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NOTE

Occlusive mycotic tracheobronchitis and systemic *Alphaherpesvirus* coinfection in a free-living striped dolphin *Stenella coeruleoalba* in Italy

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ABSTRACT: A juvenile female striped dolphin *Stenella coeruleoalba* live stranded on 4 March 2016 at Alassio, western Ligurian Sea coast, Italy. The dolphin died shortly after stranding, and a complete postmortem examination was performed. Necropsy revealed severe tracheal occlusion and unilateral bronchial stenosis with luminal accumulation of abundant green-yellow mucous-gelatinous material. Histological features suggestive of tracheobronchial aspergillosis were observed. Cultures of lung tissue and tracheo-bronchial exudate isolated *Aspergillus fumigatus*, identified by a Microseq D2 LSUrDNA fungal sequencing kit. A pan-*Herpesvirus* nested-PCR assay on frozen samples obtained from multiple organs was positive. Phylogenetic analysis on the partial *DNA polymerase* gene revealed that the striped dolphin isolate was closely related to known cetacean *Alphaherpesvirus* sequences from the same host species. Attempted virus isolation was unsuccessful. The tissue levels of different persistent organic pollutants and the toxicological stress, evaluated using a theoretical model, showed a severely impaired immune response. This study reports the first case of occlusive mycotic tracheobronchitis in a free-living cetacean and the first molecular identification of an *Alphaherpesvirus* in a free-ranging striped dolphin stranded on the coast of Italy.

KEY WORDS: *Herpesvirus* · *Aspergillus* · Dolphin · Tracheobronchitis · Immunosuppression

INTRODUCTION

Although considered rare, mycoses in marine mammals are being diagnosed and reported with increasing frequency (Dagleish et al. 2008, Delaney et al. 2012, Cassle et al. 2016). Pulmonary aspergillosis is the most commonly reported mycotic infection in marine mammals (Reidarson et al. 2001). Aspergillosis is caused by the genus *Aspergillus*, an ascomycete fungus widespread in the environment (Cassle et al. 2016). *A. fumigatus* is the most common species infecting animals (Cassle et al. 2016). The respiratory tract is usually the primary site of infection, and once inhaled *Aspergillus* spp. spores can produce several forms of diseases, including allergic aspergillosis, chronic necrotizing aspergillosis, as-

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pergillar fungal balls and invasive aspergillosis (Reidarson et al. 2001, Cassle et al. 2016).

Mycoses may be indicative of an underlying immunosuppression (Dagleish et al. 2008) and aspergillosis has been recorded in captive and free-living dolphins in association with other fungal, bacterial or viral infections (Domingo et al. 1992, Delaney et al. 2012, Cassle et al. 2016). In the Northern Hemisphere, aspergillosis has been described in bottlenose dolphins *Tursiops truncatus* and striped dolphins *Stenella coeruleoalba* with *Morbillivirus* infection (Domingo et al. 1992, Lipscomb et al. 1994, Cassle et al. 2016). Indeed, *Morbillivirus* infection and high levels of environmental contaminants are recognized mechanisms that impair host immune response in marine mammals, predisposing animals to secondary mycoses (Dagleish et al. 2008, Di Guardo et al. 2011, Cassle et al. 2016).

As far as concerns viral pathogens, *Herpesvirus* (family *Herpesviridae*, subdivided into 3 subfamilies; *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*) occur widely in nature, but little is known about the distribution and pathogenicity of these agents on cetaceans in general, and dolphins in particular (Bellière et al. 2010, Lecis et al. 2014). Although herpesviruses have been successfully cultured in the laboratory, only partial sequences of the polymerase and terminase genes are available, limiting the ability to classify them into species and to understand their epidemiology (van Elk et al. 2009). Based on phylogenetic analysis of the partial DNA polymerase coding sequence, the viruses identified in cetaceans to date belong to the *Alpha* and *Gamma* subfamilies of herpesviruses (Maness et al. 2011). We report herein the first case of occlusive mycotic tracheobronchitis by *A. fumigatus* in a wild cetacean. Additionally, a generalized *Herpesvirus* infection was confirmed, with the first molecular identification of *Alphaherspesvirus* in a free-ranging striped dolphin stranded on the coast of Italy.

MATERIALS AND METHODS

Postmortem examination, microbiology, histology and immunohistochemistry

On 4 March 2016, a juvenile female *Stenella coeruleoalba* was seen alive with signs of dyspnea near the shore of Alassio, on the western Ligurian Sea coast, northwestern Italy (Pelagos Sanctuary). The animal died soon after beaching, and a complete postmortem examination according to standard protocols (Geraci & Loundsbury 2005) was performed at Istituto Zooprofilattico Sperimentale, Diagnostic Laboratory of Imperia, 15 h after the dolphin's death (conservation code 2 according to Geraci & Loundsbury 2005). The animal was 165 cm in total length and weighted 51.5 kg. Total body length (TBL) and dentinal growth layer groups (GLGs) analysis of the teeth indicated an age between 4 and 5 yr.

Tissues were collected from multiple organs and frozen (–20 and –80°C). Samples from the brain, lung, prescapular and tracheobronchial lymph nodes, liver, spleen and kidney were processed for standard aerobic and anaerobic bacterial culture and identification. Specific bacteriological procedures followed international recommendations (OIE 2016) to screen for *Mycobacterium* spp., *Listeria* spp., *Salmonella* spp. and *Brucella* spp.. Samples from the brain, lung and tracheo-bronchial exudate were inoculated onto Saboraud's medium for attempted fungal isolation and speciation.

Tissues including skin, muscle, thymus, thyroid, prescapular and tracheobronchial lymph nodes, bronchus, lung, heart, main stomach, liver, spleen, pancreas, adrenal gland, mesenteric lymph node, kidney, urinary bladder, gonads and uterus were fixed in 10% neutral buffered formalin for histologic evaluation. Five different anatomic areas from the brain were sampled, including the telencephalon, diencephalon, mesencephalon, cerebellum and brainstem. Tissues were processed by conventional techniques and subsequently embedded in paraffin, sectioned at 4 ± 2 µm and stained with haematoxylin and eosin (H&E). Additional sections of trachea and lung were prepared with Grocott-Gomori's methenamine silver stain (GMS) to better demonstrate fungal morphology and tissue distribution.

Immunohistochemistry (IHC) for *Morbillivirus* was performed on brain, lung, lymph nodes, spleen, kidney and urinary bladder sections (Di Guardo et al. 2010). IHC for *Toxoplasma gondii* (Di Guardo et al. 2010) was conducted on brain, muscle, heart, liver and spleen, while IHC for *Listeria monocytogenes* (Rocha et al. 2013) was performed on brain. Appropriate positive and negative controls were included in the analysis.

Polymerase chain reaction (PCR), virological investigations and serology

A pan-nested PCR using degenerate primers targeting a conserved region of the DNA polymerase gene in herpesviruses was performed on samples

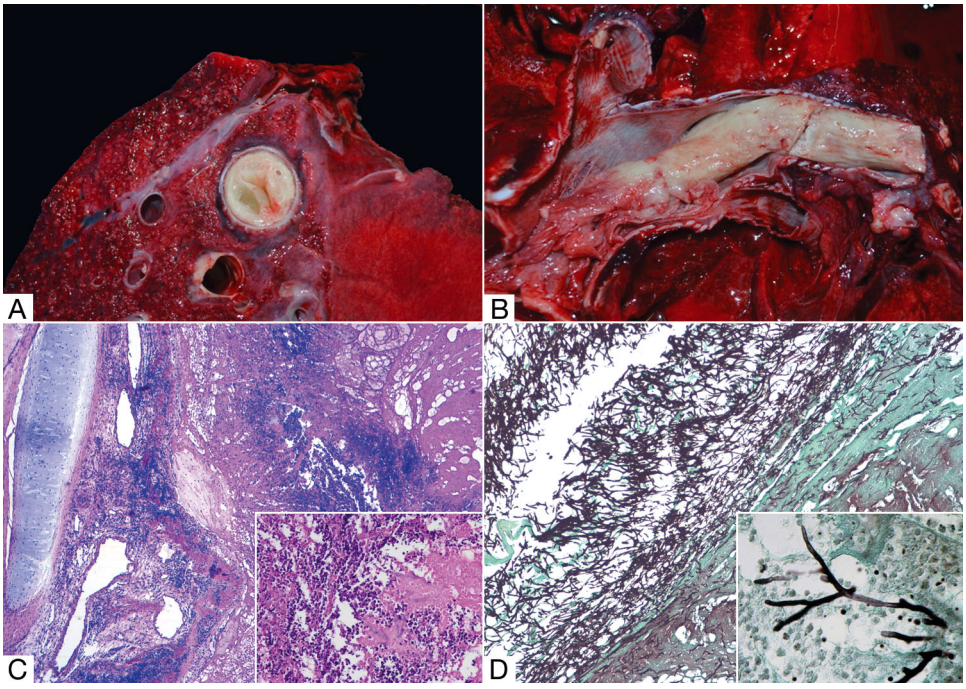

Fig. 1. *Aspergillus fumigatus* infection in striped dolphin *Stenella coeruleoalba* trachea and bronchi. (A,B) Occlusion of tracheo-bronchial lumina by green-yellowish mucous-gelatinous material within the trachea and primary bronchus of the right lung. (C) Obstructive pulmonary disease. Bronchus. Extensive necrosis and ulceration of the mucosa, associated with severe pyogranulomatous inflammation (haematoxylin and eosin [H&E]: 40×). Inset: higher magnification of the previous micrograph of the ulcerated areas with fibrinous exudate, neutrophils (intact and degenerated) and numerous fungal hyphae (H&E: 200×). (D) Fungal hyphae. Bronchus. There is a large number of septate dichotomously branching fungal hyphae, typical of *Aspergillus* spp. (Grocott-Gomori's methenamine silver stain [GMS];100×). Inset: higher magnification of the previous micrograph, illustrating the morphological features of hyphae (GMS; 600×)

from the brain, thymus, lung, prescapular and tracheobronchial lymph nodes, spleen, kidney and urinary bladder (VanDevanter et al. 1996). PCRs for *Morbillivirus*, *T. gondii* and *Brucella* spp. were also performed on specific tissues (Baily et al. 1992, Vitale et al. 2013, Verna et al. 2017). Furthermore, viral isolation was also attempted from PCR-positive frozen tissues, using confluent monolayers of Madin-Darby Bovine Kidney (MDBK) cells.

The presence of anti-*T. gondii*, anti-*Brucella* spp. and anti-*Morbillivirus* antibodies was investigated in blood serum, aqueous humour and cerebrospinal fluid (CSF) (Di Guardo et al. 2010, Hernández-Mora et al. 2008, Profeta et al. 2015).

Toxicology

Considering the opportunistic nature of fungal pathogens, and more specifically, of those colonizing the lung tissue, together with recognized immunosuppressive effects of organochlorine (OC) xenobiotics in Mediterranean cetaceans (Marsili 2000, Marsili et al. 2012), polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB) and dichlorodiphenyltrichloroethanes (DDTs) were measured in blubber. Measurements were made according to Environmental Protection Agency method 8081/8082, with modifications (Marsili & Focardi 1997), and toxicological stress was evaluated using a theoretical model (Marsili et al. 2004).

RESULTS

Postmortem examination, microbiology, histology and immunohistochemistry

The carcass was in moderate body condition, with blubber thickness varying between 19 and 20 mm. The most striking gross necropsy finding was a severe tracheal occlusion and partial bronchial stenosis. Luminal accumulation of abundant green-yellow mucous-gelatinous material (Fig. 1A,B) affected the entire length of trachea and the right lung primary bronchus. There was a lack of appre-

ciable mucosal ulceration or cartilage destruction. All thoracic cavity lymph nodes were enlarged. The heart showed a markedly dilated right ventricle, with distortion, retraction and nodules on the leaflets of the right and left atrio-ventricular valves, consistent with endocardiosis. Right and left atrioventricular valvular insufficiency was diagnosed.

Staphylococcus spp. and *Escherichia coli* were recovered from brain and lung and no *Mycobacterium* spp., *Listeria* spp., *Salmonella* spp. or *Brucella* spp. were isolated by selective media. Fungal culture yielded poor growth with a mixed culture of commensal bacterial flora (*Staphylococcus* spp. and *E. coli*) from lung, along with a pure but scant growth of fungi from tracheo-bronchial exudate. No fungi were recovered from the brain. The mould was molecularly identified as *Aspergillus fumigatus* by Microseq D2 LSUrDNA fungal sequencing kit (Life Technologies), with a 100% sequence homology.

The most remarkable histological findings were observed in the upper respiratory tract. Extensive regions of tracheobronchial mucosa and superficial submucosa were ulcerated and necrotic (Fig. 1C), and the surface was covered by a layer of fibrin with karyorrhetic cellular debris, intact and necrotic neutrophils and many fungal hyphae. The submucosa showed suppurative and pyogranulomatous inflammation, with small numbers of invading fungal hyphae. Generally, hyphae were septate, parallel-walled, argyrophilic and dichotomously branched, consistent with those of *Aspergillus* spp. (Fig. 1D). The histopathological features were suggestive of tracheobronchial aspergillosis. Multifocal pyogranulomatous bronchopneumonia with alveolar flooding of oedema and haemorrhage were observed throughout the pulmonary parenchyma. In the spleen there was sinus hypercellularity, with plasma cells, macrophages, eosinophils, rare neutrophils and scattered haemorrhagic foci. Similar changes were apparent in the prescapular and tracheobronchial lymph nodes. Mild mononuclear periportal and interstitial inflammatory infiltrates were found in the liver and in the kidney, respectively. There was moderate to severe endocardiosis of both atrio-ventricular valves with mild lymphocytic epicarditis and scattered Anitschkow cells. Sections of brain did not show any inflammatory lesions, viral inclusions or fungal elements. No antigens for *Morbillivirus*, *Toxoplasma gondii* or *Listeria monocytogenes* were demonstrated in the examined tissues by IHC.

PCR, sequence analysis, virological investigations and serology

Herpesvirus was molecularly detected in the lung, prescapular and tracheobronchial lymph nodes and spleen. BLAST analysis of the determined DNA polymerase consensus sequence retrieved *Alphaherpesvirus* sequences as first hits (GenBank accession KX822073). Determination of sequence similarity based on nucleotide homology revealed that the striped dolphin isolate shared the highest degree of nucleotide sequence identity with known cetacean alphaherpesviruses, although significant genetic variability was found, with a similarity ranging from 52.6 to 99.5%. Therefore, an alignment of DNA polymerase gene sequences of all cetacean alphaherpesviruses available in the GenBank was performed to compare the striped dolphin herpesvirus identified in the current case with other cetacean isolates. The alignment was used to construct a maximum likelihood (ML) phylogenetic tree (Fig. 2).

Virus isolation attempts failed, and no cytopathic effect (CPE) was observed. No *Morbillivirus*, *T. gondii* or *Brucella* spp. nucleic acids were detected. Anti-*Morbillivirus* antibodies (1:8) were detected in serum, with no titers in the CSF or aqueous humour. No anti-*T. gondii* nor anti-*Brucella* spp. antibodies were identified in these samples.

Toxicology

The levels of PCBs, HCB and DDTs, expressed in ng g^{-1} lipid weight basis (PCBs: 207947.6; DDTs: 179424.3; HCB:668.9; canonical variable value [CAN] = 1.41), confirmed the presence of immunotoxic levels of OC pollutants (CAN > 0.47).

DISCUSSION

To the best of our knowledge, this report represents the index case of tracheobronchial aspergillosis with systemic *Herpesvirus* infection in a striped dolphin, and the first molecular identification of an *Alphaherspesvirus* in a cetacean in Italy. A similar upper respiratory fungal infection was previously reported in captive bottlenose dolphins, but never in a free-living cetacean (Delaney et al. 2012). In this individual, the diagnosis of tracheobronchial aspergillosis was achieved through the correlation of gross and histologic findings, fungal culture and genomic sequencing. Considering the abundance,

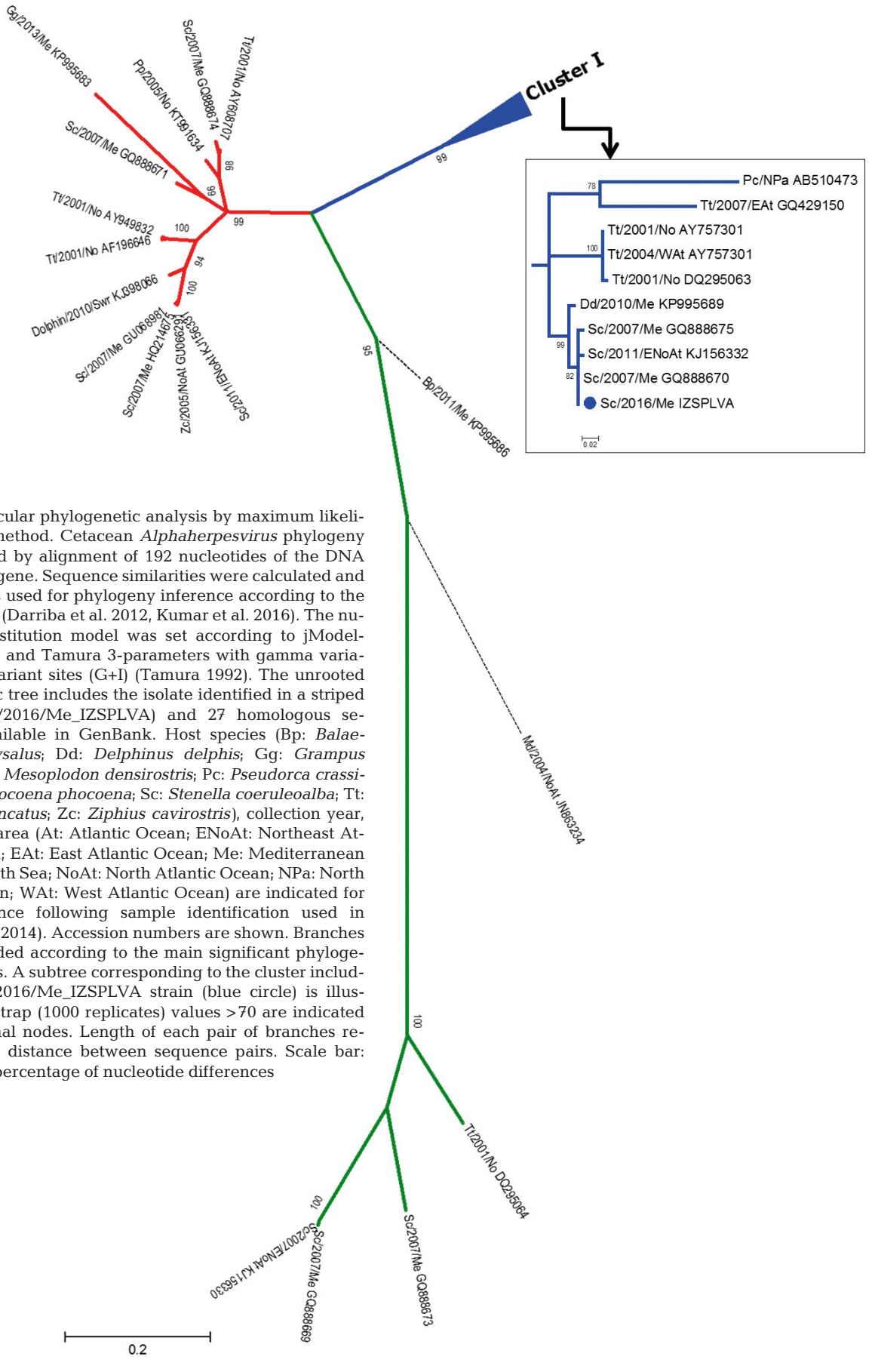

Fig. 2. Molecular phylogenetic analysis by maximum likelihood (ML) method. Cetacean *Alphaherpesvirus* phylogeny was obtained by alignment of 192 nucleotides of the DNA polymerase gene. Sequence similarities were calculated and MEGA7 was used for phylogeny inference according to the ML criterion (Darriba et al. 2012, Kumar et al. 2016). The nucleotide substitution model was set according to jModelTest2 output and Tamura 3-parameters with gamma variation and invariant sites (G+I) (Tamura 1992). The unrooted phylogenetic tree includes the isolate identified in a striped dolphin (Sc/2016/Me_IZSPLVA) and 27 homologous sequences available in GenBank. Host species (Bp: *Balaenoptera physalus*; Dd: *Delphinus delphis*; Gg: *Grampus griseus*; Md: *Mesoplodon densirostris*; Pc: *Pseudorca crassidens*; Pp: *Phocoena phocoena*; Sc: *Stenella coeruleoalba*; Tt: *Tursiops truncatus*; Zc: *Ziphius cavirostris*), collection year, geographic area (At: Atlantic Ocean; ENoAt: Northeast Atlantic Ocean; EAt: East Atlantic Ocean; Me: Mediterranean Sea; No: North Sea; NoAt: North Atlantic Ocean; NPa: North Pacific Ocean; WAt: West Atlantic Ocean) are indicated for each sequence following sample identification used in Sierra et al. (2014). Accession numbers are shown. Branches are color-coded according to the main significant phylogenetic clusters. A subtree corresponding to the cluster including the Sc/2016/Me_IZSPLVA strain (blue circle) is illustrated. Bootstrap (1000 replicates) values >70 are indicated at the internal nodes. Length of each pair of branches represents the distance between sequence pairs. Scale bar: percentage of nucleotide differences

generalized occurrence and morphological features of the fungal hyphae observed, as well as the nature of the inflammatory infiltrate, it was unlikely a chronic infection phase (Delaney et al. 2012). The marked reduction of the luminal diameter of the respiratory tract caused by gelatinous casts could have hampered ventilation, while the mechanical stress related to prolonged forceful breathing and impaired cardiopulmonary perfusion associated with the endocardiosis may have led to the pathological changes observed in the myocardial fibers (Panagiotou et al. 2017). In fact, scattered thickening of the muscular layer of intramural coronary arteries has been identified and associated with displacement of myocardial cell nuclei (Bentzon et al. 2006). These findings suggest the existence of a subacute respiratory syndrome due to pulmonary hypertension.

Anitschkow cells were also identified within the myocardium, and the origin of these cells is still not clear. They have been hypothesized to derive from connective tissue cells, histiocytes or degenerated or modified myocardiocytes. Anitschkow cells have also been found in normal, developing and pathological non-rheumatic conditions in the human heart (Satoh & Tsutsumi 1999). Moreover, mechanical stress by continuous contraction of the heart muscle contribute to aggregate the nuclear chromatin structure of either the stromal cells, cardiac muscle cells or cancer cells to form the Anitschkow-type configurations.

As supposed in the uncommon clinical form of aspergillosis in humans, the isolated invasive *Aspergillus* tracheobronchitis (iiATB), alterations of local mucosal immune function could have predisposed this dolphin to mycotic tracheobronchitis (Delaney et al. 2012). In this regard, some aspects of immune functions in cetaceans (such as respiratory tract local immunity) are still not easy to understand, and certain peculiar anatomical features, such as the lack of nasal sinuses and protective hairs, could be considered significant for their susceptibility to respiratory infections, comprising mycoses (Delaney et al. 2012).

Aspergillus spp. is known as cofactor for mortality in cases of primary dolphin morbillivirus (DMV) infection (Domingo et al. 1992, Cassle et al. 2016). However, in this case, no *Morbillivirus*-related lesions nor antigens were detected, and only a low anti-*Morbillivirus* antibody titre was noted in postmortem serum. It is possible that the systemic herpesviral infection could have played an immunosuppressive role and predisposed the animal to secondary opportunistic infection with *A. fumigatus*. However, the immunotoxicity of OC pollutants has also been well-documented (Marsili et al. 2012). As tissue levels in this case were particularly high (Marsili et al. 2004), immunosuppression in this dolphin secondary to OC contaminant exposure should also be taken into consideration as a contributing factor in the mycosis and possible *Herpesvirus* infection.

Very little is known about *Herpesvirus* infections in cetaceans (Bellière et al. 2010, Lecis et al. 2014). Generalized *Alphaherpesvirus* infections can be associated with foci of necrosis in multiple organs and tissues (Blanchard et al. 2001), or with no lesions (Bellière et al. 2010). In this case, no gross lesions indicative of *Herpesvirus*-associated disease were found (Bellière et al. 2010), and the infection may have been persistent with periods of latency and reactivation. Moreover, no characteristic histopathological changes or intranuclear inclusions were identified in the tissues examined (Blanchard et al. 2001). It is possible that *Herpesvirus*-induced lesions might have been obscured by the necrosis associated with the mycotic infection (Lipscomb et al. 1994).

Phylogenetic analysis revealed that the striped dolphin herpesvirus was closely related to other known cetacean *Alphaherpesvirus* sequences. The phylogenetic tree, inferred by using the ML method and including all homologous sequences available in GenBank, shows that cetacean *Alphaherpesvirus* forms at least 3 separate clades (color-coded in Fig. 2), supported by highly significant bootstrap values. Although there was no evidence of clustering according to geographic location or collection date, there was a tendency for isolates to group according to host species, in agreement with previously published data (Arbelo et al. 2012). This finding is consistent with previous observations (Bellière et al. 2010), with a single notable exception of Zc/2005/NoAt, identified in Cuvier's beaked whale *Ziphius cavirostris*, which clustered with 3 striped dolphin sequences. However, due to the limitations in the number and length of available GenBank sequences, caution should be exercised in the evaluation of cetacean *Alphaherpesvirus* phylogeny. With regard to the Italian striped dolphin isolate reported herein (Sc/2016/Me IZS-PLVA), the tree clearly shows that the virus forms a cluster with sequences from the same host species, and in particular with 2 sequences identified in striped dolphins stranded along the Spanish coast during the cetacean morbillivirus epidemic in 2007 and one derived from a dolphin stranded in the Canary Islands in 2011 (Fig. 2).

Even though the phylogenetic analysis points out a remarkable genetic heterogeneity among cetacean *Alphaherpesvirus* clade members, additional molec-

ular data are needed to gain further insight into their epidemiology, as well as to better define their phylogenetic and evolutionary relationships, and possibly, to unveil the existence of different circulating strains.

This report highlights the risk of severe mycosis for immunosuppressed cetaceans in the marine environment. More observations are needed to better understand the pathogenicity of *Herpesvirus* in cetaceans. Specifically, molecular studies may provide valuable insights into the spatiotemporal distribution, prevalence, epidemiology and impact of herpesviral infections on the health status and conservation of free-ranging cetaceans in the Mediterranean and other marine environments (Di Guardo et al. 2011).

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