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A Reliable and Cost-Efficient PCR-RFLP Tool for the Rapid Identification of Cetaceans in the Mediterranean Sea

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Abstract: Twenty-five species of cetaceans have been reported throughout the Mediterranean Sea, eight of them are commonly distributed in the whole basin and are regularly found beached or adrift in the sea. Stranded animals are frequently found in poor conservation status, preventing reliable identification; identification is thus often based solely on morphological features. Therewith, molecular tools are especially useful to provide taxonomic identification. In this work, a four-enzymes PCR-RFLP *in silico* protocol, based on a fragment of the mitochondrial gene *cytb*, has been designed for cetacean species occurring in the Mediterranean Sea. Moreover, beached or floating specimen samples belonging to the eight common species have been tested in the laboratory, providing evidence that this approach represents a reliable, cost- and time-effective tool for their specific identification.

Keywords: cetaceans; PCR-RFLP; Mediterranean Sea; stranded cetacean; adrift cetacean; molecular taxonomy; cetacean molecular identification

Citation: Brustenga, L.; Chiesa, S.; Maltese, S.; Consales, G.; Marsili, L.; Lauriano, G.; Scarano, V.; Scarpato, A.; Lucentini, L. A Reliable and Cost-Efficient PCR-RFLP Tool for the Rapid Identification of Cetaceans in the Mediterranean Sea. *Sustainability* **2022**, *14*, 16763. https://doi.org/10.3390/su142416763

Academic Editor: Chen-Tung Arthur Chen

Received: 6 October 2022
Accepted: 8 December 2022
Published: 14 December 2022

1. Introduction

Cetaceans are a fundamental component of marine biodiversity; as apex predators, they are direct indexes of environmental status. According to Perrin [1], eighty-nine species currently occur in the world's oceans, lakes, and rivers: some species are known to have a cosmopolitan distribution, while others have a limited distribution due to their ecological features.

Regarding the Mediterranean Sea, although it covers only 0.8% of the global ocean surface, it hosts a highly diverse marine fauna, including cetaceans, some of which are of conservational concern [2,3]. Twenty-five species are globally reported to occur in the Mediterranean (Table S1) [4–12]: eight of them are recognized as regular in the whole basin, two as regular just in specific sectors, as in the Gibraltar strait and/or the Levantine Sea, while fifteen have been occasionally sighted (visitors) or recorded very rarely (vagrant) [3,13–17].

The specific identification of humpback dolphins (*Sousa* sp. Gray, 1866) is often difficult in the Mediterranean [18,19]; although *Sousa chinsensis* (Osbeck, 1756) has been previously mentioned in the checklist of Mediterranean species [16], more recent literature referred only to *S. plumbea* (Cuvier, 1829) [3,17]. Therefore, as a cautionary approach, we included both of them in the presence list.

Moreover, in the Mediterranean Sea, the harbor porpoise (*Phocena phocoena* Linnaeus, 1758) is represented by two subspecies: the Atlantic harbor porpoise, *P. p. phocoena* (Linnaeus, 1758), which is vagrant, and the endangered Black Sea harbor porpoise *P. p. relicta* (Abel, 1905), a regular in the Aegean Sea [20].

Cetaceans of the Mediterranean Sea are facing different pressures due to several human activities, acknowledged to produce both indirect and direct effects on these marine mammals. Among these pressures, it is possible to recognize the ones related to incidental catches in fishing gear and ship collisions; both are responsible for direct, lethal, and sub-lethal effects, respectively. Moreover, acoustic and industrial pollution, chemical wastes, and prey depletion all lead to a generalized habit degradation which results in the displacement of cetacean populations from habitats. The above-mentioned threats have different extents and effects on cetaceans and a complete description of the main threats occurring in the Mediterranean Sea can be found in Johnson and colleagues' work [21].

Consequently, beached, adrift, or sunken animals are frequently detected in the Mediterranean basin, providing useful samples for both ecological and genetic population dynamic studies. However, dead individuals are often in bad conservation status, due to the degradation processes ongoing, or are not easily retrievable from the sea floor. Therefore, the taxonomic identification might be difficult or impossible to base only on a morphological approach. In the latter case, molecular tools are especially useful to provide a taxonomic identification [22,23]. Among all available techniques, a special attention should be given to those approaches reliable for degraded DNA, as for example PCR-RFLP (restriction fragment-length polymorphisms); this technique provides a simple alternative to DNA sequencing, allowing species identification even if the template DNA is deteriorated [24].

Therefore, the main scope of this work is to provide a reliable, cost-efficient PCR-RFLP tool for a rapid identification of the cetacean species that are commonly occurring in the Mediterranean Sea, to be further applied in case of degraded or unrecognizable samples.

2. Materials and Methods

2.1. Bioinformatics: In Silico Restriction Design

All the twenty-five species occurring in the Mediterranean were selected for *in silico* restriction design; unfortunately, no specific *cytb* sequences or complete mitochondrial genomes of *S. plumblea* were available from GenBank (as of July 2022), therefore the species was not included in the analysis. Further studies will be performed when sequences are available. Nevertheless, the congeneric species *S. chinensis* has been included in the alignment and analyzed.

Regarding the two subspecies of *P. phocoena,* the analysis has been performed at species level, since the *cytb* available sequences did not include the subspecies description.

Therefore, the final list of species selected for *in silico* restriction design included twenty-four cetaceans, listed in Table 1. Marked in bold, along with the features of all tissue samples and the sampling collection sites, are the eight species commonly occurring in the Mediterranean that were tested with the designed protocol.

Sequences of *cytb* fragment and the complete mitochondrial genomes of all species were collected from Genbank (https://www.ncbi.nlm.nih.gov/ (accessed on 10 April 2022)), preferring, when available, those obtained from Mediterranean specimens. Sequences were aligned using MEGA X [26].

Primers used for PCR amplifications were then fit into the alignment to trim the sequences at the desired length. All entries that did not encompass the desired fragment were discarded along with duplicates showing identical sequences, of which only one was left to declutter the alignment.

Table 1. List of twenty-four species tested in the PCR-RFLP protocol. Eight species marked in bold were subjected to both *in silico* protocol and then to sample analysis. Species and abbreviations are provided, together with the sample ID; T tissue type (M = muscle; B = blubber; S = skin); C condition of the sample (F = fresh; F-D = freeze-dried); sampling year (D) and place of collection; sex (M = male; F = female; U = undetermined); length (L) in meters of the animal; preservation status (PS) of the carcass with decomposition code (1-5) according to Geraci and Lounsbury's classification [25]: 1 = alive, later died; 2 = recently dead; 3 = decomposed; 4 = advanced decomposition; 5 = mummified. N.A. = Samples/information not available.

Species and Abbreviation	Sample Id	T	C	D	Place	Sex	L	PS
Balaenoptera acutorostrata (Bacu)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Balaenoptera borealis (Bbor)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Balaenoptera physalus (Bphy)	ID 536 BP	M+B	F	2021	Italy	F	19.77	1
	BPROSIGNANO M2013	M	F	2013	Italy	F	16.40	4
	RT145Bp M	M	F	2021	Italy	F	12.10	4
	RT91Bp	B	F	2015	Italy	M	17.30	4
Delphinus delphis (Ddel)	28/06/02Grecia	B	F	2002	Greece	U	U	U
	2/07/02Grecia	B	F	2002	Greece	U	U	U
	80377M	M	F-D	2016	Italy	M	2.2	2
	21963M	M	F-D	*N.A.*	*N.A.*	U	U	U
	19194M	M	F-D	2018	Italy	M	1.97	4
	13381 Dd A	S+B	F	2021	Italy	F	1.73	2
Eschrichtius robustus (Erob)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Eubalena glacialis (Egla)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Globicephala macrorhynchus (Gmac)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Globicephala melas (Gmel)	34251M	M	F-D	2015	Italy	F	3.95	4
	01/02/13 A	B	F	2013	Italy	M	5.60	3
	35117M	M	F-D	2018	Italy	M	5.25	3
	73948M	M	F-D	2017	Italy	F	3.50	4
Grampus griseus (Ggri)	RT 176 GG	M+B	F	2021	Italy	F	2.98	1
	2GRM	M	F-D	2007	Italy	M	2.40	U
	26153GgM	M	F-D	2012	Italy	U	U	5
	GVM226M	M	F-D	*N.A.*	*N.A.*	U	U	U
	66102M	M	F-D	2016	Italy	F	1.68	2
	55536M	M	F-D	2016	Italy	M	2.28	2
Hyperoodon ampullatus (Hamp)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Kogia sima (Ksim)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Megaptera novaengeliae (Mnov)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Mesoplodon bidens (Mbid)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Mesoplodon densirostris (Mden)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Mesoplodon europaeus (Meur)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Orcinus orca (Oorc)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*

Table 1. *Cont.*

Species and Abbreviation	Sample Id	T	C	D	Place	Sex	L	PS
Phocoena phocoena (Ppho)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
***Physeter macrocephalus* (*Pcat*)**	RT101Pm	M	F	2016	Italy	M	12.8	4
	7C	M	F	2009	Italy	M	11.20	1
	5C	B	F	2009	Italy	M	12.10	1
	6C	B	F	2009	Italy	M	10.50	1
Pseudorca crassidens (Pcras)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Sousa chinensis (Schi)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
***Stenella coeruleoalba* (*Scoe*)**	RT 166 SC	M+B	F	2021	Italy	F	2.04	2
	RT 167 SC	M+B	F	2021	Italy	F	1.49	3
	RT 169 SC	M+B	F	2021	Italy	M	1.63	3
	RT 170 SC	B	F	2021	Italy	M	1.12	3
	RT 171 SC	M+B	F	2021	Italy	M	1.93	4
	RT 172 SC	B	F	2021	Italy	U	1.97	4
	RT 175 SC	M+B	F	2021	Italy	F	2.04	2
	RT 188 Sc M	M	F	2022	Italy	F	1.52	2
	RT 187 Sc M	M	F	2021	Italy	F	1.83	3
	13546 Sc A	S+B	F	2022	Italy	F	1.52	2
	13261 M	M	F	2020	Italy	M	1.00	2
Steno bredanensis (Sbre)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Tursiops truncatus (Ttru)	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
	RT180Tt	M+B	F	2021	Italy	M	2.35	3
	RT168Tt	M	F	2021	Italy	U	2.20	4
	RT86Tt	M	F	2014	Italy	M	1.35	4
	RT189Tt M	M	F	2022	Italy	F	2.08	2
	13443Tt A	M+B	F	2021	Italy	U	1.50	3
	13283Tt M	M	F	2020	Italy	F	1.95	3
***Ziphius cavirostris* (*Zcav*)**	29794ZcM	M	F-D	2012	Italy	F	4.77	1
	ID429M	M	F-D	2017	Italy	M	5.30	2

The alignment was firstly screened visually for polymorphisms and then the most representative sequence for each species was uploaded to NEBcutter 3.0.15 (https://nc3.neb.com/NEBcutter/ (accessed on 10 April 2022)) to verify which restriction enzymes showed restriction sites within the fragment. The same sequence was then uploaded to molbiotools' restriction analyzer (https://molbiotools.com/restrictionanalyzer.php (accessed on 10 April 2022)) which allowed us to simulate a restriction reaction and check if the enzymes identified were able to cut the fragment at the expected sites, visualizing the length of the produced fragments. A double-check of the functioning of the restriction enzymes in all the available sequences was carried out manually, searching for the restriction sites in MEGA X and calculating the length of the presumptive fragments.

A four-enzyme PCR-RFLP protocol, using the enzymes *Hpy*188III (NewEngland Biolabs), *Hha*I (NewEngland Biolabs), *Alu*I (NewEngland Biolabs), and *Mwo*I (Promega), was then devised to discriminate the species commonly occurring in the Mediterranean Sea. The restriction sites of the four enzymes are reported in Table 2.

Table 2. Restriction sites of the chosen enzymes, slashes indicate the cleavage sites.

***Hpy*188III**	***Hha*I**	***Mwo*I**	***Alu*I**
TC/NNGA	GCG/C	GCNNNNN/NNGC	AG/CT

2.2. Sample Collection and Processing

Study Area and Sample Collection

Skin, blubber, and muscle samples were collected from stranded and adrift animals along the Italian coasts except for two *Delphinus delphis* specimens which were sampled in Greece (Figure 1). Ethical review and approval were waived for this study due to CITES permit number IT007.

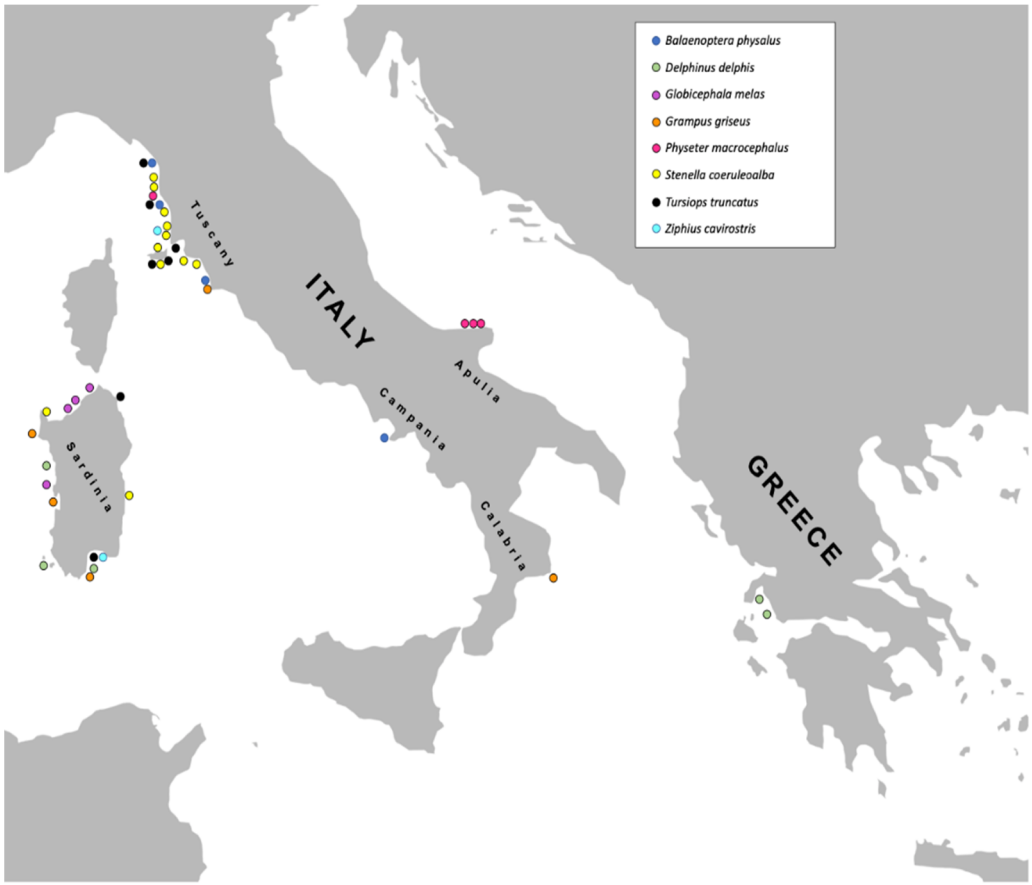

Figure 1. Sampling sites in the Mediterranean Sea.

The specimens collected were characterized by different conservation conditions, with some animals that were stranded before or right after dying, and other that had undergone pre- and post-stranding decaying processes. Figure 2 shows examples of the specimens collected. Each sample was classified for its status of preservation and a decomposition code was assigned, according to Geraci and Lounsbury's scale [25].

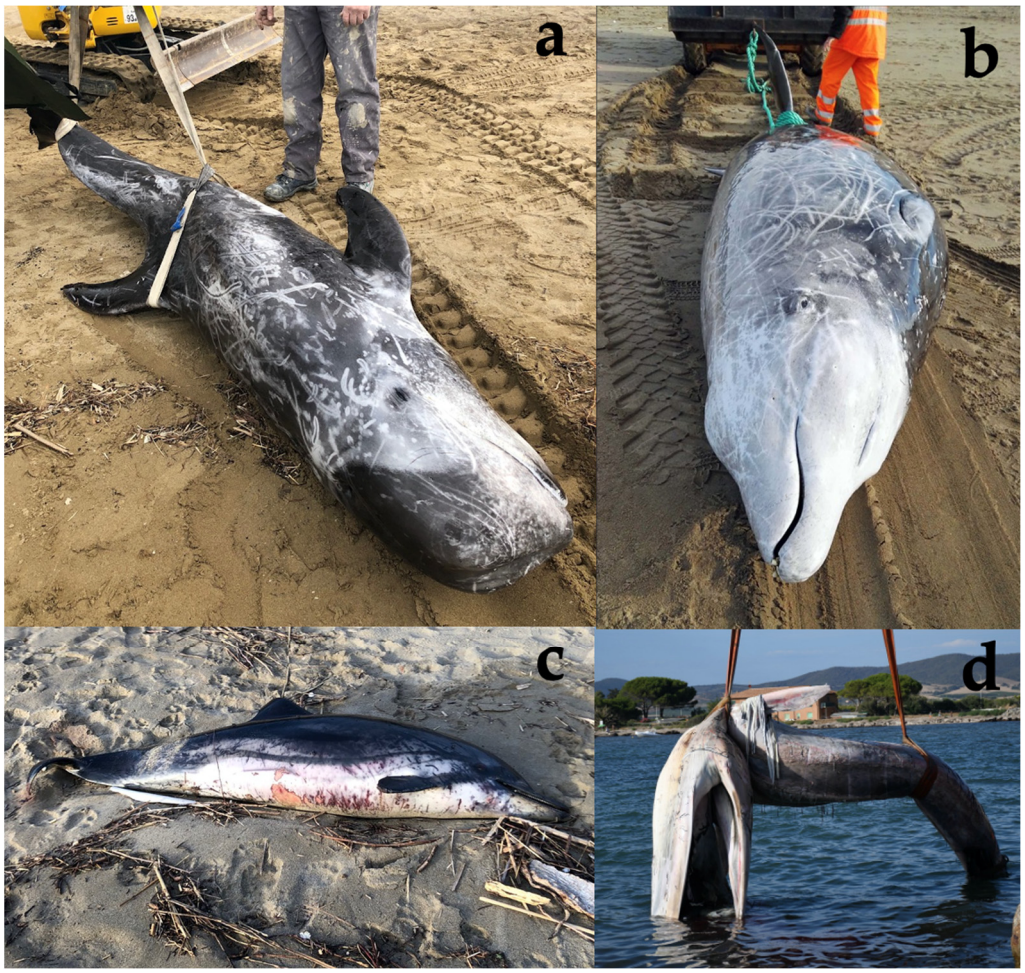

Figure 2. Examples of conservation status, with the relative decomposition code, of some collected animals from which samples were drawn: (**a**) *Grampus griseus* that died after the stranding (Code 1); (**b**) *Ziphius cavirostris* recently dead (Code 2); (**c**) *Stenella coeruleoalba* decomposed (Code 3); (**d**) *Balaenoptera physalus* retrieved in an advanced status of decomposition (Codes 4/5).

Overall, 44 tissue samples were collected; from 2 to 11 different individuals each species: 4 samples of *B. physalus*; 6 samples of *D. delphis*; 4 samples of *G. melas*; 6 samples of *G. griseus*; 4 samples of *P. macrocephalus*; 11 samples of *S. coeruleoalba*; 7 samples of *T. truncatus*; and 2 samples of *Z. cavirostris*.

All specimens used were undoubtedly taxonomically identified by morphological characters. These characters were differently selected, depending on the conservation status of the animals and on the available anatomical parts. The main features analyzed were overall animal size, rostrum shape, teeth morphology (when occurring), color pattern, and flippers' shape and dimensions.

Whenever the lower part of the abdomen was preserved, sex was visually determined. Animals which were already consumed by scavengers or already decayed were sexed upon dissection.

Tissue samples were removed with a sterile scalpel, wrapped in aluminum foil and conserved fresh at −20 °C in EtOH or freeze-dried at room temperature until further processing.

2.3. HMW DNA Extraction and Purification

High molecular weight (HMW) total genomic DNA was extracted from the forty-four biological samples described above. When multiple kinds of tissues were available for the same animal, muscle tissue was preferred for extraction.

Fresh tissue was cut with a sterile scalpel and fragmented in smaller pieces with surgical scissors; lyophilized samples already came as dehydrated small flakes that were used without further processing.

All procedures that involved sample manipulation were preceded and followed by a thorough cleansing of all possible surfaces with denaturized ethyl alcohol to avoid cross-contaminations. Moreover, all laboratory procedures were conducted under a laminar flow hood using disposable gloves and FFP-2 face masks to avoid the risk of environmental or operator contamination.

Tissue fragments were collected in 1.5 mL sterile tubes and 620 µL of a lysis solution mix made of 500 µL of Nuclei Lysis Solution (Promega), 120 µL EDTA, to be stored in the freezer until cloudy, and 20 µL of Proteinase K (Promega) was aliquoted in each sample which was then ground with an autoclaved potter to furtherly break up the tissue. Each sample was then vortex mixed for 25″ to ensure that the extraction liquid was evenly in contact with the tissue fragments. Fresh samples were processed right away using the Wizard Genomic DNA Purification Kit (Promega) following a modified and already validated protocol [27,28]. Lyophilized samples were kept overnight at 4 °C in the lysis solution to allow rehydration before processing to completion the day after following the same protocol. On the final step, 80 µL of DNA Rehydration Solution (Promega) was added to each sample to resuspend the DNA pellet to be used for downstream application, resuspension was facilitated by a 15′ water bath at 65 °C.

2.4. Amplification

PCR amplification targeting a 439 bp fragment of the mitochondrial gene cytochrome b (*cytb*), widely used for phylogenetical analyses [29,30], was performed using *L15162* (F 5′-GCTACGTACTTCCATGAGGACAAATATC-3′) and *H15549* (R 5′-AAACTGCAGCCCCTC AGAATGATATTTGTCCTCA-3′) [31]. Amplifications were performed using 2 µL of the extracted DNA, 12.5 µL of 2× PCR Master Mix (Promega), 1 µL for each of 10 µM primers and nuclease free H_2O (Promega) to a final volume of 25 µL, using the following amplification scheme: initial denaturation at 94 °C for 2′, followed by 35 cycles with denaturation at 94 °C for 1′, annealing at 48 °C for 1′, elongation at 72 °C for 1′30″, and a final extension at 72 °C for 8′. The amplified products were checked by electrophoresis in 1.5% agarose gel containing SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, Cambridgeshire, UK) run in 1×TBE buffer and later visualized under a UV transilluminator.

2.5. PCR-RFLP

All restriction mixtures were prepared as follows: 10 µL of amplified DNA; 3 µL of the restriction enzyme; 2 µL of enzyme buffer; 5 µL of nuclease-free H_2O, to reach a final volume of 20 µL. *Hpy*188III, *Alu*I and *Hha*I were all utilized along with CutSmart Buffer (New England Biolabs), whereas *Mwo*I was utilized along with Buffer Tango (Promega). Restriction reactions were all performed at 37 °C in a heated dry bath thermoblock for a running time of 3 h, all the product was then loaded in an electrophoresis run in 3.5% agarose gel containing SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, Cambridgeshire, UK) run in 1×TBE buffer and later visualized under a UV transilluminator to see the fragment-length polymorphisms.

2.6. Blind Samples for Protocol Application Testing

After the PCR-RFLP protocol assessment, a further testing of its reliability was performed; two additional adipose tissue samples were given to the laboratory team without species attribution and geographical localization, for an unbiased application test of the restriction protocol. DNA was extracted and amplified as reported in Sections 2.3 and 2.4. The restriction protocol was then tested as reported in Section 2.5.

3. Results

3.1. In Silico Restriction Simulations

A final alignment of 289 cytochrome b (*cytb*) and of complete mitochondrial genomes was obtained (Aligment S1).

Theoretical restrictions were produced for each enzyme on twenty-four species reported in the Mediterranean Sea (with the exception of *S. plumbea*, see Section 2.1). Results for fragment-length polymorphisms obtained by the *in silico* analysis of the four enzymes are reported in Table 3.

Table 3. Fragments produced by the digestion of the selected enzymes (lengths in base pairs) for each of the twenty-four designated species. Species in bold are the eight most common species occurring in the Mediterranean.

	***Hpy*188III**	***Hha*I**	***Mwo*I**	***Alu*I**
Balaenoptera acutorostrata	439 bp	306 bp + 133 bp	439 bp	439 bp
Balaenoptera borealis	439 bp	267 bp + 133 bp + 39 bp	439 bp	439 bp
Balaenoptera physalus	**226 bp + 213 bp**	**400 bp + 39 bp**	**439 bp**	**439 bp**
Delphinus delphis	**351 bp + 88 bp**	**400 bp + 39 bp**	**250 bp + 189 bp**	**439 bp**
Eschrichtius robustus	439 bp	267 bp + 133 bp + 39 bp	439 bp	439 bp
Eubalena glacialis	439 bp	306 bp + 133 bp	439 bp	439 bp
Globicephala melas	439 bp	267 bp + 133 bp + 39 bp	439 bp	247 bp + 192 bp
Globicephala macrorhynchus	439 bp	267 bp + 133 bp + 39 bp	250 bp + 189 bp	247 bp + 192 bp
Grampus griseus	439 bp	267 bp + 133 bp + 39 bp	250 bp + 189 bp	247 bp + 192 bp
Hyperoodon ampullatus	439 bp	400 bp + 39 bp	439 bp	439 bp
Kogia simus	439 bp	306 bp + 133 bp	439 bp	310 bp + 129 bp
Megaptera novaengeliae	439 bp	267 bp + 133 bp + 39 bp	439 bp	439 bp
Mesoplodon bidens	439 bp	400 bp + 39 bp	439 bp	439 bp
Mesoplodon densirostris	226 bp + 213 bp	439 bp	439 bp	439 bp
Mesoplodon europaeus	439 bp	400 bp + 39 bp	439 bp	310 bp + 129 bp
Orcinus orca	439 bp	267 bp + 133 bp + 39 bp	250 bp + 189 bp	247 bp + 192 bp
Phocoena phocoena	226 bp + 213 bp	439 bp	439 bp	439 bp
Physeter macrocephalus	**369 bp + 70 bp**	**439 bp**	**439 bp**	**439 bp**
Pseudorca crassidens	439 bp	267 bp + 133 bp + 39 bp	250 bp + 189 bp	247 bp + 192 bp
Sousa chinensis	439 bp	400 bp + 39 bp	250 bp + 189 bp	439 bp
Stenella coeruleoalba	**439 bp**	**400 bp + 39 bp**	**250 bp + 189 bp**	**439 bp**
Steno bredanensis	439 bp	306 bp + 133 bp	439 bp	439 bp
Tursiops truncatus	**439 bp**	**267 bp + 133 bp + 39 bp**	**250 bp + 189 bp**	**439 bp**
Ziphius cavirostris	**439 bp**	**439 bp**	**250 bp + 189 bp**	**439 bp**

In detail, Figure 3 shows the *in silico* restriction patterns of the eight common species of Mediterranean cetaceans. It is to be noted that virtual bands representing DNA fragments shorter than 80 base pairs are not shown in the figures, as 80 bp is the visualization threshold of the virtual digestion system.

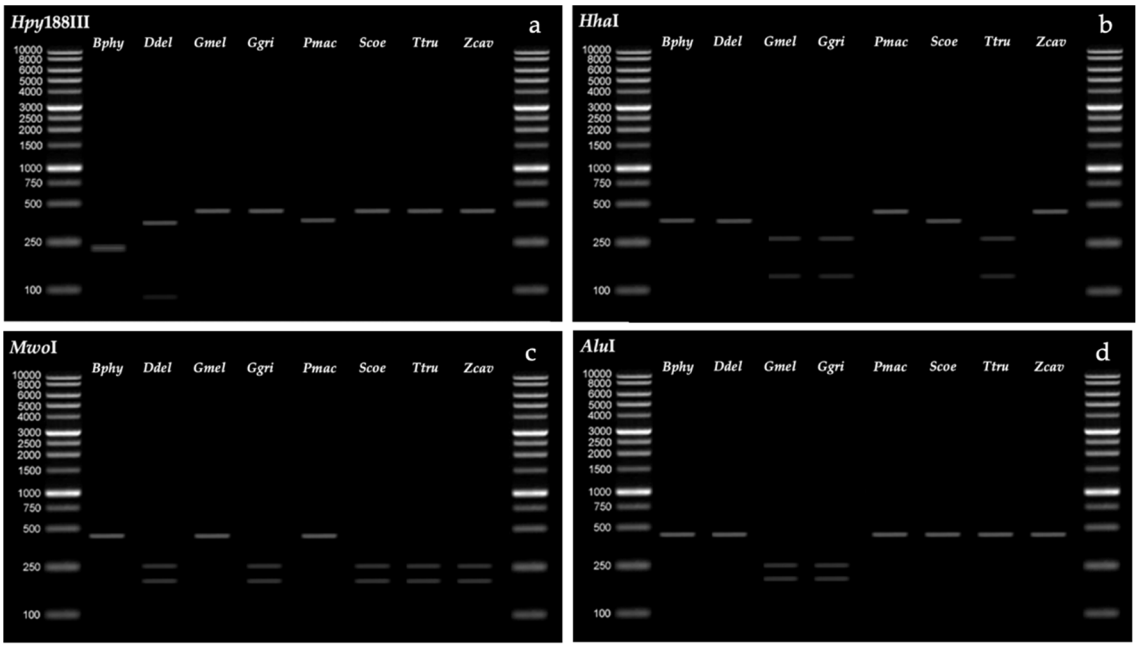

Figure 3. *In silico* simulation of restriction fragment-length polymorphism pattern of the eight common species: (**a**) simulated digestions with the enzyme *Hpy*188III; (**b**) simulated digestions with the enzyme *Hha*I; (**c**) simulated digestions with the enzyme *Mwo*I; (**d**) simulated digestions with the enzyme *Alu*I. Acronyms: *Bphy*, *Balaenoptera physalus*; *Ddel*, *Delphinus delphis*; *Gmel*, *Globicephala melas*; *Ggri*, *Grampus griseus*; *Pmac*, *Physeter macrocephalus*; *Scoe*, *Stenella coeruleoalba*; *Ttru*, *Tursiops truncatus*; *Zcav*, *Ziphius cavirostris*.

All the restriction patterns from the remaining sixteen species from Table 1 can be found in Figure S1.

Significant intraspecific polymorphism, i.e., polymorphisms that affect the restriction sites of the selected enzymes, were found in *M. densirostris* and *M. europaeus*. Specifically, in *M. densirostris,* the enzyme *Hpy*188III is able to digest 6 of the 12 sequences analyzed, as in the other 6 a thymine is substituted to a cytosine blocking the enzyme; whereas the enzyme *Hha*I is able to digest only 1 of the 12 sequences analyzed, as an adenine is substituted to a guanine allowing enzymatic digestion. In *M. europaeus*, a substitution of a cytosine to a thymine in 2 of the 7 sequences analyzed allows the enzyme *Alu*I to digest the fragments. Such polymorphisms could be confounding factors that need to be taken in consideration when testing the aforementioned species as restrictions might not produce a reliable identification pattern.

3.2. Sample Analyses

All forty-four tissue samples listed in Table 1 were successfully processed and their DNA was extracted, amplified, and restricted. Genomic DNA was also successfully obtained from epidermal, adipose, and even from lyophilized freeze-dried muscular tissue conserved at room temperature, whereas all fresh tissues were stored in ethanol at −20 °C.

Upon verification of the fragment-length polymorphisms, all taxonomic identifications were confirmed, in accordance with the theoretical patterns. Assembled agarose gel images of the fragment-length polymorphisms obtained for each enzyme are shown in Figure 4. It was possible to identify all fragments in the real DNA gel, with the exception of the smallest ones (39 bp).

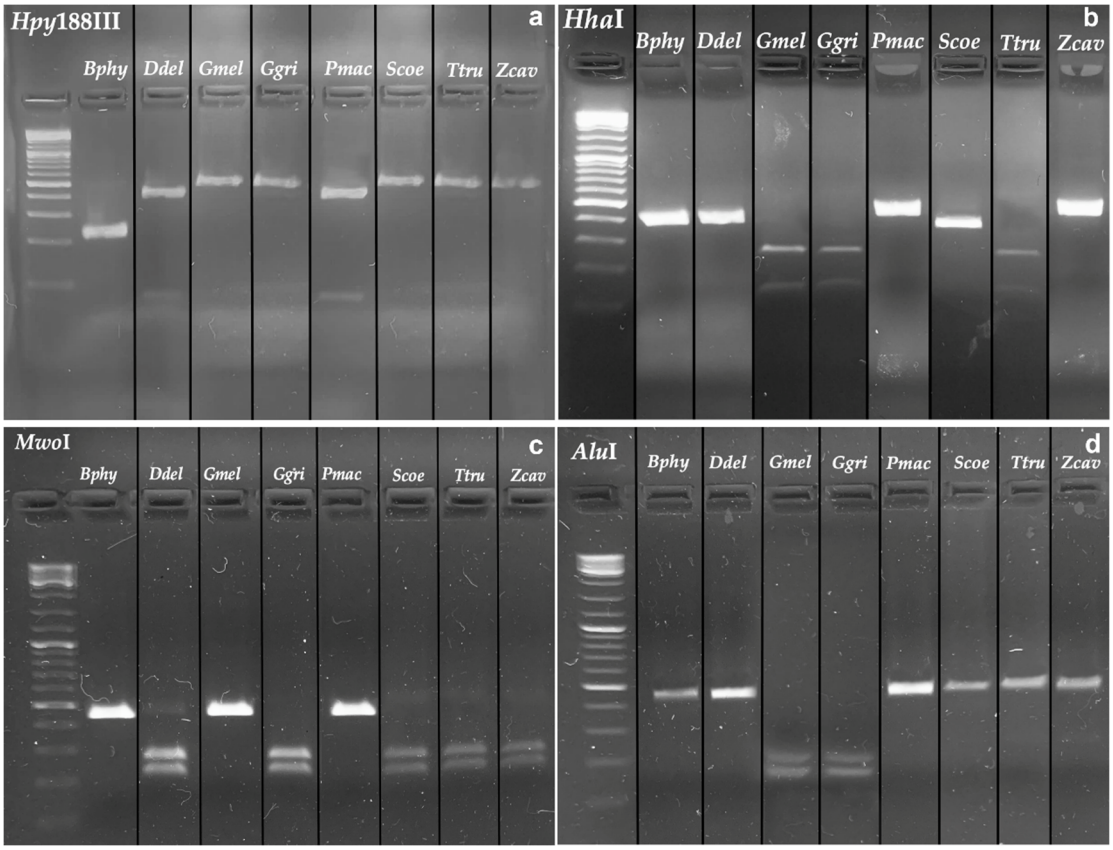

Figure 4. Assembled agarose gel electrophoretic runs showing one sample for each species of cetaceans, digested with all the 4 enzymes: (**a**) digestions with the enzyme *Hpy*188III; (**b**) digestions with the enzyme *Hha*I; (**c**) digestions with the enzyme *Mwo*I; (**d**) digestions with the enzyme *Alu*I. Acronyms: *Bphy*, *Balaenoptera physalus*; *Ddel*, *Delphinus delphis*; *Gmel*, *Globicephala melas*; *Ggri*, *Grampus griseus*; *Pmac*, *Physeter macrocephalus*; *Scoe*, *Stenella coeruleoalba*; *Ttru*, *Tursiops truncatus*; *Zcav*, *Ziphius cavirostris*.

To summarize the results obtained from sample processing, as showed in Figure 5 some species do not require digestion with all four enzymes to be identified, although the application of the full protocol gives more certainty to the identification.

	Hpy 188 III	*Hha* I	*Mwo* I	*Alu* I
Balaenoptera physalus	226bp + 213bp	400bp + 39bp	439bp	439bp
Physeter catodon	369bp + 70bp	439bp	439bp	439bp
Globicephala melas	439bp	267bp + 133bp + 39bp	439bp	247bp + 192bp
Grampus griseus	439bp	267bp + 133bp + 39bp	250bp + 189bp	247bp + 192bp
Tursiops truncatus	439bp	267bp + 133bp + 39bp	250bp + 189bp	439bp
Stenella coeruleoalba	439bp	400bp + 39bp	250bp + 189bp	439bp
Delphinus delphis	351bp + 88bp	400bp + 39bp	250bp + 189bp	439bp
Ziphius cavirostris	439bp	439bp	250bp + 189bp	439bp

Figure 5. Blocks inside the thick black line reflect the minimal required digestions to provide a reliable species identification. Blocks are also color-coded to match the number and length of the produced fragments.

3.3. Blind samples Analyses

Restriction patterns of the two unknown samples are shown in Figure 6. The application of the protocol allowed the identification of the two samples as *Balaenoptera physalus* and *Grampus griseus*, identities were later confirmed by the sampling team.

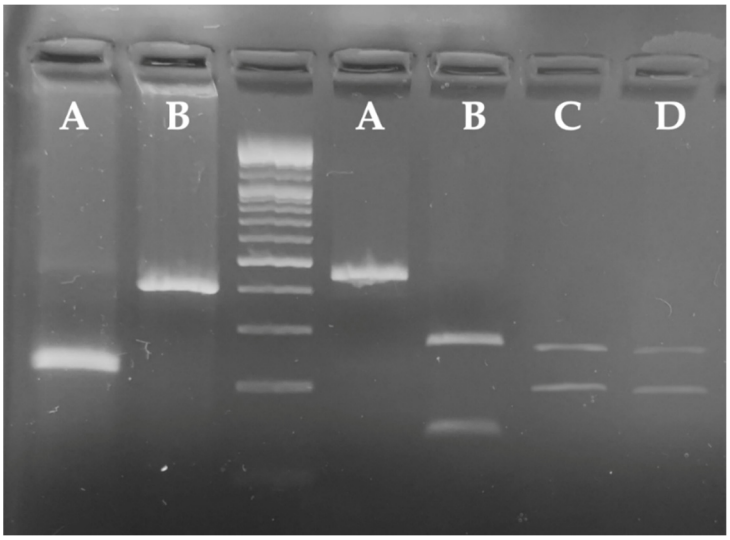

Figure 6. Electrophoretic run showing the digestion patterns for the two blind samples. Capital letters represent the digestion enzymes used: A = *Hpy*188III; B = *Hha*I; C = *Mwo*I; D = *Alu*I. Digestions of sample 1 are on the left of the DNA-ladder lane and allowed the sample to be identified as *Balaenoptera physalus*. Digestions of sample 2 are on the right of the DNA-ladder and allowed the sample to be identified as *Grampus griseus*.

4. Discussion

This PCR-RFLP protocol has been developed to provide a reliable and cost/time effective tool for taxonomic attribution of unknown or unrecognizable cetacean species of the Mediterranean Sea. The protocol is intended to be applied as follows: (i) whole genomic DNA extraction; (ii) PCR amplification targeting the desired *cytb* fragment; (iii) simultaneous digestions with all the four restriction enzymes (iv) results visualization.

Results obtained from *in silico* design for twenty-four species of cetaceans occurring in the Mediterranean Sea (*S. plumblea* was not analyzed, see Section 2.1), showed that the proposed protocol is efficient and economically advantageous to identify, at species level, all the 8 common species of Mediterranean cetaceans. The specific identification via PCR-RFLP on the selected locus, of all the 25 occurring species, would require a significantly wider panel of restriction enzymes making sequencing a much faster and more economic way to produce an identification.

The virtual fragment pattern has been clearly confirmed by the laboratory DNA analyses, showing the same restriction bands as predicted in the *in silico* design. When tested on tissue samples collected in the wild, the designed protocol confirmed its capacity to reliably discriminate their taxonomic identity.

The reliability of the PCR-RFLP protocol was also reinforced by the analysis of the two blind samples. All the evidences gathered from the theoretical process and the genomic DNA analyses support the applicability of the protocol that could be particularly useful for future applications, such as to provide a taxonomic attribution to either fragmented or bloated animal remains, or decaying carcasses that can be found both stranded on shores, floating adrift, or sunk in the sea.

Moreover, this PCR-RFLP protocol could be used as an indicator to diagnose the presence of vagrant and visitor species. This early-diagnostic tool can be of relevant use in an evolving framework of climatic changes; this may allow for a much more frequent and stable presence of cetacean species not historically detectable in the Mediterranean basin.

It is also noteworthy to mention that the effectiveness of this PCR-RFLP protocol opens new perspectives in the devising of similar molecular strategies to resolve comparable problems, such as the specific identification of sea turtle remains or monk seals (*Monachus monachus* Hermann 1779) that are often mistaken for small delphinids.

Often, the conservation status of such specimens does not allow for an identification based on the morphological analysis of the specimen, especially if key features such as the skull or teeth are missing, making the molecular approach necessary.

The selection of a small fragment of the *cytb* gene was of paramount importance to allow the successful extraction and amplification of mtDNA, even from severely damaged specimens that could undergo decaying processes after being exposed to environmental agents, contributing to the degradation of the genetic material.

It is noteworthy to mention that, among the analyzed samples, thirteen of them were freeze-dried; in particular, one sample (code 2GRM) from a Risso's dolphin (*G. griseus*) was collected and freeze-dried in 2007 and conserved for fifteen years at room temperature. In spite of this, the protocol was successfully applied and we were able to correctly identify the species.

The protocol could also find wide applications for monitoring purposes as it can provide a rapid and economic way to process a great number of samples in a short time, without the need to use expensive sequencing infrastructures. An amplification-sequencing approach can be limitative not just economically bust also logistically, as not all laboratories can afford sequencing equipment or shed funds to outsource the sequencing of amplified products to dedicated companies.

Conversely, the equipment required, along with the necessary reagents to apply the PCR-RFLP protocol, are very basic, and, nowadays, affordable to virtually all laboratories, meaning the analyses can be performed even in a well-equipped field lab. Aside from taxonomical and conservation scopes, the protocol can be useful to detect and prevent food frauds, where cetacean meat is sold to unaware customers, and environmental crimes, such as acts of deliberate poaching.

Possible limitations of this protocol should be also discussed, depending both on the molecular marker selected and on the ethology of cetaceans. The use of a mitochondrial gene as a molecular target will not produce reliable results for potentially hybridized specimens, as the genetic assessment that is described is only the one derived by maternal lineage. In spite of their high morphological variability, cetaceans exhibit an elevated karyotypic uniformity which supports the possibility of hybridization [32]. In fact, several intra- and intergeneric hybrids, both in captivity and in the wild, have been reported [33–37]. Cetaceans' ethology and the marine environment itself make for a difficult estimation, especially through molecular evidence, of the real extent of these phenomena if compared with terrestrial species.

It is possible that genetic diversity changes in space and time, and that the natural genetic variability produced genotypes not yet sequenced, and therefore not considered in this study. For example, a time-series analyses performed on Mediterranean striped dolphins showed that the patterns of genetic composition have fluctuated significantly during the last decades, presumably as a consequence of a particular resistance to morbillivirus [38]. Another source of variability might be the genetic flow, even though some authors reported the absence of haplotypes shared between Mediterranean and Atlantic areas and the existence of a very limited gene flow across the Strait of Gibraltar [39]. On the contrary, de Sthephanis and colleagues [40] described seven cetacean species regularly inhabiting the Strait of Gibraltar during summer, suggesting the possible interchange between the two units.

5. Conclusions

Twenty-five species of cetaceans have been reported throughout the whole Mediterranean basin and, among them, eight are commonly distributed and regularly found dead, stranded on shores or adrift in the sea. After being exposed to environmental agents and

decaying processes, their taxonomic identification based on morphological features could be difficult or impossible to achieve. Therefore, molecular tools could be particularly useful when species identification is problematic, to confirm or identify the taxonomic status. Although some limitations should be taken in consideration using this approach, the method herein proposed represents a viable, cost- and time-efficient tool for the identification of cetacean species occurring in the Mediterranean Sea.

Moreover, DNA analyses confirmed its capability to discriminate the eight common species of the Mediterranean, even when used with samples collected from animals in different conservation status and/or preserved lyophilized at room temperature for a long time. This approach could be particularly useful for monitoring purposes of cetacean populations, i.e., to collect data on species occurrence and distribution, and frequency of mortalities. Furthermore, molecular taxonomy gathered from this PCR-RFLP protocol could also be useful to detect environmental crimes, such as illegal catching and food fraud, where species substitution may occur.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142416763/s1, Supplementary Table S1. List of cetacean species reported in the Mediterranean Sea. Status assessed by: [1] [4]; [2] [5]; [3] [6]; [4] [7]; [5] [8]; [6] [9]; [7] [10]; [8] [11]; [9] [12]. For symbol * see [16]. Supplementary Figure S1. In silico simulation of restriction length polymorphism pattern of the sixteen accidentally occurring species: (a) Simulated digestions with the enzyme *Hpy*188III; (b) Simulated digestions with the enzyme *Hha*I; (c) Simulated digestions with the enzyme *Mwo*I; (d) Simulated digestions with the enzyme *Alu*I. Acronyms: *Bacu*, *Balaenoptera acutorostrata*; *Bbor*, *Balaenoptera borealis*; *Erob*, *Eschrichtius robustus*; *Egla*, *Eubalena glacialis*; *Gmac*, *Globicephala macrorhynchus; Hamp*, *Hyperoodon ampullatus*; *Ksim*, *Kogia sima*; *Mnov*, *Megaptera novaeangliae*; *Mbid*, *Mesoplodon bidens*; *Mden*, *Mesoplodon densirostris*; *Meur*, *Mesoplodon europaeus*; *Oorc*, *Orcinus orca*; *Ppho*, *Phocoena phocoena*; *Pcra*, *Pseudorca crassidens*; *Schi*, *Sousa chinensis*; *Sbre*, *Steno bredanensis*. Supplementary Alignment S1. MEGA alignment of the 289 analyzed sequences. For each sequence, the abbreviation, the species name, the Genbank Accession Number and the indication of *cytb* fragment/complete mt genome are provided.

Author Contributions: Conceptualization, L.B., S.C., L.M. and L.L; methodology, L.B., S.C., S.M., G.C., L.M. and L.L.; software, L.B., V.S. and L.L.; validation, L.B., S.C., S.M., G.C., L.M. and L.L; formal analysis, L.B., S.M., G.C., L.M., V.S. and L.L.; resources, S.M., G.C., L.M. and L.L.; data curation, L.B., S.C., S.M., G.C., L.M., G.L., V.S., A.S. and L.L.; writing—original draft preparation L.B., S.C., S.M., G.C., L.M., G.L. and L.L.; writing—review and editing, all authors; visualization, L.B., S.C., G.C., L.M. and L.L.; supervision, L.L.; project administration, S.C. and L.L.; funding acquisition, L.M. and L.L. All authors have read and agreed to the published version of the manuscript.

Funding: Financial support was given by "Progetto Ricerca Di Base 2020", University of Perugia (Italy) and by "Progetto NATura NEtwork Toscana–NAT.NE.T – Ambito Marino DGR N 592 del 06-05-2019" financed by Tuscany Region to University of Siena (Italy).

Institutional Review Board Statement: Ethical review and approval were waived for this study due to CITES permit number IT007.

Informed Consent Statement: Not applicable.

Data Availability Statement: Date and location of the stranding event are available for free at http://mammiferimarini.unipv.it/ (accessed on 18 July 2022).

Acknowledgments: Authors would like to thank Mazzariol S. and CERT staff (University of Padua), Denurra D. and Pintore A. (Istituto Zooprofilattico Sperimentale of Sardegna—IZSS), Mancusi C. (Agenzia Regionale per la PRotezione Ambientale della Toscana—ARPAT), Terracciano G. (Istituto Zooprofilattico Sperimentale of Lazio and Toscana—IZSLT Sez. Pisa), Cancelli F. (Accademia dei Fisiocritici, Siena). The Authors would also thank the two anonymous referees for their useful suggestions to improve the manuscript.

Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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