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► To cite this version:

Guillaume Desoubeaux, Anne Debourgogne, Nathan Wiederhold, Marie Zaffino, Deanna Sutton, et al.. Multi-locus sequence typing provides epidemiological insights for diseased sharks infected with fungi belonging to the *Fusarium solani* species complex. *Medical Mycology*, 2018, 56 (5), pp.591-601. 10.1093/mmy/myx089 . hal-02902565

HAL Id: hal-02902565

<https://hal.univ-lorraine.fr/hal-02902565>

Submitted on 3 Jun 2022

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**Multi-locus sequence typing provides epidemiological insights for diseased sharks infected
with fungi belonging to the *Fusarium solani* species complex.**

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Short-running title: Investigation of *Fusarium* infections in sharks

Word count: 2841 (typographical signs)

Figure count: 4

Table count: 2

Supplementary material: 2

Conflicts of interest: the authors have no conflicts of interest to declare

ABSTRACT

Fusarium spp. are saprobic molds that are responsible for severe opportunistic infections in humans and animals. However, we need epidemiological tools to reliably trace the circulation of such fungal strains within medical or veterinary facilities, to recognize environmental contaminations that might lead to infection and to improve our understanding of factors responsible for the onset of outbreaks. In this study, we used molecular genotyping to investigate clustered cases of *Fusarium solani* species complex (FSSC) infection that occurred in eight Sphyrnidae sharks under managed care at a public aquarium. Genetic relationships between fungal strains were determined by multi-locus sequence typing (MLST) analysis based on DNA sequencing at five loci, followed by comparison with sequences of 50 epidemiologically unrelated FSSC strains. Our genotyping approach revealed that *F. keratoplasticum* and *F. solani* haplotype 9x were most commonly isolated. In one case, the infection proved to be with another Hypocrealian rare opportunistic pathogen *Metarhizium robertsii*. Twice, sharks proved to be infected with FSSC strains with the same MLST sequence type, supporting the hypothesis the hypothesis that common environmental populations of fungi existed for these sharks and would suggest the longtime persistence of the two clonal strains within the environment, perhaps in holding pools and life support systems of the aquarium. This study highlights how molecular tools like MLST can be used to investigate outbreaks of microbiological disease. This work reinforces the need for regular controls of water quality to reduce microbiological contamination due to waterborne microorganisms.

Keywords: MLST; genotyping; Sphyrnidae sharks; outbreak; *Metarhizium robertsii*.

INTRODUCTION

Fusariosis refers to opportunistic infections due to molds belonging to the genus *Fusarium*, that is often considered saprobic due to its common occurrence in soil, but also contains water-associated lineages.¹ The taxonomy of the *Fusarium* genera is very complex, and recently new species names have been proposed.² The *Fusarium solani* species complex (FSSC) is a phylogenetic and biological group that is currently estimated to contain at least 60 distinct - but very closely related - *Fusarium* species, including some cryptic and unnamed species.³ The members of this species complex are unified by phenotypic characters such as asexual morphs with long monophialides associated with uniloculate perithecial ascomata that are usually yellow, orange-red to brown. On the basis of molecular phylogenetic relationships, the FSSC is comprised of three major clades. FSSC clade 3 includes isolates that are pathogens of plants and animals,⁴⁻⁶ and all were formerly characterized as *Nectria haematococca* because of a common unique sexual teleomorphic stage and their ability to reproduce with other members of the same species.⁷ Members of FSSC clade 3 are responsible in humans for either localized infection, especially after trauma,⁸ or systemic disease with multiple internal organ involvement, as seen in immunocompromised patients.^{9,10} Molecular analysis has demonstrated that the two most clinically-common species of clade 3 are *F. falciforme* (FSSC 3 and 4) and *F. keratoplasticum* (FSSC 2).¹¹

In vertebrate animals, fusariosis has been described in several marine species, including sea turtles, whales, dolphins and seals [Violetta GC. A case history of *Fusarium* sp. in a captive population of bonnethead sharks (*Sphyrna tiburo*). Int. Assoc. Aquatic Anim. Med., Gulfport, 1984:64; Davis MR. Successful resolution of “bonnethead shark disease”, presumptive *Fusarium* infection, with antifungal therapy and environmental manipulation. Int. Assoc. Aquatic Anim.

Med., Mystic, 2007: 128].¹²⁻²² In elasmobranchs *e.g.* sharks, pathologic findings during fusariosis can include dermatitis, with ulcers and hemorrhages with lesions containing white-to-hemorrhagic, purulent exudates. They are primarily located within the lateral line canals, along the body, and within the cephalic canals of the cephalofoil, and can easily disseminate to other anatomical sites.^{14,18,20} Perichondritis and chondritis of the cartilaginous vertebrae, as well as chronic myositis, have been occasionally described.²⁰ Overall mortality rates are greater than 90% [Davis MR. Successful resolution of “bonnethead shark disease”, presumptive *Fusarium* infection, with antifungal therapy and environmental manipulation. Int. Assoc. Aquatic Anim. Med., Mystic, 2007: 128].^{13,14,18,20} To date, cases of *Fusarium* infections in elasmobranchs are rarely reported, as the prevalence was estimated below 0.6% in a cohort of 1,546 individuals versus 15.0% and 8.9% for bacterial and parasitic infections, respectively.¹⁶ However, conventional phenotypic methods may lead to possible fungal misidentifications, and although DNA sequencing is now recognized as the most reliable method for accurate strain identification, it was not routinely practiced before the last decade, and currently it is still commonplace that veterinary samples are submitted to human laboratories for that purpose.^{14,18} Little is known about the transmission of *Fusarium*, particularly in aquatic environments, which emphasises the need for better understanding of the epidemiology of *Fusarium* infection.

In human medicine, several molecular tools have been developed to study the epidemiology of transmissible agents,^{23,24} such as multi-locus sequence typing (MLST) analysis, microsatellite genotyping, and polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis. These techniques are useful in tracing the epidemiology of microbiological strains in the context of outbreaks.²⁵⁻²⁸ Multi-locus sequence typing enables the detection of nucleotide mutations (mostly single nucleotide polymorphisms, so called SNPs) within several

specifically-chosen loci that are usually located within conserved housekeeping genes.²⁹ It increases the discriminatory power compared to single gene sequencing.¹⁴ Unlike microsatellite genotyping and PCR-RFLP, MLST is highly reproducible and facilitates the comparison of data from different laboratories.²⁵

In this study, we used MLST genotyping to evaluate isolates from eight cases of FSSC infection for possible epidemiological links. They occurred over a four-year period in Sphyrnidae sharks, *i.e.* five bonnethead (*Sphyrna tiburo*) and three scalloped hammerhead sharks (*Sphyrna lewini*), within a public aquarium..

MATERIALS AND METHODS

Study population

Context of the study

In addition to over 8,500 aquatic species within different habitats comprising 7,600,000 million litres (L) of water, Adventure Aquarium (Camden, NJ, U.S.A.) is home to the largest collection of sharks on the East Coast of the United States of America (U.S.A.), including rare scalloped hammerhead sharks which are listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).³⁰

In the present study, five bonnethead and three scalloped hammerhead sharks were diagnosed with *Fusarium*, or *Fusarium*-like, infections during a four-year period (04/22/2012 – 11/03/2016) within a 93,860 L quarantine system (Q1) and the 2,900,000 L “Ocean Realm” (OR) exhibit. Both systems were maintained at 23 °C. The life support systems (LSS) of both Q1 and OR consisted of sand filtration, protein fractionation and ozonation.

Clustering of cases was suggested as four bonnethead shark infections occurred between February 2012 to March 2013, all within Q1, and two scalloped hammerhead shark cases were diagnosed concomitantly in February 2016, but separated in Q1 and OR. Two other cases were separately observed six and eight months after the two aforementioned onsets, respectively.

Cases definition

The diagnosis of fungal infection was confirmed by either cytological or histopathological examination of dermal lesions and internal tissues.^{31,32} Definitive fungal identification of *Fusarium* species was achieved by *in vitro* mycological cultures obtained from lesions and molecular sequencing as described below.^{4,33}

Microbiological samples

Each fungal strain, isolated from the eight diseased sharks and referred to as [A – H], were sub-cultured onto BBL[®] Sabouraud-dextrose agar supplemented with gentamicin and chloramphenicol (Becton-Dickinson, Le Pont-de-Claix, France). DNA was extracted from each by using the QIAmp[®] DNA Mini kit (Qiagen, Courtabœuf, France), according to the manufacturer's instructions. Isolates were confirmed as belonging to the FSSC by DNA sequencing of the translation elongation factor 1-alpha (*TEF1*) region, as described previously.³⁴ For some isolates, additional targets were used for this purpose, including the internal-transcribed spacer region (*ITS*) and D1/D2 domains of the large subunit of the nuclear ribosomal

RNA gene, *i.e.* portions of 5.8S and 28S rRNA, (*ITS-nuLSU*) and the RNA polymerase II second largest subunit B150 gene (*RPB2*). DNA extracts were stored at -20°C until subsequent analysis.

Genotyping of *Fusarium solani* species complex

In order to detect a clonal outbreak, molecular genotyping was performed according to the previously-reported MLST technique for the FSSC.²⁹ PCR mixes were prepared in a 50 µL reaction volume containing 25 µL of TaqPurple[®] (Ozyme, Montigny-le-Bretonneux, France), 5 µL of each aforementioned fungal DNA extracts, and 0.5 µL of each forward and reverse oligonucleotide primer at 20 µM.^{29,35} Fungal DNA was amplified at five loci corresponding to the following housekeeping genes: acetylcoenzyme A carboxylase (*ACC*), isocitrate lyase (*ICL*), glyceraldehyde-3P dehydrogenase (*GPD*), mannitol-1P dehydrogenase (*MPD*), and manganese superoxide dismutase (*SOD*). The PCR assays were performed in a iCycler IQ Thermal[®] cycler apparatus (Bio-Rad, Hercules, CA, U.S.A.), under the following conditions: an initial 2 min denaturation step at 95°C, followed by 35 cycles of hybridization/elongation, including 30 sec at 95°C, 30 sec at 55 °C, and 60 sec at 72°C, with a final extension step of 5 min at 72°C, before the unlimited cooling step at 4°C. PCR products were sequenced on both strands using the aforementioned amplification primers by the Genoscreen Company (Lille, France).

The one-letter code for nucleotides from the International Union of Pure and Applied Chemistry (IUPAC) nomenclature was used for data analyses.³⁶ Forward and reverse DNA sequence chromatograms were analyzed with the BioEdit[®] software (Ibis Biosciences, Carlsbad, CA, U.S.A.). Consensus DNA sequences at the five aforementioned loci were registered in GenBank[®] (NCBI, Bethesda, MA, U.S.A.) and compared to each other for every strain in order

to determine polymorphisms and sequence type (ST). Unique numbers were assigned to allelic variants for each housekeeping gene, according to the reported method.^{29,35} These numbers were then combined to yield a five-digit ST genotype.²⁹

Phylogenetic relationships

Phylogenetic relationships between the eight fungal strains were established by MEGA[®] v6.06 software (The Biodesign Institute, Tempe, AZ, U.S.A.) according to the maximum parsimony method, using 1,000 bootstrap resampling and 50% as the cutoff value for generation of the consensus tree. The reference sequence of *Fusarium staphyleae* NRRL 22316 was used as the outgroup (GenBank[®] accession number: JX171496.1),³⁴ and sequences of 50 epidemiologically-unrelated FSSC isolates deposited at the Westerdijk Fungal Biodiversity Institute (formerly known as *Centraalbureau voor Schimmelcultures*-Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands) were downloaded from GenBank[®] database to serve as controls (Suppl. Material 1).^{29,35}

Statistical analysis

XLStat[®] v.2016.6.04 software (Addinsoft, Paris, France) for Windows[®] (Microsoft, Issy-les-Moulineaux, France) was used for statistical analysis. The α -risk was adjusted at 0.05.

Ethics

No animal was anesthetized, euthanized, or sacrificed for the specific purposes of this study. The

biological samples used herein were the result of the routine veterinary care of these sharks. Adventure Aquarium is accredited by the Association of Zoos and Aquariums (AZA) and is held to the absolute highest standards in animal care and exhibition.

RESULTS

Study population and case confirmation

Clinical, pathological and mycological findings of the eight diseased Sphyrnidae sharks are summarized in Table 1. Gross findings at post-mortem examination included epidermal erosions, ulcers, haemorrhages, and vesicles containing white-to-hemorrhagic exudate, located on the dorsal and ventral aspects of the cephalofoil, progressing from the cutaneous, lateral line and cephalic canals, and ampullae of Lorenzini, to disseminated involvement (Figure 1A-C).

Cytological findings from skin exudate obtained from one female bonnethead shark provided evidence of fungal infection. Histopathological findings from tissue samples of the cephalofoil from the other seven sharks were very similar to one another, and consisted of severe, multifocal and focally extensive-to-regional, chronic, necrotizing dermatitis and cellulitis with ulceration of the skin, haemorrhage, and intra-lesional filamentous fungal hyphae. The inflammatory infiltrate in areas of dermatitis and cellulitis was dominated by macrophages and, in some cases, was noted to include degenerate and non-degenerate granulocytes, as well as a few multinucleate giant cells. Fungal hyphae extended into ampullae of Lorenzini, invaded blood vessels with and without vasculitis, and extended into nerves in some instances. Hyphae were 2-4 μm wide in histologic section, and appeared hyaline with parallel walls, septa, and right angle-branching (Figure 2). All eight cases were confirmed by positive mycological cultures of the lesions

showing *Fusarium*-like features (Figure 3). Molecular identification confirmed that seven of the eight fungal isolates belonged to FSSC clade 3, further classified with three to FSSC 2 species (*Fusarium keratoplasticum*) and four to FSSC 9 (*Fusarium* haplotype 9x). Interestingly, strain *C* was identified as *Metarhizium robertsii*, although the clinical and pathologic features in the infected shark were similar to those of the other bonnetheads (Figure 1D-E). All the sharks died between one and 37 days after the onset of clinical signs, except for one scalloped hammerhead shark that is alive to date while still undergoing treatment.

***Fusarium solani* species complex genotyping and phylogenetic relationship**

Results of MLST genotyping for each strain are reported in Table 2, except for strain *C*, which was excluded as it did not belong to FSSC. All isolates were successfully sequenced in the regions of the five housekeeping genes (accession numbers: KY780124 - 58; Suppl. Material 1-2). No locus haplotypes were identical in all the strains, indicating that each of the five genes was useful in allowing identification of distinct genotypes. Few polymorphisms were detected with only five distinct FSSC ST identified. Unique ST1, ST4 and ST5 genotypes were found for FSSC strains *D*, *E* and *H*, respectively, while the same MLST sequence type ST2 was observed for strains *B* and *F* which displayed 99.8% sequence similarity; *A* and *G* strains both exhibited ST3 genotype, with 99.9% sequence similarity.

Phylogenetic analysis indicated that the sequences of the 50 control FSSC clade 3 strains downloaded from the Westerdijk Institute were heterogeneously dispersed, confirming that these isolates were epidemiologically-unrelated, whereas *Fusarium* strains in sharks referred to as *A* and *G* were tightly clustered, supported by 95% bootstrap-value, confirming that they were not independent (Figure 4). In the same way, *B* and *F* isolates were also tightly clustered.

Transmission map

The first five cases of fusariosis or *Metarhizium* infection due to [A – E] strains were diagnosed in bonnethead sharks that all arrived at the same time to the aquarium (Table 1). Except for the shark infected with FSSC strain E, which was diagnosed in OR exhibit and was previously housed in Q1, all other FSSC strains were diagnosed while sharks were managed in Q1.

There was no contact between the three scalloped hammerhead sharks infected with FSSC strains [F – H] and the five bonnethead sharks, as they arrived at the aquarium three years later after the bonnethead infections and subsequent mortalities. Except for the shark with FSSC isolate F, which was diagnosed while it was still managed in Q1, the scalloped hammerhead sharks were diagnosed while in OR, after moving from Q1. The OR exhibit had also been occupied six months before by the bonnethead shark infected with the strain E, but this strain was genetically distinct from all the others.

DISCUSSION

Despite a lack of published literature, there is an increasing incidence of fusariosis in animals, especially in sharks of the genus *Sphyrna*.^{13,16,20} Since the late 1980's, 19 cases in bonnethead sharks [Violetta GC. A case history of *Fusarium* sp. in a captive population of bonnethead sharks (*Sphyrna tiburo*). Int. Assoc. Aquatic Anim. Med., Gulfport, 1984:64; Davis MR. Successful resolution of “bonnethead shark disease”, presumptive *Fusarium* infection, with antifungal therapy and environmental manipulation. Int. Assoc. Aquatic Anim. Med., Mystic, 2007: 128],^{16,20,37} and 15 in scalloped hammerhead sharks have been reported whether isolated or

grouped within six clinical clusters,^{13,14,18} including the five bonnethead and three hammerhead cases from the present study. Herein, *F. solani* haplotype 9x (FSSC 9) and *F. keratoplasticum* (FSSC 2) were most commonly isolated. In light of the previously published works,¹¹ the frequent occurrence of *F. keratoplasticum* was expected, but that of *F. solani* haplotype 9x was not. Our findings in sharks may simply reflect the specific presence of these two species in this environment. Risk factors for developing fusariosis in aquarium settings are not well known, but appear to be multifactorial, including water temperature, exhibit size and design, substrate and décor, and co-exhibiting animal interactions leading to increased susceptibility to opportunistic infections.¹⁸ It is noteworthy that our work is the first report of *Metarhizium robertsii* inciting infection in sharks. *M. robertsii* belongs to *M. anisopliae* complex which has been described as pathogen causing sclerokeratitis,³⁸⁻⁴⁰ and cutaneous infection in humans.⁴¹ A recent study evidenced that among the 11 human cases caused by *M. anisopliae* complex species, six were actually due to *M. robertsii* and none to *M. anisopliae stricto sensu*.⁴²

Previously, molecular analysis of *Fusarium* strains isolated from elasmobranchs has been performed in two recent studies and consisted of *ITS* and D1/D2 (*ITS*-nuLSU gene) sequence analyses.^{14,18} The current study describes a different MLST analysis for the genotyping of the fungal strains isolated over a four-year period from the eight *Sphyrna* sharks studied herein. As shown in other infectious diseases,^{24,25,28} MLST is considered to be the standard by which to investigate outbreaks.⁵ Recently, Debourgogne *et al.* demonstrated that a simple cost-efficient MLST scheme relying on sequencing fungal DNA at five loci (*ACC*, *ICL*, *GDP*, *MPD* and *SOD*) provided sufficient discriminatory power to reliably differentiate FSSC isolates and, thereafter, for epidemiological investigations of clustered cases.²⁹ This method is not intended to support current taxonomic classification, but in comparison to sequencing of the *ITS*-nuLSU alone, the

five-locus MLST approach allowed complete analysis of 1,616 nucleotides instead of 430;¹⁴ hence, its typing efficiency, *i.e.* ability to discriminate the highest number of isolates, was estimated at 0.48 versus 0.30.³⁵ While ITS-nuLSU sequencing is probably sufficient for phylogenetic species recognition in other genera,³⁴ it is too conserved to obtain a reliable species-level identification within *Fusarium* genus, and may not be suitable to distinguish similar strains, and to accurately address epidemiological links of clustered cases caused by a unique phylogenetic species. For example, sequencing the *ITS*-nuLSU region was not able to distinguish between strains *A*, *G* and *B* (100% similar sequences; data not shown). Likewise, it was previously shown that the MLST method is characterized by a higher discriminatory power than the reference three-locus typing scheme (0.991 versus 0.980, measured with Simpson's index of diversity, calculated according to Hunter and Gaston's modification),⁴³ which means that several FSSC isolates which shared the same sequence through the three-locus scheme were correctly differentiated by the five-locus scheme.³⁵ Using this method, the current study reliably demonstrated that two of the FSSC strains, isolates *B* and *F*, were genetically different from the other strains but were similar to each other (whereas at *TEF1* region, sequences of isolates *B* and *F* were similar to strain *G*; data not shown), although they were isolated from different diseased sharks 1,269 days apart. On the contrary, such sharp distinction was not possible with the three-locus scheme which erroneously encompassed the strain *D* within the same genotype (data not shown). Because the DNA ST data for the five loci combined were sufficient to discriminate between 50 unrelated FSSC control isolates, this method could reliably characterize a single genotype as responsible for the infections in these two individuals. The presence of a common FSSC sequence type, which was found to be ST2 according to the previously reported five-digit MLST genotyping classification,²⁹ in two sharks at different times suggested the persistence of

this strain within the aquarium environment. As with other fungal genera,⁴⁴ *Fusarium* spp., including members of the FSSC, are well adapted to aquatic environments. For instance, 45.5% of the samples collected from coastal waters of the Mediterranean Sea yielded at least one *Fusarium* sp.⁴⁵ Similarly, water maintained at a constant mild-to-moderate 20-35°C temperature,^{46,47} commonly found in aquaria, was shown to support fungal growth. *Fusarium* spp. were also reported to grow from 4 to 40°C,⁴⁸ and to persist nearly six years in water distribution systems.⁴⁹

The bonnethead and the scalloped hammerhead sharks that were infected with the strains *A* et *G*, displaying a single FSSC ST3 genotype (*i.e.* a distinct sequence type than ST2 that was found for strains *B* and *F*), raised an additional question, since diagnosis of fungal infections in these two sharks was separated by 1,424 days and the sharks were never maintained together in the same pool. This finding may underline a possible persistent circulation of this FSSC strain within the environment or LSS of the aquarium, although the probability of a new introduction with the same strain, but from an external source, could not be ruled out. *Fusarium* spp. are able to produce conidia that can remain in suspension in water, whereas the mycelium, which is generally associated with organic particles, is usually eliminated by filtration.⁴⁷ Thus, through suspended conidia, one might hypothesize that the FSSC ST3 strain could possibly enter the plumbing and LSS of the aquarium, form biofilms on pipe surfaces, and could secondarily colonize downstream, while subsequently exposing sharks at separate times.⁴⁷ Unfortunately due to the retrospective nature of this study, water samples were not cultured within the aquarium, and therefore the accurate determination of an environmental source of infection by various FSSC strains was not possible.

Our results suggest that reducing the risk of fungal infections caused by environmental molds should be an emphasis in the care of aquatic animals. Unlike the clinical scenarios in human hospitals,⁵⁰⁻⁵³ systematic prophylaxis with antifungal drugs is impractical for sharks because of logistics, cost, and the risks of promoting drug resistance. Rather, prevention should be based on managing water quality and temperature, and by regularly monitoring the biofilms within the plumbing, especially those with stagnant or unused sections which can be colonized by molds.⁴⁶ Some authors anecdotally attempted to decrease the water temperature of the enclosure by 5–8°C.¹⁷ Installation of small-mesh filters, *i.e.* ≤ 0.2 μm diameter, at different points of the water pipes provides good protection against filamentous fungi,⁵⁴ but this would not be functional with water turnover rates within LSS in the aquarium. Further information regarding the threshold of contamination at which there is an actual risk of fungal infection is needed to better define a routine monitoring plan.

Fusarium spp. are associated with pathologic lesions, morbidity and mortality in some elasmobranch species. Through this study, the clinical application of MLST genotyping at five loci has been shown to be useful in tracing FSSC strains to better understand the epidemiological aspects of fungal disease in a clustering of cases in bonnethead and scalloped hammerhead sharks, more accurately than with molecular identification to phylogenetic species level only. Specialized veterinary laboratories may wish to implement MLST for other genera of fungi as a tool for epidemiologic studies.

ACKNOWLEDGMENTS

The authors are grateful to Martha E. O'Dowd for her technical help in handling the mycological strains, and to Manon Dominique for her protocol describing the phylogenetic study.

FUNDING

This work was supported by internal laboratory funding.

DISCLOSURE OF CONFLICT OF INTEREST

None.

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FIGURE LEGENDS

Figure 1: Gross lesions in diseased Sphyrnidae sharks with confirmed fusariosis due to *Fusarium solani* species complex (FSSC) species or *Fusarium*-like species. A - Dorsal aspect of the head of the bonnethead shark infected with FSSC strain *E* (this isolate was thereafter identified as *F. solani* haplotype 9x) showing multiple, extensive and coalescing, serpentine and haemorrhagic skin lesions on the cephalofoil (black thick arrows); B – Lateral aspect of the body of the bonnethead shark infected with FSSC strain *E* (*F. solani* haplotype 9x) showing haemorrhagic lesions that follow the lateral line system (black thin arrow); C – Ventral view of the head of the scalloped hammerhead shark infected with FSSC strain *G* (afterwards identified as *F. keratoplasticum*) showing multiple and coalescent ulcerative lesions accompanied by hyperemia of the skin of the cephalofoil. Overall, very similar lesion patterns were found in all the diseased sharks of the present study: localization of the lesions to the ampullae of Lorenzini or lateral line suggests that these could have been portals of entry for the fungi. Skin lesions have also been postulated to spread infection from one individual to another, since sensory organs along the head are used to probe the environment and to contact other individuals.¹⁴ Similarly, involvement of the skin has been observed in marine mammals infected with *Fusarium* spp.^{17,37,55,56}; D – Dorsal lateral aspect of the body of the bonnethead shark infected with fungal strain *C* identified as *Metarhizium robertsii*; ventral view of the head of the bonnethead shark infected with fungal strain *C* identified as *Metarhizium robertsii*; E – Multiple, severe and extensive hemorrhagic and ulcerative lesions were present on the cephalofoil involving ampullae

of Lorenzini and cephalic canals. These lesions were very similar to those incited by species of the *Fusarium solani* species complex in the other cases.

Figure 2: Histopathological photomicrographs of fungal hyphae within lesions of the cephalofoil of diseased Sphyrnidae sharks. A – Hyphae of *Fusarium solani* species complex (FSSC) strain A (the isolate was thereafter identified as *F. keratoplasticum*) appeared filamentous (black thin arrow) with lightly basophilic cytoplasm that was occasionally vacuolated, and were located among macrophages, necrotic cells, protein and erythrocytes (hematoxylin-phloxine-saffron trichrome stain, bar = 20µm); B – Hyphae of FSSC isolate E (afterwards identified as *F. solani* haplotype 9x) were highlighted magenta among inflammatory cells and erythrocytes (Periodic acid-Schiff / Light Green SF Yellowish stain, bar = 20 µm). Septa were clearly visible (gray tips), as well as hyphae ramifications (black thin arrow).

Figure 3: Morphologic observations of the *Fusarium solani* species complex (FSSC) species in *in vitro* culture. A – Colonial morphology of FSSC strain D (*F. solani* haplotype 9x), after seven days incubation at 25°C on potato flakes agar (prepared in-house). Note moist sporodochial areas forming in the central portion of the colony; B - A cream-colored sporodochium (dark thin arrow), *i.e.* small, compact mass of hyphae that bears the conidiophores on which the asexual characteristic spores or conidia are formed, of FSSC isolate D (*F. solani* haplotype 9x), forming on carnation leaf agar (prepared in-house) after seven days incubation at 25°C (magnification x25); C – Microscopic morphology from a slide culture on potato flakes agar (prepared in-house) after seven days incubation at 25°C of FSSC strain D (*F. solani* haplotype 9x), demonstrating crescent-like septate macroconidia (dark thin arrow) and

unicellular microconidia forming at the apex of a long monophialidic conidiogenous cell (grey thick arrow) (lactophenol cotton blue stain, magnification x400, bar=100µm).

Figure 4: Phylogenetic tree showing the relationships of the seven *Fusarium solani* species complex (FSSC) strains isolated in diseased Sphyrnidae sharks compared to other members of clade 3. The tree was inferred from the nucleotide sequences of the *ACC*, *ICL*, *GPD*, *MPD* and *SOD* gene regions, according to maximum parsimony mean. Maximum parsimony is based on the number of character-state changes to construct all possible trees and give each a score. The strains isolated from the clustered cases of FSSC infection in sharks [A - B; D - H] are individually labelled with a dark diamond, and written in bold font. For controls, the sequences of 50 epidemiologically-unrelated FSSC isolates were uploaded from GenBank[®] database (all accession numbers are given in the Supplementary Material No. 1). The outgroup reference sequences are from *F. staphyleae* NRRL 22316 (GenBank[®] accession number: JX171496.1). Minimum bootstrap values were set at 1,000. High scores, expressed in percentage at nodes, demonstrate the reliability of embranchment.

Abbreviations: CBS, *Centraalbureau voor Schimmelcultures*; NRRL, Northern Regional Research Laboratory's Agricultural Research Service Culture Collection Database.