ISOLATION AND MOLECULAR CHARACTERIZATION OF *FUSARIUM* SPECIES (FUNGI, ASCOMYCOTA) FROM UNHATCHED EGGS OF *CARETTA CARETTA* IN TUSCANY (ITALY)

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Abstract – Fungal infectious diseases have dramatically increased in marine ecosystems during the past two decades and actually represent one of the main threats to biodiversity, likely due to the occurrence of emerging pathogens in new environments and the stress conditions induced by global climate change. In this context, the loggerhead sea turtle (*Caretta caretta* L.) is a vulnerable species according to the International Union for Conservation of Nature (IUCN) and it is included as a protected species under several international conventions. Sea Turtle Egg Fusariosis (STEF) is a worldwide emergent fungal disease associated with egg and embryos mortality in endangered sea turtle nests such as those of *C. caretta*. The disease can lead to a significant mass mortality in the infected nests and is caused by a complex of species belonging to *Fusarium* genus with isolates included in the Fusarium Solani Species Complex (FSSC); however, many questions regarding the aetiology and epidemiology of this disease as well as the biology and ecology of the causal agents are still open. *C. caretta* is the only sea turtle species nesting along the Tuscan archipelago where nests are becoming more numerous and widespread. At the same time, in the recent years a continuous monitoring of nesting and hatching sites allowed to record an increased number of affected nests, probably due to STEF. During the monitoring activities conducted in 2019-2020 in several localities on the Tuscany coast (province of Grosseto), a large number of eggs showing symptoms resembling those caused by STEF were found. Symptomatic eggs were so collected from nests located in three beaches and a total of 32 fungal isolates were obtained and submitted to a morphological identification followed by a molecular characterization. Amplicons were sequenced and used to assign the species, thus allowing to identify our isolates as *Fusarium solani*, *Fusarium oxysporum* and *Fusarium nodosum*. Finally, the phylogenetic relationships between our strains and those already known was rebuilt. While *F. solani* and *F. oxysporum* were already associated with *C. caretta* eggs showing symptoms of fungal infection, as far as we know, this is the first time that *F. nodosum* was isolated from affected eggs. Furthermore, this work represents the first report of STEF on Tuscan coast. Although Tuscany does not represent a primary nesting area of *C. caretta* in the Mediterranean basin, the record of the disease on this coastline, in line with what is happening across the globe, confirms that STEF may represent a major risk for the conservation of the loggerhead sea turtle also in this region.

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Introduction

Fusarium represents one of the most important genera of plant-pathogenic fungi known in agriculture, currently including approximately 300 species distributed in 23 monophyletic groups, referred to as species complexes [1]. It is responsible for a range of diseases on hundreds of plant species, and it is commonly recognized as one of the most relevant pathogens based on scientific and economic importance [1, 2]. Furthermore, many *Fusarium* species can produce relevant quantities of mycotoxins, such as trichothecenes, zearalenone and fumonisins, that can adversely affect the marketability of the product and cause adverse effects on human and animal health [3, 4]. In addition, under specific environmental conditions, a variety of species also cause infection (opportunistic mycosis) in humans with consequences that can be devastating, particularly in immunocompromised patients [5, 6]. In addition, some pathogenic *Fusarium* species are now widely recognized as a major threat to animal health and biodiversity conservation [7, 8].

Emerging infectious diseases caused by fungi have dramatically increased in marine ecosystems during the past two decades and actually represent one of the main threats to biodiversity, likely due to the occurrence of emerging pathogens in new environments and the stress conditions induced by global climate change [7, 9]. In this context, the loggerhead sea turtle (*Caretta caretta* L.) is a vulnerable species according to the Internationsal Union for Conservation of Nature (IUCN) [10] and it is included - as a protected species - under several international conventions. Sea Turtle Egg Fusariosis (STEF) is a worldwide emerging fungal disease associated with eggs and embryos mortality in endangered sea turtle nests such as those of *C. caretta* [11, 12]. The disease can lead to a significant mass mortality in the infected nests and is caused by a complex of species belonging to *Fusarium* genus with isolates mainly belonging to the Fusarium Solani Species Complex (FSSC) [13, 14]. This situation has recently increased concerns about the developing of fungal infection caused by *Fusarium* in endangered sea turtles, which causes hatching failure in sea turtle eggs [11, 12]. Pathogenic fungi can infect and grow within *C. caretta* nests by first creating a mycelial network on eggs, whose surface results completely covered; at a later time, they produce enzymes and organic acids that destroy the shells by dissolving organic substrates and calcium carbonate [12, 13, 15]. Affected eggs show coloured infection zones, which can turn into necrotic lesions and kill the surviving embryos [11, 12].

However, many questions regarding the aetiology and epidemiology of this disease, as well as the biology and ecology of the causal agents, are still open. As an example, it is unclear whether these pathogens are invasive species or natural nest inhabitants able to cause disease under a changing environmental scenario [12]. It is worthy of attention that species belonging to the FSCC are globally recognized also as among the most important plant pathogenic fungi, causing severe diseases on several cultivated, thus representing a significant threat to human food supply and agricultural biosecurity. This trend in the transmission of emerging pathogens into new environments, such as marine one, reinforces the importance of deeply investigating those factors responsible of this situation [12]. In addition, nevertheless the environmental conditions may not be the only aspect determining pathogenic fungi development, during embryonic development, the eggs survive for a long period under constant conditions of high temperature and humidity, parameters that favour the growth of soil-borne fungi [12, 13, 14].

C. caretta is the only sea turtle species nesting along the Tuscan coastline and the Tuscan archipelago, where nests are becoming more numerous and widespread. At the same time, in the recent years a continuous monitoring of nesting and hatching sites allowed to record an increased number of affected nests, presumably due to STEF. During the samplings carried out in 2020 in several localities on the Tuscan coast (province of Grosseto), a large number of eggs showing symptoms resembling those caused by STEF were found. In this work, we analysed eggs from natural nests of *C. caretta* that showed visual symptoms of egg fusariosis, with the aim to (i) isolate *Fusarium* spp. isolates present in unhatched eggs, (ii) morphologically and molecularly characterize the isolated fungi and, finally, (iii) reconstruct the phylogenetic relationships between our isolates and those already known (animal and plant pathogens).

Materials and Methods

Symptomatic eggs, characterized by an unusual, coloured area compared with healthy ones and/or covered with mycelium, were collected from nests located in three beaches along the coast (Table 1) and placed in sterile containers. Egg portions were plated on Sabouraud Dextrose Agar (SDA, Biolife Italiana S.r.l., Milan, Italy) in order to isolate associated fungi. By transferring individual hyphal tips, following a first step of mass isolation, to Potato Dextrose Agar (PDA, Biolife Italiana S.r.l., Milan, Italy), or, when sporulating, by monoconidial isolations, we were able to obtain axenic cultures of the fungal outgrowths. Pure cultures of the isolates are actually stored at the fungal collection of the University of Pisa, Italy (Department of Agriculture, Food, and Environment).

Fungal gDNA was extracted from all pure cultures according to the Chelex 100 (Chelex® 100 sodium form, MERCK SERONO S.P.A., Rome, Italy) protocol [16]. To identify and analyse the genetic variability within *Fusarium* isolates, a region including the Translation elongation factor 1 alpha (Tef1-α) gene, a useful region for fungal taxonomic and phylogenetic studies [16], was amplified. PCR reaction (25 µL) contained 2 µL of gDNA (around 10 ng), 2.5 µL of each primer (0.5 µM), 5.5 µL nuclease free H_2O and 12.5 µL GoTaq® Green Master Mix (Promega Italia S.r.l, Milan, Italy). Primers Tef1-α (5'-CATCGAGAAGTTCGAGAAGG-3' as forward and 5'-TACTTGAAGGAACCCTTACC-3' as reverse) were used. The amplification program consisted in 2' of preliminary denaturation (95 °C), 30 cycles of amplification (1' at 94 °C for denaturation, 1' at 55 °C for the annealing and 1' at 72 °C for the extension) and a final extension at 72 °C for 5 min. All amplifications were performed in a Q-Cycler 24 (HAIN, Lifescience, Nehren, Germany). The PCR products were checked by 2.0 % gel electrophoresis run and purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). PCR products were sequenced (both forward and reverse direction) by the Bio Molecular Research Genomics (BMR Genomics, Padova, Italy).

For phylogenetic analyses, reference Tef1-α sequences of strains belonging to the *Fusarium* species were retrieved from previously published works [17]. All the sequences obtained were aligned using MAFFT v. 7.402 [18]. Multiple sequence alignments were trimmed to get comparable sequences and exported to MEGAX [19] where the best-fit substitution model was calculated. Using MrBayes 3.2.6 [20], the Markov chain Monte Carlo (MCMC) algorithm was performed to generate phylogenetic trees with Bayesian posterior probabilities for sequence dataset, using the nucleotide substitution models previously

determined. Four MCMC chains were run simultaneously for random trees for 2 000 000 generations and sampled every 1000 generations (p-value reached a value lower than 0.01). The first 25 % of trees were discarded as burn-in phase of each analysis and posterior probabilities were determined from the remaining trees.

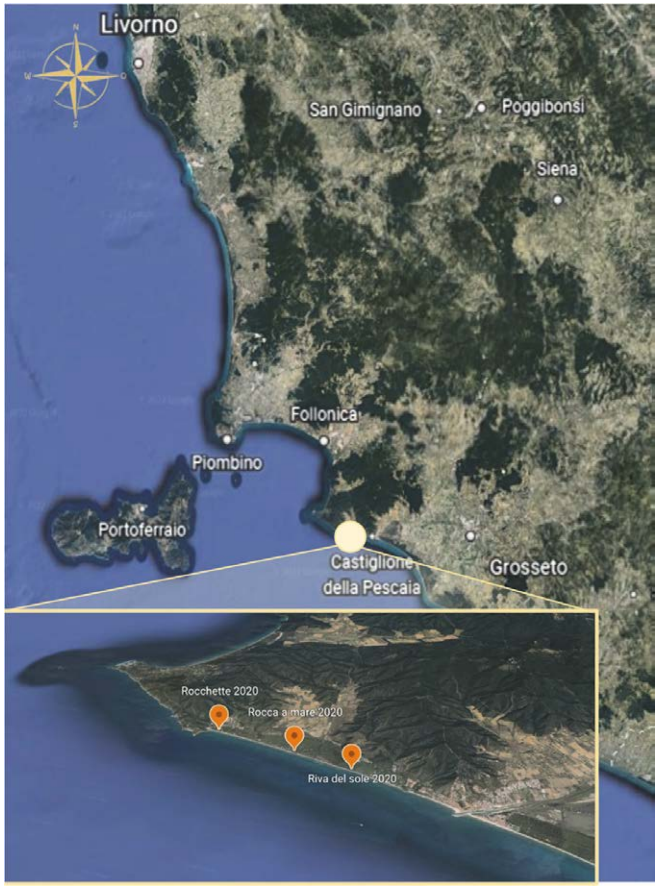

Figure 1 − Geographical distribution of the nests sites.

Table 1 − List of sampling locations and corresponding nest ID number, sampling period, geographic coordinates (GCS) and number of eggs collected in each site.

Nest site (ID)	Sampling period	GCS	Eggs n°
Rocchette (2080970)	before hatching	42°46'9.75" N | 10°50'43.07" E	4
Rocchette (20106236)	after hatching	42°46'9.75" N | 10°50'43.07" E	4
Rocca a mare (20106837)	after hatching	42°46'22.04" N | 10°49'31.76" E	5
Riva del sole (20106864)	after hatching	42°46'35.28" N | 10°47'50.08" E	5

Results

After the development of fungal colonies, a total of 32 isolates (Table 2) were obtained and submitted to a morphological identification followed by a molecular characterization. Morphological identification, by microscopic observation, allowed to preliminary assign all the isolates to the *Fusarium* genus, according to size and shape of conidia, conidiogenic cells and conidiophores structures (Figure 2). In order to assign a species to the isolates, a molecular approach, consisting in the amplification and sequencing of Tef1-α regions was performed. Amplicon analysis allowed to identify our isolates as *F. solani*, *F. oxysporum* and *F. nodosum* (Table 2). Specifically, at the Rocchette sites, a total of 14 different isolates (t7, t2, t6, 1A, 1B, 1C, 2A, 2B, 2C1, 2C2, 3A, 3B, 3C), belonging to *F. solani* (*Fusarium solani* Species Complex), *F. oxysporum* (*Fusarium oxysporum* species Complex) and *F. nodosum* species, were collected from eggs taken before hatching (ID 2080970), while only two isolates (t21, t5; *F. solani*) were found in samples taken after hatchling (ID 20106236). A total of eight isolates were found from samples collected at the Rocca a Mare site (ID 20106837): five of these were found to belong to the *F. oxysporum* Species Complex (t11, t12, t14, t19, t22), while the remaining three to the *F. solani* ones (t16, t13, t18). The remaining nine isolates were collected from the Riva del Sole site samples (ID 20106864), and they included five isolates of *F. solani* (t3, t8, t9, t10, t15) and three of *F. oxysporum* (t1, t4, t17, t20). Overall, 18 strains of *F. solani* (56 %), 12 of *F. oxysporum* (38%) and two of *F. nodosum* (6 %) were identified (Figure 3).

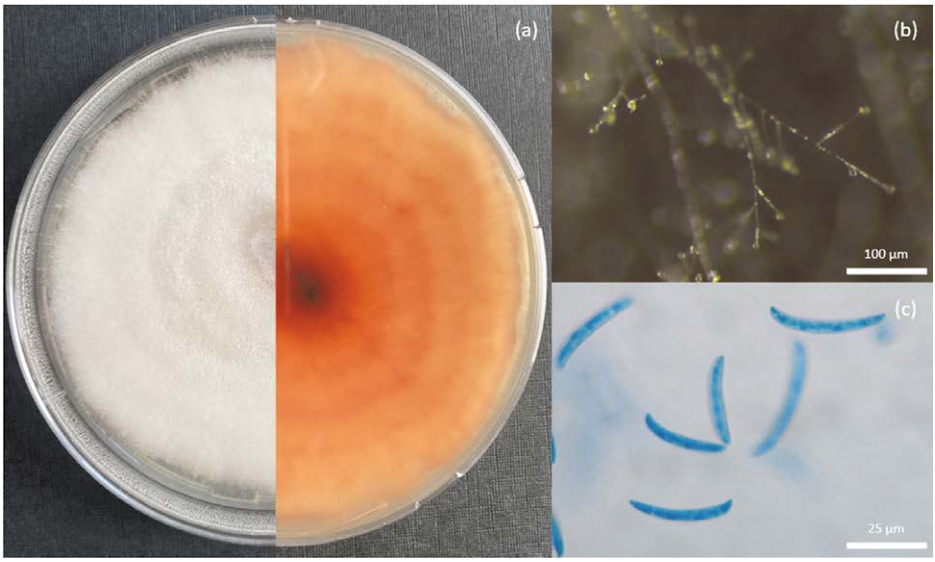

Figure 2 − Morphological features of a *Fusarium oxysporum* isolate from unhatched sea turtle eggs. Front and reverse of mature colony (a), conidiophores (b) and macroconidia (c)

Table 2 – Identification of *Fusarium* spp. isolates, according to Tef1-α DNA sequence, collected from sea turtle eggshells. Abbreviation: Egg ID, identification number of nest site and unhatched egg number (nest | egg); Sample ID, identification number of fungal isolates.

Egg ID (nest | egg)	**Sample ID**	**Species**
2080970 | 70	70_1a	*Fusarium oxysporum*
2080970 | 70	70_2a	*Fusarium nodosum*
2080970 | 70	70_3a	*Fusarium solani*
2080970 | 1	1a	*Fusarium solani*
2080970 | 1	1b	*Fusarium solani*
2080970 | 1	1c	*Fusarium solani*
2080970 | 2	2a	*Fusarium solani*
2080970 | 2	2b	*Fusarium nodosum*
2080970 | 2	2c1	*Fusarium oxysporum*
2080970 | 2	2c2	*Fusarium oxysporum*
2080970 | 2	2d	*Fusarium oxysporum*
2080970 | 3	3a	*Fusarium solani*
2080970 | 3	3b	*Fusarium solani*
2080970 | 3	3c	*Fusarium solani*
20106236 | 16	36_16b	*Fusarium solani*
20106236 | 18	36_18a	*Fusarium solani*
20106837 | 1	37_1b	*Fusarium oxysporum*
20106837 | 1	37_1a	*Fusarium oxysporum*
20106837 | 2	37_2b	*Fusarium solani*
20106837 | 2	37.2a	*Fusarium oxysporum*
20106837 | 3	37.3a	*Fusarium solani*
20106837 | 4	37_4c	*Fusarium oxysporum*
20106837 | 5	37_5a	*Fusarium oxysporum*
20106837 | 5	37_5b	*Fusarium solani*
20106864 | 18	18a	*Fusarium solani*
20106864 | 19	19b	*Fusarium oxysporum*
20106864 | 92	92b	*Fusarium solani*
20106864 | 92	92a	*Fusarium solani*
20106864 | 93	93b	*Fusarium solani*
20106864 | 93	93c	*Fusarium solani*
20106864 | 93	92a	*Fusarium oxysporum*
20106864 | 104	104b	*Fusarium oxysporum*

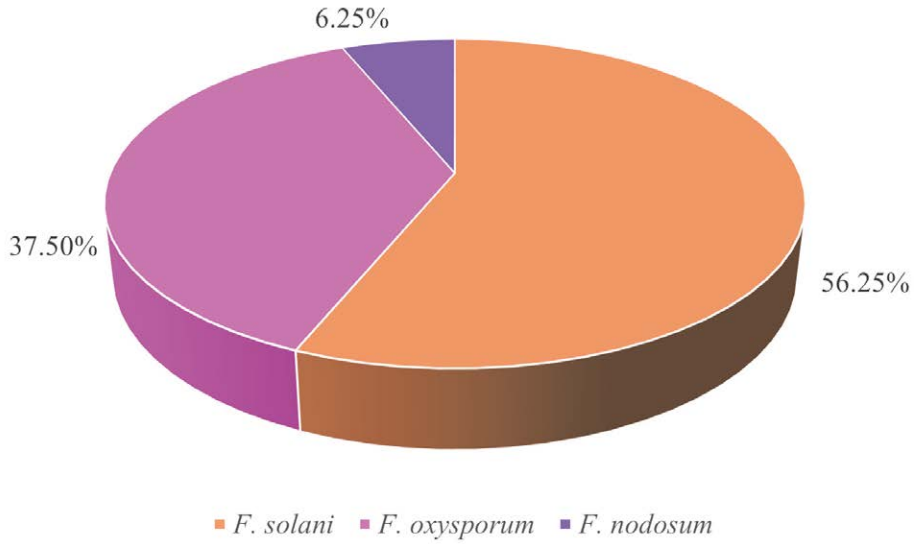

Figure 3 − Distribution of *Fusarium* species among the 32 fungal isolates from unhatched eggs.

Discussion

This work represents the first report of STEF on Tuscan coast. Although Tuscany does not represent a primary nesting area of *C. caretta* in Italy and in the Mediterranean area, the record of the disease on this coastline, in line with what is being recorded across the globe, confirms that STEF may represent a major risk for the conservation of the loggerhead sea turtles also in this region, especially considering how natural systems can be affected by the present climate change perspective [12]. While *F. solani* and *F. oxysporum* were already associated with *C. caretta* eggs showing symptoms of fungal infection, together with other FSSC members such as *F. keratoplasticum* and *F. falciforme* [13, 14, 21], as far as we know, this is the first time that *F. nodosum* was isolated from affected eggs. This is a noteworthy result, since *F. nodosum* is a mycotoxigenic plant-pathogenic fungus, belonging to the complex of *Fusarium* species causal agents of Fusarium head blight of wheat, recently reported for the first time in Italy on *Triticum durum* [22].

These results confirm a global spread of the problem and the need for further studies concerning the biology and ecology of the pathogenic agents, as well as the aetiology and epidemiology of the disease. As the frequency of fungal infections in marine habitats is severely increasing, it is critical to identify the biological and ecological components that contribute to disease epidemic outbreak and severity. In this context, further studies focused on pathogen phylo-biogeography, mechanisms of dispersion and colonization of coastal habitats, and environmental and physiological parameters for infection are needed. For these reasons, isolation and characterization of fungal pathogens will help us to reveal their biology and epidemiology and will allow a better management of disease and to better understand the current and future impact of STEF on sea turtles' conservation worldwide.

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