MYCELIAL FUNGAL DIVERSITY ASSOCIATED WITH LEATHERBACK SEA TURTLE (*Dermochelys coriacea*) NESTS IN THE MAYAGÜEZ-AÑASCO BAY COAST, WESTERN PUERTO RICO

by

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ABSTRACT

Dermochelys coriacea (leatherback) is one of the four sea turtles found in Puerto Rico and has the highest nesting frequency on the island. This species is currently listed as endangered due to habitat contamination, erosion, predation, and other anthropogenic pressures. Factors that influence its reproductive success include high relative humidity in the nests, relatively low temperatures, and infection by bacteria, protozoa, and fungi. The main objective of this work was to study mycelial fungal diversity associated with leatherback sea turtle nests and eggs from Mayagüez-Añasco Bay Coast (MABC), Puerto Rico. Comparison is made of conditions (i) previous to leatherback nesting season (August-December 2008), (ii) during leatherback nesting season (February-May 2009), and (iii) during nest hatching season (May-August 2009). Prior to *D. coriacea* nesting season the fungal community along the MABC showed a normal distribution (P=0.098) by One-Way ANOVA. We found that *Aspergillus* was the most frequent genera (0.15), followed by *Cladosporium* (0.09), and *Curvularia* (0.08). At the time of oviposition we found that *Penicillium* was the most frequent isolate (0.15), followed by *Cladosporium* (0.11), *Aspergillus* (0.11), and *Fusarium* (0.07). No fungi were isolated from nesting leatherback's ovipositor. During the third sampling period fungal diversity was evaluated from the sand of hatched nests and from failed eggs. *Fusarium solani* was the most frequent isolate (0.57) from nest sand and was the only species isolated from failed eggs. Fungal abundance of sand from nests and failed eggs exhibited a strong and positive correlation (r=0.853, P=0.00000173). This was the first attempt to study fungal diversity associated with *D. coriacea*'s nest and eggs in Puerto Rico.

RESUMEN

Dermochelys coriacea (tinglar) es una de las 4 tortugas marinas que existen en Puerto Rico con mayor frecuencia de anidaje en la isla. Esta especie se encuentra actualmente en peligro de extinción debido a la contaminación de hábitat, erosión, depredación y otras presiones antropogénicas. Entre los factores que influyen en el éxito reproductivo de esta especie se encuentran alta humedad relativa en el nido, temperaturas relativamente bajas e infección por bacterias, protozoos y hongos. El objetivo principal de este trabajo fue estudiar la diversidad de hongos miceliales asociados a los nidos del tinglar en el Complejo de Bahías Mayagüez-Añasco (MABC por sus siglas en inglés), Puerto Rico (i) previo a la temporada de anidaje de la tortuga (agosto-diciembre 2008), (ii) durante la temporada de anidaje (febrero-mayo 2009) y (iii) durante la temporada de eclosiones (mayo-agosto 2009). Previo a la temporada de anidaje de *D. coriacea* la comunidad de hongos miceliales mostró tener una distribución normal (P=0.098). *Aspergillus* fue el género aislado con mayor frecuencia (0.15), seguido por *Cladosporium* (0.09) y *Curvularia* (0.08). Durante el segundo periodo de muestreo estudiamos la diversidad de hongos asociados a los nidos del tinglar al momento del desove. *Penicillium* fue el género aislado con mayor frecuencia (0.15) de las muestras de arena, seguido por *Aspergillus* (0.11), *Cladosporium* (0.11) y *Fusarium* (0.07). No se obtuvo hongos de las muestras procedentes de la cloaca de la tortuga. Durante el tercer periodo de muestreo encontramos que *Fusarium solani* fue el aislado con mayor frecuencia (0.57) de la muestras de arena y huevos, y fue el único recuperado del interior de los huevos dañados. Correlacionamos la abundancia de hongos miceliales en la arena con la aparición de huevos dañados en los nidos, donde encontramos una correlación fuerte y

positiva (r=0.853, P=0.00000173). Este trabajo representa el primer esfuerzo por estudiar la diversidad de hongo miceliales en los nidos del tinglar en Puerto Rico.

DEDICATION

To the woman that gave me wings and taught me to fly…

Ana M. Rodríguez Martínez

Because this achievement

belongs to you.

I love you Mom.

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There are several people that I would like to thank for their collaboration and guidance during my graduate research, without them this work would not have been possible. First of all I have to mention the most important people in my life: I am the most grateful to God and my family, for their unconditional love and support.

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1 INTRODUCTION

Puerto Rico's shorelines are frequently visited by four of the eight globally distributed species of sea turtles. Three of these, the hawksbill turtle (*Eretmochelys imbricata* Linnaeus), the green turtle (*Chelonia mydas* Linnaeus) and the loggerhead turtle (*Caretta caretta* Linnaeus) have hard shells. The leatherback (*Dermochelys coriacea* Vandelli), unlike the others, is characterized for the lack of a bony shell. The carapace is covered with black skin and the turtle's oily flesh. *Dermochelys coriacea* is the largest sea turtle (up to 2.5 meters long and 800 kilograms in weight). The leatherback's reproductive season extends from April to October nesting on sandy, coral free, low wave energy beaches, like those found in western Puerto Rico. This turtle is the one with the highest occurence of nesting in Puerto Rico, never the less it has been listed as an endangered species due to habitat contamination, erosion, and predation. Other factors contributing to risk include the loss of eggs due to high relative humidity in the nests, relatively low temperatures, and nest infection and colonization by microorganisms such as bacteria, protozoa, and fungi (Eckert and Eckert, 1990).

Because the leatherback is an endangered species, effective management requires an understanding of the factors that negatively affect its reproduction. Beside the present study, there are no studies that provide information about the mycelial fungi associated with leatherback's nests, nor of the influence of relative abundance on *D. coriacea* reproductive success in Puerto Rico. The main objective of this study is to determine the diversity and abundance of mycelial fungi associated with leatherback turtle nests in the beaches of western Puerto Rico, particularly along the Mayagüez-Añasco Bay Coast (MABC). Characterization of these fungi will provide key information about the biological risk factors that sea turtle's eggs are facing and could help us to define strategies for the exclusion or reduction on the number of harmful fungal species in the nests. Similar works have been completed with other endangered species such as the Puerto Rican parrot *Amazona vittata vittata* (Arroyo-Rojas, 2004).

2 LITERATURE REVIEW

Despite the fact that the leatherback sea turtle has the highest nesting frequency in Puerto Rico, no previous studies have described the diversity and abundance of mycelial fungi that could be affecting their nests. Hatching success of the turtle's eggs is limited by either direct or indirect disturbances such as human intrusion, environmental conditions, fungal and bacterial infections, and predation (Eckert and Eckert, 1990; Bjorndal, 1995). A study in Australia showed that, in fact, there are mycelial fungi that affect the productivity of sea turtle's nests. Some fungi may produce lytic enzymes that could degrade the eggshell (Phillot and Parmenter, 2001b; 2006). These researchers conclude that the fungi found in the nests come from the nutrients provided by damaged eggs. From this source, mycelial networks extended to viable eggs, covering them completely and consequently causing their loss.

Three other possible ways the mycelium might cause debilitation and developmental retardation of embryos have been proposed: 1) gas exchange impediment due to pore obstruction of the eggshell, 2) fungal spore transfer from the allantois to the embryonic tissue, and 3) calcium depletion (Solomon and Baird, 1980). Phillot and Parmenter (2001a) demonstrated that it is not necessary for the mycelium to cover the entire egg to damage it; but is enough to cover the egg's north pole, where the exchange takes place, to cause the egg loss (Phillot and Parmenter, 2001a). It has been suggested that egg ultrastructure may provide a surface for fungal development. There is evidence of fungi directly associated with leatherbacks eggs from Malaysia (Chan and Solomon, 1989), but Phillot and Parmenter (2006) concluded that the egg ultrastructure does not contribute directly to fungal infection in turtles from Australia. They found that the size of the spaces between the inorganic and organic matrices of the eggs does not

allow spore diffusion, neither the penetration of hyphae (Phillot and Parmenter, 2006). This type of data has not been studied in Puerto Rico.

Leatherback egg composition has been studied in an effort to provide more information about possible causes of egg loss. The eggshell is composed of an inner organic membrane and an outer inorganic layer of calcium carbonate deposited as aragonite (Chan and Solomon, 1989). The main component found in eggs is albumin, comprising nearly two-thirds of the egg and approximately eighty percent of the mass of every clutch (Wallace et al., 2006). Albumin has antifungal properties, similar to the mucus secreted by the turtle during oviposition (Phillot and Parmenter, 2006). Regardless, *Fusarium oxysporum*, *F. solani*, and *Pseudallescheria boydii* have been regularly isolated from the exterior of failed eggs and from embryonic tissue (Phillot et al, 2001b; 2004). These species produce enzymes that degrade the inorganic and organic components of the sea turtle eggshells and therefore are able to penetrate and colonize the egg (Phillot, 2004).

2.1 Possible Fungal Pathogens

Fusarium solani (Mart.) Saccardo

Fusarium solani is a widely distributed common soil-borne fungus comprising over 45 phylogenetic and/or biological species (Zhang et al., 2006; O'Donnell et al., 2008). This saprotroph is among the most isolated fungi from soil and responsible for serious plant diseases, infecting over 111 plant species in 87 genera (Kolattukudy & Gamble, 1995). The fungus is well-known as an opportunistic pathogen in immunocompromised humans and has also being reported causing serious infections in leatherback hatchlings and hyalohyphomycosis in

loggerhead sea turtles (Miller et al., 2009; Cabañes et al., 1997). Other *Fusarium* species have been also isolated from sea turtle nests and eggs, including *F. moliniforme* and *F. oxysporum,* which are also associated with the occurrence of bronchopneumonia in adult sea turtles (Elshafie et al., 2007; Phillot et al., 2001; Güclü et al., 2010).

Aspergillus Micheli

The genus *Aspegillus* includes very powerful mycotoxigenic fungal species. These fungi are cosmopolitan, very common in soil and marine environments. Their mycotoxigenic properties have been extensively studied for their detrimental effect on humans living of working under sick buildings conditions. Here, volatile compounds can be easily spread via ventilation systems (Tuomi et al., 2001). Members of this genus have been previously reported as plant and animal opportunistic pathogens and recently, as responsible for sea turtles egg failure (Elshafie et al., 2007). *Aspergillus* is the most common fungi reported from soil at nesting sites followed by *Fusarium* (Solomon and Bair, 1980; Eckert and Eckert, 1990; Phillot 2001; Phillot and Parmenter, 2001). Several species from this genus are known to produce potent mycotoxins such as aflatoxins (*A. flavus*), malformic C and nigragillin (*A. niger*), ochratoxin (*A. ochraceous*) and citrinin and patulin (*A. terreus*) (Elshafie et al., 2007).

Penicillium Link

Along with *Fusarium* and *Aspergillus*, *Penicillium* is another very common soil fungus. It has a widespread distribution as an organic matter decomposer, extensively studied for its economic significance in pharmacological and food industries. It is also of great relevance for the production of mycotoxins, in sick buildings syndrome, where volatile compounds produced by *Penicillium* have been found (Tuomi et al., 2001). This fungus has been isolated from sea turtle nests and eggshells and is able to produce citrinin and penicillic acid under nest conditions (Elshafie et al., 2007). *Penicillium* is one of the most common cloacal contaminants found in nesting sea turtles, occurring simultaneously with *Fusarium* and *Aspergillus* (Phillot et al., 2002). This fungus was found fossilized intact on a sea turtle clutch from the lower Cretaceous, giving us hint of the fungal-animal associations and possible ecological interactions among them (Jackson et al., 2009).

3 OBJECTIVES

Our main objective was to:

- Document the diversity of mycelial fungi associated with *Dermochelys coriacea*'s nests.

Secondary objectives include:

- Development of an effective methodology for the isolation and study of fungi associated to sea turtle nests.
- To study the relationship between fungal abundance and the potential reproductive success of the nests.

4 GENERAL MATERIALS AND METHODS

4.1 Description of research area

The study site is located near 18º16'53"N and 67º11'59"W along the Mayagüez-Añasco Bay Coast (MABC), west of Mayagüez and Añasco. The MABC is located on the west side of Puerto Rico with a total area of 100 km^2 (Alfaro, 2002). The area experiences great amount of precipitation, due to the convergence between the afternoon sea breeze and easterly winds. This results in a large annual mean precipitation of 200 to 250 cm (Morelock et al., 1983). Most rainfall occurs from April to October, being May the month of lower rainfall and a peak occurring in September.

Añasco River discharges onto the bay and produces a wide distribution of sediments. Fluctuation in annual rainfall activity results in a maximum discharge during the months of May to November. The average discharge in May is 12m^3/sec and the peak is in October with 24m^3/sec (Alfonso, 1995).

Figure 4.1 Study site location map. Mayagüez-Añasco Bay Coast, Añasco, PR. Stations from top to bottom: Tres Hermanos (TH), La Puente (LP), and Jardines del Caribe (JC). Map modified from GoogleEarth©2008.

4.2 Sampling periods

Data were collected during three periods. The first samples were taken between August to December 2008. During this period, mycelial fungal diversity along the MABC was studied to establish pre leatherback nesting conditions. Fungal diversity along MABC was determined by traditional culturing techniques.

The second sampling period extended from March to June 2009 (leatherback reproductive season). These samples established mycelial fungal diversity associated with the leatherback nests and the ovipositor of nesting females during oviposition.

The last set of samples was taken from the sand and eggs of hatched and failed nests. After incubation period, between 60-80 days, we collected two different samples from each nest: sand and failed eggs. Collection permit NUPE: OVS-PVS15-MD-00005-640309 was obtained from the Department of Natural and Environmental Resources of Puerto Rico (DNER).

5 ASSESSMENT OF THE FUNGAL COMMUNITY AT THE MABC SITES PRIOR TO LEATHERBACK NESTING SEASON

5.1 *Materials and Methods*

A total of three study plots were established using data from the last two MABC reproductive seasons of the leatherback turtles. These were provided by the DNER (Milagros Justiniano, pers. com.). Each plot was subdivided in three sub-plots, where samples (sand cores) were collected from three different parts at three different depths using a disinfected probe. Sand cores were separated in three fractions: top (T), middle (M), and bottom (B) according to its position on the nest (Figure 5.1). Individual sand fractions were placed in sterile bags (WhirlPak ®) and processed the same day when possible.

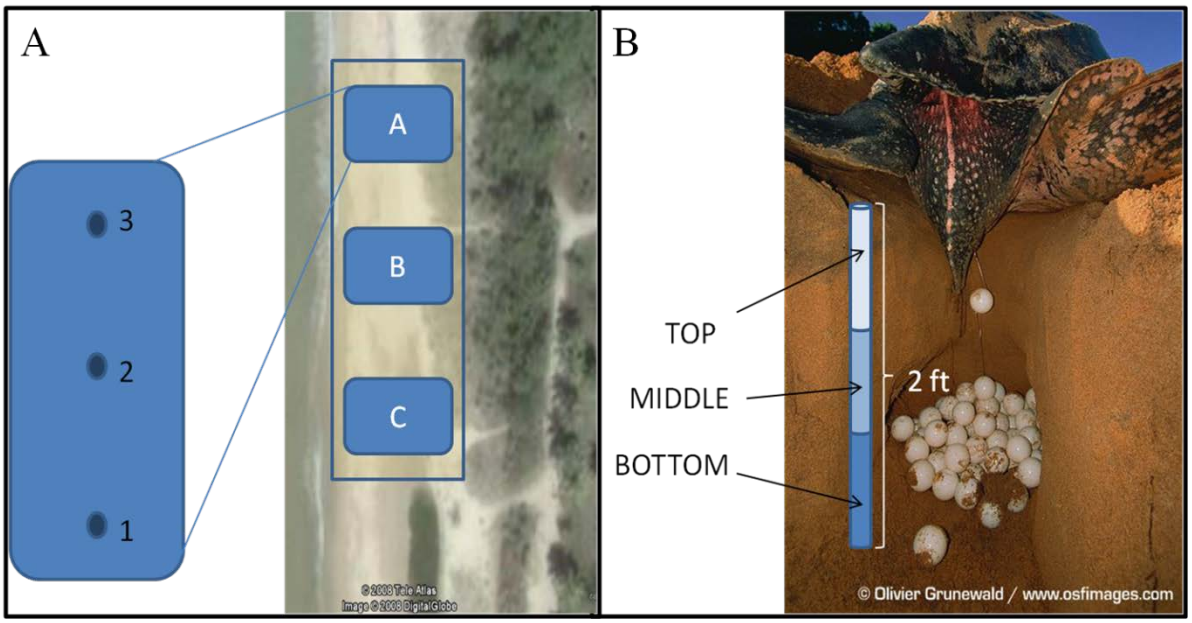

Figure 5.1 Station illustration and sand core sub-divisions. Study plot showing sub-plots and sand cores position (A). Sand core representation and layering based on leatherback's nest depth (B).

A 10^{-1} dilution of each fraction was prepared by adding ten grams of sand to ninety milliliters of sterile phosphate buffer (1X), and then agitating for ten minutes at 250 rpm. Successive extractions of 1 ml from this suspension, and then from the following, were performed until a 10^{-4} dilution was obtained (Davet and Rouxel, 2000). The remaining volume of suspension (99mL) was filtered through a 0.45µm nitrocellulose membrane (Millipore, Billerica, MA) and individual membranes were stored at -20°C for DNA extraction.

5.1.1 Isolation of filamentous fungi from samples

A volume of 0.5 ml from each dilution was spread on the surface of solidified agar medium in the Petri dishes. The culture media used included Glucose-Yeast Agar (GYA) and Marine Agar (MA) with 100µg/ml chloramphenichol to inhibit bacterial growth. Plates were incubated at 25ºC ± 2ºC in duplicate for a period of 5 to 30 days. Pure cultures were obtained from individual colonies using different selective culture media. Fungi were characterized based on morphological features to the level of species when possible.

5.1.2 Statistical analysis

Statistical analysis of the data collected was performed by One-Way ANOVA using SigmaStat 3.5 software. Plots were analyzed first independently and then as single plot. Shannon's and Simpson's diversity indexes were calculated using BioDiversity Professional (version 2) software (McAleece, 1997). To study the fungal diversity similarity among plots a Bray-Curtis Cluster Analysis was performed.

5.2 *Results*

A total of 85 morphotypes were isolated from sand previous to leatherback nesting season (Table 5.1). The most common genera isolated were *Aspergillus*, *Cladosporium* and *Curvularia* with relative frequencies of 0.15, 0.09 and 0.08 (Figures 5.2 and 5.3), respectively followed by *Penicillium* (0.06), *Trichoderma* (0.06) and *Fusarium* (0.05). There were no differences in fungal diversity among sand cores between plots (P=0.563) and the fungal population showed a normal distribution (P=0.098). Shannon's diversity values were 0.689, 0.852 and 0.806 for Caño La Puente, Tres Hermanos and Jardines del Caribe transects, respectively. Simpson's diversity values were 0.384 for Caño La Puente, 0.176 for Tres Hermanos and 0.341 for Jardines del Caribe. Bray-Curtis Cluster Analysis showed that Tres Hemanos and Caño La Puente transects had the highest similarity in fungal population (35% between them) (Figure 5.4).

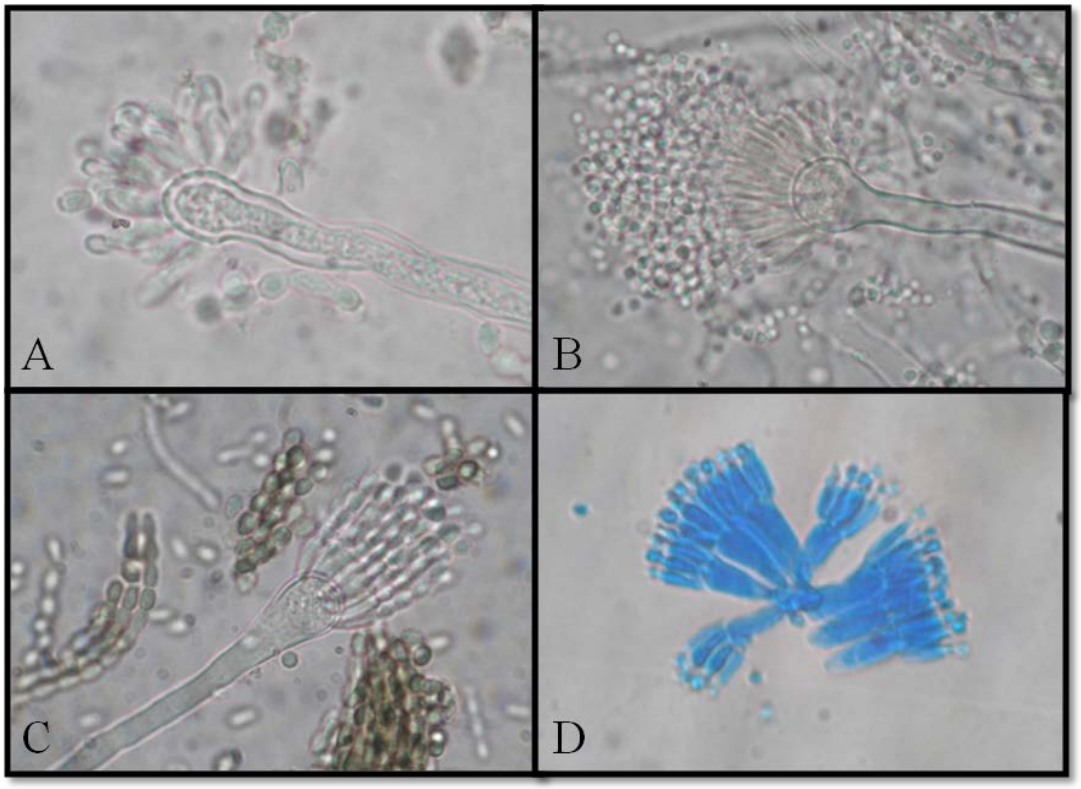

Figure 5.2 Light microscopy of *Aspergillus penicillioides* (A), *A. terreus* (B), *A. restrictus* (C) and metulae and phialides of *A. ochraceus*(D) isolated during the first sampling period.

Table 5.1 Mycelial fungi isolated from sand previous to leatherback nesting season at MABC.

Genus	Number of isolates	Genus	Number of isolates
Aspergillus		*Cladosporium*	8
A. alliaceus	1	*Curvularia*	7
A. ochraceus	1	*Fusarium*	4
A. penicillioides	8	*Gloeosporium*	2
A. restrictus	1	*Penicillium*	5
A. terreus	2	*Pestalotia*	1
Aposphaeria	1	*Phoma/Phillosticta*	1
Cephalosporium	2	*Sphaeropsis*	1
Chaetomella	1	*Trichoderma*	5
Chaetomium cancroideum	1	Other fungi	6
Rhizoctonia	1	Unidentified isolates	26

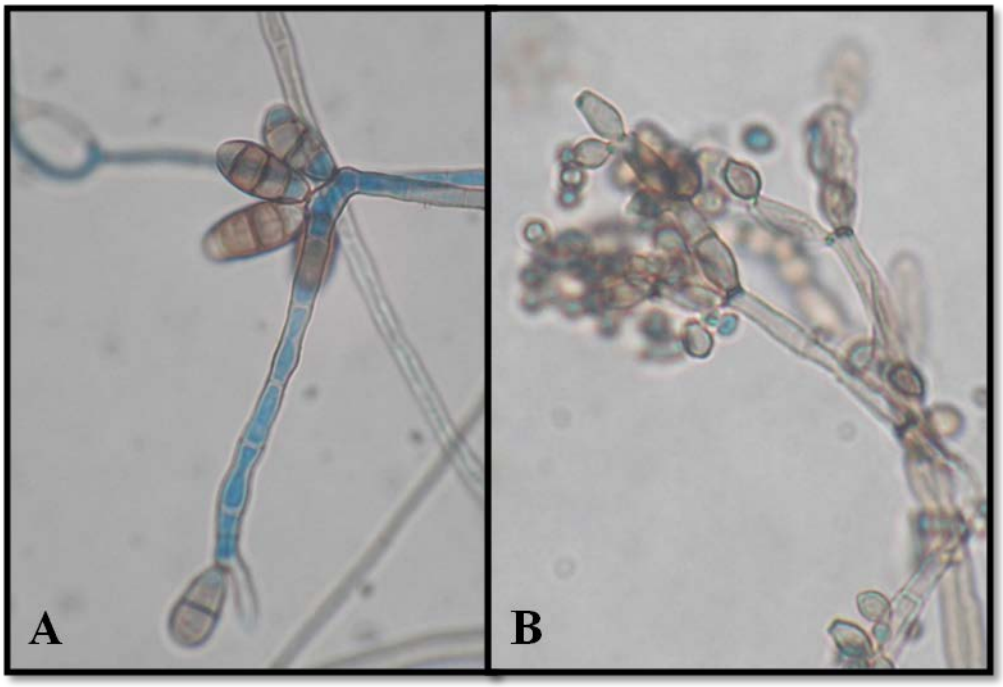

Figure 5.3 Light microscopy of conidiophores and conidia of *Curvularia lunata* (A) and conidiophores and conidia of *Cladosporium* sp. (B).

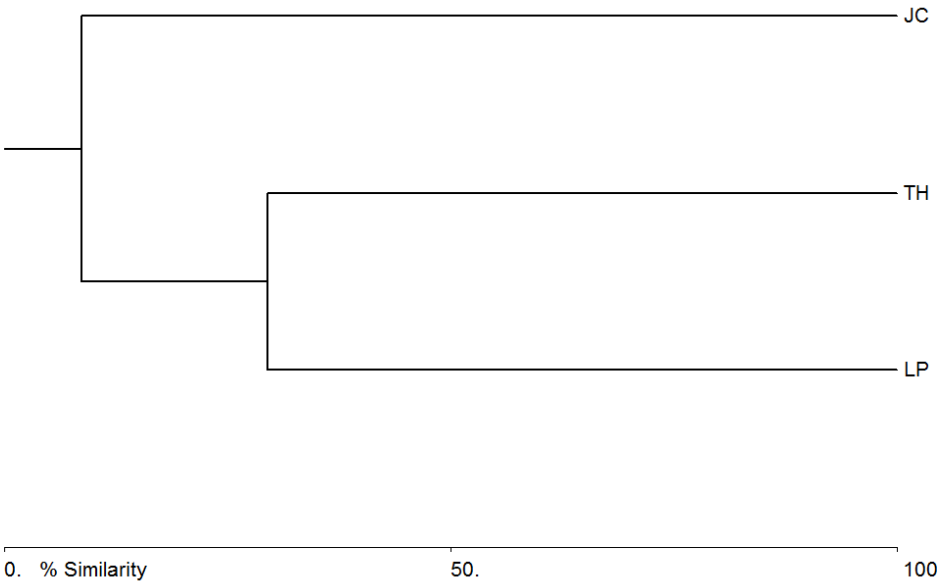

Figure 5.4 Bray-Curtis Cluster Analysis of fungal diversity from study sites at the MABC. Jardines del Caribe, Mayagüez PR (JC), Tres Hermanos beach (TH), and Caño La Puente (LP), Añasco PR.

5.2.1 Some fungal isolates of special interest

Aspergillus terreus Thom

This species is characterized for producing pale brown, fast growing colonies (60.0mm) in seven days cultures on Czapek Yeast Agar (CYA) at 25˚C. Colonies on Czapek Yeast 20% Sucrose Agar (CY20S) and Malt Extract Agar (MEA) were >40 mm and 50.3 mm in diameter respectively. Production of yellowish-brown exudate was observed on CYA cultures. Pale brown soluble pigment was observed on MEA, CYA and CY20S media. Smooth walled conidiophores averaged 100.0 µm long and vesicles were spherical, 12.5 µm wide. Aspergilla biseriate, metulae tightly packed over the upper tree quarters of the vesicle, 7.0 × 2.5 µm; phialides acerose 5.0 × 1.5 µm. Conidia were smooth-walled, spherical; 2.0 µm in diameter (Figure 5.5). Globose to ovoid, hyaline aleuriconidia were also produced.

This species has been commonly isolated from soils in tropical regions from cultivated soils and forests (Klich, 2002). Among the mycotoxins produced by this species are: patulin (genotoxic and antibiotic) (Hopmans, 1997; Bennett and Klich, 2003), citrinin (nephrotoxic) (Bennett and Klich, 2003), citreoviridin (neurotoxic and specific inhibitor of the mitocondrial adenosine triphosphatase) (Ueno and Ueno, 1972; Linnett et al., 1978), and gliotoxin (immunosuppressive) (Pahl et al., 1996).

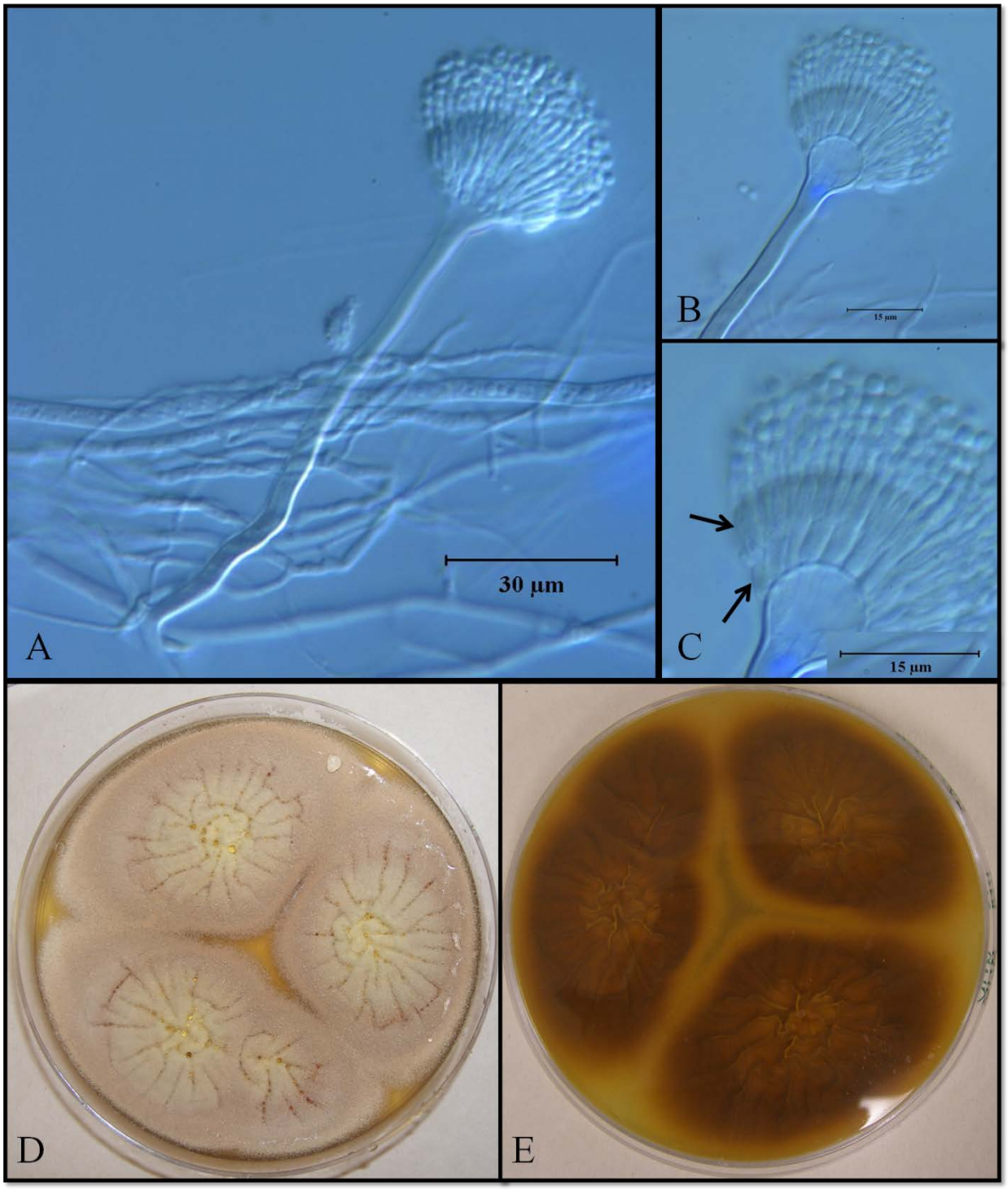

Figure 5.5 Nomarski microscopy of *Aspergillus terreus* showing short conidiophore (A), spherical vesicle (B), and tightly packed metulae and phialides in the upper tree quarters part of the vesicle (C). Seven day cultures on CYA 25˚C showing exudates (D), and brown reverse and yellowish-brown soluble pigment (E).

Aspergillus restrictus Smith

This species is characterized for producing slow growing colonies (<1 cm) on seven day cultures on MEA and CYA. Optimal growth was obtained in Malt 40% Sucrose Yeast Agar (M40Y) for which this fungus is characterized as highly osmophilic. Colonies were 40 mm in diameter on fourteen days cultures on M40Y 25˚C. Colonies were grey-green, reverse uncolored. Smooth-walled, short conidiophores averaged 90.5 μm in length and vesicles were hemispherical, 10.0 μm wide. Aspergilla uniseriate, phialides restricted to the upper quarter of the vesicle, 7.2 × 3.0 μm. Conidial heads columnar. Conidia were rough-walled, ovoid to cylindrical; 3.9 × 2.6 μm (Figure 5.6).

This species has been isolated in high frequency from desert soils with low water activity (Klich, 2002; Itabashi et al., 2006). *Aspergillus restrictus* can produce cristatin A (immunosuppressive) and a cytotoxic ribotoxin, resctictocin, which inhibits protein synthesis in eukaryotes (Yang and Moffat, 1996; Itabashi et al., 2006). Other proteins have been recently isolated from these xerophilic fungi including arestrictins A and B, with unknown properties at the moment (Itabashi et al., 2006).

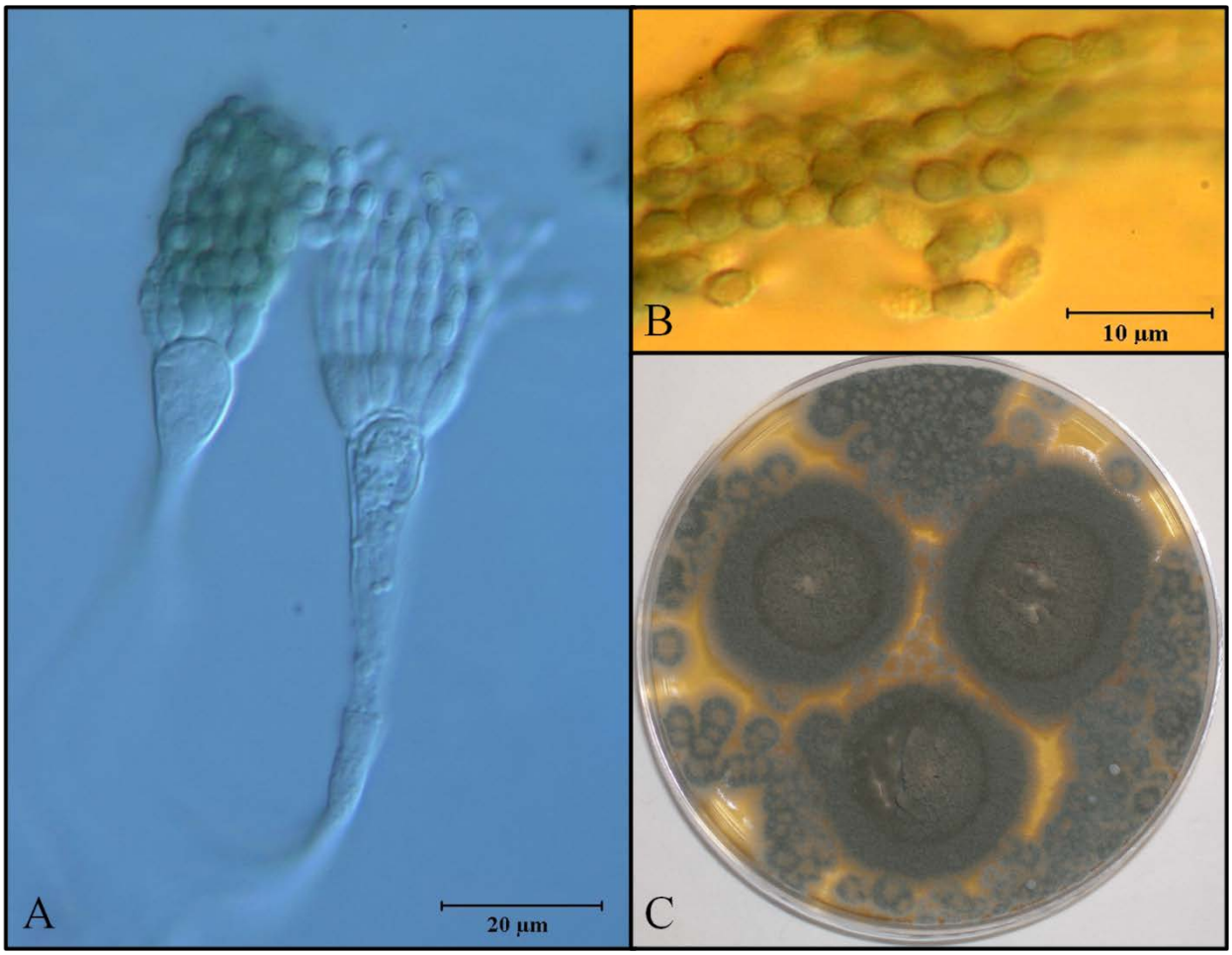

Figure 5.6 Nomarski microscopy of *Aspergillus restrictus* showing short conidiophores with pyriform vesicles and phialides, conidial heads columnar, restricted to the upper quarter of the vesicle (A). Rough-walled, ellipsoid to cylindrical conidia in chains (B), and fourteen days cultures on M40Y at 25˚C (C).

Aspergillus penicillioides Spegazzini

This species is characterized for producing slow growing colonies (0-4 mm) on seven day cultures on MEA and CYA, where no sporulation occurred. Optimal growth was obtained in CY20S for which this species is characterized as highly osmophilic. Colonies were 4.0-7.0 mm in diameter on seven days cultures on CY20S at 25˚C. Colonies were green, reverse uncolored. Smooth-walled, short conidiophores averaged 100.0 µm in length and vesicles were hemispherical, 10 µm wide. Aspergilla uniseriate, phialides covering upper two to three quarters of the vesicle, 7.0 × 2.5 µm. Conidial heads radiate. Conidia were rough-walled, ovoid to cylindrical; 4.0 × 3.0 µm (Figure 5.7).

This species is xerophilic and reported mostly from desert soils and other dried habitats such as dried food and human skin (Klich, 2002; Tamura et al., 1999). Novel compounds such as arestictins A and B, isolated from *A. restrictus*, have been also isolated from *A. penicillioides* (Itabashi et al., 2006).

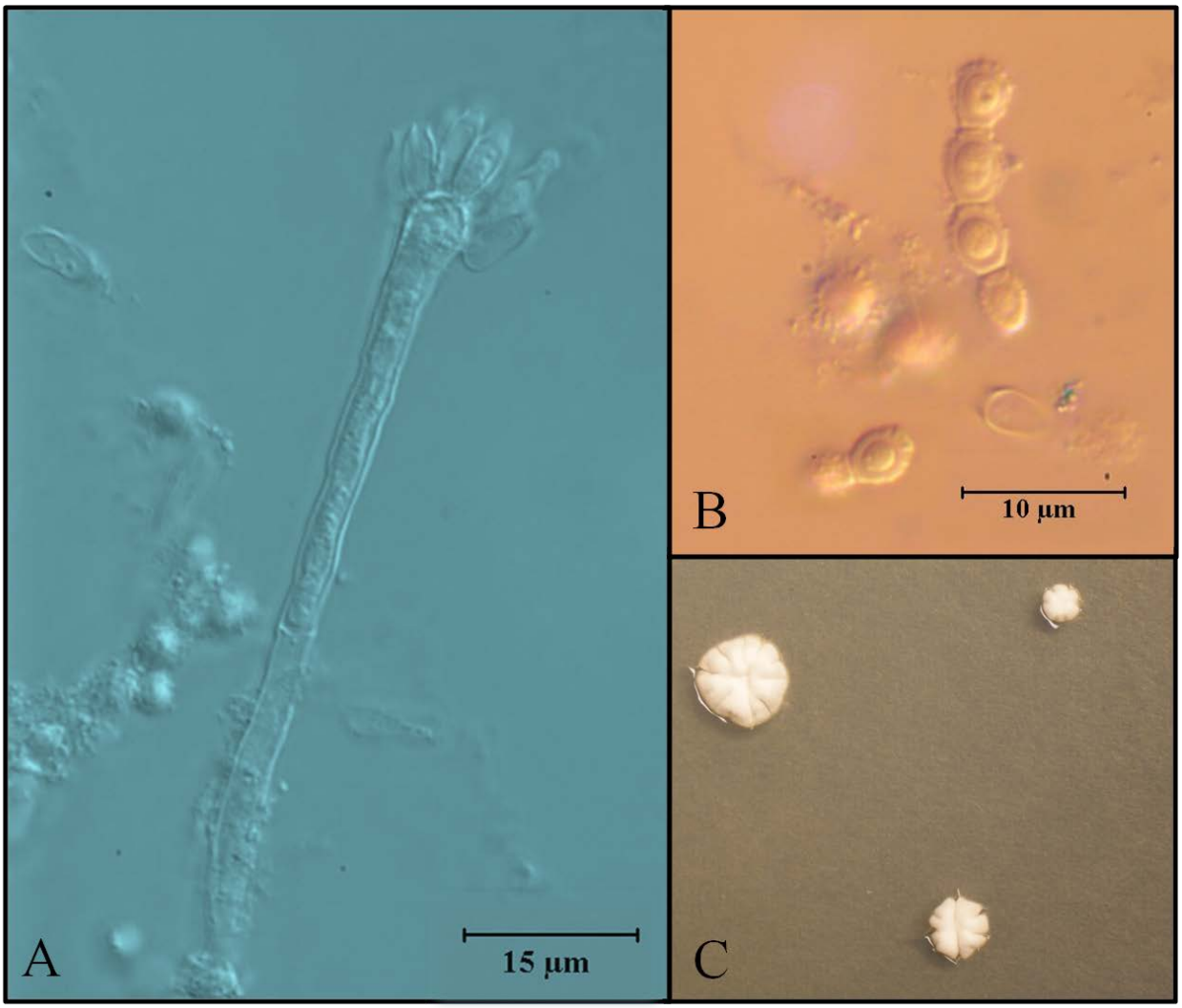

Figure 5.7 Nomarski microscopy of *Aspergillus penicillioides* showing conidiophores with phialides covering more than the half of the vesicle (A), and rough-walled, ellipsoid conidia (B). Seven days cultures on CYA showing slow growing colonies (C).

Unidentified species M84

This dematiaceous species grows as olive-brown colonies, 15.0 mm in diameter on fourteen days PDA cultures at 25˚C, reverse dark brown. Exudates or soluble pigments were not observed. Conidiophores distinct, polyblastic, up to 56.0 µm in length, composed of pyriform cells (variable in number) 8.0 × 4.0 µm showing broad septa between them. Conidia sub-spherical 4.0 × 3.0 µm, unicellular, produced as blastoconidia in acropetal arrangement (Figure 5.8).

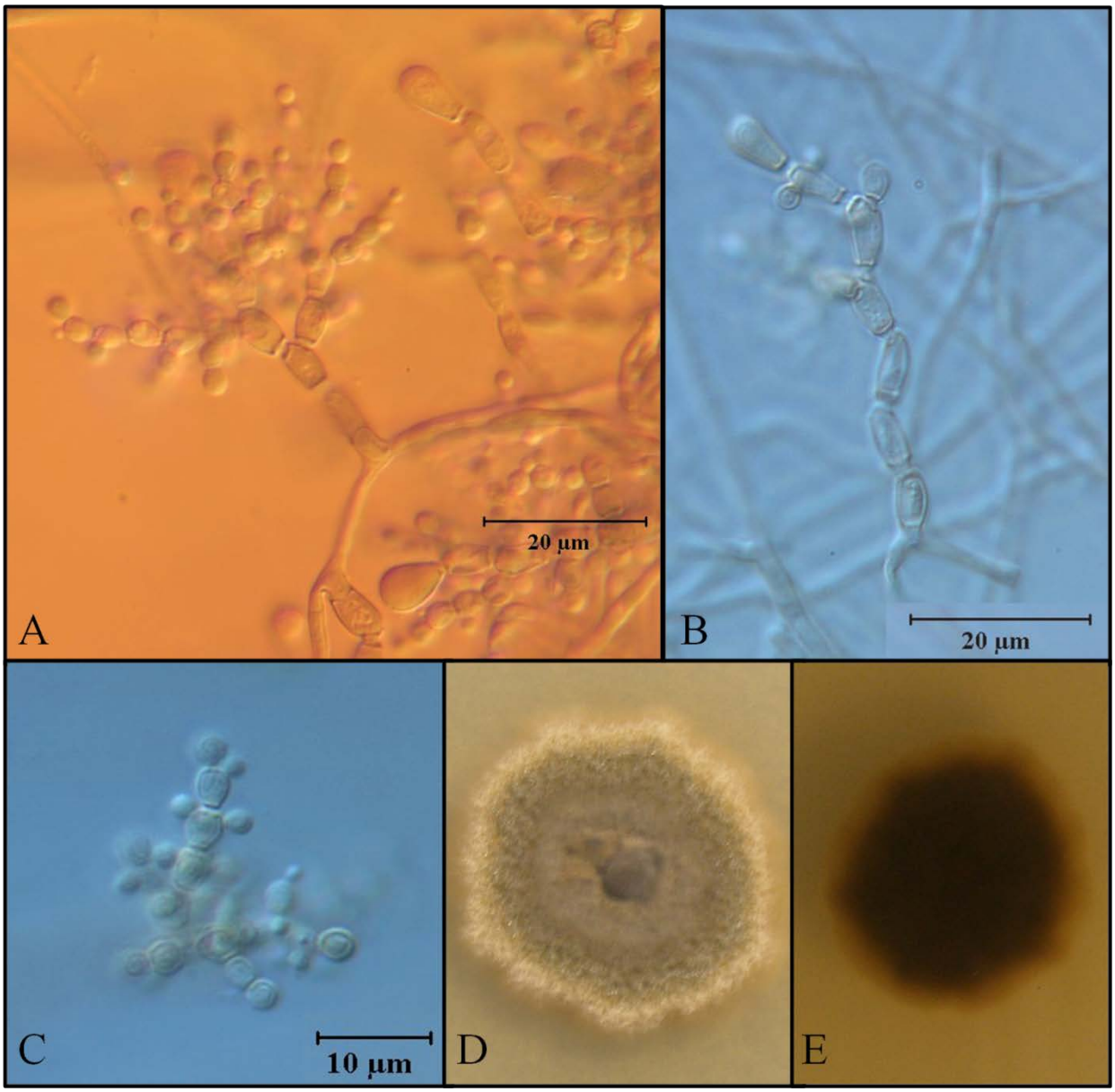

Figure 5.8 Nomarski microscopy of M84 showing polyblastic conidiophores (A), pyryform cells of the conidiophores with broad septa (B), and blastoconidia in acropetal arrangement (C). Fourteen days culture on PDA at 25˚C (D), and dark brown colony reverse (E).

5.3 *Discussion*

A total of 85 mycelial fungi representing at least 15 identified genera and unidentified isolates were recovered from MABC sand prior to the nesting season. Most of the fungi we identified are common in soil, saprophytic or common plant pathogens. The most abundant genus was *Aspergillus* with 13 isolates distributed in 5 species (Table 1). The presence of these fungi represents a threat to nesting leatherback females since it has been previously reported causing loss of turtle eggs during incubation (Elshafie et al., 2007; Solomon and Bair, 1980; Eckert and Eckert, 1990; Phillot 2001; Phillot and Parmenter, 2001). *Penicillium* (0.06) and *Fusarium* (0.05) are of special interest as well for their potential as egg pathogens. *Penicillium* is well known for its mycotoxigenic properties, which could be detrimental for eggs under incubating conditions. *Fusarium* has been previously reported causing egg and hatchling mass mortality in nests (Phillot, 2004; Phillot et al., 2004; Phillot and Parmenter, 2001b; Sarmiento et al., 2010). All the other fungi were isolated in lower frequencies (0.01) during sampling. This can be explained in terms that sand is not a nutrient-rich source limiting the diversity of fungi that can colonize the substrate.

Based on Shannon's Diversity Index (H') and Simpson's Diversity (D), Tres Hermanos transect showed the highest mycelial fungal diversity (H'= 0.852 and D= 0.176) while Caño La Puente showed the lowest diversity with H'= 0.689 and D= 0.384. Similarity among transect's fungal diversity determined by Bray-Curtis Cluster Analysis showed that Caño La Puente and Tres Hermanos transects had a low similarity of 35%, while these two were 8% similar to Jardines del Caribe. These differences in fungal diversity might be explained in terms of the geographical barrier that the coastal currents that distribute freshwater and sediments north and south of the Río Grande de Añasco river mouth may represent, which separates Jardines del

Caribe transect from the other two. Furthermore, Tres Hermanos and Caño La Puente have a higher human impact than Jardines del Caribe since the first two are located at the Tres Hermanos public beach.

6 ASSESSMENT OF FUNGAL COMMUNITY ASSOCIATED WITH LEATHERBACK NESTS DURING NESTING SEASON

6.1 Materials and Methods

During this period, samples were taken at the time of oviposition from 16 nesting females. Once the turtle finished digging the nest, a sample of sand from the bottom was collected on a Whirlpak ™ using a sterile spoon. A sample from the ovipositor was collected using sterile transport swabs. Sand samples were processed as previously described in section 5.1. Swabs containing ovipositor samples were streaked over solidified Marine Agar (MA) plates and incubated for 5 to 30 days at 25˚C ± 2˚C.

6.2 Results

A total of 27 morphotypes were isolated during this period. No fungi were isolated from turtles' ovipositor. Fungi identified include *Penicillium* (0.15), *Cladosporium* (0.11), *Aspergillus* (0.11), and *Fusarium* (0.07). Identification of all of the other 15 morphotypes was not possible by traditional culturing techniques since no reproductive structures were produced.

Table 6.1 Mycelial fungi isolated from sand collected from *D. coriacea*'s nests during oviposition.

Genus	Number of isolates
Penicillium	4
Cladosporium	3
Aspergillus	3
Fusarium	2
Unidentified	15

6.3 Discussion

Organisms identified during this period were previously recorded from the preliminary assessment, except for *A. niger*. Most of the isolates could not be identified since no reproductive structures were produced. No fungi were isolated from cloacal samples. This can be explained in terms of the antifungal properties of the mucose secreted by nesting females during oviposition as previously documented by Phillot and Parmenter (2006). These results suggest that fungi associated with egg loss may then be from the established MABC fungal community. Our results differ from those of Phillot et al. (2002) were nesting and inter-nesting turtles had a great occurrence and diversity of cloacal fungi (*Acremonium, Aspergillus, Chrysosporium, Fusarium, Mucor, Penicillium, Phialophora, Sporothrix,* and *Stachybotrys*).

7 ASSESMENT OF MABC MYCELIAL FUNGI ASSOCIATED WITH LEATHERBACK HATCHED NEST AND FAILED EGGS

7.1 Materials and Methods

After incubation period, nests were opened and a sample of sand associated to the eggs was collected from the bottom on a Whirlpak ™ using a sterile spoon. Hatched and failed eggs were counted in order to determine de hatching success of the nests. Failed eggs were collected in sterile bags and taken to the laboratory for processing following the dilution method previously described in section 5.1.

7.1.1 Isolation of filamentous fungi from sand samples

A volume of 0.5 ml from each dilution was spread on the surface of solidified agar medium in Petri dishes. Culture media used were the Glucose-Yeast Agar (GYA) and Marine Agar (MA) with 100µg/ml chloramphenichol to inhibit bacterial growth. Plates were incubated at 25ºC ± 2ºC in duplicate for a period of 5 to 30 days. Pure cultures were obtained from individual colonies using different selective culture media. Fungi were characterized based on morphological features to the species level, when possible.

7.1.2 Isolation of filamentous fungi from surface and interior of the eggs

Eggs were surface-washed with 100mL of sterile phosphate buffer and agitated for 15 minutes in order to remove any fungal propagule attached to it. Dilutions from the wash solution were performed from 10^{-1} to 10^{-4} and 100µL of each dilution and stock solution were spread over solidified MA medium on 50mm Petri plates in duplicate.

After the surface-wash protocol, eggs were superficially disinfected by submerging each one on hydrogen peroxide (H_2O_2) for 1 minute, then in 95% ethanol for 1 minute and followed by two washes in sterile distilled water for 1 and 5 minutes, respectively. This method was developed taking under consideration H_2O_2 and ethanol disinfecting success on hen eggs (Shane and Faust, 1996). Eggs were then cut open carefully with a sterile scalpel. Eggs content were separated based on thickness, resulting in two subsamples: soft content (SC) and thick content (TC). A 10^{-1} dilution of each sample was prepared by adding 10mL of the sample to 90mL of sterile phosphate buffer and agitating for 10 minutes. Successive extractions of 1mL from this suspension and then from the following were performed until 10^{-4} dilutions were obtained. A volume of 100µL from each dilution was spread over solidified MA medium on 50mm Petri plates in duplicate. All plates were incubated at 25˚C ± 2˚C for 7 to 14 days.

7.1.3 DNA extraction, PCR amplification and sequencing of ITS region from pure cultures

For molecular characterization, DNA extraction was performed by growing the fungi on Potato Dextrose Broth on 1.5 mL tubes for seven days at 25˚C. Micelium was then removed from tubes and processed with the Fast DNA® Spin Kit for Soil (MP Biomedicals, USA). DNA fragments containing internal transcribed spacers ITS1 and ITS2, including 5.8S, were amplified and sequenced with primer pair ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). An initial cycle of denaturalization at 95°C for 3 minutes was followed by 35 cycles of denaturalization at 95°C for 30 seconds, annealing at 56˚C for 1 minute, and extension at 72˚C for 2:30 minutes. A final step of extension at 72˚C for 10 minutes was done. Samples were sent for sequencing to High-Throughput Genomics Unit (HTGU), Department of Genome Sciences, University of Washington. Sequences were edited with

Sequencher 4.10 (©Gene Codes Corporation). Nucleotide BLASTN searches were used to compare sequenced obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases.

7.1.4 Total DNA extraction from sand and egg samples

Total DNA extraction from sand samples was performed to study the fungal diversity in the nests by culture independent techniques. A sand wash solution was prepared by adding 300g of sand sample to 300mL of sterile phosphate buffer and agitated on a shaker at least for 12 hours. After agitation period, the solution allowed to settle at the bottom of the flask to avoid excess particulate in the suspension. The suspension was filtered through 0.45µm nitrocellulose membranes (Millipore, Billerica, MA). Membranes containing microorganisms were stored at -20˚C until DNA extraction was performed using the FastDNA Spin Kit for Soil® with modifications (Apendix A). DNA extractions were documented in 0.8% agarose gel after staining with ethidium bromide (10µg/ml). Total DNA extraction from failed eggs content was performed with the protocol described by Couto (2009).

7.1.5 PCR Amplification of fungal ITS from environmental DNA samples

PCR reactions were performed with Green Master Mix (Promega Corp., Madison, WI) and fungal primers ITS1 and ITS4 according to manufacturer's specifications following protocol on section 7.1.3. PCR products were documented in 2% agarose gel after staining with ethidium bromide (10µg/ml). These products were purified with the MinElute Gel Extraction Kit (Qiagen Inc., USA) according to manufacturer's protocol. PCR products were stored at -20˚C until clone libraries were constructed.

7.1.6 Construction of PCR amplified ITS region clone libraries

PCR products were ligated into the pGEM®-T cloning vector and transformed into *E. coli* JM109 high efficiency competent cells as described by the manufacturer (Promega Corp., Madison, WI). Transformants were recovered in Luria-Bertani (LB) agar plates supplemented with ampicillin (100µg/ml). Transformants were analyzed by colony PCR to confirm the presence of the cloned fragments. Primers used for the PCR amplifications were T7 promoter and SP6 promoter (Promega Corp., Madison,WI), which targets the plasmid vector's multiple cloning site. PCR products were documented in 1.5% agarose gel after staining with ethidium bromide (10µg/ml). After confirmation of amplicons of expected size (~650 pb), restriction fragment length polymorphism (RFLP) community analysis was performed.

7.1.7 Restriction fragment length polymorphism community analysis

RFLP profiles were constructed following the protocol described by Viaud et al. (2000) modified using 400ng of sample DNA. The endonuclease utilized was Hinf I (New England BioLabs) following manufacturer's recommendations. Restriction reactions were performed in a termocycler at 37°C for 1 hour followed by 80°C for 2 minutes to allow inactivation of the enzyme. Electroforesis of the total volume of the reactions were done in 3% agarose gel for 12 hours at 20V and verified after staining with ethidium bromide (10µg/ml). Samples with different RFLP patterns were selected for sequencing analysis.

7.1.8 Phylogenetic analysis of Fusarium solani isolates from leatherback failed eggs

The program BioEdit version 7.0.9.0 (Hall, 1999) was used for alignment of the ITS sequences of the *Fusarium solani* isolates from leatherback failed eggs and selected sequences obtained from the NCBI nucleotide databases. For the external group, a *Fusarium staphyleae* (AF178423) was selected based on a previous phylogenetic analysis of the genus *Fusarium* (O'Donnell, 2000). The program MEGA 5 (Tamura et al., 2011) was used for phylogenetic analysis. We applied Neighbor-Joining analysis following the heuristic search and bootstrap support with 2,000 replications.

7.2 Results

A total of 39 morphotypes were isolated from sand of hatched nests and failed eggs of *D. coriacea* at MABC. Most of the fungi isolated were identified as *Fusarium* (0.57), *Aspergillus* (0.13), *Penicillium* (0.10), *Cladosporium* (0.08), and *Scedosporium* (0.05) genera. All five genera were isolated from sand samples and from the surface of failed eggs. Only *Fusarium solani* was isolated from the interior of failed eggs. Correlation between sand fungal colony forming units (CFU's) and failed eggs in the nest was determined by Pearson Product-Moment Correlation Coefficient. We found a strong and positive correlation (r=0.853, P=0.00000173) between fungal CFU's and the number of failed eggs in the nest (Figure 7.1).

Twenty one failed eggs of *D. coriacea* were collected from hatched and failed nests after incubation period (for nest locations see Table 7.1). Fungal structures were observed developing on the surface of most of these eggs. Dark spots, suggesting the presence of fungi, were observed in the outer inorganic layer and on the inner organic layer of some of the eggshells analyzed (Figure 7.2). Debilitation of the calcareous layer of infected eggs was also observed. Mycelial fungi were isolated from 95% of the eggs sampled. Egg content was separated based on thickness, resulting in two subsamples: soft content (SC) and thick content (TC). Mycelial fungi were isolated from the interior of 52% of the eggs analyzed. All the eggs had mycelium their surface. Fungi were isolated from 48% of soft content and 43% of thick content of the eggs (Table 7.2). *Fusarium solani* was the only fungi isolated from these samples (Figure 7.3). Thirty-eight percent of the failed eggs had fungi associated to their surface but no fungi were isolated from the interior. Eggs with no fungal structures their surfaces were free of fungi in the interior as well.

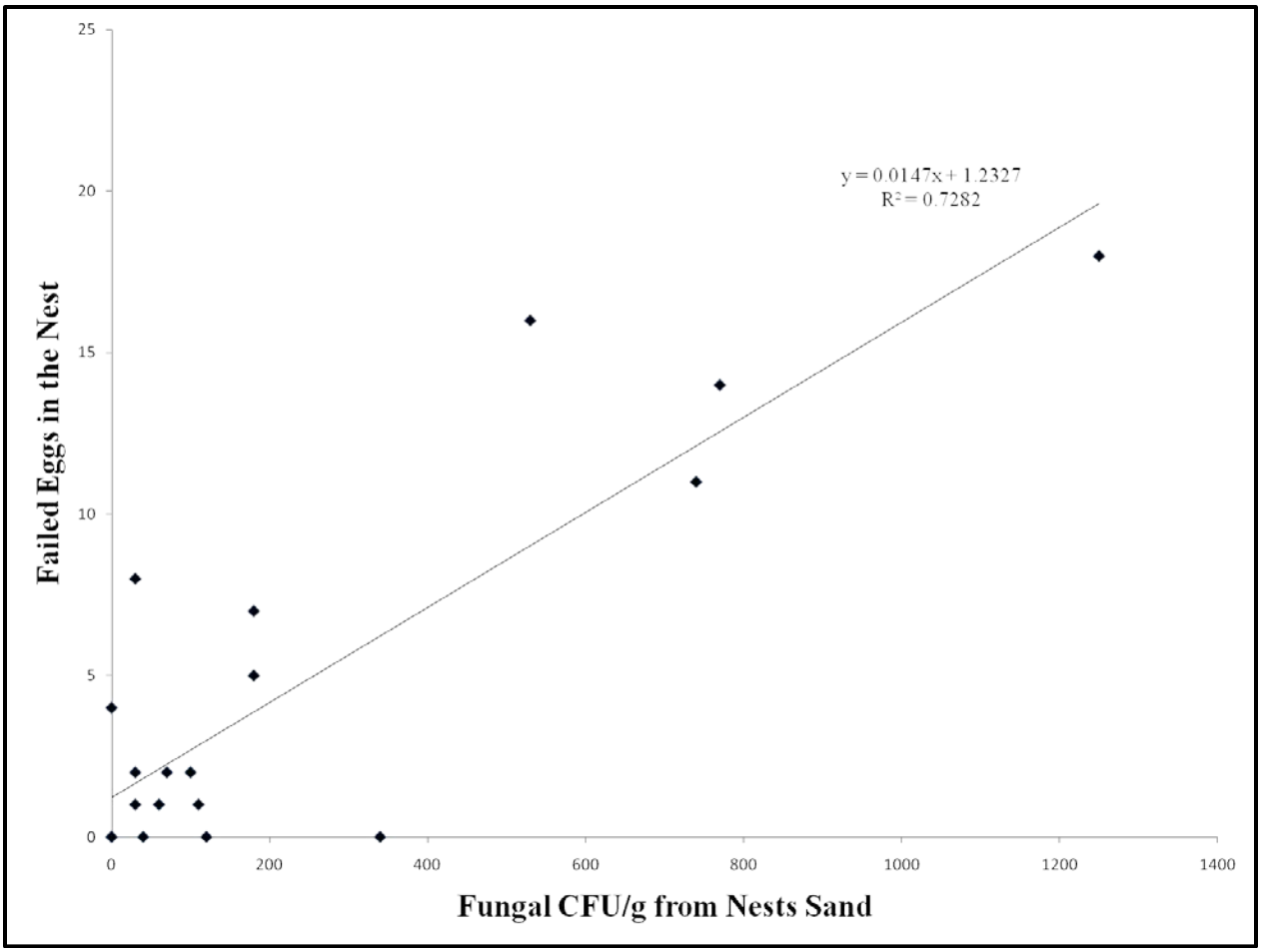

Figure 7.1 Correlation plot for fungal CFU's of sand from nests and the occurrence of failed eggs in the nests.

Table 7.1 MABC Locations of hatched nest sampled.

Nest Code	Location	Coordinates
ETH-SM	At Samaris, Tres Hermanos	18°16'50"N - 67°11'23"W
ELP-AG	At Gangui access, Caño La Puente	18°16'22"N - 67°11'23"W
ELP-AR	At the arch, Caño La Puente	18°16'34"N - 67°11'23"W
ELP-CC	Close to camp, Caño La Puente	18°16'47"N - 67°11'22"W
ELP-CR	At the round house, Caño La Puente	18°16'40"N - 67°11'21"W
ELP-CV	At the summer house, Caño La Puente	18°16'29"N - 67°11'21"W
ELP-LC	At the pond, Caño La Puente	18°16'07"N - 67°11'22"W
ELP-LP	At La Puente, Caño La Puente	18°16'10"N - 67°11'23"W
ELP-PN	At the pines, Caño La Puente	18°16'24"N - 67°11'23"W
ELP-RG	At Rancho Grande, Caño La Puente	18°16'36"N - 67°11'23"W
ELP-YU	At El Yukayeke, Caño La Puente	18°16'31"N - 67°11'23"W
ETH-PS	At La Pescadería, Tres Hermanos	18°16'58"N - 67°11'26"W

Table 7.2 Fungal counting from sand samples of nests along with the species isolated and hatching success.

Nest Code	CFU/g	Species	Hatching Success
ELP-AR	60	*Cladosporium* sp.	98%
ELP-T4217R	740	*Fusarium solani*	83%
ELP-AGII	40	*Aspergillus ochraceus, Aspergillus sclerotiorum*	99%
ELP-AGI	770	*Fusarium solani*	18%
ETH-PS	720	*Fusarium solani*, *Penicillium* sp., *Cladosporium* sp.	25%
ELP-PN	30	*Trichoderma longibrachiatum,* Unidentified M131, Unidentified M130	63%
ELP-YU	1250	*Scedosporium aurantiacum, Fusarium solani*	80%
ELP-LP	110	*Cladosporium* sp.	89%
ELP-LCII	180	*Penicillium islandicum, Aspergillus ochraceus,* Unidentified M143	91%
ELP-YUII	100	*Fusarium solani*, *Cladosporium* sp.	89%
ELP-LC	180	*Aspergillus* sp, *Fusarium solani*	88%
ELP-AGIII	30	*Fusarium solani*, *Cladosporium* sp.	88%
ELP-RG	530	*Fusarium solani*	70%
ELP-CR	70	*Fusarium solani*	94%
ELP-CC	30	*Cladosporium* sp.	99%
ELP-CV	340	*Aspergillus niveus, Fusarium solani*	96%
ETH-SM	0	-	98%
ELP-RGII	0	-	91%
ELP-PNII	120	Unidentified M158	99%
ELP-LPII	0	-	67%
ELP-LCIII	0	-	91%

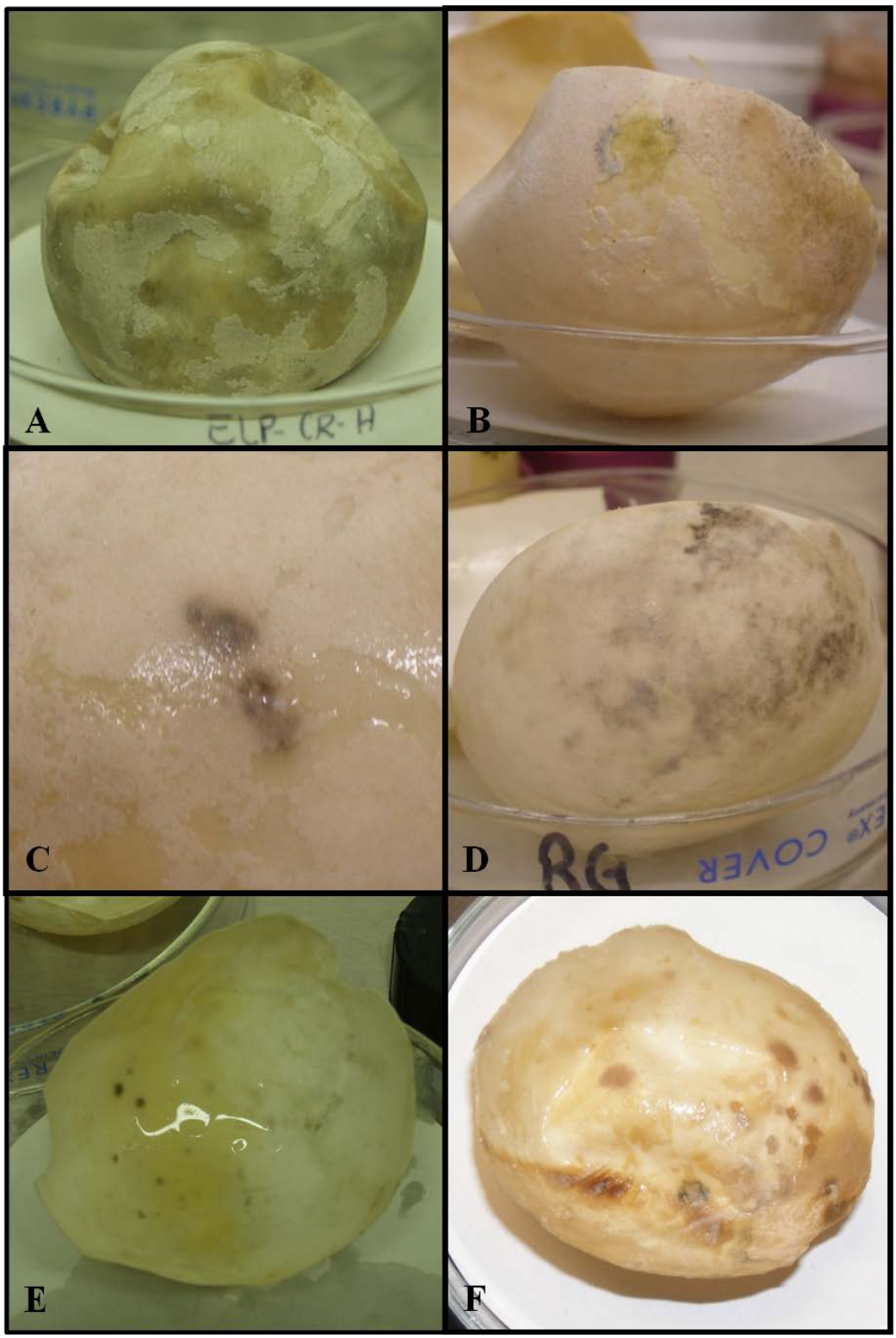

Figure 7.2 *Dermochelys coriacea* eggshells from fail eggs showing dark spots associated to fungal infection. Deterioration of outer inorganic layer of ELP-CR-H (A) and ELP-PN-H (B). Fungal spots on the outer inorganic layer of ELP-YUII-H (C) and ELP-RG-H (D). Fungal spots on the inner organic layer of ELP-CV-H (E) and ELP-LP-H eggshells (F).

Table 7.3 Summary of fungal infection on failed eggs of *D. coriacea* from hatched nests from MABC. (+) Indicates presence of fungi, while (-) indicates absence of fungi.

Egg Code	Egg Surface	SC	TC
ETH-SM-H	+	-	+
ELP-AG-H	ND	+	+
ELP-AGIII-H	+	-	-
ELP-AR-H	ND	-	-
ELP-CC-H	-	-	-
ELP-CR-H	+	-	-
ELP-CV-H	+	+	+
ELP-LC-H	+	+	+
ELP-LCII-H	+	-	-
ELP-LCIII-H	+	-	-
ELP-LP-H	+	+	+
ELP-LPII-H	+	-	-
ELP-PN-H	+	-	-
ELP-PNII-H	+	+	-
ELP-RG-H	+	+	+
ELP-RGII-H	+	-	-
ELP-YU-H	+	+	-
ELP-YUII-H	+	-	-
EYU-T4217R-H	ND	+	+
ETH-PS-H1	ND	+	+
ETH-PS-H2	ND	+	+

ND=No Data

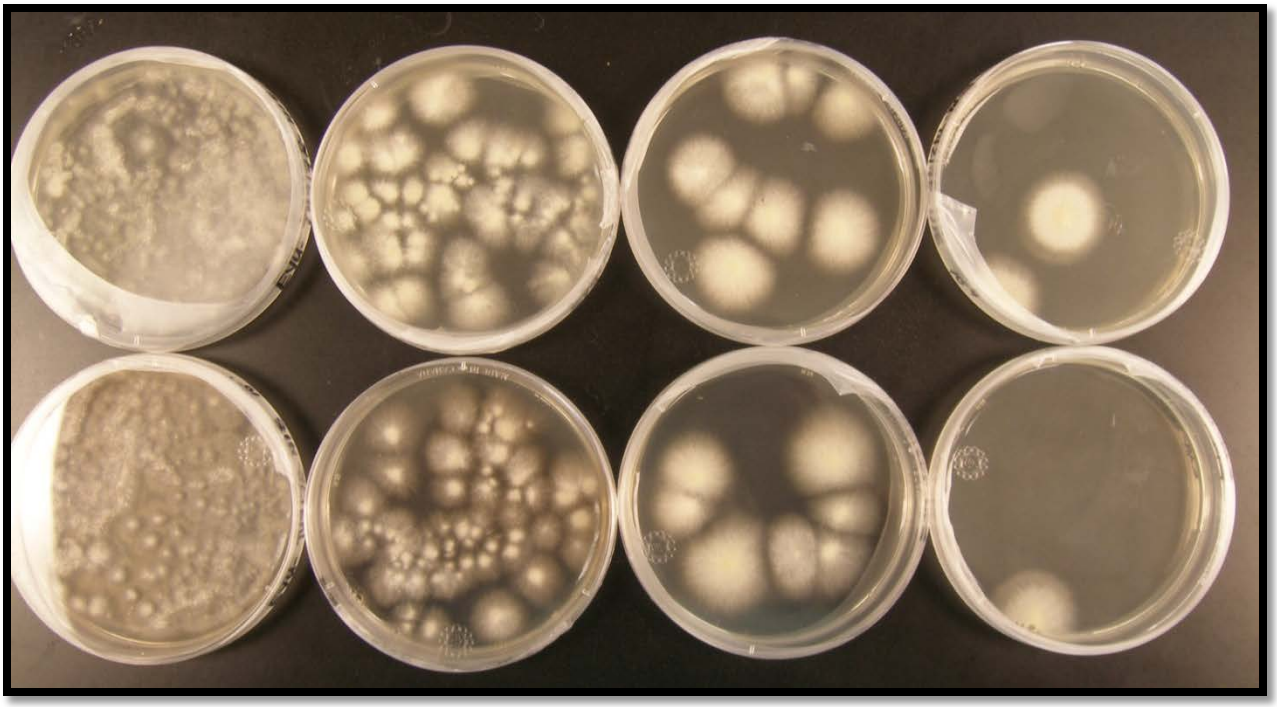

Figure 7.3 Serial dilution (10^{-1} to 10^{-4} from left to right) of EYU-T4217R-H thick content in Marine Agar after 5 days incubation at 25°C. All colonies belong to *Fusarium solani* (isolate M126).

7.2.1 Description of fungal isolates from sand and failed eggs

Aspergillus ochraceus K. Wilh.

These isolates were characterized for producing cream-buff, fast growing colonies (63.0mm) in seven days cultures on CY20S medium at 25˚C. Colonies on CYA and MEA were 50.6 mm and 63.3 mm in diameter respectively. Production of exudate was not observed. Soluble ochraceus pigment was observed on CYA and CY20S media. Rough walled conidiophores averaged 360.0 µm long and vesicles were round, 30.5 µm in diameter. Aspergilla biseriate, metulae covering the entire surface of the vesicle, 11.0 × 5.0 µm; phialides 7.0 × 3.0 µm. Conidia were smooth-walled, spherical; 2.0 µm in diameter (Figure 7.4).

This species have been commonly reported from desert soils (Klich, 2002). Major mycotoxins produced by this fungus include: penicillic acid (carcinogenic), ochratoxin A (nephrotoxic) (Bennett and Klich, 2003), xanthomeginin (uncoupler of oxidative phosphorylation) (Stack and Mislivec, 1978), vioellein and vioxantin (nephro- and hepatotoxic) (Kocic-Tanackov et al., 2010).

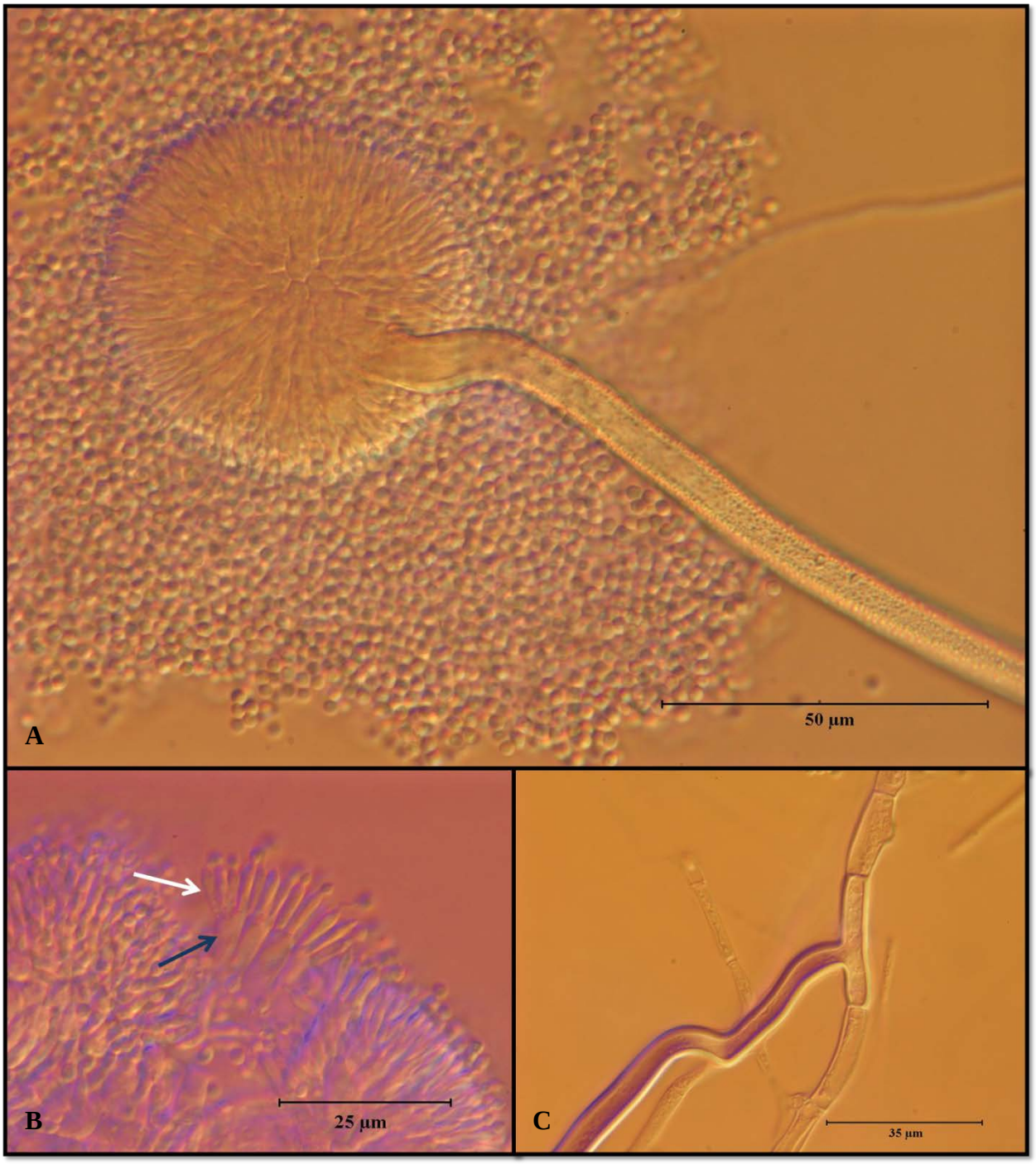

Figure 7.4 Nomarski microscopy of *Aspergillus ochraceous* showing rough-walled stalks, biseriate aspergilla and smooth spherical conidia (A), metulae (blue arrow) and phialides (white arrow) (B), and "T" shaped basal foot (C).

Scedosporium aurantiacum Gilgado, Cano, Gené, et Guarro

These isolates were characterized for the production of fast growing, dense cottony colonies on PDA at 25˚C seven days cultures. Mycelia brownish-gray at the center of the colony, and whitish, slightly fimbriate at the margins. Reverse was brownish-gray at the center and colorless to the periphery. Soluble yellowish pigment was not observed. Conidiogenous cells cylindrical, growing laterally from undifferentiated mycelium or branched forming verticils of two to three cells. *Graphium* synanamorph (conidiogenous cells arranged as synnemata) was not observed. Two conidial types were observed: (i) from solitary conidiophores, sub-hyaline, sub-ovoid to sub-cylindrical and (ii) from short lateral protrusions, solitary, brown, smooth and thick-walled, mostly ovoid to ellipsoid (Figure 7.5).

Scedosporium aurantiacum belongs to the *Pseudallescheria boydii* species complex (Gilgado et al., 2005; Gilgado et al, 2008). This species complex is characterized for being opportunistic pathogens, particularly in immunocompromised individuals, and the mortality rate is extremely high (Alastruey-Izquierdo et al., 2007; Rainer et al., 2000; Gilgado et al., 2006). Most of the isolates of *S. aurantiacum* come from clinical samples (Gilgado et al., 2005).

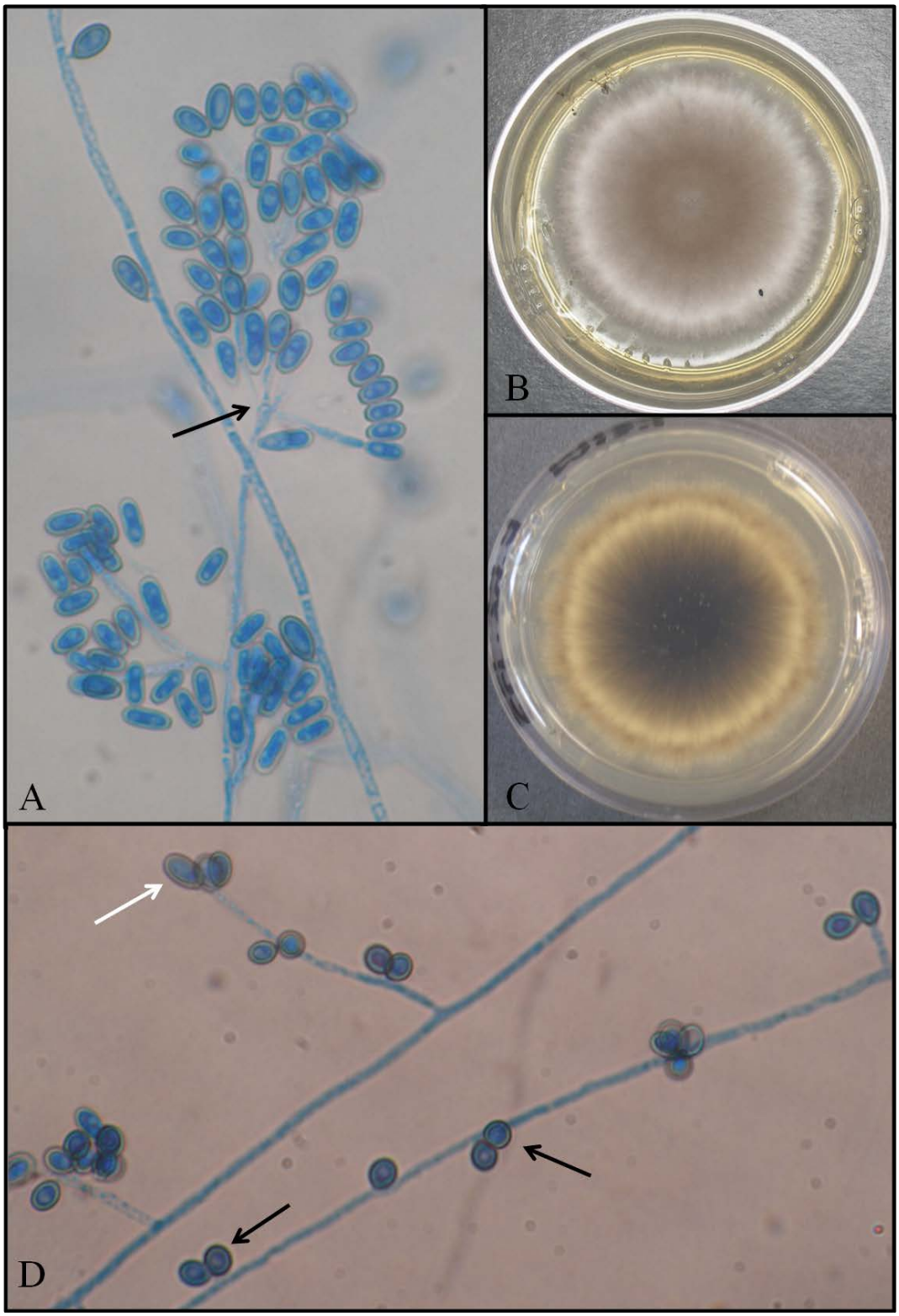

Figure 7.5 Conidiophores of *Scedosporium aurantiacum* showing verticils of conidiogenous cells (arrow) and sub-hyaline ovoid to sub-cylindrical conidia (A). Seven day culture on PDA (B-C). Conidia produced laterally from undifferentiated hyphae (black arrow) and from solitary conidiophores (white arrow) (D).

Aspergillus candidus Link

These isolates were characterized by lightly sulcate, white to cream colonies on CYA (32.5 mm), MEA (30.5 mm), CY20S (13.5 mm) and M40Y (30.0 mm) at 25˚C on seven days cultures. Colonies reverse were pale brown on all media and soluble amber pigment was observed solely on CYA. Isolates were able to growth at 37˚C (23.0 mm). Yellowish-brown exudate was produced on CYA only. Aspergilla biseriate, metulae 6.0 × 2.5 µm, radiate on spherical vesicles. Phialides 5.0 × 2.0 µm. Stipes were smooth-walled, uncolored, 250.0 × 10.0 µm. Conidia spherical, 2.0 µm in diameter, smooth-walled (Figure 7.6).

This species occurs predominantly on tropical and subtropical regions and has been reported from dung, fruits, various foodstuff, indoor environments and soils (Klich, 2002). Isolates of *A. candidus* are able to produce a variety of metabolic compounds, including terphenyl and terprenins which exhibits immunomodulating capabilities and are highly cytotoxic (Takahashi et al., 1976; Varga et al., 2007). These capabilities have also been found in isolates from marine environments (Wei et al., 2007).

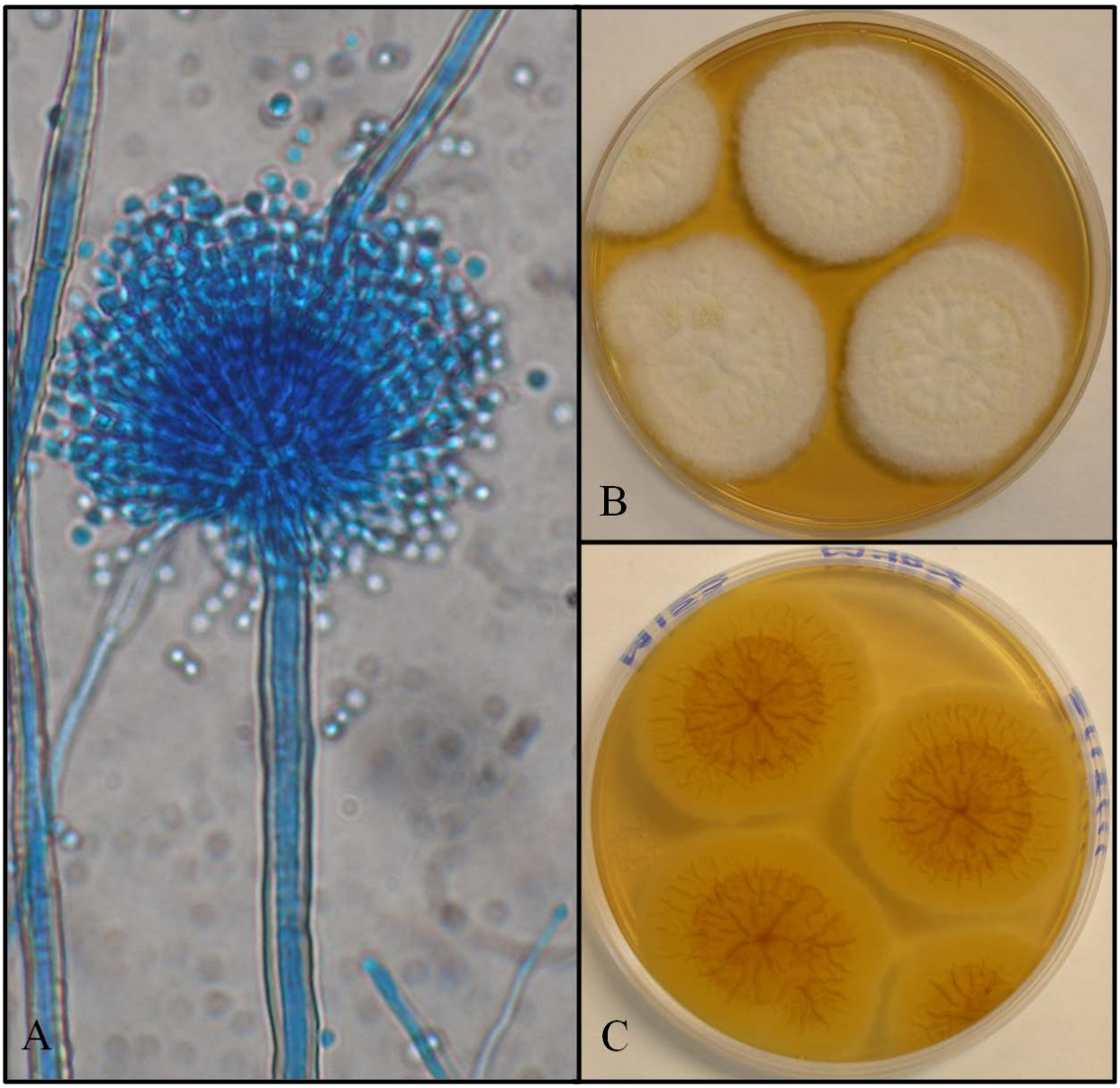

Figure 7.6 Biseriate conidophores and conidia of *Aspergillus candidus*. Seven day cultures on M40Y at 25˚C showing whitish-cream, lightly sulcate colonies (B-C).

Fusarium solani (Mart.) Saccardo

Colonies on PDA growing rapidly, with white to cream-colored aerial mycelium, reverse brown. Bluish exudates were observed when sporodochia were present. Conidiophores arise laterally from aerial hyphae. Macroconidia were abundant on Carnation Leaf Agar (CLA) produced on shorter, branched conidiophores, moderately curved, with short, blunt apical and indistinctly pedicellate basal cells, mostly four-celled, 28-42 × 4-6 μm, occasionally six-celled. Microconidia were abundant, produced on elongate, sometimes verticillate conidiophores, 8-16 × 2.0-4.5 μm. Chlamydospores were frequent, singly or in pairs, terminal or intercalary, mostly rough-walled, 6-10 μm diameter (Figure 7.7).

This fungus belongs to the *Fusarium solani* species complex (FSSC), which comprises plant and animal pathogenic fungi (O'Donnell et al., 2008). Among the mycotoxins produced by this species is cyclosporin A, an immunosuppressive compound that may influence its pathogenic potential, and the novel cytotoxic compound neo-*N*-methylsansalvamide (Sugiura et al., 1999; Song et al., 2010).

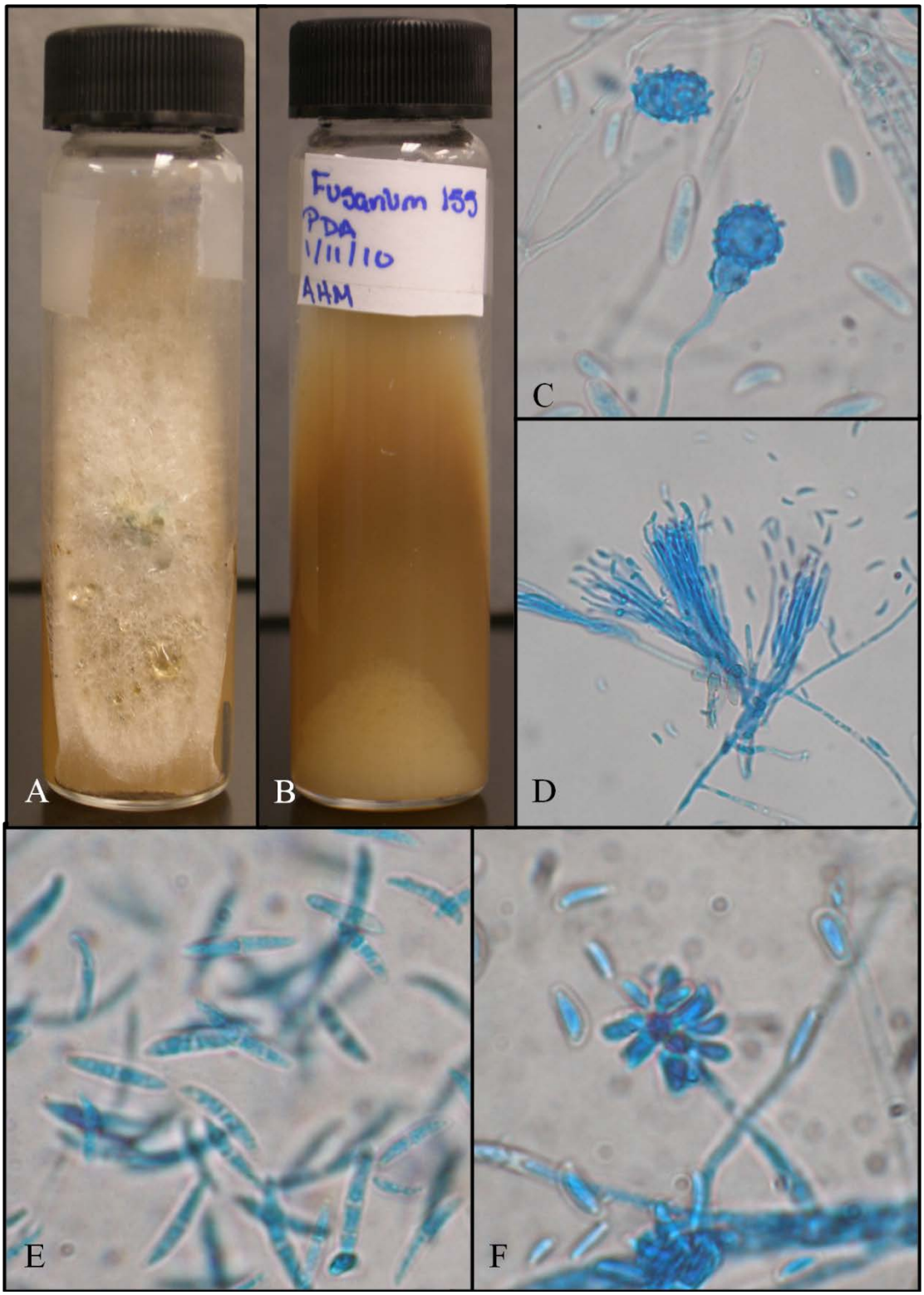

Figure 7.7 Seven days cultures of *Fusarium solani* on homemade PDA at 25˚C (A-B). Microscopic features of *F. solani* showing rough-walled, terminal chlamidospores (C), sporodochia (D), macroconidia from CLA seven days cultures (E), and microconidia on monophialides (F).

7.2.2 Molecular analysis of selected cultures

After morphological characterization of fungi isolated from sand and egg samples, isolates that were morphologically distinct were selected for molecular characterization. DNA extractions were performed with the Fast DNA® Spin Kit for Soil and PCR amplification was performed using ITS1 and ITS4 primers. Figure 7.8 demonstrates the quality of genomic DNA from selected isolates. Species identity confirmation by BLASTN searches is showed on Table 7.3.

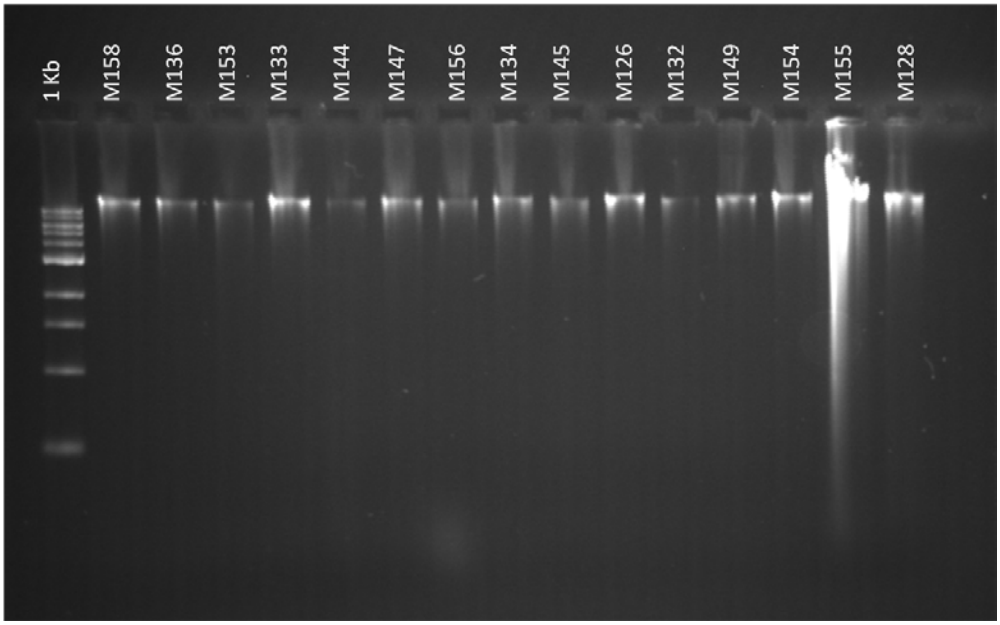

Figure 7.8 Genomic DNA extraction of fungal isolates from sand and eggs of hatched and failed nests of *D. coriacea* from MABC beach showing high concentration yields.

Table 7.4 Molecular characterization of fungal isolates from sand and eggs of leatherback sea turtle from MABC.

Isolate	Source**	BLASTN*		
		Species	Max. Id.	GenBank Accession Number†
M31	S	*Aspergillus restrictus*	99%	AY373864.1
M38	S	*Aspergillus terreus*	100%	GQ461911.1
M39	S	*Aspergillus penicillioides*	99%	AY373862.1
M59	S	*Cladosporium sphaerospermum*	100%	HQ263345.1
M60	S	*Penicillium sumatrense*	99%	HM469404.1
M65	S	*Gibberella fujikuroi*	95%	FJ755697.1
M68	S	*Fusarium equiseti*	100%	HQ380774.1
M71	S	*Trichoderma longibrachiatum*	99%	HQ717793.1
M83	S	*Acremonium* sp.	92%	GU973795.1
M84	S	Unidentified	95%	AY251086.1
M85	S	Unidentified	96%	HQ125832.1
M95	S	*Penicillium commune*	100%	HM366606.1
M116	S	Hypocreales	99%	GU017508.1
M128	S	*Aspergillus ochraceus*	98%	DQ336712.1
M136	ES	*Sedosporium aurantiacum*	100%	AJ888439.1
M139	ES	*Cladosporium sphaerospermum*	99%	AM176735.1
M141	S	*Cladosporium sphaerospermum*	96%	DQ093662.1
M145	S	*Aspergillus* sp.	98%	EF661405.1
M146	EI	*Nectria haematococca*	99%	GU066713.1
M154	EI	*Fusarium solani*	100%	FR691775.1
M155	EI	*Nectria haematococca*	99%	GU066713.1
M157	EI	*Fusarium solani*	100%	JF323002.1

* BLAST option of the NCBI nucleotide database

** S, sand; ES, egg surface; EI, egg interior

† GenBank accession numbers of the isolates with maximum identity to the studied isolate

7.2.3 Phylogenetic analysis of Fusarium solani isolates from leatherback failed eggs

In the resulting phylogram from the Neighbor-Joining phylogenetic analysis tree mayor clades can be distinguished. The first one is composed only by *Fusarium oxysporum*, which is considered as member of the *Fusarium solani* Species Complex (FSSC). The second clade contains other *Fusarium* species that have been recently segregated of the FSSC. The third and larger clade contains all *F. solani* included in the analysis. In the *F. solani* clade two sub-clades (A and B) were labeled. Subclade A contains isolates from animals and plants and our isolates from failed eggs, subclade B comprises isolates from *Solanum tuberosum*.

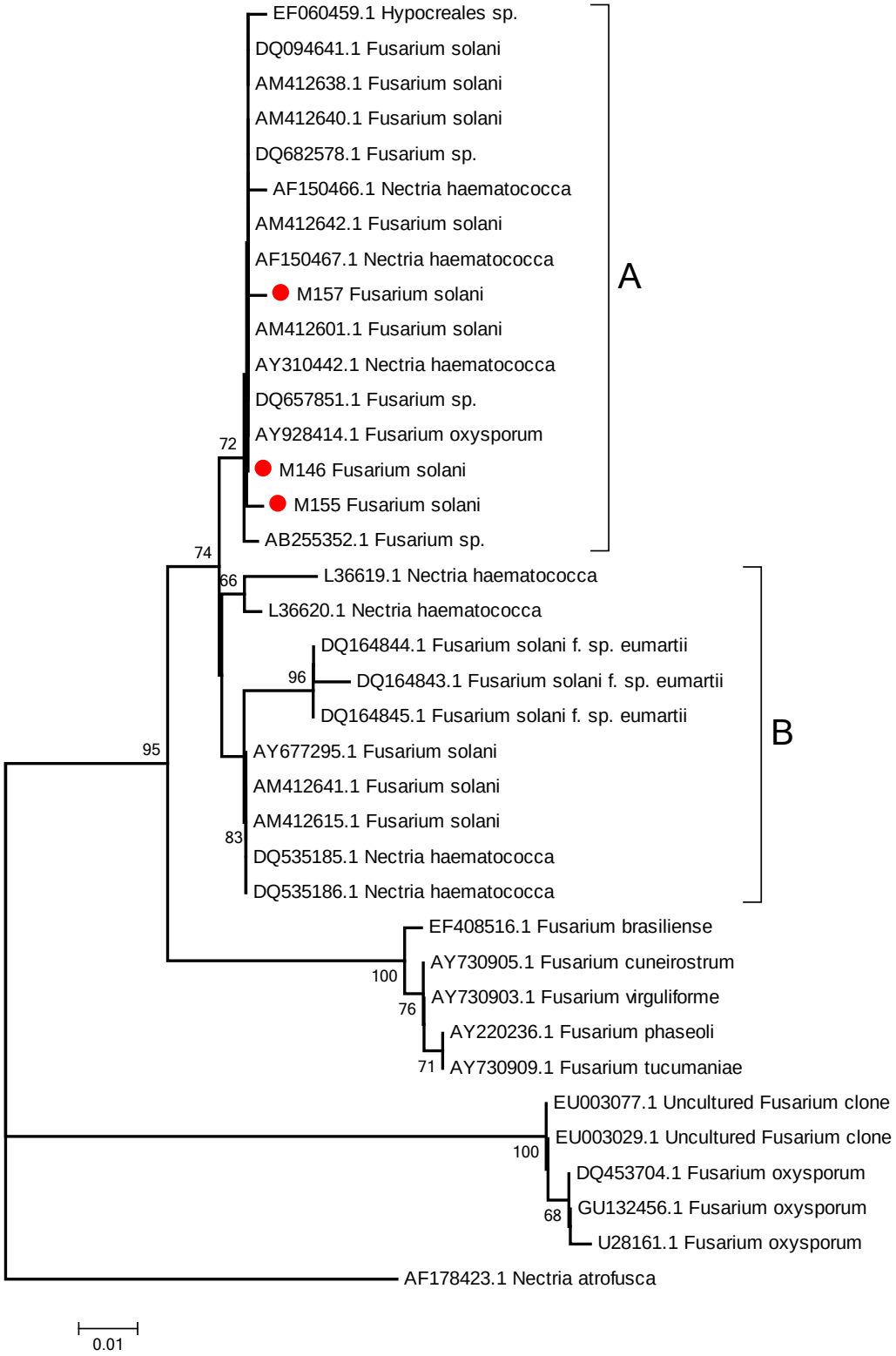

Figure 7.9 Neighbor-Joining out-group-rooted phylogram inferred from ITS gene sequences of *Fusarium* spp. Numbers placed on branches represent bootstrap values >50%. Our *Fusarium solani* isolates from leatherback failed eggs are labeled with the red marks.

7.2.4 Construction of PCR amplified ITS region clone libraries

DNA was successfully extracted from membranes containing microorganisms (Figure 7.9). A concentration curve was performed to determine the DNA concentration from environmental extractions required for PCR amplification of fungal ITS region. We determined that the optimum DNA concentration required from our samples for successful amplification was 90.0 ng (Figure 7.10). PCR products showed 4 different amplified fragments of 1,000 bp, 750 bp, ~650 bp, and ~550 bp (Figure 7.11). The desired fragments of ~550 bp were purified by gel extraction and then cloned as previously described on section 7.1.6. Colony PCR from clones showed amplicons of expected size (~650-700 bp) (Figure 7.12). RFLP was successful with the modification to the Viaud et al. (2000) protocol. Figure 7.13 shows the different restriction profiles from randomly selected clones.

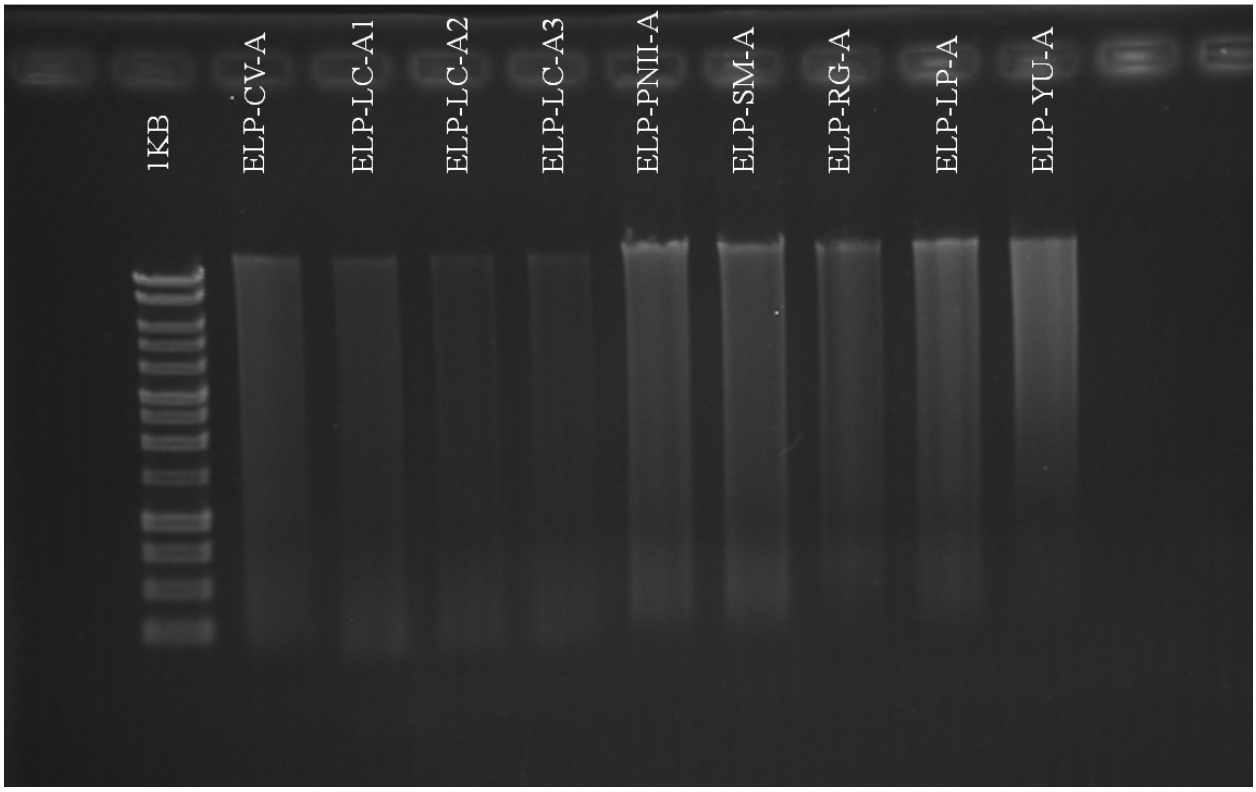

Figure 7.10 DNA extractions from membranes containing microorganisms from hatched nests sand samples from MABC.

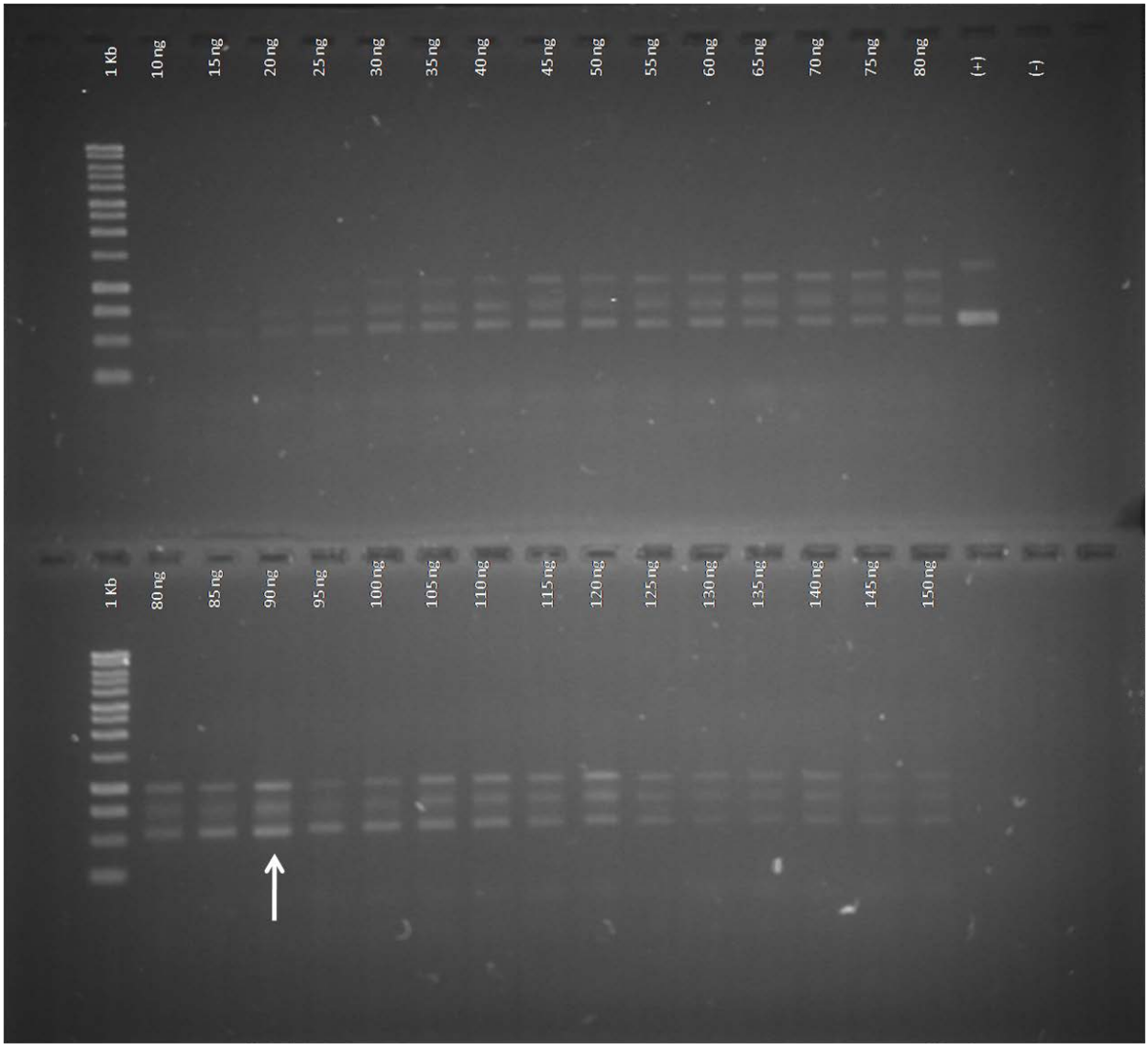

Figure 7.11 DNA concentration curve from environmental extractions demonstrating the optimum concentration required for PCR amplification (arrow).

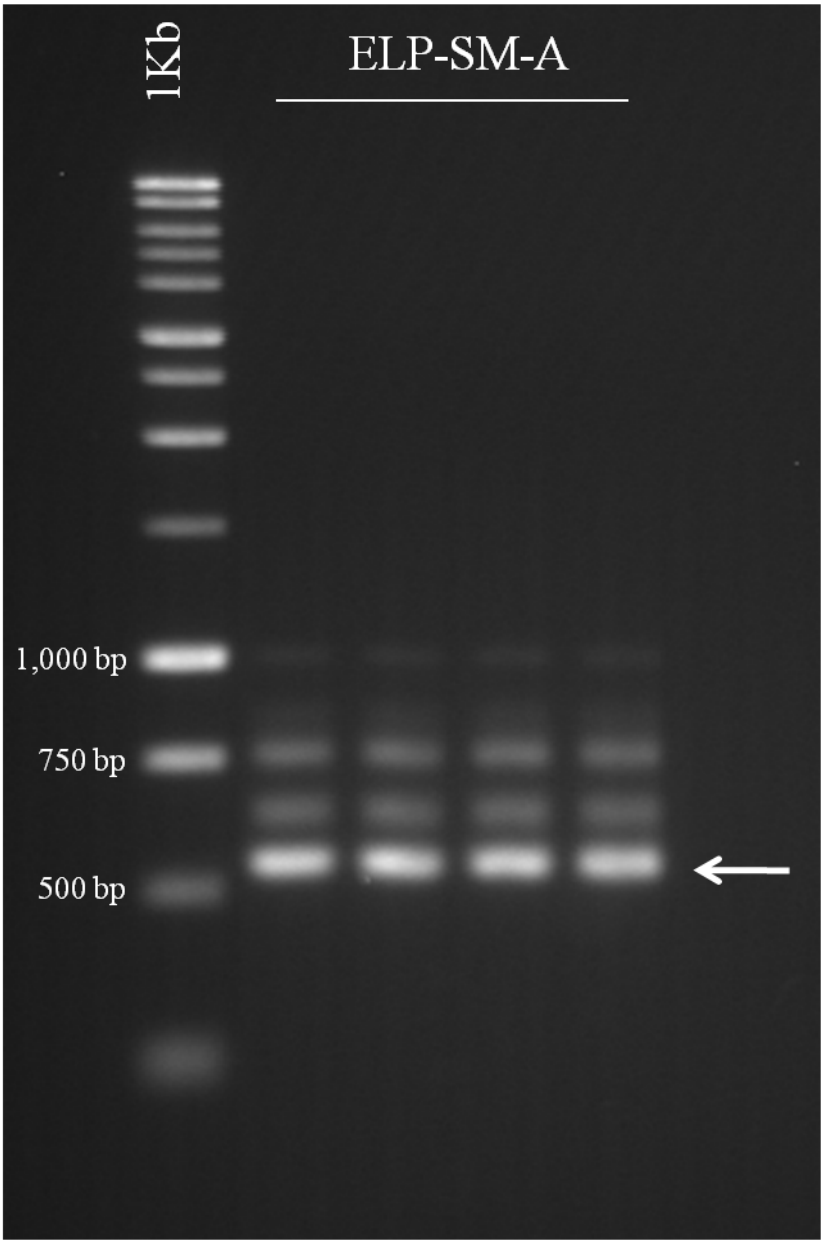

Figure 7.12 Electrophoresis on 3% agarose gel of PCR from environmental samples showing different amplified products and our band of interest for cloning at ~550 bp (arrow).

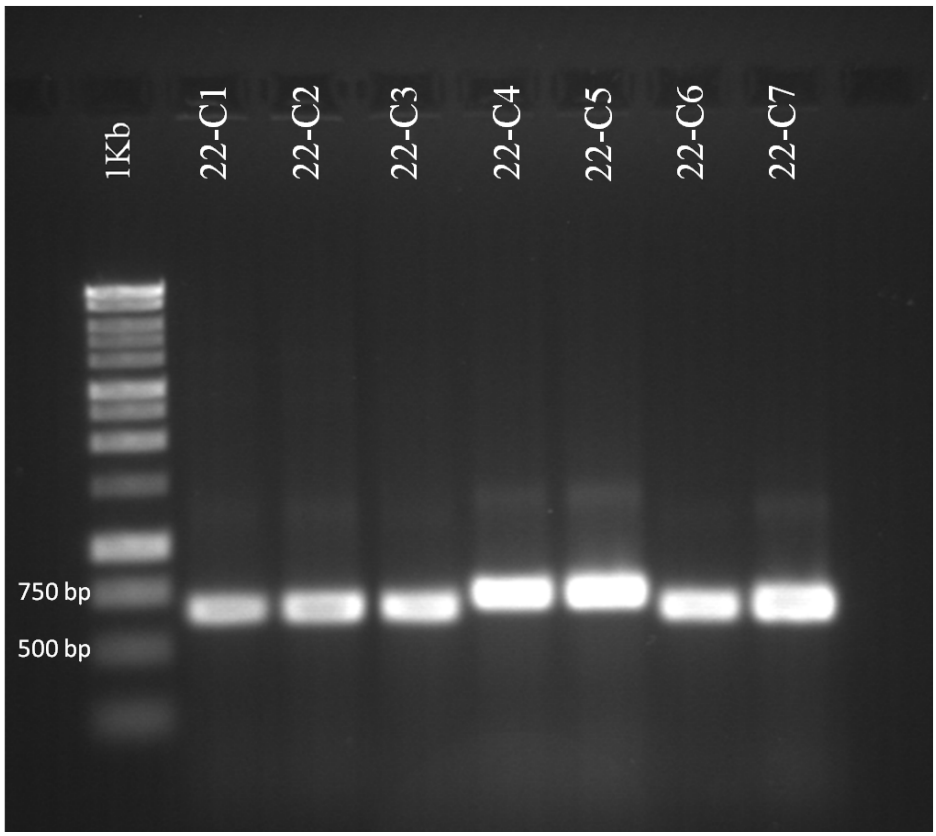

Figure 7.13 Colony PCR of clones from environmental clone library from sand from ETH-SM (library #22) nest at MABC. Amplicons of expected size (~650-700 bp) are shown.

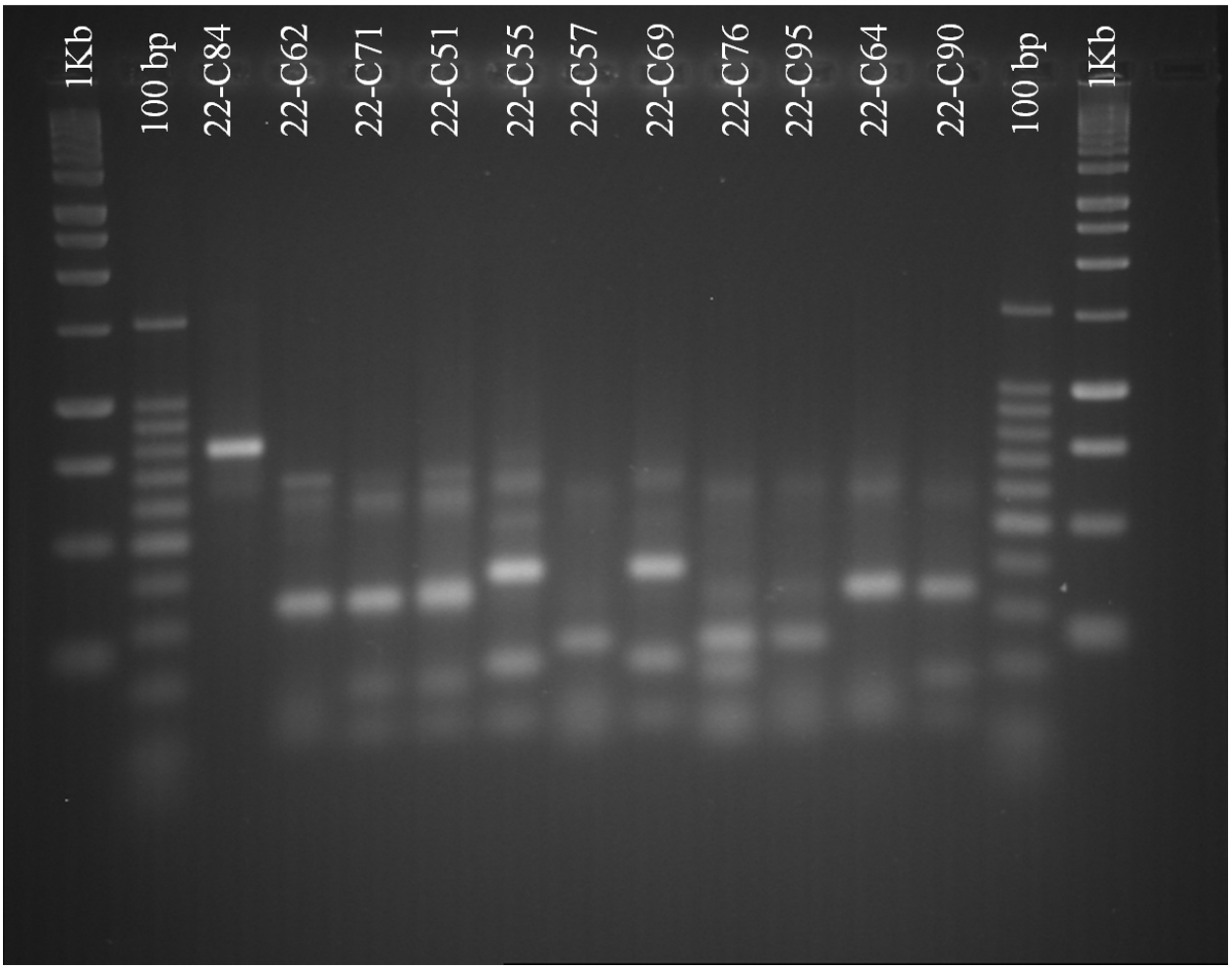

Figure 7.14 RFLP profiles from clones from ELP-SM library obtained from single digestion with Hinf I.

7.3 *Discussion*

A total of 39 fungi representing 5 identified genera and unidentified isolates were recovered from MABC sand and failed eggs from *D. coriacea* nests. During this period we focused on isolating mycelial fungi from nests sand and failed eggs. *Fusarium solani* was isolated with higher frequency (0.57) during this sampling period. It was isolated from 50% of nests sampled and it was the only species able to infect the egg completely (surface and interior). Most of the nests from which *F. solani* was isolated had a low hatching success. Miller et al. (2009) reported this species from dead leatherback hatchling's skin and carapace. It has been also documented by Phillot et al. (2004) along with *Fusarium oxysporum* and *Pseudallescheria boydii* occurring in other sea turtles clutches. These three fungi have been reported to cover the entire surface of the eggs and to produce enzymes that degrade their inorganic and organic compounds (Phillot and Parmenter, 2001b; Phillot, 2004). *Fusarium solani* has been confirmed to be extremely detrimental to sea turtle eggs, and that some strains may act as primary pathogens (Phillot et al., 2006; Sarmiento et al., 2010). Other species that we isolated from the surface of eggs was *Scedosporium aurantiacum*, member of the *Pseudallescheria boydii* species complex (Gilgado et al., 2005; 2008). It is unclear if our species is the same that Phillot et al. (2001) reported in their study, since it was not until 2005 that *S. aurantiacum* was proposed as a species within the *P. boydii* species complex. Also, *S. aurantiacum* has been regularly reported from clinical samples unlike its sister species *S. prolificans*, which has been mostly isolated from environmental samples (Gilgado et al., 2005).

Other species identified in our study include *Aspergillus ochraceus, A. niveus, A. sclerotiorum, Penicillium islandicum, Cladosporium* sp., and *Trichoderma longibrachiatum*. All of these fungi were isolated from nests sand and eggs surface, but were not able to colonize the

interior of the eggs. *Aspergillus* species have been reported as a threat to sea turtle clutches for their mycotoxigenic properties and has been the most frequent species isolated from sea turtle nests in other studies (Elshafie et al., 2007). Our *Aspergillus* isolates, may then contribute to egg failure during incubation, not by direct colonization, but by the production of mycotoxins that could negatively affect embryo development.

By Pearson Product Moment Correlation Coefficient we determined that fungal abundance may be strongly related to egg loss in *D. coriacea* nests. This does not necessarily means that fungi are the main cause of egg loss since high humidity and low temperatures, are not only detrimental to egg development, but are favorable for fungal proliferation. According to Morelock et al. (1983), most of the MABC rainfall occurs between April and October, which coincides with leatherback nesting season. The combination of these two factors and the presence of the fungal isolates may then be the cause for leatherback eggs loss during our study.

Environmental clone libraries were constructed in order to have a better understanding of fungal diversity on leatherback nests. Diversity assessment by RFLP analysis of clones showed distinct profiles, which suggests that diversity of fungi in the nest might be higher than recorded by traditional culturing techniques.

8 CONCLUSIONS

- This study was the first attempt to assess mycelial fungal diversity associated to *Dermochelys coriacea* sea turtle nests in Puerto Rico.
- The most common MABC fungi occurring prior to leatherback nesting season were identified as ubiquitous soil dwellers.
- It was determined that the most frequent isolates prior to nesting season were *Aspergillus*, represented by 5 species (*A. alliaceus*, *A. ochraceus*, *A. restrictus*, *A. penicillioides*, and *A. terreus*), *Fusarium*, represented by 3 species (*F. solani*, *F. equiseti*, and *F. moniliforme*), *Cladosporium* sp., *Curvularia lunata*, and *Trichoderma longibrachiatum.*
- Isolation of *Aspergillus*, *Penicillium* and *Fusarium* is of great relevance since they have previously been reported causing sea turtle egg loss.
- No fungi were isolated from nesting leatherback females' cloacae, which suggests that fungi related to egg loss might be present in sand at the moment of egg laying.
- A moderate positive correlation was found between mycelial fungi abundance and eggs loss in the nests.
- *Aspergillus*, *Penicillium*, *Cladosporium* and *Scedosporium* were able to colonize egg's surface only.
- This work reports the species *Scedosporium aurantiacum* for the first time occurring on leatherback sea turtle eggs.
- *Fusarium solani* was the only species isolated from the interior of *D. coriacea* eggs, suggesting that our isolates might have the necessary mechanisms to deplete the eggshell.

- RFLP community analysis trials suggests that fungal diversity associated with leatherback nests at MABC might be higher than recorded by traditional culturing techniques.

9 RECOMMENDATIONS

- To have a better understanding of mycelial fungal diversity a year-round study is recommended.
- In order to determine if the absence of cloacal mycelial fungi in the present study is due to environmental factors, a similar study on other leatherback populations in the island is suggested.
- Fungal diversity associated to other leatherback nesting populations in the island should be performed.
- Assessment of fungal infection and presence of mycotoxins on dead-in-nest hatchlings is strongly recommended.
- Analysis of metabolic compounds produced by *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, and *Scedosporium* is suggested.
- Sequencing and phylogenetic analysis of environmental clone libraries from *D. coriacea* nests from MABC should be done.

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APPENDIX A: ITS sequences of *Fusarium solani* isolates from leatherback failed eggs

> M146_Fusarium_solani

TGGTGAACCTGCGGAGGGATCATTACCGAGTTATACAACTCATCAACCCT

GTGAACATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACA

CGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCT

TCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGG

CTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG

CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT

ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCG

GGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTCC

CCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAAC

ACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCT

GAATGTGACCTCGAATCAG

> M155_Fusarium_solani

GTGAACCTGCGGAGGGATCATTACCGAGTTATACAACTCATCAACCCTGT

GAACATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACG

GGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCCATAATGTTTCTTC

TGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCT

CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA

GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT

TCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGG

CCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTCCCC

CAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACAC

CTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGA

ATGTGACCTC

>Contig_M157

GAGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCC

TAACTCTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCAAATAAATTAA

AACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCG

AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT

TGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGC

GTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAG

CCCCCTGCGGGCACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGC

AGCTTCCATTGCGTAGT