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New record of *Hexanchus griseus* in the northwestern Mediterranean Sea with insights into its biology and feeding ecology

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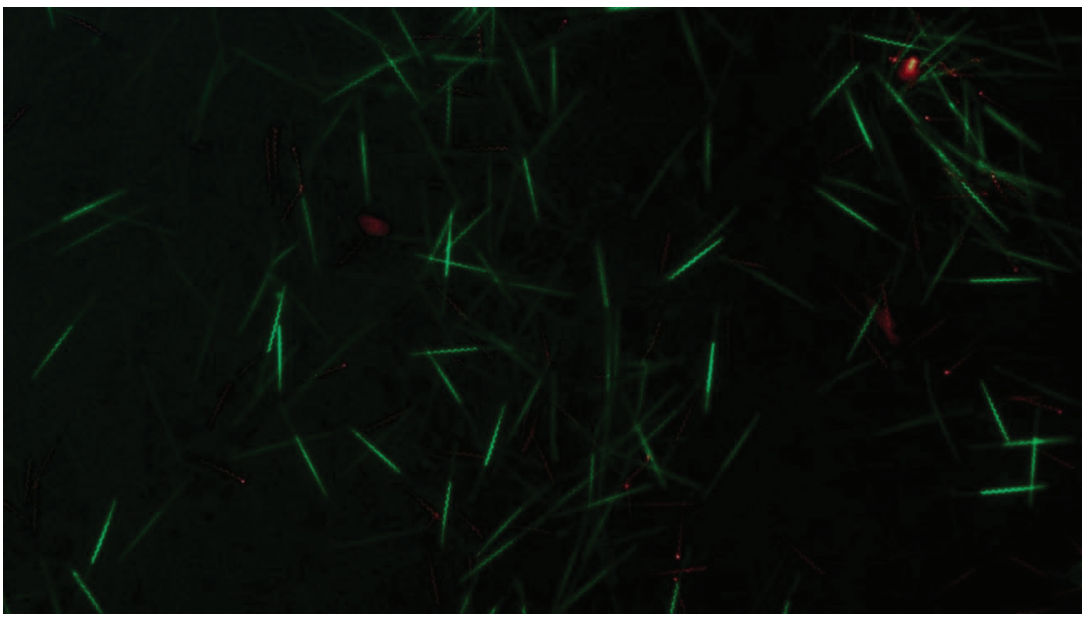

***Fig. S1*:** Hexanchus griseus spermatozoa stained to assess their plasma membrane integrity. SYBR-14 stains intact cells in green, and propidium iodide (PI) stains damaged cells in red. Stained samples were observed under a fluorescence microscope (Nikon Eclipse 80i) at 10x magnification.

Supplementary Methods

DNA was extracted from two 50 mg samples of muscle tissue preserved in DMSO, using the Silica based NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol. The D-Loop region of the mitochondrial DNA was isolated using standard PCR, following the protocol described in Gray et al. (2018). PCR was carried out in a Biometra TAdvanced thermocycler, with ramping rate of 3ºC/s. All other parameters followed the conditions described in Gray et al. (2018). Sequences were aligned against the D-loop region of Odontocete mitogenomes from the NCBI nucleotide database (Table S1).

Table S1: GenBank accession numbers and original references, for all reference mtDNA sequences used in the phylogenetic analysis show in Figure S2.

Species	GenBank Accession Number	Reference
Platanista minor	AJ554058	Arnason *et al.* (2004)
Inia geoffrensis	AJ554059	Arnason *et al.* (2004)
Lipotes vexilifer	AY789529	Yan *et al.* (2005)
Monodon monoceros	AJ554062	Arnason *et al.* (2004)
Phocoena phocoena	AJ555063	Arnason *et al.* (2004)
Orcinus orca	GU187192; GU187173	Morin *et al.* (2010)
Globicephala macrorhynchus	HM060333, JF339976	Vilstrup *et al.* (2011)
Globicephala melas	HM060334; JF339972	Morin *et al.* (2010), Vilstrup *et al.* (2011)
Pseudorca crassidens	HM060332; JF289173; JF289174	Morin *et al.* (2010), Vilstrup *et al.* (2011)
Feresa attenuata	JF289171; JF289172	Vilstrup *et al.* (2011)
Peponocephala electra	JF289176	Vilstrup *et al.* (2011)
Orcaella heinsohni	JF339977	Vilstrup *et al.* (2011)
Orcaella brevirostris	JF289177	Vilstrup *et al.* (2011))
Grampus griseus	EU557095	Xiong *et al.* (2009)
Lagenorhynchus albirostris	AJ554061	Arnason *et al.* (2004)
Sousa chinensis	EU557091	Xiong *et al.* (2009)
Stenella atenuata	EU557096	Xiong *et al.* (2009)
Stenella coeruleoalba	EU557097	Xiong *et al.* (2009)
Steno bredanensis	JF339982	Vilstrup *et al.* (2011)
Delphinus capensis	EU557094	Xiong *et al.* (2009)
Tursiops aduncus	EU557092; KF570335	Xiong *et al.* (2009), Moura *et al*. (2013)
Tursiops truncatus	EU557093; KF570379	Xiong *et al.* (2009), Moura *et al*. (2013)
Tursiops cf. australis	KF570363	Moura *et al*. (2013)

A Bayesian phylogenetic tree was then reconstructed using MrBayes, as implemented in Geneious R7 (https://www.geneious.com). Four independent MCMC runs of 1 000 000 iterations following 100 000 iterations of discarded burn-in were set. The best fitting substitution model was identified using the software Topali v2 (Milne et al. 2009), as the GTR+I+G nucleotide substitution model.

Both sequences produced from each of the tissue samples obtained from the right flipper found in the shark's stomach content resulted in identical nucleotide sequences. Therefore, only one of the sequences was used in the phylogenetic reconstruction. The shark stomach content derived sequence (Genbank accession number OP902517) grouped more closely with the one of *Stenella coeruleoalba* (derived from mitogenome with accession number EU557097; Fig. S2). Pairwise nucleotide similarity between the shark stomach remains and *Stenella coeruleoalba* was 96.2 %, which was higher than to any other Odontocete species tested (65.9% - 96.1%). The only species with nucleotide similarity of 95% or greater were *Tursiops truncatus*, *Tursiops aduncus*, *Delphinus capensis* and *Stenella coeruleoalba*. From these species, *T. aduncus* can be excluded because it does not occur in the Mediterranean Sea. The remaining species were therefore tested using microsatellite loci, as described below. *Delphinus capensis* is currently not formally recognized as an independent species of *D. delphis* and also does not occur in the Mediterranean, and therefore *D. delphis* was used instead.

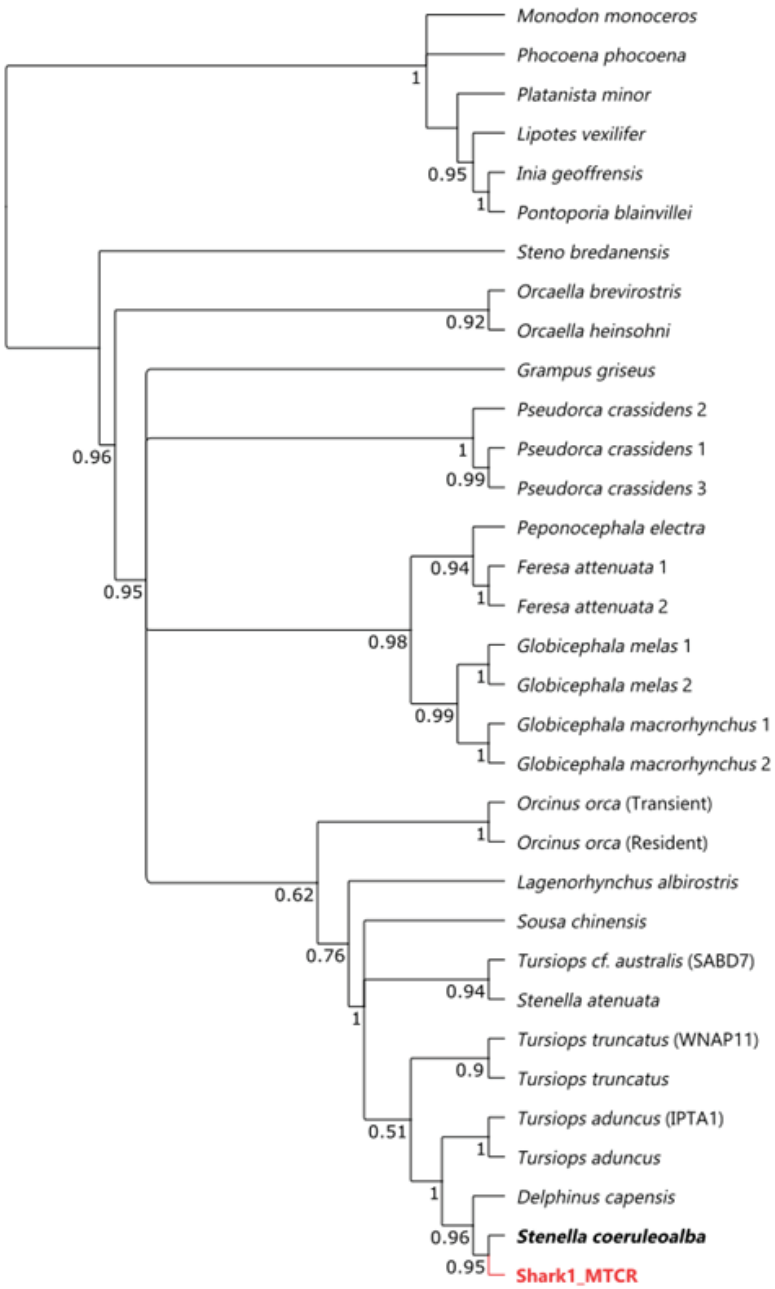

***Fig. S2*:** Bayesian phylogenetic tree based on cetacean mtDNA D-Loop sequences extracted from mitogenome datasets (see Moura *et al*., 2013 for details). Posterior probabilities are presented next to the nodes. Only one of the mtDNA D-Loop sequences obtained from the suspected dolphin muscle found in the shark's stomach content was used for the reconstruction of this tree, and is shown in red.

Microsatellite loci were amplified through two multiplex PCR reactions in a Biometra TAdvanced thermocycler with ramping rate of 6ºC/s. Multiplex reactions were carried out using Qiagen Multiplex PCR kit with the thermocycling conditions: 95°C for 15 min, 40 cycles at 52°C (primer set A)/57°C (primer set B) for 90 s, 72°C for 1 min and a final extension at 60°C for 30 min. Primer set A consisted of loci D08 (Shinohara *et al*., 1997), Dde66 (Coughlan *et al*., 2006), TtrRH1, TtrC12, Ttr04, Ttr11, Ttr34 (Rosel *et al*., 2005), KWM2a, KWM1b, KWM2b, KWM12a (Hoelzel *et al*., 1998). Primer set B consisted of loci Dde09, Dde59, Dde65, Dde69, Dde70, Dde84 (Coughlan *et al*., 2006), AAT44 (Caldwell *et al*., 2002), Ttr19, Ttr58, Ttr63 (Rosel *et al*., 2005). Successful amplifications were genotyped through capillary electrophoresis using an ABI 3500 genetic analyzer. Microsatellite allele sizes were determined by visual inspection in Geneious R7. A principal component analyses was then carried out using GenalEx 6.5 based on pairwise genetic distances between individuals. Microsatellite data is available upon request.

The results for both the mtDNA phylogenetic tree and the microsatellite PCA grouped both Delphinidae remains samples (which showed identical genotypes for both marker types) more closely to the reference stripped dolphin (*Stenella coeruleoalba*) samples, which were always well differentiated from other phylogenetically close dolphin species (Fig. S3).

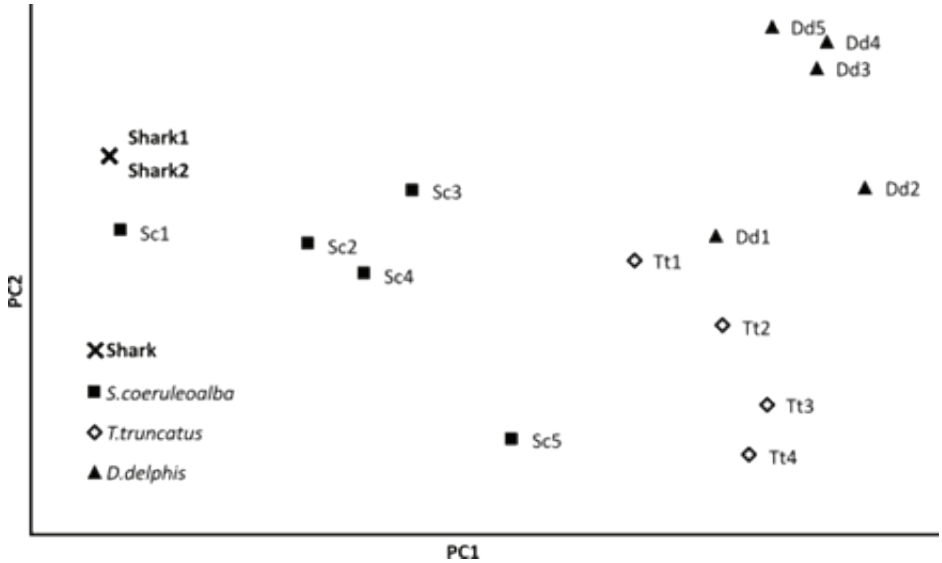

***Fig. S3*:** PCA based on 21 microsatellite loci genotyped from the dolphin muscle found inside the shark stomach, compared to the phylogenetically close species *Stenella coeruleoalba*, *Delphinus delphis* and *Tursiops truncatus*. Both shark stomach content extracts had identical genotypes, and grouped more closely to striped dolphin (*Stenella coeruleoalba*) than the other reference species.

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