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Isolation of fungal pathogens from eggs of the endangered sea turtle species *Chelonia mydas* in Ascension Island

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Fungal emerging pathogens are one of the main threats for global biodiversity. Sea turtles do not seem to be an exemption, and recent studies on important nesting areas worldwide have shown that two fungal pathogens, i.e. Fusarium falciforme *and* Fusarium keratoplasticum*, are involved in low hatching success in nests of sea turtle species. Although the presence of these pathogens has been detected in Ascension Island, there are no investigations on the distribution of these two pathogens in main nesting beaches in the island. In this study, we analysed 109 eggshells of the species* Chelonia mydas *from four nesting areas in Ascension Island. We have isolated and identified a total of 46 fungal isolates. A phylogenetic analysis, of the ITS nrDNA region, with a number of reference sequences of the* Fusarium solani *species complex, showed that 23 of these isolates corresponded to the pathogen* F. keratoplasticum*. The analyses on isolation frequency, that included other previously obtained isolates, i.e. 11* F. keratoplasticum *and one* F. falciforme*, showed that* F. keratoplasticum *was the species most frequently isolated in Ascension Island and it was found in all nesting beaches, while* F. falciforme *was only isolated from Pan Am beach. When compared with other nesting areas worldwide, the abundance of* F. keratoplasticum *over* F. falciforme *was higher than any other nesting region tested. These findings are important in order to evaluate the potential threat of this pathogen to nests of the sea turtle population of Ascension Island, and to develop future control strategies.*

Keywords: Distribution, fungal pathogens, sea turtle eggs, nesting areas, conservation

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INTRODUCTION

Emerging pathogens are affecting a wide number of species worldwide, representing an important negative factor in the current global biodiversity crisis (Daszak *et al.*, 1999, 2000). In the marine ecosystem, the occurrence and severity of diseases have dramatically increased during the last 20 years mainly due to emerging pathogens and global climate change (Altizer *et al.*, 2003; Harvell *et al.*, 2009). Studies to identify the pathogens responsible for these diseases, combined with information on environmental drivers have helped to better understand these diseases. For example, aspergillosis is a fungal emerging disease in corals, mainly caused by *Aspergillus sidowii*, and it has produced massive mortalities in Caribbean populations. Recent studies on diseased coral species in the Tropical Eastern Pacific (TEP) have revealed that *A. sidowii* is the most frequently isolated species and it might be implicated in mortalities in TEP coral populations (Barrero-Canosa *et al.*, 2013).

In sea turtles, the pathogens *F. keratoplasticum* and *F. falciforme* cause a potentially lethal disease (Sarmiento-Ramírez *et al.*, 2010, 2014a). These species possess a number of biological features that are similar to those of known fungal pathogens involved in emerging infectious diseases and host extinctions, which impose an enormous threat for all sea turtle species (Sarmiento-Ramírez *et al.*, 2014a). These pathogenic species seem to have a global distribution and are present in the main nesting areas for at least six of the seven existing sea turtle species, including Ascension Island. Ascension Island hosts one of the most important and well protected nesting populations of green sea turtles (*Chelonia mydas*) in the world (Broderick *et al.*, 2006; Weber *et al.*, 2014). Thus, it is very important to identify the presence of fungal pathogens such as *F. keratoplasticum* and *F. falciforme* and therefore assess the threat that these pathogens might represent to the nesting population at Ascension Island.

In order to understand the threat that *F. keratoplasticum* and *F. falciforme* represent for sea turtle populations it is also key to detect environmental drivers of *Fusarium* disease. In eggs, this disease seems to be highly dependent on environmental factors (Sarmiento-Ramirez *et al.*, 2014a). However, there are no investigations of the potential source of these pathogens in nesting beaches. The species *F. keratoplasticum* and *F. falciforme* belong to the *Fusarium solani* species complex (FSSC), and are some of the most clinically important pathogens (Short *et al.*, 2011, 2013). Studies on the distribution and source of these pathogens have revealed that a large majority of isolates of *F. keratoplasticum* are

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present worldwide, mostly from indoor aquatic biofilms and infections of animals (Zhang *et al.*, 2006; Mehl & Epstein, 2008; Short *et al.*, 2011). Moreover this species is mainly isolated from environments significantly impacted by human activity (Short *et al.*, 2013). Regarding *F. falciforme*, it is a pathogen rarely found to cause animal diseases in tropical areas and it is not a common fungus of food, household plant material, garden soil or domestic water, as *F. solani* is (Summerbell & Schroers, 2002). However, the main source of infection for *F. falciforme* seems to be the soil and plant debris particles (Zhang *et al.*, 2006). In this study we will also discuss the potential source of *F. keratoplasticum* and *F. falciforme* in nesting beaches at Ascension Island.

To sum up, in order to understand the potential threat that *F. keratoplasticum* and *F. falciforme* represent for the green sea turtle nesting population nesting at Ascension Island, it is key to understand the distribution of these species in the nesting locations and the source of infection. With this purpose, we carried out fungal isolations and molecular characterization of the isolates from egg shells of the sea turtle species *C. mydas* collected at Ascension Island.

MATERIALS AND METHODS

Fungal isolations and molecular characterization

A total of 109 egg shells, of the green sea turtle, showing symptoms of fungal infection, i.e. egg shells that exhibited unusual coloured spots (yellow, blue, greyish) (Sarmiento-Ramírez *et al.*, 2010), were collected from sea turtle nests laid at English bay (29), North East bay (30), Pan Am beach (15) and Long beach (35) at Ascension Island (Figure 1). All samples were taken after the nest emergence. Fungi were isolated by placing fragments of the egg shells on peptone glucose agar (PGA) with ampicillin (100 mg l^{-1}). Pure cultures are kept in the culture collection of the Real Jardín Botánico-CSIC, Madrid, Spain.

For molecular characterization, DNA was extracted from pure cultures using the DNA Easy PlantMini Kit (Qiagen, Valencia, CA). 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). Amplification of the ITS nrDNA was carried out with the Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, Buckinghamshire, UK). The amplification program was initial denaturalization at 94°C for 5 min; 5 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min; followed by 33 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 1 min; with a final extension at 72°C for 10 min (Martin & Winka, 2000). The amplification products were purified using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced by MACROGEN (Inc. Seoul, Korea). Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI) was used to identify the consensus sequence from the two strands of the ITS nrDNA of each isolate. For initial identification of the isolates, the ITS nrDNA sequences were compared with those of the National Center of Biotechnology Information (NCBI) nucleotide databases using the Nucleotide BLASTN tool.

Phylogenetic analyses

For precise identification of the *Fusarium* spp. phylogenetic analyses, including the isolates initially identified as members of the *F. solani* species complex (FSSC), were carried out. The generated ITS nrDNA sequences from isolated *Fusarium* (Table 1), 146 NCBI-GenBank sequences of *Fusarium* turtle egg isolates from previous studies from Ecuador (44) and Cape Verde (68), and 60 selected sequences of *Fusarium* spp. from other hosts and environments were included (Sarmiento-Ramirez *et al.*, 2014a, b). The program Se-Al 2.0a11 Carbon (Rambaut, 2002) was used for manual alignment of the sequences. Maximum parsimony (MP) (Swofford, 2003) was inferred using the heuristic search option in PAUP*4.0b10. The bootstrap proportion was used to assess confidence for a specific node (Felsenstein, 1985; Lutzoni *et al.*, 2004). Phylogenetic trees were edited with TreeView (Page, 1996). Newly obtained sequences of the *F. falciforme* and *F. keratoplasticum* isolates were submitted to GenBank with accession numbers KJ944397 through KJ944419.

Differences in isolation of *F. keratoplasticum* and *F. falciforme* in nesting areas in Ascension Island

In order to analyse the differences in distribution of *F. keratoplasticum* and *F. falciforme* and other fungi between nesting areas, the isolation frequency of these fungi was calculated. The isolation frequency of these species and other fungi in the Island was calculated as the number of isolates of each species over the number of overall fungal isolates. The isolation frequency of each species per nesting area was also calculated. The analyses also included isolates of *F. keratoplasticum* (11) and *F. falciforme* (1), obtained in a previous study (Sarmiento-Ramirez *et al.*, 2014a).

RESULTS

Fungal isolations

A total of 46 fungal isolates were obtained from 109 eggshells (Table 1). A total of 23 of these isolates were initially identified as members of the FSSC, based on the NCBI BLAST analysis of the ITS nrDNA sequences (Table 1). Phylogenetic MP and Bayesian analyses showed that these isolates belonged to the species *F. keratoplasticum* (Table 1 and Figure 2). The other 21 fungal isolates were identified as members of the genera *Fusarium* (2), *Pseudallescheria* (10), *Scedosporium* (1), *Aspergillus* (3), *Phoma* (2), *Alternaria* (1), *Gymnascella* (1) and *Pleosporales* (1). The genera of other two fungal isolates were undetermined (Table 1).

Differences in isolation of *F. keratoplasticum* and *F. falciforme* in nesting areas in Ascension Island

The analyses included all the fungal isolates obtained, to date, from sea turtle eggs at Ascension Island, i.e. a total of 34 *F. keratoplasticum* isolates, one *F. falciforme* and 23 fungal isolates from other genera. These analyses showed that 58% (34 out

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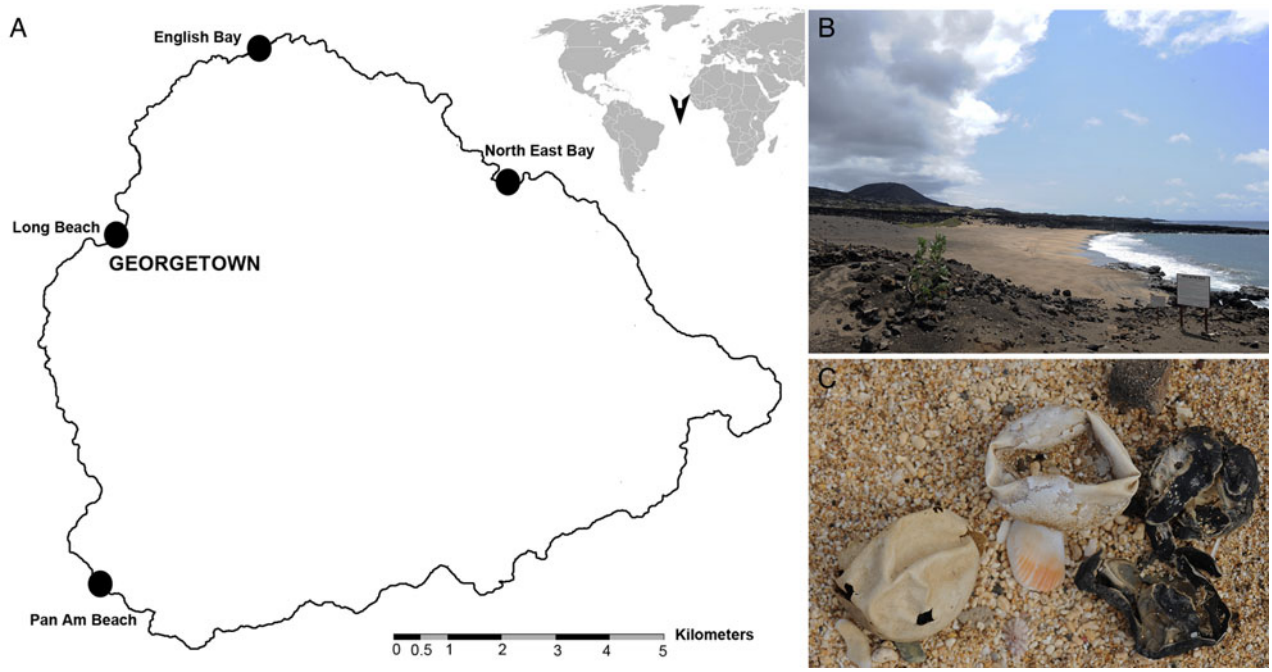

Fig. 1. Sea turtle nesting areas sampled for isolation and characterization of *Fusarium* species at Ascension Island: (A) Nesting areas sampled at Ascension Island; (B) North East Bay; (C) egg shells with symptoms of *Fusarium* disease and dead embryos found in the sampled nests (Long Beach).

of 58) of the isolates belonged to the species *F. keratoplasticum*, 40% (23 out of 58) belonged to other fungi, and the species *F. falciforme* represented 2% (1 out of 58) of the isolates (Table 1 and Figure 3).

The species *F. keratoplasticum* was isolated from all locations sampled and *F. falciforme* was only isolated from Pan Am Beach (Table 1). 47% (16 out of 34) of *F. keratoplasticum* isolates was obtained from Long beach, 23% (8 out of 34) from North East Bay, 15% (5 out of 34) from Pan Am Beach, 9% from English Bay and 6% from an undetermined nesting area (Table 1).

DISCUSSION

In this study, we found that the pathogenic species *Fusarium keratoplasticum* is the *Fusarium* spp. most frequently isolated from egg shells of the green sea turtle species nesting at Ascension Island. The phylogenetic analyses of ITS nrDNA region demonstrated that the majority, i.e. 97% (34 out of 35) of the *Fusarium* isolates that belong to the *Fusarium solani* species complex obtained from sea turtle egg shells at Ascension Island, corresponded to the species *F. keratoplasticum* and only one isolate corresponded to the species *F. falciforme*. A previous study on the global distribution of the sea turtle egg pathogens *F. keratoplasticum* and *F. falciforme* showed that these pathogens are distributed in major sea turtle nesting regions including Ascension Island (Sarmiento-Ramírez *et al.*, 2014a). However, this is the first time that differences in distribution of both *Fusarium* spp. are described among nesting locations in Ascension Island.

The analyses on distribution of the *Fusarium* spp. on Ascension Island showed that *F. keratoplasticum* is present in all locations sampled and this species was most frequently isolated from Long Beach (16 out of 34). *Fusarium keratoplasticum* is mainly isolated from environments significantly impacted by human influence (Short *et al.*, 2013). Interestingly, Long Beach is located near to Georgetown, the largest settlement on the Island, which might play a role in the high presence of *F. keratoplasticum*. This beach also has by far the greatest density of nests in the island, which can influence the presence of this pathogenic fungus. Regarding *F. falciforme*, it was only found at Pan Am Beach, which is not subject to a large amount of human activity. Indeed, *F. falciforme* is usually not a common fungus in areas impacted by humans (Summerbell & Schroers, 2002). The presence of *F. falciforme* in this beach might be influenced by the major presence of plant debris compared with other nesting beaches in the island. Further studies on the source of both *Fusarium* spp. should be addressed in order to reduce the potential impact of these species on nesting areas of Ascension Island.

If compared with other nesting areas worldwide, the isolation frequency of the pathogenic species *F. keratoplasticum* (97%) over *F. falciforme* (3%) is higher than any other nesting region tested, i.e. Cape Verde and Ecuador, where the isolation frequency of *F. keratoplasticum* over *F. falciforme* is 75 and 23% respectively (Sarmiento-Ramirez *et al.*, 2014a, b). These data might suggest a prevalence of the species *F. keratoplasticum* over *F. falciforme* in nesting areas located in the Atlantic (Ascension Island and Cape Verde) compared with those located in the Pacific (Ecuador). Although *F. keratoplasticum* is known to cause diseases worldwide (Short *et al.*, 2013), this is the first time that this species is described as the most represented *Fusarium* spp. on the sea nesting regions located in the Atlantic Ocean. Although *F. falciforme* is considered a serious pathogen, it is rarely found causing disease in tropical and subtropical areas (Guarro *et al.*, 1997); this study demonstrates that this fungal species has a broader distribution and is most frequent in tropical areas. Certainly, in recent years, there has been evidence that *F.*

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Table 1. Fungal isolates found in eggshells of the sea turtle species *Chelonia mydas* in Ascension Island.

Line number	Strain[a]	Species	GenBank accession[b]*	Location[c]
1	223 FUS	*F. keratoplasticum*	KC574007*	North East Bay
2	227 FUS	*F. keratoplasticum*	KC574008*	Long Beach
3	228 FUS	*F. keratoplasticum*	KC574009*	Undetermined
4	230 FUS	*F. keratoplasticum*	KC574010*	Undetermined
5	233 FUS	*F. keratoplasticum*	KC574011*	Pan Am Beach
6	235 FUS	*F. keratoplasticum*	KC574013*	Pan Am Beach
7	236 FUS	*F. keratoplasticum*	KC574014*	Pan Am Beach
8	237 FUS	*F. keratoplasticum*	KC574015*	Pan Am Beach
9	238 FUS	*F. keratoplasticum*	KC574016*	Long Beach
10	239 FUS	*F. keratoplasticum*	KC574017*	Long Beach
11	240 FUS	*F. keratoplasticum*	KC574018*	Long Beach
12	241 FUS	*F. keratoplasticum*	KJ944397	Long Beach
13	242 FUS	*F. keratoplasticum*	KJ944398	Long Beach
14	243 FUS	*F. keratoplasticum*	KJ944399	Long Beach
15	244 FUS	*F. keratoplasticum*	KJ944400	Long Beach
16	245 FUS	*F. keratoplasticum*	KJ944401	Long Beach
17	246 FUS	*F. keratoplasticum*	KJ944402	Long Beach
18	247 FUS	*F. keratoplasticum*	KJ944403	Long Beach
19	248 FUS	*F. keratoplasticum*	KJ944404	Long Beach
20	249 FUS	*F. keratoplasticum*	KJ944405	Long Beach
21	250 FUS	*F. keratoplasticum*	KJ944406	Long Beach
22	251 FUS	*F. keratoplasticum*	KJ944407	Long Beach
23	252 FUS	*F. keratoplasticum*	KJ944408	English Bay
24	253 FUS	*F. keratoplasticum*	KJ944409	English Bay
25	255 FUS	*F. keratoplasticum*	KJ944410	English Bay
26	259 FUS	*F. keratoplasticum*	KJ944411	North East Bay
27	261 FUS	*F. keratoplasticum*	KJ944412	North East Bay
28	263 FUS	*F. keratoplasticum*	KJ944413	North East Bay
29	265 FUS	*F. keratoplasticum*	KJ944414	North East Bay
30	266 FUS	*F. keratoplasticum*	KJ944415	North East Bay
31	268 FUS	*F. keratoplasticum*	KJ944416	North East Bay
32	270 FUS	*F. keratoplasticum*	KJ944417	North East Bay
33	298 FUS	*F. keratoplasticum*	KJ944418	Long Beach
34	300 FUS	*F. keratoplasticum*	KJ944419	Pan Am Beach
35	234 FUS	*F. falciforme*	KC574012*	Pan Am Beach
36	269 FUS	*F. equiseti*		North East Bay
37	257 FUS	*F. chlamydosporum*		English Bay
38	256 FUS	*Pseudallescheria boydii*		English Bay
39	258 FUS	*Pseudallescheria boydii*		English Bay
40	262 FUS	*Pseudallescheria boydii*		North East Bay
41	264 FUS	*Pseudallescheria boydii*		North East Bay
42	301 FUS	*Pseudallescheria boydii*		English Bay
43	302 FUS	*Pseudallescheria boydii*		English Bay
44	304 FUS	*Pseudallescheria boydii*		North East Bay
45	305 FUS	*Pseudallescheria boydii*		North East Bay
46	306 FUS	*Pseudallescheria boydii*		North East Bay
47	308 FUS	*Pseudallescheria boydii*		North East Bay
48	297 FUS	*Scedosporium aurantiacum*		Long Beach
49	225 FUS	*Aspergillus tamarii*		Undetermined
50	299 FUS	*Aspergillus sclerotiorum*		Long Beach
51	307 FUS	*Aspergillus* sp.		North East Bay
52	226 FUS	*Phoma multirostrata*		Long Beach
53	267 FUS	*Phoma* sp.		North East Bay
54	232 FUS	*Alternaria* sp		Undetermined
55	224 FUS	*Gymnascella hyalinospora*		Undetermined
56	254 FUS	Pleosporales		English Bay
57	229 FUS	Undetermined		Undetermined
58	231 FUS	Undetermined		Undetermined

[a]Code of the fungal isolates deposited in the fungal collection of the Real Jardín Botánico-CSIC.
[b]GenBank accession number of the fungal isolates. The asterisks indicate the isolates obtained in a previous work (Sarmiento-Ramírez *et al.*, 2014a).
[c]Location undetermined means that it was not possible to track the origin of the samples from which those fungi were obtained.

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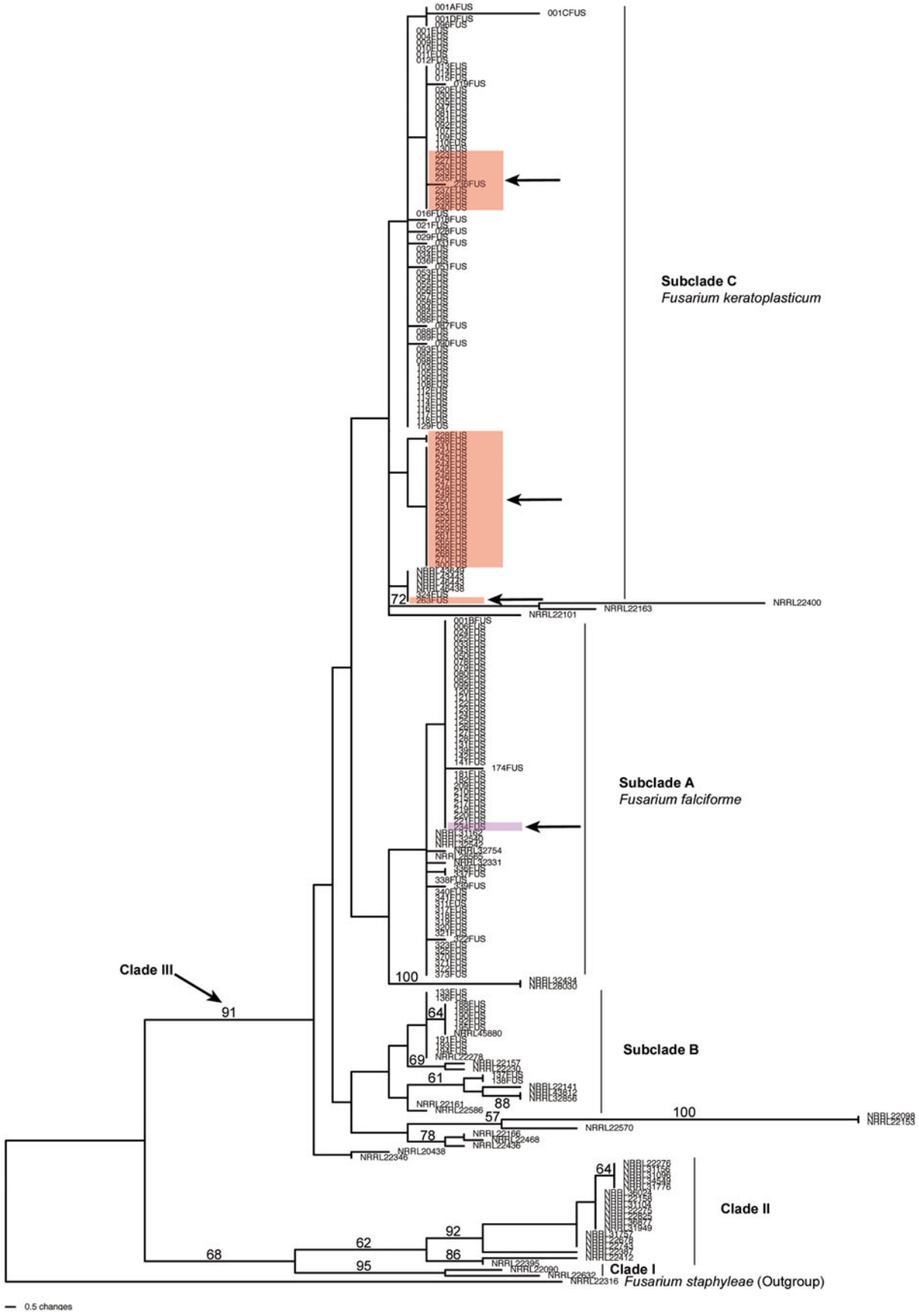

Fig. 2. Out-group rooted cladogram of the ITS nrDNA region of isolates within the *Fusarium solani* species complex (FSSC). One of the most parsimonious trees inferred from the ITS nrDNA sequence data of 146 sea turtle fungal isolates and 60 non-sea turtle fungal isolates. The numbers on the internodes indicate the bootstrap values (BS) of the parsimony analysis. Highlighted isolates correspond to those obtained from sea turtle egg shells collected at Ascension Island (N = 35).

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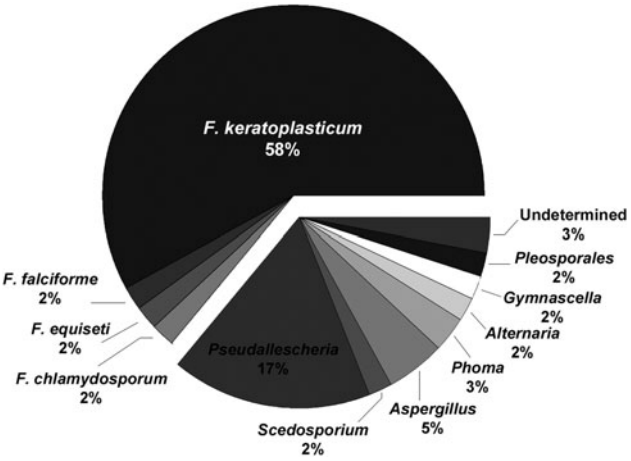

Fig. 3. Pie chart showing the isolation frequency of the fungal isolates found in eggshells of the sea turtle species *Chelonia mydas* from several nesting areas at Ascension Island.

falciforme is emerging as an agent of infections (Van Etta *et al.*, 1983; Miró *et al.*, 1994; Lau *et al.*, 1995; Noble *et al.*, 1997), thus overlapping more broadly in pathogenic potential with *Fusarium solani*. *Fusarium ketatoplasticum* and *F. falciforme* are capable of causing disease on sea turtle eggs and further studies on prevalence of both species and the potential impact of this prevalence on nesting populations are necessary.

Fungi are highly sensitive to environmental conditions and especially to changes in weather and climate, in particular temperature, humidity and wind that can directly influence their growth, spread and survival (Harvell *et al.*, 2002). Thus, the potential prevalence of *F. keratoplasticum* in Atlantic nesting areas and *F. falciforme* in those located in the Pacific might respond to the microclimatic conditions that they share. However, climate change seems to influence dispersion of fungal pathogens mainly as a consequence of current global warming (García-Solache & Casadevall, 2010). Thus, under current climate change scenarios, sea turtles could be threatened because these conditions might favour the dispersion and development of the *Fusarium* disease caused by *F. keratoplasticum* and *F. falciforme*. Further studies on environmental conditions responsible for prevalence of both species on sea turtle nesting areas and the implications of this prevalence to sea turtle conservation should be addressed.

To sum up, *F. keratoplasticum* seems to be the main pathogenic species present on sea turtle eggs in Ascension Island and in nesting regions located in the Atlantic. Moreover, *F. falciforme* is rarely found in the Atlantic and seems to be most dominant in nesting areas of the Pacific. Further studies on the source of these species and prevalence should be carried out to establish strategies directed at controlling these pathogens and their pathogenic effect on sea turtles.

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