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M. NORCIA[1,†], A. ILLIANO[1,†], B. MUSSI[2], F. DI NOCERA[3], E. ESPOSITO[3], A. DI COSMO[1,4], D. FULGIONE[1], V. MASELLI[1]

[1]Department of Biology, University of Naples Federico II, Via Cinthia 26, 80126 Napoli
[2]Oceanomare Delphis APS, Viale Rimembranze 14, 47924 Rimini
[3]Experimental Zooprophylactic Institute of Southern Italy, Via Salute, 2, 80055 Portici
[4]MNESYS - PNRR partenariato esteso
[†] These authors contributed equally to this work.
corresponding authors: barbara@oceanomaredelphis.org; valeria.maselli@unina.it

HIGH-RESOLUTION MELTING AS A MOLECULAR TOOL FOR CETACEAN SPECIES IDENTIFICATION IN THE MEDITERRANEAN SEA

ANALISI DI MELT AD ALTA RISOLUZIONE COME STRUMENTO MOLECOLARE PER L'IDENTIFICAZIONE DELLE SPECIE DI CETACEI NEL MAR MEDITERRANEO

Abstract - *Effective species identification underpins cetacean conservation in the Mediterranean Sea, where several populations are threatened. We developed a High-Resolution Melting (HRM) protocol to rapidly and cost-effectively identify four representative cetacean species: the bottlenose dolphin (*Tursiops truncatus*), the striped dolphin (*Stenella coeruleoalba*), the sperm whale (*Physeter macrocephalus*), and the fin whale (*Balaenoptera physalus*). Species-specific primers targeting mitochondrial cytochrome b and D-loop regions produced distinct melting profiles. Validation on tissue and fecal samples confirmed high sensitivity, reproducibility, and discrimination, with an accuracy of over 95% in identifying unknown samples. Compared to sequencing, HRM is faster, cheaper, portable, and effective on degraded material, including samples from stranded individuals. This method represents a valuable tool for non-invasive genetic surveys and real-time monitoring, enhancing conservation efforts and enforcement of regulations against illegal trade.*

Keywords: *High-Resolution Melting analysis, species assignment, cetacean DNA genotyping*

Introduction - The Mediterranean basin is a recognized cetacean biodiversity hotspot, hosting both resident and migratory species despite growing anthropogenic threats (Bianchi & Morri, 2000; Pace *et al.*, 2015; Notarbartolo di Sciara, 2016; Gnone *et al*., 2023). The Mediterranean Sea hosts nine regularly occurring species (Notarbartolo di Sciara & Tonay, 2021) and others that are present only sporadically. These populations are often reported to be genetically distinct from their Atlantic counterparts and face varying levels of conservation concern. The striped dolphin, *Stenella coeruleoalba* (Meyen, 1833) and the bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821), are widely distributed and classified as Least Concern (LC) in the IUCN list; other species, including the common dolphin (*Delphinus delphis* Linnaeus, 1758), Risso's dolphin, *Grampus griseus* (Cuvier, 1812), long-finned pilot whale, *Globicephala melas* (Traill, 1809), sperm whale (*Physeter macrocephalus* Linnaeus, 1758), and fin whale, *Balaenoptera physalus* (Linnaeus, 1758), show declining trends and are classified as Endangered (EN). Major threats include bycatch, ship strikes, chemical and noise pollution, plastic ingestion, and epizootic events. Monitoring for the conservation of these populations requires accurate species identification and reliable data on distribution and abundance. Traditional approaches include line-transect surveys, photo-identification, and passive acoustic monitoring. While valuable, these methods have limitations, especially for elusive or deep-diving species. Genetic approaches have therefore become increasingly important, especially in cases where morphological identification is impossible, such as in stranded or degraded specimens. Invasive sampling (e.g., biopsies) offers high-quality DNA but raises ethical and practical challenges, whereas non-invasive alternatives, such as sloughed skin, feces, blow, or

environmental DNA, are expanding rapidly. Molecular markers, especially the mitochondrial cytochrome b and COX-I genes, are widely used in cetacean taxonomy and forensic investigations; however, sequencing can be costly, time-consuming, and unsuitable for degraded material. High-Resolution Melting (HRM) analysis offers a rapid, economical, and contamination-resistant alternative for real-time species identification (Wittwer, 2009). In this study, we developed and validated an HRM protocol for both tissue and fecal samples targeting mtDNA D-loop and cytochrome b regions for four representative Mediterranean cetaceans: bottlenose dolphin, striped dolphin, sperm whale, and fin whale.

Materials and methods - Muscle tissue samples (N=28) were collected from four cetacean species in the Mediterranean Sea: *T. truncatus* (N=5), *S. coeruleoalba* (N=15), *P. macrocephalus* (N=4), and *B. physalus* (N=4). Samples were provided by the Experimental Zooprophylactic Institute of Southern Italy and the Department of Life and Environmental Sciences, Polytechnic University of the Marche (CITES permit no. 4814/2019/PAB). Fecal samples (N=2) were collected from sperm whales in the "waters of Ischia and Ventotene" Important Marine Mammal Area, by the research vessel Oceanomare Delphis team aboard Jean Gab. Floating feces were collected using a fine nylon mesh net, avoiding direct contact with animals to minimize stress and disturbance. Fecal samples were immediately placed in sterile tubes and stored at −20 °C. Genomic DNA was extracted from 200 mg of fecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN), and from 25 mg of tissue samples using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's protocol. To facilitate species identification, cytochrome b and the D-loop were targeted to design primers using Geneious Prime (v2024), with subsequent validation using Primer-BLAST. A total of 21 primer pairs were tested and four were selected based on species specificity and distinct amplicon profiles (Table 1): B1F (5'-TGGAACTTCGGCTCCCTACT-3') and B1R (5'-AATTCACGTCTCGGCAGATG-3'); S1F (5'-CACAGCATTAGCAGCCGTTC-3') and S1R (5'-CCTAGTAGGTCGGGGGTGAA-3'); P1F (5'-TGAGCTCTCGGATCAGACCA-3') and P1R (5'-GCAGGTGCCTCGAGTTATGA-3'); T1F (5'-TGCGCATGCTAATATTTAGTCTCT-3') and T11R (5'-TCGTATGGAAAATAAATGAATGCACAA-3').

Primer pairs were validated in multiplex reactions, including all four primer pairs and DNA from each species, to exclude cross-amplification. PCR reactions (20 µL) included 100 ng DNA, 10 µL of 2X Invitrogen™ Platinum™ Hot Start PCR Master Mix, and 10 µM primer mix. Thermocycling was set at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s, and 68 °C for 15 s. PCR products were sequenced (Eurofins Genomics) and aligned to reference sequences in GenBank. HRM was performed on a MIC qPCR Cycler (Bio Molecular Systems) in 20 µL reactions containing 100 ng DNA, 10 µL of 2X Clara™ HRM Mix (Biosystems), and 10 µM primer mix. Cycling conditions: initial denaturation at 95 °C for 2 min; 45 cycles at 95 °C for 5 s and 64 °C for 30 s; followed by melting from 55 °C to 95 °C in 0.025 °C increments (2 s/step). Data were analyzed using BMS Workbench v1.4.2. Unknown samples were assigned to species by comparing melting curves with reference profiles. Species identification based on morphology was confirmed by Sanger sequencing. Negative and positive controls were included in all PCR and HRM runs to monitor contamination and validate amplification. HRM analyses were performed in duplicate to confirm reproducibility of the profiles.

Results - The study analyzed mitochondrial DNA (mtDNA) to explore genetic patterns in Mediterranean cetaceans, focusing on the cytochrome b and D-loop regions, chosen for their optimal fragment lengths (80–220 bp) suitable for HRM analysis. Of the 21 primer pairs tested, four species-specific primer pairs were selected to produce distinct, reproducible melting profiles. All samples belonged to the four target species, and a subset with known identity was treated as unknown to evaluate the accuracy of HRM-

based species assignment. Each amplified region was unique in sequence and evaluated for nucleotide composition and GC content, both of which influence melting temperature (Tm) during HRM (Tab. 1).

Tab. 1 – Amplicon characteristics obtained with primer pairs selected.
Caratteristiche degli ampliconi ottenuti usando le coppie di primers selezionati.

Species	Name	Size (bp)	GC content (%)	Region
Balaenoptera physalus	B1F + B1R	133	47.7	Cytochrome b
Stenella coeruleoalba	S1F + S1R	189	43.1	Cytochrome b
Physeter macrocephalus	P1F + P1R	175	29.1	D-loop
Tursiops truncatus	T1F + T11R	86	53.6	D-loop

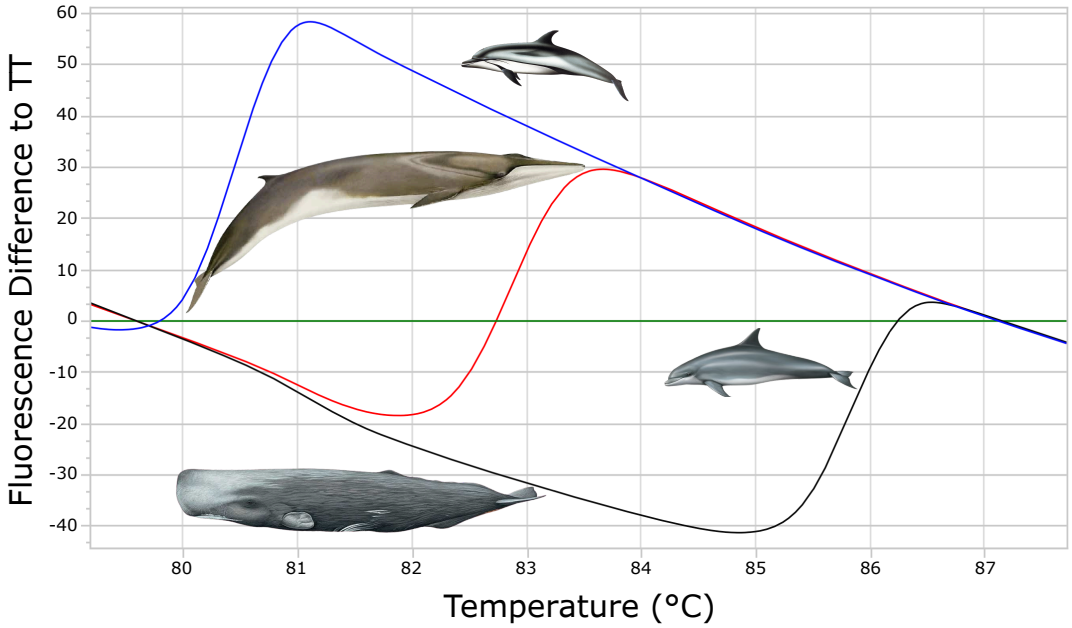

Fig. 1 - Normalized difference plot of High-Resolution Melting (HRM) curves generated using species-specific primer pairs targeting mitochondrial cytochrome b or D-loop regions. HRM profiles were obtained using the following primer sets: B1F/B1R for *Balaenoptera physalus* (red), S1F/S1R for *Stenella coeruleoalba* (blue), T1F/T11R for *Tursiops truncatus* (green), and P1F/P1R for *Physeter macrocephalus* (grey). The graph highlights differences in fluorescence relative to *Tursiops truncatus*, used as a reference species.
Grafico delle differenze normalizzate tra le curve di High-Resolution Melting (HRM) generate utilizzando coppie di primers specie-specifiche che considerano le regioni del citocromo b o D-loop mitocondriale. I profili HRM sono stati ottenuti utilizzando i seguenti set di primer: B1F/B1R per Balaenoptera physalus *(rosso), S1F/S1R per* Stenella coeruleoalba *(blu), T1F/T11R per* Tursiops truncatus *(verde) e P1F/P1R per* Physeter macrocephalus *(grigio). Il grafico evidenzia le differenze di fluorescenza rispetto a* Tursiops truncatus*, utilizzato come specie di riferimento.*

Initial tests on four reference samples revealed clear species-specific melting profiles. Sequencing confirmed species identity through NCBI BLAST with a 96–100% match. Primer selection prioritized specificity, using both intraspecific and interspecific SNP analyses to reduce cross-species amplification and enhance accuracy. The optimized protocol was then applied to unknown samples, including fecal DNA samples. Melting profiles matched predicted patterns from reference species, and over 95% of the analyses were successful. Data reliability was supported by high sequence identity values (>90%) in BLAST results. Distinct Main Melting Peaks (MMPs) were observed for each species (mean ± standard deviation): *B. physalus* 83.05 ± 0.18 °C, *S. coeruleoalba* 80.76 ± 0.28 °C, *T. truncatus* 72.66 ± 1.00 °C, and *P. macrocephalus* 86.08 ± 0.32 °C (Fig. 1).

Conclusions - This study highlights the potential of HRM analysis as a rapid and cost-efficient tool for cetacean species identification in the Mediterranean Sea. Using species-specific primers and a portable HRM platform, we were able to discriminate four representative taxa: two abundant species of the Delphinidae family and the two largest whale species, across both muscle tissue and fecal samples, including those with degraded DNA. The protocol, with a per-sample cost of approximately 2.8 € , represents a complementary approach to sequencing and addresses key challenges in cetacean conservation by enabling rapid identification in strandings or compromised material. Its successful application to sperm whale feces underscores the potential of non-invasive sampling, thus having no impact on animal welfare (Papastavrou & Ryan, 2023).
A universal cetacean-specific primer coupled with HRM fingerprinting represents an attractive long-term goal. However, closely related Mediterranean species often show limited variation in conserved mitochondrial regions, which may generate overlapping or ambiguous melting profiles, especially in degraded or low-template DNA. For this reason, we adopted a species-specific primer strategy to ensure robustness, sensitivity, and reproducibility under real-world conservation and forensic conditions. Beyond conservation monitoring, HRM also has applications in wildlife forensics. The ongoing illegal trade of cetacean-derived products underscores the growing need for reliable molecular tools in enforcement protocols.
Future applications should extend HRM assays to all regular Mediterranean species and the global diversity of cetaceans, including the development of eDNA protocols (Suarez-Bregua *et al.*, 2022) and the integration of machine learning for automated profile classification. Overall, HRM represents a methodological advancement that combines scientific rigor with practical utility, supporting both conservation management and the fight against illegal trade, ultimately contributing to the protection of cetaceans and the conservation of marine biodiversity.
This work benefited from collaboration with Oceanomare Delphis APS, whose long-term commitment to cetacean research and the use of their research vessel were instrumental in enabling sample collection and field validation.

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