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FIRST REPORT OF *Fusarium* SPECIES AT NESTING SITES OF ENDANGERED SEA TURTLES IN TERENGGANU AND MELAKA, MALAYSIA

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ABSTRACT

In recent years, turtle eggs incubated *in situ* or in protected hatcheries in Malaysia have been reported to show symptoms and signs of fungal colonisation. However, there are no studies addressing this problem and potential relationship with frequent hatching failures. We identified *Fusarium* species from nesting sites of green turtle (*Chelonia mydas*) and hawksbill turtle (*Eretmochelys imbricata*) situated in Terengganu and Melaka, as well as the environments surrounding those sites. The eggs were incubated on the nesting beach (*in situ*) or relocated to the beach hatchery and styrofoam boxes (*ex situ*) in Peninsular Malaysia. Samples were collected from infected eggs, sand, plant roots and debris around the egg chambers. One-hundred and six strains of *Fusarium* spp. were isolated. They were identified morphologically as member of the *Fusarium solani* species complex (FSSC, 101 strains), *F. oxysporum* (four strains) and *F. proliferatum* (one strain). We conducted phylogenetic analysis based on nucleotide sequences of translation elongation factor 1-alpha gene (TEF-1α). The strains of the FSSC were further separated into three lineages, *F. falciforme*, *F. lichenicola* and *F. keratoplasticum*. This is the first report on *Fusarium* species isolated from symptomatic green and hawksbill turtle eggs in Peninsular Malaysia. *Fusarium* colonisation in sea turtle nests poses a serious risk to the survival of endangered sea turtles in Malaysia. It is, therefore, important to examine the nature of such colonisation and their relationship to hatching failures of the turtles in Malaysia or elsewhere in the region to mitigate pathogenic fungi impact.

Key words: *Fusarium solani* species complex, green turtle, hawksbill turtle, hatching failure and Malaysia

INTRODUCTION

Sea turtles are marine reptiles widely distributed in tropical, subtropical and temperate seas throughout the world. Out of the seven species of sea turtles, four species nest in Malaysia: the green turtle (*Chelonia mydas*), hawksbill (*Eretmochelys imbricata*), leatherback (*Dermochelys coriacea)* and olive ridley (*Lepidochelys olivacea*) (Chan, 2006). The green turtle is the most abundant sea turtle species in Malaysia, nesting in Sabah, Sarawak, Terengganu, Pahang, Perak and Johor. Redang Island is one of the largest nesting sites for green turtles in Peninsular Malaysia, with 1000–2500 nests per year. The hawksbill turtles nest in Sabah, Melaka, Johor and Terengganu (Chan, 2006). Both species are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Seminoff, 2004). The International Union for Conservation of Nature (IUCN, 2004) has listed the green turtle as endangered and the hawksbill turtle as critically endangered. This study focused on the green and hawksbill turtles in Peninsular Malaysia.

Most conservation efforts try to restore the depleted populations by protecting nests and improving hatching success. Like many other conservation programmes in the world, they cannot

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protect all sea turtle eggs because of poaching, as well as predation by monitor lizards, ants, ghost crabs, and maggots (Morita *et al.,* 2008; Garcia *et al*., 2003). Fungal disease is another threat to survive especially due to the increasing number of pathogenic fungi that threatens wildlife and domesticated species worldwide (Fisher *et al.,* 2012; Sarmiento-Ramírez *et al.,* 2014). Examples of some fungal diseases that pose substantial threats to animal such as chytridiomycosis (*Batrachochytrium dendrobatidis*) that contributed to nearly half of all amphibian species being in decline worldwide (Fisher *et al.,* 2009; Hof *et al.,* 2011) and white-nose syndrome (*Geomyces destructans*) found in six species of North American bats that has killed millions of bats (Frick *et al.,* 2010; Foley *et al.,* 2011). There has been several reports of fungi that have infected sea turtle eggs and is responsible for the sea turtles' decline in the Atlantic, Indian and Pacific Oceans from 2005-2012 (Sarmiento-Ramírez *et al.,* 2014).

Researchers in Costa Rica (Mo *et al.,* 1990), the United States (Eckert and Eckert, 1990), Australia (Phillott *et al.,* 2004), Turkey (Güçlü *et al.,* 2010) and Spain (Sarmiento-Ramírez *et al.,* 2010) have demonstrated the association of fungi with unhatched turtle eggs. The loggerhead turtle (*Caretta caretta*) is susceptible to the ascomycete fungus (*Fusarium solani*) (Sarmiento-Ramírez *et al.,* 2010). The genus *Fusarium* has been widely recognized as plant pathogens or soil inhabitants and plays an important role in animal pathology and mycotoxicology (Siti Nordahliawate *et al.,* 2012; Geiser *et al.,* 2013). It has been demonstrated that approximately 70 species of *Fusarium* have been involved in infections in humans and other animals that were grouped into about ten phylogenetic species complexes (O'Donnell *et al.*, 2010; Guarro, 2013). Sarmiento-Ramírez *et al.* (2014) found *F. falciforme* and *F. keratoplasticum* members of *Fusarium solani* species complex (FSSC) in six species of sea turtles; *Chelonia mydas* (Raine Island, Australia; Isla de la Plata at Machalilla National Park, Ecuador and Ascension Island), *Caretta caretta* (Boa Vista Island, Cape Verde), *Eretmochelys imbricata* and *Lepidochelys olivacea* (La Playita, Machalilla National Park), *Dermochelys coriacea* (La Playona, Colombia and Pacuare Nature Reserve, Costa Rica) and *Natator depressus* (Crab Island, Australia). Nevertheless, *Fusarium solani* species complex (FSSC) is the most common group of fusaria associated with human infectious diseases and to date, 60% responsible for fusariosis (Short *et al.,* 2013). Fusariosis is the second most common cause of mould infections in human after aspergillosis (Guarro, 2013).

However, the *Fusarium* isolates were morphologically indistinguishable and cultures requires extensive molecular techniques especially monophyletic "species complex" such as *Fusarium solani* species complex (FSSC) (O'Donnell, 2000; Short *et al.,* 2013). Thus, to identify phylogenetic species to date, based on a partial sequence from translation elongation factor-1a (TEF1), and DNA-directed RNA polymerase II largest (RPB1) and second largest subunit (RPB2) has proven to be the most reliable (O'Donnell *et al.,* 2015).

Very few studies of microbial contaminants of sea turtle eggs have been conducted in Malaysia. An unknown species of fungus on leatherback turtle eggs at Rantau Abang, Terengganu was reported in 1989 (Chan and Solomon, 1989). In 2010, Daraup (2010) identified morphologically of *Fusarium* species isolated from green turtle eggs incubated at Chagar Hutang, Redang Island and Terengganu. However, both studies were rather limited and did not identify the fungal species. Our study recorded for the presence of *Fusarium* species and identified *Fusarium* isolates from the shells of unhatched eggs, sands surrounding the egg chamber and debris and plant roots around the turtle nests on natural nesting beaches and in hatcheries of Peninsular Malaysia. We believe that this study supplies useful data on the fungi isolated from sea turtle eggs and nests. Our results might help the efforts to increase the hatching success and replenish the depleted populations of sea turtles in Malaysia.

MATERIALS AND METHODS

Ethics statement

Collection of sea turtle eggshells was done under permissions Sea Turtle Research Unit (SEATRU) and Department of Fisheries Terengganu and Melaka. None of the experiments involved sacrificing animals and, therefore, we did not require a specific approval from any institutional animal research ethics committee.

Sampling sites

Three sites in Peninsular Malaysia, using different methods of turtle nest incubation (*in situ* and *ex situ*), were selected for sampling (Fig. 1). Samples from *in situ* nests were obtained from Redang Island, Terengganu, in 2010. Samples of green turtle eggs were also obtained from *ex situ* nests at the Ma'Daerah hatchery, Terengganu. Samples of hawksbill turtle eggs were obtained from Tanjung Kemunting, Melaka, in 2011. Eggs incubated in Tanjung Kemunting were collected from nearby nesting beaches, namely from Pulau Upeh, Kem Terendak and Balik Batu. The nesting sites in Redang Island were near vegetation areas (0–10.76 m) with an average nest depth of 70 cm. *Ex situ* nesting hatcheries were located far (500 m)

Fig. 1. Sampling of green and hawksbill sea turtle eggs colonised with fungus at Redang Island, Ma' Daerah and Tanjung Kemunting, Melaka (nests at the hatcheries were in Pulau Upeh, Kem Terendak and Balik Batu).

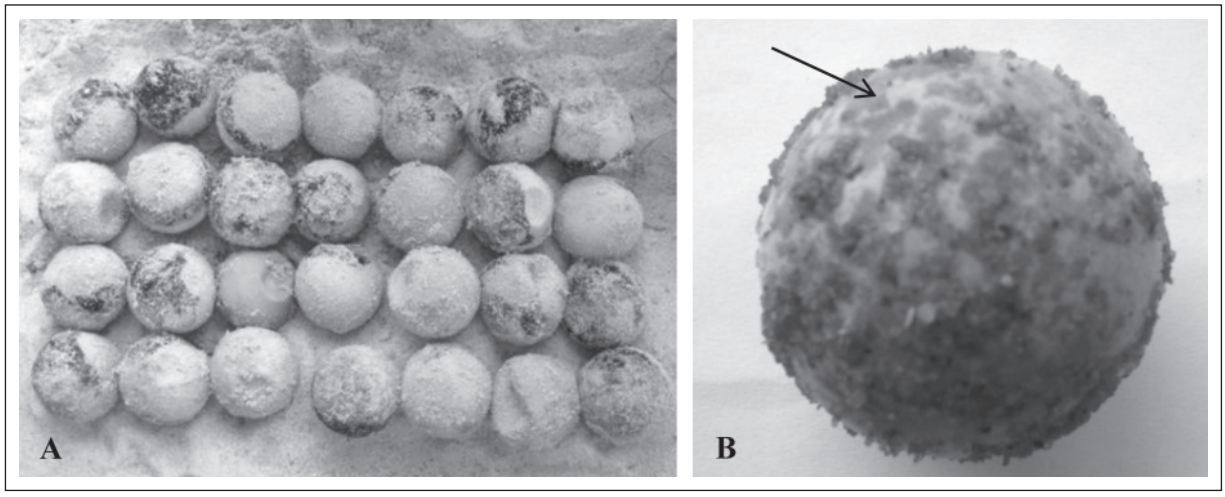

Fig. 2. Green turtle eggs at Redang Island. (A) Characteristic appearance of fungal colonisation on external areas of eggshells, showing black-like fungi infection spots and (B) bluish spots of internal symptoms.

from vegetation areas. The nest depths in these hatcheries were 60–70 cm for green turtles and 40 cm for hawksbill turtles, to imitate the average natural nest depth.

Sample collection

Upon emergence of hatchlings, both *in situ* and *ex situ* nests were excavated to determine hatching success of each nest by counting hatched and unhatched eggs. Eggs showing signs of fungal invasion (Fig. 2) were swabbed using sterile cotton sticks. The deposit was then smeared onto the half-strength potato dextrose agar (PDA) and semi-selective peptone pentachloronitrobenzene agar (PPA) (Leslie and Summerell 2006) prepared in 1.5 mL microtubes. Eggs adjacent to the contaminated eggs, but without obvious signs of fungi, were sampled in the same manner. Samples of sand, plant roots and organic debris from the egg chambers were collected and kept in sterile paper envelops for fungus isolation.

Isolation of fungi

The sand from each nest was air-dried at room temperature (27±1ºC) for 5 days and passed through a 0.5-mm sieve to separate the large debris particles. Aliquots (10 mg) of sieved sand were spread evenly on the surface of PDA or PPA plates. Organic debris was soaked in running water overnight to remove soil particles and air-dried on sterile filter papers.

Samples of plant roots were surface-sterilized with 0.5% sodium hypochlorite for three minutes, rinsed twice in sterile water, blotted on sterile filter paper and plated in the same manner as sand samples. Plates were incubated for 7 days under standard conditions (12 h under fluorescent and NUV lights, 12 h in the dark, at 28±2ºC) (Salleh and Sulaiman, 1984). Axenic cultures from colonies grown from eggshell, nest substrate, organic debris and plant roots were obtained using the single-spore isolation technique (Salleh and Sulaiman, 1984) and incubated in the same manner.

Visual identification and morphological characterisation of fungal isolates

Axenic cultures were transferred onto full-strength PDA and carnation leaf agar (CLA) plates for examination of macroscopic (formation of mycelium and pigmentation) and microscopic characteristics (macroconidia and microconidia, phialides and chlamydospores) under an advanced Nikon Eclipse 80i compound microscope. Images were analysed using NIS-Elements D 2.30 software (Fisher *et al.,* 1982). The morphological features were examined and species were identified based on Short *et al.* (2013) and Leslie and Summerell (2006).

DNA extraction, PCR amplification and DNA sequencing

Isolates were grown in PDA broth (PDB) (Booth 1977) for 5–6 days under the standard growth conditions (Salleh and Sulaiman, 1984). The resulting mycelial pellet were harvested from the surface of the PDB and lyophilized. Lyophilized mycelia were ground with liquid nitrogen (N_2) to a fine powder and weighed approximately 0.22–0.25 g. Genomic DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN).

Reactions were carried out in a total volume of 25 µl, containing 4 µl each of 5X Green GoTaq® Flexi Buffer, 4 µl of 25 mM magnesium chloride ($MgCl_2$), 0.5 µl of 10 mM deoxynucleotide triphosphate mix (dNTP; Promega) and 0.75 units of GoTaq® DNA polymerase (Promega). The reaction mixture also contained 4 µl each of 5µM primer EF1 (forward primer; 5'-ATG-GGT-AAG-GAG-GAC-AAG-AC-3') and EF2 (reverse primer; 5'-GGA-AGT-ACC-AGT-GAT-CAT-GTT-3') (O'Donnell *et al.,* 1998), 8.05 µl of double distilled water (ddH_2O) and 6 ng of DNA template. Every reaction mix was overlaid with 25 µl of paraffin oil to prevent evaporation. The amplification was carried out in a programmable thermal cycler (DNA Engine Peltier Thermal Cycler PTC-200®) under the following cycles: an initial denaturation at 94ºC for 85 s, followed by 35 cycles of 95ºC for 35 s, 46ºC for 55 s, and 72ºC for 90 s and final extension at 72ºC for 10 min. PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. The purified PCR products obtained from each isolate were sent to a service provider for sequencing. The aligned sequences were BLAST against sequences in the GenBank database. In this study, phylogenetic tree was generated using maximum likelihood (ML) in MEGA 6.0. The program Molecular Evolutionary Genetic Analysis software, ver. 6.0 (MEGA6.0; http://www.megasoftware.net) was performed in order to edit and align the sequence files (Tamura *et al.,* 2013), which were manually adjusted. *F. incarnatum*, (CBS 133024) obtained from GenBank was treated as the outgroup.

RESULTS AND DISCUSSION

Our results showed that all unhatched eggs at Redang Island (*in situ* nests), Ma' Daerah (*ex situ* nests) and Tanjung Kemunting (*ex situ* nests) were colonised with *Fusarium* species. Eighty-five unhatched eggs of green and hawksbill turtles showed bluish discolorations (Figure 2) similar to those reported by Sarmiento-Ramírez *et al.* (2010) at Boavista Island, Cape Verde. Fungal growth can occur on the external surface of unhatched turtle eggs and the internal shell membrane and embryonic tissue, as reported by Elshafie *et al.* (2007) and Sarmiento-Ramírez *et al.* (2010). Sarmiento-Ramírez *et al.* (2010) had performed Koch's postulates on *F. solani* and after 6 days of inoculation, the healthy eggs showed similar symptoms to those in the field and the mortality rate was 83.7%.

In this study, the greatest number of isolates was identified as *Fusarium solani* species complex (FSSC, 95.28%) (Table 1 and Table 2) with 101 isolates from eggshell, nest substrate, organic debris and plant roots. Four isolates of *F. oxysporum* and one isolate of *F. proliferatum* were isolated from eggshells (Table 1). On PDA, FSSC growth was rapid with abundant mycelia with yellow and cream pigmentation. A few isolates showed sporodochia, which produced massive uniform macroconidia. On CLA, FSSC formed banana-shaped macroconidia with blunt ends (Figure 3A), with the length of 28.4–37.6 µm and 3–4 septa. We observed many microconidia, oval and ellipsoidal, with 0–1 septum (Figure 3B). Chlamydospores were abundant and usually produced singly (Figure 3C) but some were found in pairs or clusters. Another important characteristic was the phialides; the phialides produced by FSSC and *F. oxysporum* were easily distinguishable. *Fusarium solani* species complex produced long monophialides bearing microconidia (Figure 3D) and *F. oxysporum*, short monophialides. *F. proliferatum* produced microconidia in chains (Leslie and Summerell 2006).

Table 1. Frequency of *Fusarium* species isolated from turtle eggs shell, debris, nest sands and plant roots in the year 2010 and 2011 based on the visual and morphological identification

Fusarium species	Natural and relocated nests area (%)			Percentage	Total isolates and source			
	[a]Redang Island, Terengganu	[a]Ma'Daerah, Terengganu	[b]Tanjung Kemunting, Melaka		Eggs shell	Nest sands	Debris	Root
F. oxysporum	0.94	2.83	0	3.77	4	0	0	0
F. proliferatum	0.94	0	0	0.94	1	0	0	0
F. solani	39.62	6.60	49.06	95.28	80	13	2	6
Total number of isolates	44	10	52	106 isolates	85	13	2	6

[a]Green turtle
[b]Hawksbill turtle

Table 2. Identification of *Fusarium* species (106 isolates) based on morphological data and TEF-1α sequences obtained from various sources near *in situ* and *ex situ* nesting sites of green turtles and hawksbill turtles

Isolates	Morphological identification	TEF-1α sequences (% similarity)	Sources	Origin	GenBank Accession number
T9839PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF035992
T9840PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF035981
T9841PY	*F.* cf. *solani*	*F. falciforme* (99%)	Debris	[a]Redang Island, Terengganu	KF035990
T9842PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF035995
T9843PY	*F.* cf. *solani*	*F. falciforme* (99%)	Debris	[a]Redang Island, Terengganu	KF020509
T9845PY	*F. oxysporum*	*F. oxysporum* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF429207
T9846PY	*F. oxysporum*	*F. oxysporum* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF429206
T9847PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF035973
T9848PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF020508
T9850PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF035972
T9851PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF035984
T9852PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF035994
T9853PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF020507
T9854PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Nest sands	[a]Redang Island, Terengganu	KF429211
T9855PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF020497
T9856PY	*F. oxysporum*	*F. oxysporum* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF429208
T9857PY	*F. oxysporum*	*F. oxysporum* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF429209
T9859PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF035985
T9860PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF035988
T9861PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF035991
T9862PY	*F.* cf. *solani*	*F. falciforme* (96%)	Nest sands	[a]Redang Island, Terengganu	KF429197
T9863PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF020506
T9864PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035978
T9865PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF020505
T9866PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF020498
T9867PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF020504
T9868PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF020502
T9869PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF035993
T9870PY	*F. proliferatum*	*F. proliferatum* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429210
T9871PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[a]Redang Island, Terengganu	KF035980
T9872PY	*F.* cf. *solani*	*F. falciforme* (100%)	Eggs shell	[a]Redang Island, Terengganu	KF020503
T9873PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035970
T9874PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035975
T9876PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF460430
T9877PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF437474
T9878PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035969
T9879PY	*F.* cf. *solani*	*F. falciforme* (100%)	Eggs shell	[b]Ma' Daerah, Terengganu	KF020501
T9880PY	*F.* cf. *solani*	*F. falciforme* (99%)	Root	[a]Redang Island, Terengganu	KF035975
T9881PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429222
M9882PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429213
T9883PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035977
T9890PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429223
T9891PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429220
T9892PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429218

Table 2 continued...

Isolates	Morphological identification	TEF-1α sequences (% similarity)	Sources	Origin	GenBank Accession number
T9893PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035971
T9894PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035967
T9895PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF020500
T9896PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429216
T9897PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429214
T9898PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF429217
T9899PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035986
T9900PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035983
T9907PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035987
T9908PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035996
T9909PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[a]Redang Island, Terengganu	KF035982
M9910PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429219
M9912PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF437479
M9913PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429212
M9914PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429228
M9916PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429229
M9917PY	*F.* cf. *solani*	*F. falciforme* (100%)	Eggs shell	[c]Pulau Upeh, Melaka	KF020496
M9919PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429215
M9920PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429227
M9921PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429230
M9922PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF035997
M9923PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF020499
M9924PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF437473
M9925PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF035999
M9926PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF437472
M9927PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF437471
M9928PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF437470
M9929PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF437469
M9931PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Kem Terendak, Melaka	KF036000
M9932PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437477
M9933PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437475
M9934PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437478
M9935PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF020510
M9936PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF035998
M9937PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437468
M9938PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437467
M9939PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437466
M9940PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF437464
M9941PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF020512
M9942PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF035974
M9943PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429198
M9944PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429202
M9945PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429200
M9946PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429204
M9947PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429205
M9949PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429201
M9951PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF020513
M9952PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429231
M9953PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429203
M9954PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF035989
M9956PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429232
M9959PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429233
M9960PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429225
M9961PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429226
M9962PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429197
M9965PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Nest sands	[c]Kem Terendak, Melaka	KF437476
M9966PY	*F.* cf. *solani*	*F.* cf. *solani* (99%)	Nest sands	[c]Kem Terendak, Melaka	KF437465
M9967PY	*F.* cf. *solani*	*F. falciforme* (99%)	Nest sands	[c]Pulau Upeh, Melaka	KF035968
M9968PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Nest sands	[c]Pulau Upeh, Melaka	KF429234
M9969PY	*F.* cf. *solani*	*F. keratoplasticum* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429221
M9970PY	*F.* cf. *solani*	*F. falciforme* (99%)	Eggs shell	[c]Balik Batu, Melaka	KF020511
M9971PY	*F.* cf. *solani*	*F. lichenicola* (99%)	Eggs shell	[c]Pulau Upeh, Melaka	KF429199

a = samples from *in situ* nests; b = samples from *ex situ* (relocated) nests; c = *ex situ* samples obtained from Tanjung Kemunting, Melaka (nests at the hatcheries were in Pulau Upeh, Kem Terendak and Balik Batu).

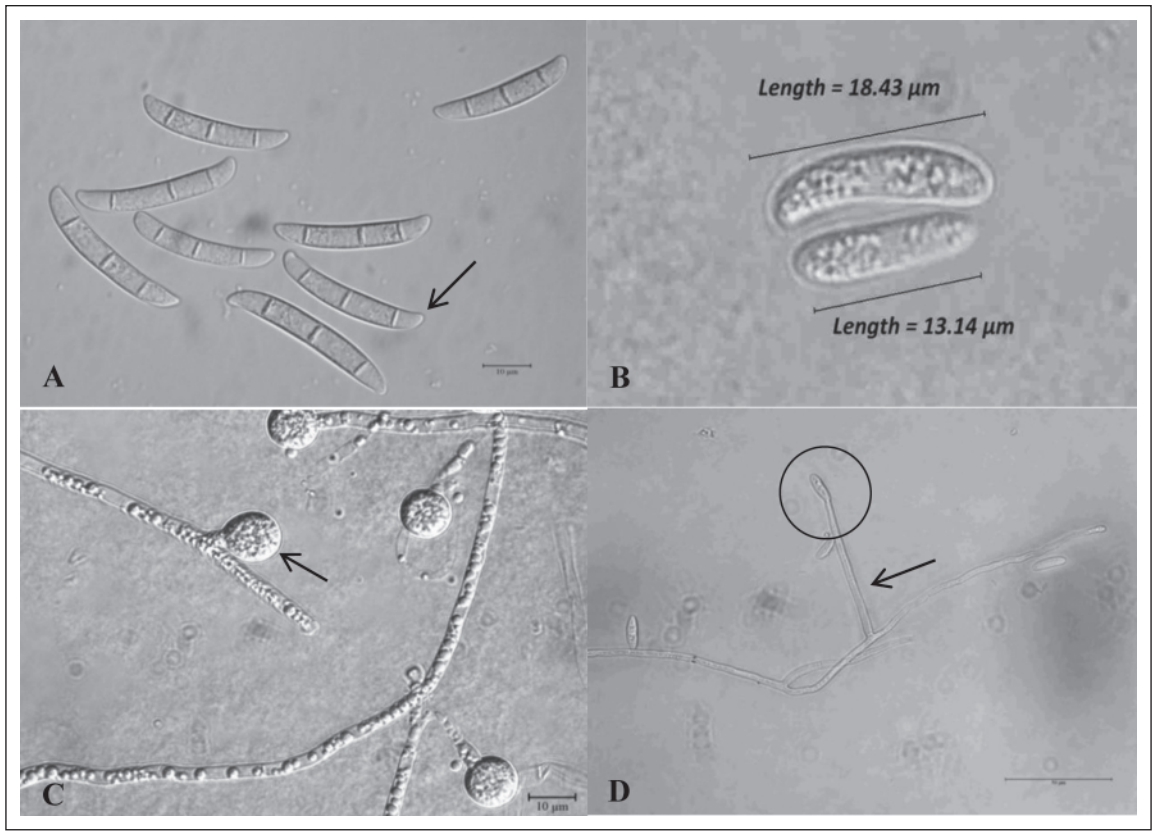

Fig. 3. Morphological characteristics of *F. solani.* A) Blunt end of macroconidia (arrow) with 3 septa (bar: 10 µm), B) microconidia with 1 septum, of oval and reniform shape (bar: 10 µm), C) single chlamydospores (arrow) (bar: 10 µm) and D) long monophialides (arrow) with microconidia (circle) (bar: 50 µm).

Fusarium solani, defined on the basis of morphological characteristics, is actually a diverse complex of approximately 60 phylogenetically distinct species (Short *et al.,* 2013). These species are morphologically similar and are incorrectly classified under the name of *F. solani*.

However, our molecular phylogenetic analysis of FSSC demonstrated that Main Clade I consisted of FSSC isolates, divided into four sub-clades (Figure 4): A, B, C and D. Sub-clade A consisted of *F. falciforme* isolates, mainly from Terengganu (40 isolates) and from Melaka (12 isolates). The isolates in sub-clade A from Terengganu came from Redang Island and Ma' Daerah sites for green turtles. Isolates from Melaka were from a hawksbill turtle hatchery site. Sub-clade B consisted of 23 isolates of *F.* cf. *solani*, mainly from Melaka (18 isolates), and five from Terengganu (Redang Island). Sub-clade C consisted of 17 isolates of *F. keratoplasticum* from Terengganu (4 isolates) and from Tanjung Kemunting, Melaka (13 isolates). Sub-clade D consisted of nine isolates of *F. lichenicola* from Melaka (eggshells collected from nests in Pulau Upeh). *Fusarium falciforme* and *F. keratoplasticum* have been isolated from six species (*Chelonia mydas*, *Caretta caretta*, *Eretmochelys imbricata*, *Lepidochelys olivacea*, *Dermochelys coriacea* and *Natator depressus*) of sea turtle eggs in nesting beaches of the Atlantic, Indian and Pacific Oceans, and the Caribbean Sea (Sarmiento-Ramírez *et al.,* 2014). These fungi are considered a threat to the endangered sea turtles (Sarmiento-Ramírez *et al.,* 2014). Main Clade II consisted of two sub-clades with isolates only from Terengganu (Figure 4). These were sub-clades E and F: *F. proliferatum* (one isolate from eggshells collected in Redang Island, Terengganu) and *F. oxysporum* (four isolates from eggshells collected in Ma' Daerah hatchery, Terengganu), respectively. Only *F. lichenicola* (8.5%) and *F. oxysporum* (3.8%) were isolated from the eggshells obtained from Tanjung Kemunting (Melaka) and Ma'Daerah hatchery, respectively.

There are three major clades of the FSSC and all the veterinary sources (including sea turtle) are nested within clade 3 (Zhang *et al.,* 2006), the most evolutionarily diverse and species-rich clade (MP=100%; ML=83%) (O'Donnell *et al.,* 2008). A strong association of FSSC species with marine animals has been reported.

The FSSC members adapt well to the marine environment, causing infections in marine animals (Sarmiento-Ramírez *et al.,* 2014; Zhang *et al.,* 2006). Various environmental stressors (frequent tidal inundation, unsuitable substrates, beach erosion and

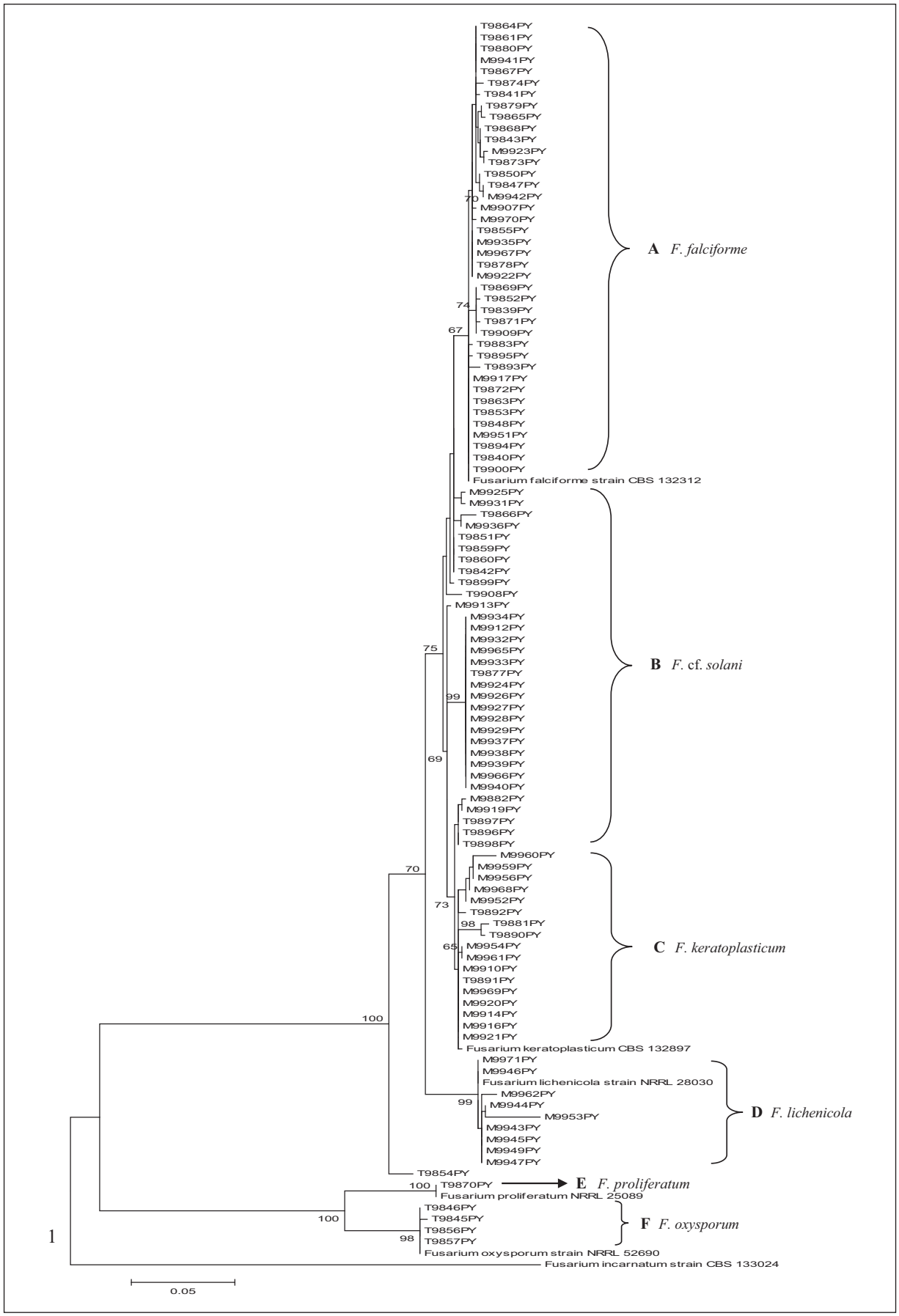

Fig. 4. Maximum Likelihood tree of *Fusarium* spp. based on sequence information for the gene encoding translation elongation factor 1-alpha (TEF). *Fusarium incarnatum* (CBS 133024) obtained from GeneBank was treated as the outgroup.

changes in sand composition) might increase the opportunities for adaptation of these pathogenic *Fusarium* species and increase their ability to colonise the sea turtle eggs. Additionally, endangered species whose populations are reduced in terms of genetic diversity could be easily being diseased when environmentally stressed or combination of other threats (Aguirre & Tabor, 2008). A single pathogenic species may become the factor of a host toward extinction (Heard *et al.,* 2013). A recent study has reported that *F. falciforme* and *F. keratoplasticum* were always found in dead turtle eggs and can kill up to 90% of the embryos; the optimal temperature for pathogen germination is similar to the thermal environment of turtle nests, 29.7°C (Sarmiento-Ramírez *et al.,* 2014). In Redang Island, the temperature of turtle nests range between 25.6°C to 33.6°C were recorded and we also noticed that most nests near to vegetation (<3m) had more number of infected eggs (Siti Nordahliawate *et al.,* 2017; unpublished data). Recently, apart from *Fusarium* species we had isolated other fungi from soil and debris where a few numbers had been identified as *Aspergillus* species (Siti Nordahliawate *et al.,* 2017; unpublished data).

Although the purpose of eggs relocation is to reduce the threat to the turtle eggs, it might also affect the sea turtle population because of differences in environmental conditions, especially the temperature of egg incubation (Chan, 2006). It has been reported that the hatching success in relocated nests (*ex situ*) is lower than in undisturbed natural nests (*in situ*) (Wyneken *et al.,* 1988). It is possible to spread the fungus to a new nest location unwillingly, particularly if proper handling procedures and sterile conditions are not ensured. Another factor that needs some consideration is the reuse of substrate in the hatcheries over several seasons, which might cause accumulation of the fungal inocula. Recently, we found that there is no relation between fungal infection and hatchling success even though turtle nests contained eggs with symptoms of fungal infection (Siti Nordahliawate *et al.,* 2017; unpublished data).

Members of the FSSC can be a serious plant and human pathogens (Short *et al.,* 2013; Zhang *et al.,* 2006). Thus, anyone working with *Fusarium* isolated from turtle eggs should be aware of the risk. Infection can be prevented by wearing masks, gloves or maintaining sterile conditions.

CONCLUSION

This study confirms the presence of several species of *Fusarium* on infected green and hawksbill turtle eggs and nests. All infected egg samples (incubated *in situ* and *ex situ*) showed similar symptoms at all sampling sites in Terengganu and Melaka. The fungi were also isolated from sands, debris and roots found in the nests. Members of FSSC constituted the greatest proportion of the isolated fungi. The turtle eggs that were damaged by predators such as monitor lizards are suitable substrate to colonise by *Fusarium* and could progressively move to the neighboring eggs. Furthermore, *Fusarium* has necrotroph phase to its lifecycle, the prominence of *Fusarium* species on damaged eggs is likely becasuse it is an opportunist pathogen. We also know that the *Fusarium falciforme* and *F. keratoplasticum* implicated in sea turtle egg fusariosis have been isolated from symptomless eggs in nests with high hatching success, suggesting FSSC that present in Terengganu and Melaka nests with little to no impact on egg mortality. The FSSC has been described as an emerging animal pathogen but has not been previously reported from sea turtle eggs of any nesting sites in Malaysia. This is the first report of the occurrence of *Fusarium* species on the green turtle and hawksbill turtle eggshells in Peninsular Malaysia and confirms *F. falciforme, F. keratoplasticum, F. lichenicola* and *F.* cf. *solani* as members of the FSSC on the molecular level. In order to better understand, further study is needed to investigate the fungi fully on the turtle nesting beaches and in the hatcheries in Malaysia. We need to assess the diversity and population structure of *Fusarium* species associated with sand, ocean and sea turtle nests as well as environmental factors that favour this opportunistic pathogens. We hope that our findings will aid in the development of conservation programmes and increase the awareness of potential pathogens threatening endangered sea turtle species.

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