Mediterranean samples analysed in this study correspond to CR and CytB haplotypes previously described (Chivers *et al.*, 2005; Viricel, 2012). Both the phylogenetic analysis, carried out combining CR and CytB sequences, and the haplotype network, built using only CR sequences, show that they clustered within the Atlantic *K. sima*-A clade demonstrating that it is distributed also in the Mediterranean Sea. Some Atlantic individuals enter this basin through the Strait of Gibraltar for reasons that remain unclear, a behaviour observed also in other cetaceans such as the minke whale (*Balaenoptera acutorostrata*; Maio *et al.*, 2016) and the gray whale (*Eschrichtius robustus*; Manfrini *et al.*, 2023). However, it cannot be ruled out that a relict population of dwarf sperm whales inhabits the Mediterranean Sea but shares mtDNA haplotypes with Atlantic individuals, as already noted in the sperm whale (*Physeter microcephalus*; Drouot *et al.*, 2004). The presence of a local population of *K. sima* inhabiting this basin or the frequent use of the Mediterranean Sea by vagrants for feeding purposes is supported by parasitological analysis. In fact, the examination of gastrointestinal content of the *K. sima* stranded near Agropoli in 2017 (Santoro *et al.*, 2018) showed the occurrence of anisakid species (*Anisakis physeteris* and *A. pegreffii*) and cephalopod beaks that are usually found in Mediterranean sperm whales, suggesting that the individual had fed in the Mediterranean Sea.

In this study, we used GLG count in tooth sections, a widely practiced odontocete age-determining method (Evans *et al.*, 2007; Read *et al.*, 2018), to assess the age of *K. sima*. So far, few studies have been carried out on this species using this method. Based on the section of a tooth, Nagorsen & Stewart (1983) reported the age of about 12 years for a sexually mature female of about 2.3 m (tip of the snout to the centre of the eye) stranded along the coast of Vancouver Island. In samples of 45 strandings of *K. sima* analysed along the South African coastline (except for one, in Australia) between 1880 and 1995, Plön (2004) found that the largest female and male measured 2.64 m and 2.60 m, respectively; furthermore, the estimated maximum age was 16 years for a male, about 2.52 m TBL, and 22 years for a female, about 2.45 m TBL. Plön (2004) used ground sections of undecalcified teeth, since standard methods for determining age in delphinids, such as decalcification and formic acid etching, up to then had yielded unsatisfactory results when applied to *K. sima*. Luque *et al.* (2009) also raised questions about the reliability of age determination of *K. sima* using decalcified tooth sections obtained using rotary microtome, after paraffin embedding, or cryostat because the sections were of poor quality. Unlike these previous works, we obtained technically satisfactory paraffin sections although we encountered strong difficulties in interpreting the actual GLGs due to accessory lines. The latter are sub-annual incremental layers associated with life-history events of the species and may confuse GLG readings and lead to age inflated estimates (Evans *et al.*, 2007; Read *et al.*, 2018). Plön (2004) had already found that the number of accessory layers present in the tooth sections of *K. sima* is high. Indeed, the misinterpretation of GLGs due to the presence of accessory lines represents one of the major sources of error in age determination of odontocetes (Hohn *et al.*, 1989; Plön, 2004; Murphy *et al.*, 2014; Read *et al.*, 2018). However, it is intriguing that the age estimates from the present study were much higher from those obtained by Plön (2004) for individuals of a similar body length. While we estimated an age of 12 and 22-24 years for a female with a TBL of 1.95m and a male with a TBL of 2.07 m, respectively, Plön (2004) obtained an age lower than 5 yrs for females and males of similar length, respectively. This difference could be due to the different method of preparation between our study and the one of Plön (2004). In fact, there is some evidence that unstained ground sections, as those used by Plön (2004), may result in an underestimated GLG count when compared to decalcified stained sections from the same individual (Hohn & Fernandez, 1999). However, other factors, such as different geographical areas, could explain the differences in growth rates of *K. sima* obtained from different studies but they cannot be discerned from the information at hand.

Sampling of stranded cetaceans' organs, including gonads, usually occurs a long time after the death of the animal so that histological analysis of gametogenesis is difficult but the results are often reliable (Maio *et al.*, 2023). In our study on stranded individuals of *K. sima,* the histological analysis of the gonad allows us to conclude that both individuals were sexually mature. Furthermore, the condition of the ovaries suggests a previous pregnancy of the examined female but because there was no evidence of lactation, we hypothesize that one fetus may have been aborted. According to Plön (2004) the sexual maturity in the male of *K. sima* is usually attained by their 3rd year and 1.97 m of body length, and in the female by their 5th year and 2.15 m, while physical maturity occurs at 16 years in males and 13 years in females. Therefore, while our findings agree with previous bibliographical data (Plön, 2004; Plön & Baird, 2021) regarding the sexual maturity of *K. sima* males they indicate that females may actually attain sexual maturity at a much lower body length which corresponds to a much higher age than previously reported. As for the male, it was plausible to expect it to be sexually mature but not to be of such a high age when compared to literature data (Plön, 2004). In conclusion, molecular analyses performed on dwarf sperm whales stranded along Mediterranean coastlines have allowed us to identify them as belonging to the Atlantic *K. sima* - A clade. This result suggests that Atlantic individuals visit Mediterranean waters probably for feeding purposes or that a relict population inhabits this basin, which is a question that can only be resolved by conducting further studies with more powerful genetic markers to discriminate populations (e.g., SNPs, microsatellites). Age determination using tooth sectioning combined with gonad analysis has also shown that the female of *K. sima* reaches sexual maturity at a much lower length (but not age) than that reported so far. Finally, this study reaffirms the importance of collecting as much information as possible at the time of stranding, especially in the case of very elusive cetacean species.

Competing interests: The authors declare that they have no conflict of interest.

Authors' contributions: NM and TF contributed equally to this work. FMG, NM, VCB designed and supervised the study. FMG, NM, TF, LL, AS conducted the laboratory work. FMG, NM, TF, MM, GI, FP, AP, VCB, analysed the data. FMG, NM, TF, MM, VCB wrote the research note. All authors have read and approved the final version of the manuscript.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. GenBank accession numbers of sequences used to built the Median-Joining network, sequences that match after trimming (~400 bp) are included in the same haplotype. Haplotypes are named as in the network and letters at the beginning of the accession number are linked to reference papers: AY=Chivers *et al.* (2005); MZ=Piboon *et al.* (2022); LC=Nishida *et al.* (2023); OR=Plön *et al.* (2023) (see references in the main text of the manuscript). GenBank accession numbers in red are those of sequences obtained in this study.