Fusarium solani is responsible for mass mortalities in nests of loggerhead sea turtle, Caretta caretta, in Boavista, Cape Verde

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Abstract

The fungus Fusarium solani (Mart.) Saccardo (1881) was found to be the cause of infections in the eggs of the sea turtle species Caretta caretta in Boavista Island, Cape Verde. Egg shells with early and severe symptoms of infection, as well as diseased embryos were sampled from infected nests. Twenty-five isolates with similar morphological characteristics were obtained. Their ITS rRNA gene sequences were similar to the GenBank sequences corresponding to F. solani and their maximum identity ranged from 95% to 100%. Phylogenetic parsimony and Bayesian analyses of these isolates showed that they belong to a single F. solani clade and that they are distributed in two subclades named A and C (the latter containing 23 out of 25). A representative isolate of subclade C was used in challenge inoculation experiments to test Koch postulates. Mortality rates were c. 83.3% in challenged eggs and 8.3% in the control. Inoculated challenged eggs exhibited the same symptoms as infected eggs found in the field. Thus, this work demonstrates that a group of strains of F. solani are responsible for the symptoms observed on turtle-nesting beaches, and that they represent a risk for the survival of this endangered species.

Introduction

The main threats to marine turtles during their life cycle occur in the sea (e.g. drowning due to fishing gear, pollution, or ingestion of plastics) and at nesting beaches (both during the egg-laying period and embryonic development in the nest). During the embryonic stages, turtle nests are exposed to a number of risks that may critically affect their hatching success (Bustard, 1972; Fowler, 1979; Whitmore & Dutton, 1985). This is usually attributed to beach erosion, depredation, plant root invasion, excessive rainfall, tidal inundation, developmental abnormalities as well as pathogenic infections (Phillott et al., 2001). In the past 30 years, an abrupt decline in the number of nesting beaches of sea turtles, breeding females, hatching success and the survival rate of the hatchlings has been noted worldwide. The reasons for this are related to human impact, such as coastal development, and juvenile and adult by-catch (Marco et al., 2006). In a number of cases, this decline is also suspected to be due to pathogenic microorganisms. However, there are few studies regarding the impact of microorganisms on sea turtle eggs (Abella et al., 2008) and recent investigations are pointing to the role of Fusarium species as a possible reason of nesting decline during the embryonic stage of development (Phillott & Parmenter, 2001; Abella et al., 2008).

The fungal species Fusarium solani (Mart.) Saccardo (1881) (teleomorph = Nectria haematococca; Rossman et al., 1999) belongs to the Ascomycetes and represents a diverse complex of over 45 phylogenetic and/or biological species (Zhang et al., 2006; O'Donnell et al., 2008). This species complex is widely distributed and comprises soil-borne saprotrophs that are among the most frequently isolated fungal species from soil and plant debris. Under conducive conditions, this fungus can cause serious plant diseases, infecting at least 111 plant species spanning 87 genera (Kolattukudy & Gamble, 1995), and has also been shown to cause disease in immunocompromised animals (Booth, 1971; Summerbell, 2003). Interestingly, isolates of F. solani have been previously reported as a cause of infection on shells and skin of juvenile marine turtles (Rebell, 1981).

Fig. 1. Diseased eggs of sea turtle Caretta caretta infected in the natural environment by Fusarium solani. The characteristic appearance of disease symptoms: (a) reddish and (b) bluish; (c) healthy eggs; and (d) dead embryo infected by F. solani.

Research on F. solani–sea turtle interactions has gained increasing interest because this fungus has being isolated from dead eggs in natural nests of several different sea turtle species at different locations (Phillott & Parmenter, 2001; Phillott et al., 2001; Marco et al., 2006; Abella et al., 2008).

Identification of potential pathogens threatening endangered sea turtle species (IUCN, 2009) is crucial for the development of conservation plans. In this study, we have morphologically and molecularly characterized 25 isolates of F. solani associated with egg mass mortalities of loggerhead sea turtle, Caretta caretta, on Boavista Island. This island represents one of the most important nesting regions for this species. The hatching success of this species is currently severely threatened as a high number of their nests contained eggs with symptoms of fungal infection. This has resulted in a high hatching failure rate.

Materials and methods

Fungal isolation

Loggerhead sea turtle eggs showing symptoms of fungal infection were collected from sea turtle nests located in Ervatao, Joao Barrosa and Curral Velho beaches on Boavista Island (Cape Verde, Africa). The fungus was isolated from internal and external symptomatic areas of egg shells that exhibited unusual colored spots (yellow, blue, grayish) compared with healthy ones, from eggs shells with severe symptoms of infection characterized by grayish mycelium covering the shell (Fig. 1a–c), and also from infected embryos (Fig. 1d). For isolations, a glass-ring technique was used according to the methodology of Cerenius et al. (1987), and pure cultures were maintained on peptone glucose agar (PGA) (Söderhäll et al., 1978) with penicillin (100 mg L^{-1}). Cultures were labeled as 001AFUS through 058FUS in the culture collection of the Real Jardín Botánico (Madrid, Spain) (see Table 1).

Morphological characterization of fungal isolates

Fungal spores and mycelia were examined microscopically under an Olympus BX-51 compound microscope (Olympus Optical, Tokyo, Japan) and species characterization was performed following the manuals for Fusarium spp. identification of Booth (1977) and Nelson et al. (1983). Light micrographs were captured using a Micropublisher 5.0 digital camera (Qimaging, Burnaby, BC, Canada) and the software SYNCROSCOPY-AUTOMONTAGE (Microbiology International Inc., Frederick, MD) as described in Diéguez-Uribeondo et al. (2003).

DNA extraction, PCR amplification and sequencing

For molecular characterization, DNA extraction was carried out by growing the mycelium as drop cultures (Cerenius & Söderhäll, 1985). Genomic DNA was extracted from these cultures using an E.Z.N.A-Fungi DNA miniprep kit (Omega Biotek, Doraville, GA). DNA fragments containing internal transcribed spacers ITS1 and ITS2 including 5.8S were amplified and sequenced with primer pair ITS5/ITS4 (White et al., 1990) as described in Martín et al. (2004). Nucleotide

Table 1. Fusarium solani isolates from eggs of the sea turtle species, Caretta caretta

Isolate	Year of isolation	Source**	BLAST*		Subclade[z]
			Maximum identity (%)	GenBank accession number[w]	
001AFUS	2005	EIS, SIS, IE	100	DQ535186.1	C
001BFUS	2005	SIS	100	AM412642.1	A
001CFUS	2005	EIS, SIS, IE	98	DQ535186.1	C
001DFUS	2005	EIS, SIS, IE	100	DQ535186.1	C
004FUS	2007	SIS	94	AM412641.1	C
009FUS	2008	SIS	98	AM412641.1	C
010FUS	2008	SIS	99	DQ535186.1	C
011FUS	2008	EIS	99	DQ535186.1	C
012FUS	2008	EIS	99	DQ535186.1	C
013FUS	2008	EIS	99	DQ535186.1	C
014FUS	2008	EIS	99	DQ535186.1	C
015FUS	2008	SIS	99	DQ535186.1	C
016FUS	2008	SIS	99	DQ535186.1	C
018FUS	2008	EIS	100	DQ535186.1	C
019FUS	2008	SIS	100	DQ535186.1	C
020FUS	2008	SIS	95	AM412641.1	C
021FUS	2008	SIS	99	DQ535186.1	C
050FUS	2009	EIS	99	FJ719812.1	A
051FUS	2009	EIS	99	FJ948133.1	C
053FUS	2009	EIS, SIS, IE	99	DQ535186.1	C
054FUS	2009	EIS, SIS, IE	99	DQ535186.1	C
055FUS	2009	SIS	99	DQ535186.1	C
056FUS	2009	EIS, SIS, IE	99	DQ535186.1	C
057FUS	2009	EIS	100	DQ535186.1	C
058FUS	2009	EIS	99	DQ535186.1	C

BLAST maximum identity, GenBank accession number and phylogenetic subclade.

*BLAST option of the NCBI nucleotide database.

[w]GenBank accession number of the Fusarium solani isolates with maximum identity to the studied isolate.

[z]Subclades within the clade III, which grouped all the rRNA gene ITS F. solani sequences.

**EIS, early-infected shell; SIS, severely infected shell; IE, infected embryo.

BLASTN searches with option standard nucleotide BLAST of BLASTN 2.6 were used to compare the sequences obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases.

Phylogenetic analyses

The program SE-AL 2.0A11 CARBON (Rambaut, 1996) was used for alignment of the ITS sequences of the sea turtle infecting fungal isolates and selected sequences obtained from the NCBI nucleotide databases (Table 2). For the external group, a sequence of Fusarium staphyleae (AF178423) was selected based on a previous phylogenetic study of the genus Fusarium (O'Donnell, 2000). The programs PAUP 4.0b10 (Swofford, 2003) and MR. BAYES 3.1 (Ronquist & Huelsenbeck, 2003) were used for phylogenetic analyses. In the analysis with PAUP, we applied maximum parsimony analysis following the heuristic search and bootstrap support (BS) as a method of support (Felsenstein, 1985). The fast Stepwise addition with 10 000 replicates was used. For the Bayesian analysis, the GTR1I1G (for 2 000 000 generations and 12 simultaneous chains) evolution model was followed. The first 1000 trees obtained were discarded and a consensus tree was obtained with the last 19 000 trees.

Challenge inoculation experiments

Freshly oviposited eggs of C. caretta showing no signs of infection were collected directly from cloacae of four nesting females (six eggs per female) to prevent fungal contamination from contact with the sand. The eggs were collected on Boavista Island in a location close to where infected nests had previously been observed. Eggs were maintained in plastic containers (c. 500 mL) with sterile vermiculite as an incubating substrate and were incubated in two artificial incubators (FB 80-R-Reptiles, Jaeger Bruttechnik) at 29.5 ± 0.5 1C. This is the pivotal temperature for loggerhead egg development (Wibbels, 2003) and adequate for artificial incubation (Booth, 2004) until hatching, which takes approximately 53–63 days (Fig. 2). To maintain a constant

Table 2. GenBank sequences of Fusarium spp. (teleomorph = Nectria spp.) included in the phylogenetic analyses

GenBank accession number	Species	Source	Clade/subclade
AF178423	F. staphyleae	Staphylea trifolia	Outgroup
DQ164845	F. solani f. sp. eumartii	Solanum tuberosum	III/C2
AY043470	F. solani f. sp. eumartii	Solanum tuberosum	III/C2
DQ164844	F. solani f. sp. eumartii	Lycopersicon sculentum	III/C2
DQ164843	F. solani f. sp. eumartii	Solanum tuberosum	III/C2
AY043477	F. solani	Solanum tuberosum	III/C2
AY043469	F. solani	Solanum tuberosum	III/C2
AY677295	F. solani	Vitis spp.	III/C
AM412641	F. solani	Human keratitis	III/C
AM412615	F. solani	Human keratitis	III/C
DQ535185	Nectria haematococca	Human cornea	III/C
DQ535186	Nectria haematococca	Human cornea	III/C
AY043471	F. solani	Solanum tuberosum	III/B
AY043474	Nectria haematococca	Solanum tuberosum	III/B
L36619	Nectria haematococca	Unknown	III/B
L36620	Nectria haematococca	Unknown	III/B
EF060459	Hypocreales sp.	Marine environment	III/A
AB255352	Fusarium sp.	Pterocarpus macrocarpus	III/A
AM412601	F. solani	Nematode	III/A
AM412638	F. solani	Human ulcer	III/A
AM412640	F. solani	Human skin lesion	III/A
AM412642	F. solani	Human keratitis	III/A
AY310442	F. solani	Beetroot	III/A
DQ094641	F. solani	Mycetoma	III/A
DQ682578	F. solani	Coffea arabica	III/A
AF150466	F. solani f. sp. radicicola	Solanum tuberosum	III/A
AF150467	F. solani f. sp. radicicola	Solanum tuberosum	III/A
DQ657851	Nectria haematococca	Dioscorea zingiberensis	III/A
AY928414	F. oxysporum	Watermelon	III/A
AY220236	F. phaseoli	Phaseolus vulgaris	II
AY730909	F. tucumaniae	Glicine max	II
EF408516	F. brasiliense	Glicine max	II
AY730905	F. cuneirostrum	Phaseolus vulgaris	II
AY730903	F. virguliforme	Glicine max	II
DQ002550	F. oxysporum	Nelumbo nucifera	I
DQ016211	F. oxysporum	Gymnosperm	I
DQ453704	F. oxysporum	Unknown	I
GU132456	F. oxysporum	Solanum tuberosum	I
U28161	F. oxysporum	Pinus sp.	I
EU003077	Fusarium sp.	Apple seedling	I
EU003029	Fusarium sp.	Apple seedling	I
GU233852	Fusarium sp.	Leptinotarsa decemlineata	I

temperature of c. 29.5 1C in the incubators, temperatures were monitored by data loggers (Stoway TidbiT Onset ± 0.3 1C) placed in the incubators. Temperature data were downloaded from the data loggers every 4 days, and, if necessary, the incubator temperatures were adjusted accordingly. Each plastic container was covered with a plastic lid. Each incubator contained six eggs (from two different females). One container was used as a control and the eggs were not exposed to fungal inoculum. In the other container, the eggs were challenged with inoculum. The inoculum consisted of egg shells previously incubated for 24 h at room temperature with conidia of the cultured F. solani isolate (001AFUS). Four pieces of the inoculum (c. 1 cm × 1 cm) were added to the upper side of the healthy eggs placed in the incubators (Fig. 2). The eggs were exposed to the inoculum on day 36 of incubation. The experiment was carried out twice.

On day 45, the plastic lid was removed and exchanged for perforated polyethylene plastic wrap in order to allow for better oxygenation and to diminish condensation due to the increased embryonic metabolic heating during the last period of incubation (Carr & Hirth, 1961; Miller, 1985). Eggs were checked once a week until day 45 after initiation

of incubation in order to detect any change or fungal growth. From day 45, the eggs were observed three times a day. Eggs of the controls were always checked first in order to avoid contamination. Fungal virulence was assessed as the mortality rate based on hatching failure, i.e., the number of dead embryos out of the total number of eggs challenged with inoculum. We conducted analysis in tables 2×2 to evaluate egg mortality among treatments in laboratory experiments.

All animals used in the study were cared for in accordance with the principles and guidelines of the Cape Verde Environmental Laws.

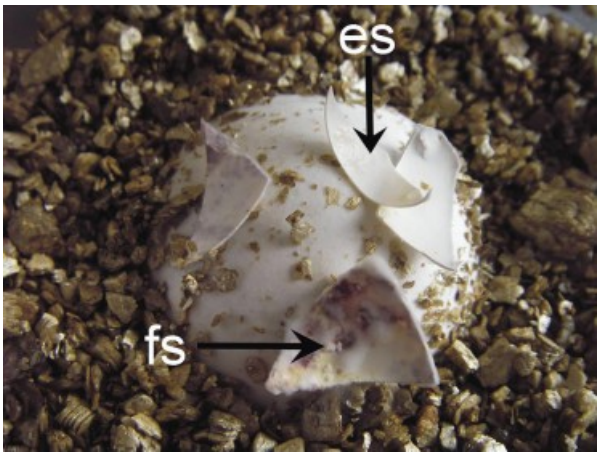

Fig. 2. Inoculation experiment. Healthy eggs of sea turtle Caretta caretta inoculated with Fusarium solani-infected pieces of turtle egg shell (es). Fusarium solani spores (fs).

Results

Fungal isolation and morphological characterization

From the infected material studied, i.e. egg shells and embryos, c. 25 isolates were obtained. All isolates produced septated microconidia, macroconidia and chlamydospores (Fig. 3a–c). The microconidia had an oval morphology and a size of c. 9–15 × 2–4 μm. Their monophialides were elongated, c. 50–70 μm long × 2–3 μm wide and bore microconidia. The macroconidia were inequilaterally fusoid, with the widest point above the center and the chlamydospores were usually globose or elliptic with smooth walls of about 9–12 × 8–10 μm, borne singly or in pairs on short lateral branches or intercalary. Occasionally, some chlamydospores

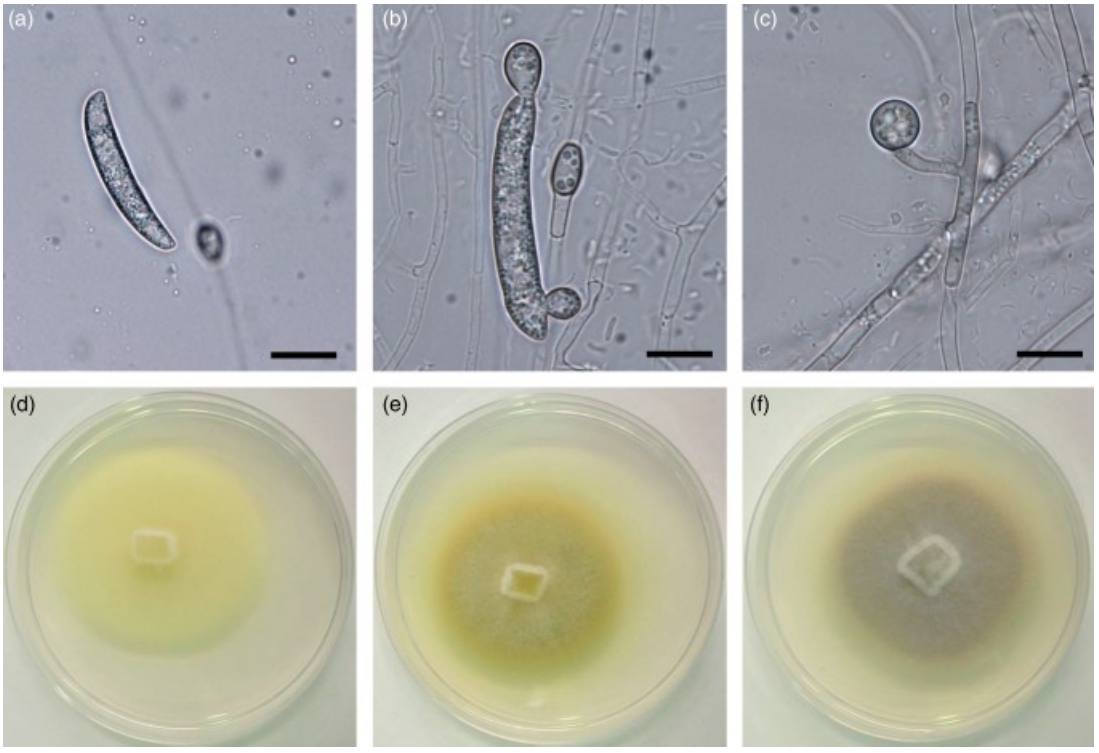

Fig. 3. Characteristic asexual structures of a strain of Fusarium solani isolated from sea turtle eggs of Caretta caretta: (a) fusoid macroconidia; (b) two chlamydospores, one macrochlamydospore with an elongated shape and another with an elliptic shape; (c) characteristic globose chlamydospore. Scale bars = 10 μm. Characteristic pigmentation of colonies of F. solani isolates growing on potato glucose agar medium: (d) cream, (e) yellow and (f) blue-green.

of an elongated shape were seen (Fig. 3b). The isolates presented characteristic colony pigmentation patterns of a cream, blue-green or blue color on PGA (Fig. 3d–f). These characteristics are typical of F. solani as described by Booth (1977) and Nelson et al. (1983).

BLAST search and phylogenetic analyses

The 100 equally parsimonious trees obtained had 133 changes. Parameters of verisimilitude of the Bayesian analysis were as follows: LnL = — 2072.222 (± 0.47); the frequencies of the bases were as follows: p(A) = 0.269 (± 2.85E — 4), p(G) = 0.224 (± 2.67E — 4), p(C) = 0.291 (± 2.99E — 4), p(T) = 0.219 (± 2.38E — 4), substitution rate r(AC) = 0.110 (± 5.16E — 4), r(AG) = 0.256 (± 1.50E — 4), r(AT) = 0.134 (± 8.64E — 4), r(CG) = 3.33E — 2 (± 1.86E — 4), r(CT) = 0.379 (± 1.753E — 3), r(GT) = 0.101 (± 7.63E — 4), a(P) = 8.799E — 2 (± 1.5E — -5) and the proportion of invariables sites P(invar) = 0.458 (± 1.753E — 3).

The phylogeny of the Bayesian and the strict consensus of the heuristic search had the same topology. Figure 4 shows the Bayesian analysis. Posterior probabilities (PP) of the Bayesian analysis are shown above the internodes and BS values ≥ 50% are indicated below. The Fusarium spp. sequences grouped in three clades named I, II and III. These clades were highly supported by PP (0.98–1.00) and

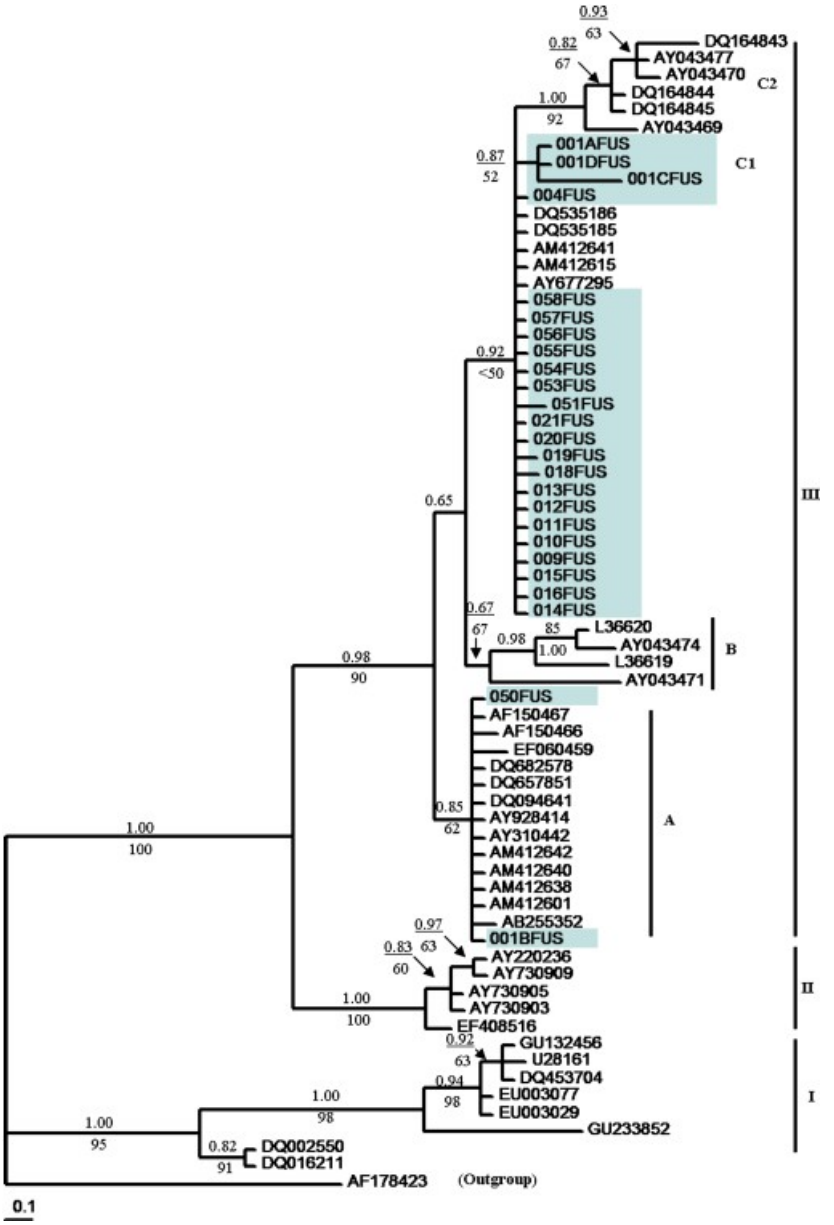

Fig. 4. Bayesian out-group-rooted cladogram inferred from ITS rRNA gene sequences of Fusarium spp. Numbers placed above and below the internodes are, respectively, PP and BS ≥ 50% of the Bayesian and parsimony analyses. Fusarium solani isolates from sea turtles are highlighted.

BS (90–100%) (Fig. 4). The Fusarium oxysporum isolates grouped in clade I, other Fusarium spp. recently segregated of the F. solani species complex (Aoki et al., 2003) grouped in clade II and the isolates of F. solani grouped in clade III. Clade III comprised three subclades (A–C). Subclade A included two sea turtle isolates grouped with isolates from animals and plants, subclade B comprised isolates from Solanum tuberosum and subclade C contained the majority of the sea turtle isolates (23 out of 25), in addition to isolates from other animals and S. tuberosum (Table 2). The isolates within this last subclade formed two distinct groups (C1 and C2), which are highly supported by PP (0.92–1.00) and BS (52–92), respectively. The group C1 included sea turtle isolates and C2 included S. tuberosum isolates (Fig. 4). Most of the nongrouped isolates of subclade C were obtained from different infections of animals (Fig. 4).

Infection challenge experiments

Eggs exposed to inoculum had a mortality rate of 83.3% (10 out of 12). Symptoms of fungal infection on the eggs resembled those observed in the field and were first seen 6 days after inoculation. Infected areas were characterized by a yellow, bluish color. The size of the infected area increased during incubation and eventually turned into a large necrotic lesion that resulted in the death of the embryos and hatching failure. Fungi were isolated from infected areas and dead embryos, and their morphological study and molecular analysis revealed that all isolates were identical to the original strain used for inoculation. In control eggs, mortality rate was 8.3% (1 out of 12). These mortality rates were statistically significantly different (Fisher exact two-tailed, $P = 0.03$). From control eggs shells, isolation attempts did not yield any fungus.

Discussion

In this work, we demonstrate that a number of isolates of F. solani are responsible for embryonic mortality in the nesting areas of the sea turtle C. caretta in Boavista, Cape Verde. Although this fungal species has been described previously in association with different infections in animals, including sea turtles (Rebell, 1981; Cabañes et al., 1997), its role as a pathogen and its relationship with hatching success has never been investigated until the present study.

The fungal isolates involved in the infection of C. caretta eggs in Boavista have been characterized morphologically and molecularly. Although the isolates were morphologically indistinguishable, their ITS sequences fell into two different subclades within F. solani clade III (A and C). In subclade A, some of the isolates were obtained from animals (5 out of 12) including two from sea turtles and the rest from plants (7 out of 12). In contrast, subclade C contained the majority of the animal isolates (24 out of 34), including those from sea turtles. Thus, there seems to be some animal host specificity in subclade C as it happens in other fungal groups (Berbee, 2001) and fungal-like organisms (Diéguez-Uribeondo et al., 2009). Despite this, further studies are needed to demonstrate possible host specificity.

Inoculation challenge experiments with a representative sea turtle infecting F. solani isolate from subclade C indicate that they are pathogenic to C. caretta eggs, because the inoculations met Koch postulates; i.e., the F. solani isolates were constantly associated with the disease; they were isolated from infected eggs and grown in pure culture; symptoms characteristic of the original disease occurred in healthy eggs when they were inoculated with the fungal isolates from pure culture, and the pathogen was reisolated from challenge inoculated eggs under experimental conditions.

Although the role of some F. solani isolates as pathogens is shown here, the presence of this fungus does not necessarily lead to the development of disease. During embryonic development, the eggs spend a long period covered by sand under conditions of high humidity and a warm and constant temperature, which are known to favor the growth of soilborne fungi such as Fusarium spp. However, these conditions may not be the only factors determining disease development. We have also examined and detected the presence of F. solani in nests with asymptomatic eggs (E. Abella et al., unpublished data). This seems to suggest that other factors such as specific microclimatic conditions, sand composition, natural immunosuppression, because the developing immune system gains full maturity and competence only during and after embryonic development of embryos, or additional immunosuppression, e.g. due to accumulation of toxic substances in turtles and their eggs, etc, may be determining the development of the disease. With regard to microclimatic conditions leading to disease symptoms, these have been extensively investigated and modelled in other ascomycete systems such Colletotrichum spp. in their host (see reviews by Wharton & Diéguez-Uribeondo, 2004; Peres et al., 2005). These studies have led to disease-forecasting systems that are very useful for preventing diseases and minimizing their economic impacts. Therefore, further studies need to be focused on investigating the conditions conducive to disease development in sea turtles.

The finding that some F. solani strains may act as a primary pathogen in loggerhead sea turtles is of considerable relevance because these pathogenic strains are currently infecting nests of loggerhead sea turtles in Cape Verde and threatening their populations, occasionally resulting in 100% mortality of the turtle eggs (E. Abella, pers. obs.). This represents an extremely high risk to the conservation of loggerhead see turtle in at least this nesting area. The description of those particular fungal strains causing this infection may help in developing conservation programs

based on artificial incubation and also on developing preventative methods in the field to reduce or totally erase the presence of F. solani in turtle nests. Isolation and characterization of these fungal strains will help us decipher their biology and epidemiology, and will allow to better understand the possible ways to prevent this disease. Further studies need to be focused on strain biogeography, mechanism of dispersion, and microclimatic and physiological parameters of the strains and/or eggs conducive for infection.

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