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Nest microbiota and pathogen abundance in sea turtle hatcheries

Daphne Z. Hoh [a, b, c], Yu-Fei Lin [a], Wei-An Liu [a], Siti Nordahliawate Mohamed Sidique [d], Isheng Jason Tsai [a, b, *]

[a] Biodiversity Research Center, Academia Sinica, 115 Nangang, Taipei, Taiwan
[b] Biodiversity Program, Taiwan International Graduate Program, Academia Sinica and National Taiwan Normal University, Taipei, Taiwan
[c] Department of Life Science, National Taiwan Normal University, 116 Wenshan, Taipei, Taiwan
[d] Laboratory for Pest, Disease and Microbial Biotechnology, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia

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ABSTRACT

Hatchery practices are pivotal to conservation success. In sea turtle hatcheries, reusing sand is a norm, but it remains unclear whether such an approach increases the risk of *Fusarium solani* species complex (FSSC) infection and mortality in sea turtle eggs. We employed 16S and ITS amplicon sequencing in sands and isolated *Fusarium* strains from diseased eggs across seven turtle hatcheries and their neighbouring beaches in Malaysia. FSSC was isolated from all sampled hatcheries, and *Fusarium falciforme* was the predominant species (82.9%). All but one hatchery had a distinct microbiota and higher abundance of FSSC (mean = 5.2%) than the nesting beaches (mean = 1.3%). The hatchery that maintained the most stringent practice by not reusing sand had a microbiota resembling that of nesting beaches, and the lowest FSSC abundance. The results of the current study imply that sand should not be reused in sea turtle hatcheries.

1. Introduction

The establishment of hatcheries has increased the conservation success of endangered sea turtles. For instance, hatcheries can reduce egg poaching, serve as a shelter from extreme weather, and facilitate conservation management (Limpus et al., 1979; Mortimer, 1999). However, hatcheries are an example of "all one's eggs in one basket", in which a single incident can destroy all the eggs (Pritchard, 1980). Hence, hatcheries must maintain strict practices from their initiation (Mortimer, 1999). To maximize hatching success, extensive research efforts have been carried out on egg handling procedures (Limpus et al., 1979; Parmenter, 1980) and incubation conditions (McGehee, 1990; Ackerman, 1996; Hewavisenthi and Parmenter, 2001). Despite variation and constant revisions to these practices, no consensus has been reached on suitable substrates and environments for sea turtle egg incubation in a hatchery setting. Provided that the eggs from a nesting beach are properly handled, relocating nests to a hatchery is a good strategy for improving hatching success (Clusella Trullas and Paladino, 2007). Nonetheless, human interference in natural processes such as egg relocation is usually discouraged (Mortimer, 1999). One potential anthropogenic impact is that pathogens can contaminate eggs in the hatchery. Yet, little is known about the link between the relative abundance of pathogens in the environment and disease prevalence on eggs in sea turtle hatcheries.

Fungi have previously been isolated from the eggs of sea turtles such as *Fusarium solani*, *Pseudallescheria boydii*, *Fusarium oxysporum*, and several *Aspergillus* spp. (Phillott et al., 2001; Elshafie et al., 2007; Sarmiento-Ramírez et al., 2010). Of these, the most frequently isolated and studied species are *Fusarium falciforme* and *Fusarium keratoplasticum*, which belong to the *Fusarium solani* species complex (FSSC; O'Donnell et al., 2008). These two pathogenic species are responsible for sea turtle egg fusariosis (STEF; Smyth et al., 2019), and are known causative agents in other host species—including human, animals, and plants (Zhang et al., 2006; Jain et al., 2011; Fernando et al., 2015; Vega-Gutiérrez et al., 2019). Some of the first detailed studies regarding fungal pathogens on unhatched sea turtle eggs were reported in Australia. These fungi were initially identified as *F. solani* based on morphology (Phillott et al., 2001, 2004; Phillott and Parmenter, 2001; Phillott, 2002),

* Corresponding author. Biodiversity Research Center, Academia Sinica, 115 Nangang, Taipei, Taiwan.
E-mail address: ijtsai@gate.sinica.edu.tw (I.J. Tsai).

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but molecular methods have since revealed that this fungal pathogen can be distinguished into the two species mentioned above. During the initial stage of infection, sea turtle eggs with fusariosis develop abnormal colours such as pink-reddish, purple, yellow, and blue spots on the inner and outer eggshell surfaces, and turn blackish during advanced stages of infection, covering almost the whole egg (Fig. 1; Sarmiento-Ramírez et al., 2014). This emerging fungal disease has been isolated from unhatched sea turtle eggs with STEF symptoms from most continents in recent years (Sarmiento-Ramírez et al., 2014; Rosado-Rodríguez and Maldonado-Ramírez, 2016; Sidique et al., 2017).

Microbes in the incubation environment play a crucial role in sea turtle embryo development. The fungal pathogen *F. keratoplasticum* was observed to directly infect living loggerhead turtle (*Caretta caretta*) eggs. Additionally, sea turtle eggs may have died prior to disease development from other causes involving the indirect influence of microbes. The substrate microbiota was suggested to cause unfavourable incubation conditions such as high temperature and low oxygen availability (Bézy et al., 2015), and microbes covering the egg surface are believed to inhibit gas exchange in the egg (Phillott and Parmenter, 2001). Fungi subsequently utilize the organic materials from the dead eggs. Uncertainty remains as to which case is more prevalent under the setting and practice of relocating eggs into new hatcheries. Most of the observed infections occur at natural nesting beaches (Phillott et al., 2004; Sarmiento-Ramírez et al., 2014) and few have been reported in hatcheries (Patino-Martinez et al., 2012; Sidique et al., 2017). However, it is still unclear to what extent these pathogens, which are ubiquitous in both natural and incubation environments, affect sea turtle eggs.

To improve disease management, an increasing number of studies are being carried out to determine how the relative abundance of pathogens within the microbiota relates to disease prevalence. These studies typically attempt to limit the abundances of specific pathogens in the environment. Examples range from the characterization of microbial communities in agricultural farms after suppressive soil is used (Shiomi et al., 1999; Weller et al., 2002) to post-probiotic treatment in an oyster hatchery (Stevick et al., 2019). Previous studies on the microbial community in sea turtle nesting beaches focused on the *arribada* event, which is the mass nesting of Olive Ridley turtles (*Lepidochelys olivacea*) in Costa Rica (Honarvar et al., 2011; Bézy et al., 2015). These studies have primarily aimed to determine the association between microbial abundance or richness and hatching success. Since it is believed that FSSC infection begins in the sand (Patino-Martinez et al., 2012), and eggs that are artificially incubated using sand from nesting beaches exhibited symptoms of fungal colonization (Phillott and Parmenter, 2001), there is a need to determine the relative abundances of pathogens and microbial community structure in the environment to improve egg incubation techniques. Past studies have successfully cultured FSSC from nest sand and debris from nest surroundings (Rosado-Rodríguez and Maldonado-Ramírez, 2016; Sidique et al., 2017). However, the ubiquitous nature of FSSC (Zhang et al., 2006) suggests that successful infection depends on how much natural nests experience environmental stressors such as tidal inundation and unsuitable substrates (Sarmiento-Ramírez et al., 2014). One unintended anthropogenic stressor may be the re-establishment of nests in hatcheries, and little is known about how this contributes to disease occurrence.

In Peninsular Malaysia, sea turtle eggs laid on natural nesting beaches are mostly relocated to the nearest *ex-situ* hatchery to reduce poaching (Mortimer et al., 1993; Chan, 2006). However, these hatcheries typically reuse the same sand within the fenced hatchery for several nesting seasons, and this may allow FSSC to accumulate. This may be the reason behind the recent discovery of STEF in some hatcheries (Sidique et al., 2017). It has long been suspected that sand containing egg residues from previous seasons acts as a reservoir for pathogens or toxic by-products (Pritchard, 1980). We hypothesized that microbial communities and FSSC abundance in the sand of egg chambers in hatcheries that reuse

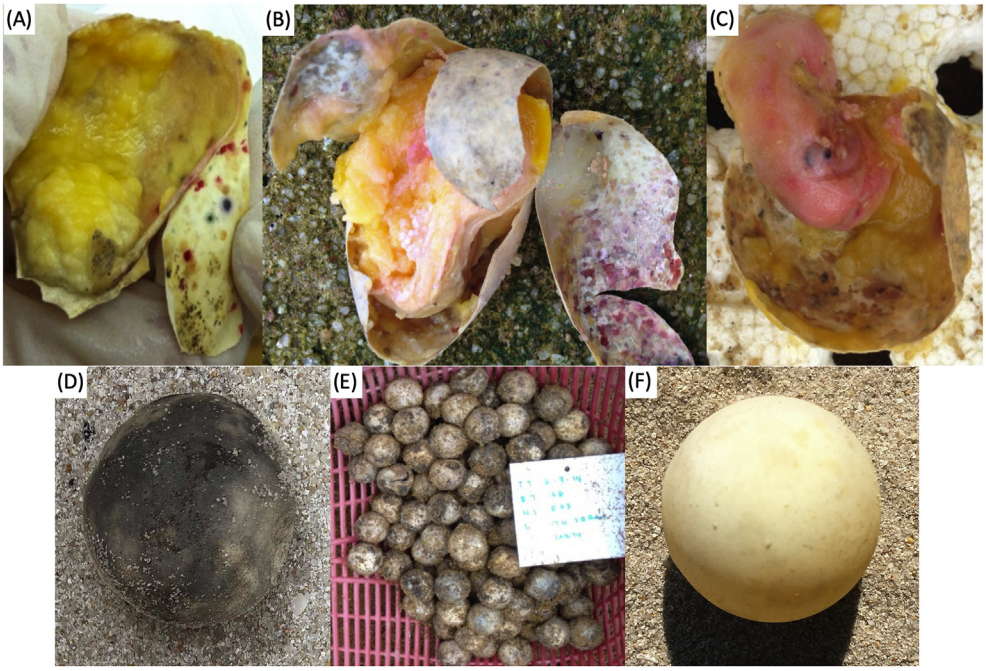


Fig. 1. Morphological symptoms of STEF on unhatched eggs. (A–C) Abnormal coloration showing pink to reddish, purple, yellow, and blue spots on the eggshell, yolk, and dead embryo. (D–E) Advanced sign of infection showing blackish mat covering almost whole egg. (E) Whole clutch of nest with low hatching success exhibited serious signs of infection. (F) Symptomless unhatched egg.

sand differ from those of natural nesting beaches. In this study, we investigated whether FSSC can be identified from diseased eggs across sea turtle hatcheries of Peninsular Malaysia. We profiled and compared the microbial composition of sands collected in hatcheries and their residing nesting beaches. Together, this study identifies how microbiomes differ based on hatchery practices and offers new insights to inform conservation practices in sea turtles.

2. Materials and methods

2.1. Site description and sampling

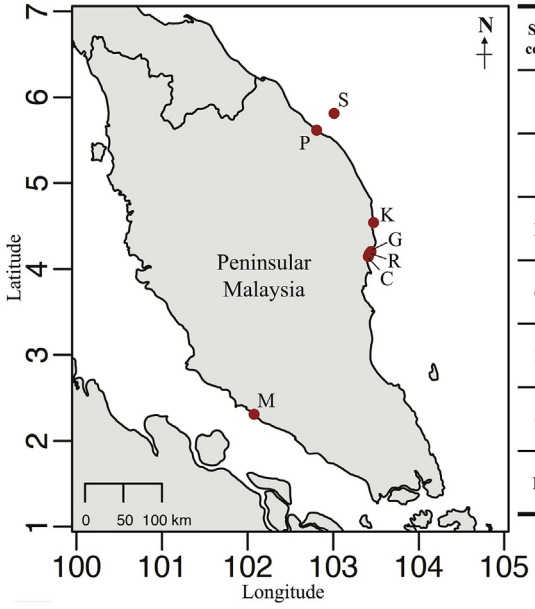
We collected samples from seven sea turtle hatcheries across Peninsular Malaysia in July 2018. Sampling permissions were obtained through verbal consent for all sites. Descriptions of these sites are shown in Fig. 2. Information on nest density and clutch size of each hatchery was estimated based on available reports (Table S1). Sampled nests from all hatcheries were of green sea turtles (*Chelonia mydas*) except the Padang Kemunting (M) site, which was made up of hawksbill turtles (*Eretmochelys imbricata*). A total of 123 sand samples from turtle nests of each hatchery (henceforth referred to as hatchery sand) and the nearest turtle nesting beach to each hatchery (control sand) were collected. Hatchery sands were collected within 2 days of hatchlings emerging from a nest. The only exception was the Geliga (G) site, where permission was given to collect sands only from nests with no successfully hatched eggs. Before each nesting season, the Penarik (P) site randomly relocates the hatchery to a new part of the nesting beach for egg incubation, making it the only hatchery that does not reuse sand from the previous season. All control sands were collected approximately 60 cm deep from the beach surface to mimic natural nesting settings and avoid areas with vegetation. Two to three technical sample replicates were collected from each nest in the hatchery or nesting beach. All sand samples were stored at −20 °C until DNA extraction, except during transportation. Samples were brought into Taiwan with permission from the Bureau of Animal and Plant Health Inspection and Quarantine under permit No. 107-F-501.

2.2. Isolating and identifying Fusarium strains from diseased eggs

For each excavated nest, we randomly collected three unhatched eggs with symptoms of *Fusarium* infection as previously described (Fig. 1, Sarmiento-Ramírez et al., 2014) to isolate *Fusarium* species. Eggshells were rinsed with sterilized distilled water to remove sand particles, cut into approximately 1 × 1 cm using sterilized scissors and incubated on Potato Dextrose Agar with 50 μg/mL chloramphenicol at 30 °C. Subcultures were made on the same medium until pure culture was obtained. DNA extraction of pure cultures was performed using ZYMO Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO Research, Irvine, CA, USA, Cat. #D6005). The primer pairs SR6R/ITS4 (Irinyi et al., 2016), LR0R/LR6 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994; Sarmiento-Ramírez et al., 2014), and fRPB2-7cF/fRPB2-11aR (Liu et al., 1999) were used to run PCR amplification of the nrDNA internal transcribed region, large subunit of the ribosomal DNA, and RNA polymerase II (*RPB2*) gene region, respectively, for all isolates. PCR conditions followed Liu et al. (1999) for all primers. Phylogenetic analysis was performed on all isolates along with selected *Fusarium* sequences obtained from NCBI GenBank nucleotide database (Table S2) and Sarmiento-Ramírez et al. (2014; Table S3). *Fusarium* sequences were first aligned and trimmed for each of the three gene regions respectively as described below, and processed sequences were concatenated before performing phylogenetic analysis. Sequences were aligned using MAFFT for 1000 iterations (v7.310; Katoh and Standley, 2013), then trimmed by trimAl (v1.2rev59; Capella-Gutiérrez et al., 2009). The final length of the concatenated sequence was 2814 bp. Phylogenetic analysis was performed using IQ-TREE (v1.6.6; Nguyen et al., 2015) with 1000 ultrafast bootstraps (Hoang et al., 2018) under the model TIM2e+R3 (Kalyaanamoorthy et al., 2017). Tree visualization was made with FigTree software (v1.4.4; Rambaut, 2018).

2.3. DNA extraction and amplicon library generation for high-throughput sequencing

Total genomic DNA was extracted from sand samples using



Site code	Site	Hatchery type	Host species (egg)	Mean hatching success (%)	Hatchery manager
S	Chagar Hutang, Redang Island	*In-situ*	*Chelonia mydas*	87.6	SEATRU
P	Penarik Beach, Terengganu	*Ex-situ*	*Chelonia mydas*	91.4	WWF
K	Ma Daerah TH, Terengganu	*Ex-situ*	*Chelonia mydas*	81.5	DoF & WWF
G	Geliga TH, Terengganu	*Ex-situ*	*Chelonia mydas*	*0	DoF
R	Rimbun Dahan TH, Terengganu	*Ex-situ*	*Chelonia mydas*	83.5	Private
C	Cherating TH, Pahang	*Ex-situ*	*Chelonia mydas*	69.6	DoF
M	Padang Kemunting TH, Melaka	*Ex-situ*	*Eretmochelys imbricata*	58.5	DoF &WWF

Fig. 2. Map, location, and metadata of the sampled sea turtle hatcheries and neighbouring beaches in Peninsular Malaysia. Mean hatching success was calculated from sampled nests only. *Asterisk indicates that no eggs hatched successfully at this site. TH, turtle hatchery; SEATRU, Sea Turtle Research Unit, Universiti Malaysia Terengganu; WWF, World Wide Fund for Nature, Malaysia; DoF, Department of Fisheries Malaysia.

DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany, Cat. #12888) with minor modifications to the manufacturer's instructions. One gram of homogenized sand was used in each extraction. PowerLyzer 24 homogenizer (MoBio Laboratories, Carlsbad, CA, USA, Cat. #13155) was set to 5000×*g* for 10 min. DNA was eluted with 60 μL of Solution C6. Bacterial 16S rRNA and fungal internal transcribed spacer (ITS) amplicons were generated using tagged primer pairs V3/V4 (Kozich et al., 2013) and ITS3ngs(mix)/ITS4 (Tedersoo et al., 2014), respectively. PCR reaction and thermocycling conditions were as previously described (Tedersoo et al., 2014). Positive and no-template controls (elution buffer) were included in the PCR reaction and amplicon pool. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research Corporation, Irvine, CA, USA, Cat. #D6305) was used as the mock community control in 16S amplicon preparation. For ITS amplicons, equal DNA concentrations of *F. solani*, *Mycena venus*, and *Mortierella elongata* were used as single organism control. Amplicons were normalized with SequalPrep Normalization Plate 96 Kit (Invitrogen Corporation, Carlsbad, CA, USA, Cat. #A10510-01). Normalized amplicons were pooled at equal volumes and concentrated using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA, Cat. #A63881). An ITS amplicon library was prepared using the Illumina TruSeq DNA Prep Kit. Sequencing was performed by the NGS High Throughput Genomics Core at Biodiversity Research Center, Academia Sinica, Taiwan. All amplicon libraries were sequenced using Illumina MiSeq with paired-end 2 x 300 bp chemistry.

2.4. Sequence data processing

Raw FASTQ sequences were demultiplexed according to their respective barcode using sabre (v1.0; https://github.com/najoshi/sabre), allowing for one nucleotide mismatch. Adaptors and primer sequences were trimmed using USEARCH (v10; Edgar, 2010). Sequence reads were processed following the UPARSE pipeline (Edgar, 2013). In brief, forward and reverse reads were merged and filtered using the default settings in USEARCH. Operational taxonomic unit (OTU) clustering at 97% sequence identity and minimum OTU abundance was set to 8 during the OTU clustering to denoise the data and remove singletons. An OTU table was created using the *usearch_global* option. OTUs were classified into taxa against the RDP training set (v16) and UNITE database (v7.1) for 16S and ITS reads, respectively, using the SINTAX algorithm (Edgar, 2016, 2018).

2.5. Fusarium solani species complex (FSSC) species-level analyses

ITS region sequences were not sufficient for species identification, especially within the *F. solani* species complex, so 38 OTU sequences assigned as Nectriaceae were manually re-identified by comparing them against the NCBI nucleotide database using BLAST with e-value cutoff of less than $1e^{-100}$ and sequence identity of more than 98%. BLAST results indicate that four of the 38 OTUs were three *F. solani* and one *F. keratoplasticum*. These four OTUs were then grouped as a single OTU in FSSC (Short et al., 2013) for subsequent analyses of microbial community since they are known to infect sea turtle eggs (Sarmiento-Ramírez et al., 2014; Smyth et al., 2019).

2.6. Microbiota community analyses

Prior to analysis, the validity of sequence data was confirmed by comparing the known identity of positive controls against the taxonomic classification results from the UPARSE pipeline. R package *decontam* was used to remove contaminants on both 16S and ITS datasets using the "prevalence" method (Davis et al., 2018). Technical replicates were merged because there was no significant variation in their microbial compositions. Unclassified ITS OTU sequences were manually curated by comparing against the NCBI nucleotide database using BLAST. Non-fungal eukaryotic OTUs were then removed from further analyses. The bulk of the analyses were carried out using R-Studio (v1.1.463; Rstudio Team, 2016). Amplicon data were analysed with the *phyloseq* package (v1.28.0; McMurdie and Holmes, 2013). For beta diversity analysis, OTUs that appeared fewer than three times in at least 20% of all samples were filtered from the analysis. Distances between samples were calculated using Bray-Curtis dissimilarity. A heatmap was plotted using *pheatmap* package (v1.0.12; Kolde, 2015) using the "correlation" option to measure distances and perform sample clustering. Differentially abundant taxa were determined using DESeq2 (v1.24; Love et al., 2014) at the genus and OTU levels for bacterial and fungal amplicons, respectively. Sample filtering criteria for differentially abundant taxa were the same as in the beta diversity analysis, except that hatchery sand samples from site P were omitted.

Having determined differentially abundant OTUs using DESeq2 analyses, we further constructed correlation networks to determine their relationships. Fastspar (v0.0.9; Watts et al., 2019); a Sparse Correlations for Compositional Data (Friedman and Alm, 2012) algorithm was used to estimate correlations between each bacterial genus and fungal OTUs from the combined 16S and ITS compositional data. The output matrices were filtered to keep correlation value > 0.3 and p-value < 0.05. Networks were visualized using Cytoscape (v3.7.2; Shannon et al., 2003).

2.7. Data availability

Sequences of all isolates were deposited into NCBI GenBank as listed in Table S4. Amplicon sequence data were deposited in the NCBI Sequence Read Archive under BioProject PRJNA560331.

3. Results

3.1. Data characteristics

To determine the effect of reusing sand on microbiota structure and pathogen abundance, we sampled 63 *Fusarium*-infected unhatched eggs (three from each sampled nest) and 123 sand samples (63 hatchery and 60 control) from seven sea turtle hatcheries across Peninsular Malaysia (Fig. 2). All sampled hatcheries were *ex-situ* except Chagar Hutang (S), which is the only hatchery that uses *in-situ* practices. Among the hatcheries, Penarik (P) relocates to a new location every nesting season, making it the only hatchery that does not reuse the same sand for egg incubation. All sampled nests contained unhatched eggs with symptoms of fungal colonization as previously described (Sarmiento-Ramírez et al., 2010). Molecular characterization of isolated fungi identified 82 isolates, and the *F. solani* species complex (FSSC) was identified from all sampled hatcheries (Table S4; Fig. S1). Of these, 68 (82.9%) were *F. falciforme*, 12 (14.6%) were *F. keratoplasticum*, one *F. oxysporum*, and one *Fusarium* sp.

3.2. Distinct microbial community structure between beach and hatchery sands

Overall, sand microbiota in sea turtle nesting beaches and hatcheries were composed of 7267 bacterial and 3229 fungal OTUs. Phyla compositions of bacteria and fungi were similar across all sand types (Fig. S2). Bacterial species richness and community evenness were lower in hatcheries than nesting beach sands (Fig. 3A). The nesting beaches had on average 764.5 fewer bacterial OTUs than the hatcheries. The most dominating bacterial phylum

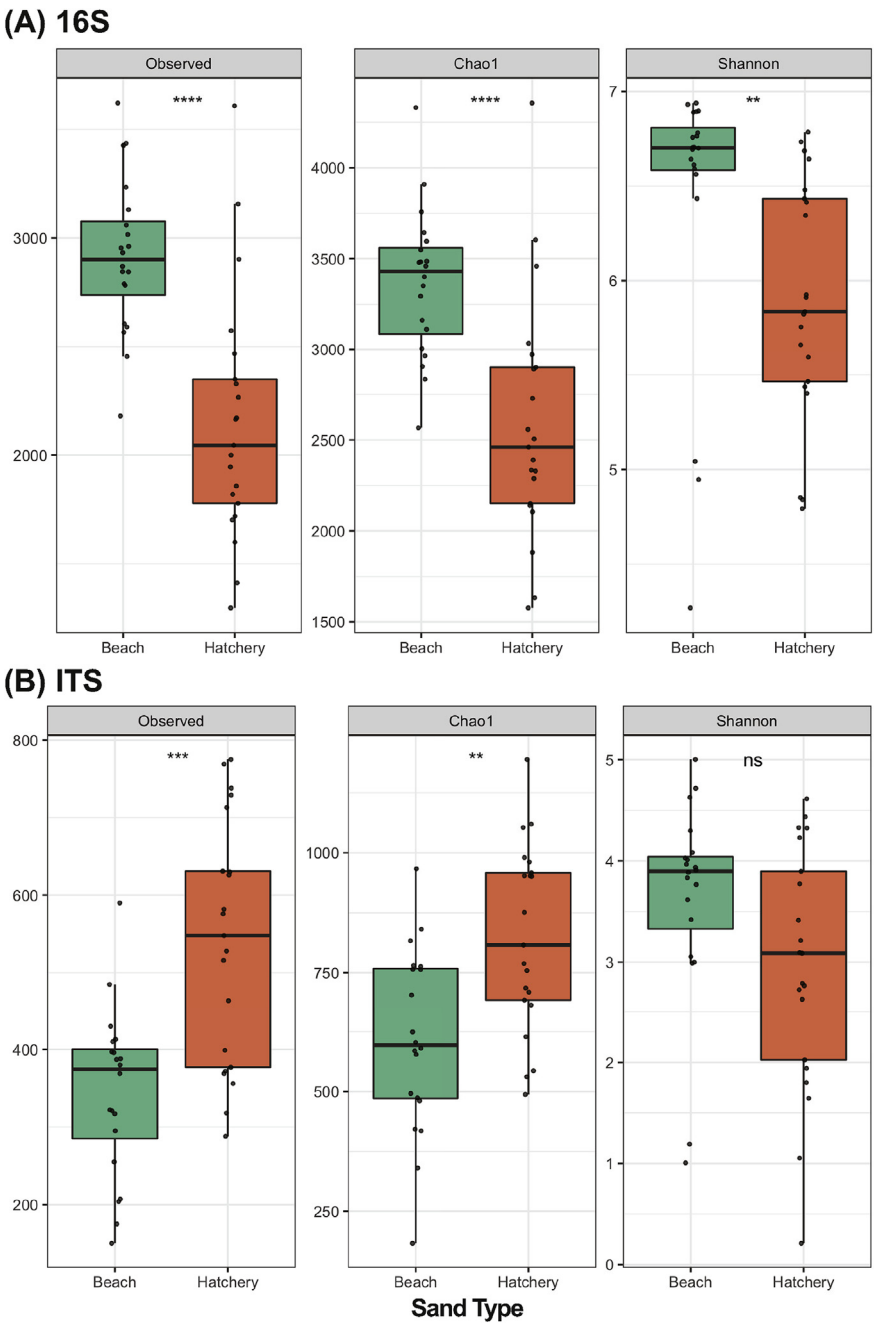


Fig. 3. Alpha diversity measures of bacterial (A) and fungal (B) community in different sand types. Statistical significance was calculated using unpaired-Wilcoxon test between sand types for each measurement (ns: $P > 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

across all samples was *Proteobacteria* (averaging 36.6%), followed by unclassified bacteria (15.4%) and *Firmicutes* (13.3%) (Fig. S2A). Most bacterial phyla were significantly different in relative abundance across sand types (Fig. S3A). In particular, *Bacteroidetes* was almost twice as abundant in hatcheries (13.3%) than nesting beaches (5.7%). This was due to an elevated *Bacteroidetes* relative abundance of over 20% in four nests from three hatcheries (MH2, MH3, SH1, and RH1; Fig. S2A), but no single *Bacteroidetes* OTU was responsible for the increased proportion. In the case of fungi, the phylum Ascomycota had the highest mean relative abundance (37.7%), consistent with the frequent observation of dominant fungal species present in soil communities (Egidi et al., 2019), followed by unclassified fungi (36.4%) and Basidiomycota (22.3%) (Fig. S2B). In contrast to the bacterial community, hatchery sand harboured a higher fungal richness but lower community evenness (Fig. 3B). The hatcheries had on average 193.7 more fungal OTUs than the beach. Mean relative abundances of four major fungal phyla were similar between sand types (Fig. S3B). Together these suggest large inter- and intra-site variation.

Both bacterial and fungal community structures showed significant differences between sites and sand types (permutational analysis of variance (PERMANOVA) of Bray–Curtis beta diversities; Table S5). Inter-site differences explained 26% of fungal taxonomic variations (permutation test, $P < 0.001$, 999 permutations) followed by 18% between nesting beaches and hatcheries ($P < 0.001$). The major inter-site variation were seen in four nests and three beach

samples in hatcheries G and P, which showed higher abundances of Basidiomycota than Ascomycota due to the dominance of a single OTU, *Inocybe* sp., ranging from 49.7% to 97.5%. A principal coordinate analysis plot shows that these samples were separated from others (Fig. 4). Site M hatchery harboured a different host species (*E. imbricata*) than the other sites (*C. mydas*), and contributed a significant host effect in bacterial but not fungal community composition (Table S6).

In addition to PERMANOVA (Table S5), Fig. 4 also demonstrated that variations in microbial communities of sand also depended on whether they originated from hatcheries or nesting beaches. Compared to five *ex-situ* hatcheries that reuse hatchery sand, hatchery P stood out because it changes location every season and exhibited a similar bacterial community to its nearby nesting beach (Fig. S4). Statistical tests showed significant difference in bacterial community composition between hatcheries and nesting beaches (after removing hatchery P sands; ANOSIM R = 0.92, $P = 0.001$; PERMANOVA $R^2 = 0.36$, $P < 0.001$; betadisper $P = 0.2$). In the case of the fungal community, PC1 (21.4%) set apart the outlier samples which contained a high proportion of *Inocybe* sp. while PC2 (15.5%) separated hatcheries and nesting beaches (Fig. 4B; Fig. S4B). After excluding samples with a high proportion of *Inocybe* sp., fungal community composition and dispersion were significantly different between sand type (ANOSIM R = 0.79, $P = 0.001$; PERMANOVA $R^2 = 0.18$, $P < 0.001$; betadisper $P = 0.04$).

3.3. Differentially abundant taxa in hatchery sands

Relative abundances of the top ten major bacterial or fungal taxa clustered the samples based on sand type—except for hatchery P, which grouped with nesting beaches (Fig. 5). The FSSC pathogens, *Pseudallescheria boydii,* and an unclassified fungus (OTU5) were present in high abundances in all hatcheries except for P. The public database matched OTU5 to the order Capnodiales, which contains fungal species that are plant pathogens (Crous et al., 2009). *Saccharomyces cerevisiae* was most frequently detected in the S and M nesting beaches. Interestingly, nesting beach S had a relatively higher abundance of various *Bacillus* spp. (mean 9.7% vs. 7.2% average in all samples) and FSSC (3.0% vs. 1.4%) compared to other nesting beaches.

Analysis of differentially abundant taxa through DESeq2 analysis revealed 168 bacterial genera and 54 fungal OTUs were significantly more abundant in hatchery sand (Fig. S5 and Fig. 6). Conversely, nesting beaches harboured 89 and 100 higher abundance of bacterial genera and fungal OTUs, respectively. The aforementioned *P. boydii* and Capnodiales sp. were indeed significantly more abundant in the hatcheries ($P = 4.3e^{-15}$ and $5.8e^{-11}$, respectively). Interestingly, FSSC was not significantly more abundant in hatcheries ($P = 0.5$). Upon closer inspection, FSSC also exhibited relatively high proportions in the beach sand of sites S (mean 3.0%) and C (mean 2.4%), implying that this fungal group can have high abundance in nature. Further independent analysis supported the differential abundances of taxa in different sand types. For instance, multivariate analysis also showed FSSC and *P. boydii* had high abundances in hatchery sands (Fig. S6).

3.4. Higher abundance of FSSC and P. boydii in reused sand

FSSC had a higher mean relative abundance in hatcheries than nesting beaches (excluding hatchery P; unpaired-Wilcoxon test, nesting beach vs. hatchery, 1.3% vs. 5.2%, $P = 0.0015$; Fig. 7). FSSC comprised 94.9% of all *Fusarium* sequences in the hatcheries. *P. boydii* also showed higher mean relative abundance in hatcheries (excluding hatchery P; unpaired-Wilcoxon test, nesting beach vs. hatchery, 0.3% vs. 11.5%, $P = 7.1e^{-10}$; Fig. 7). *P. boydii* was consistently higher in abundance across hatcheries (most samples are at least > 5%) and on average 6.4% higher than FSSC. To confirm that there was a true interaction between these two species and the correlation was not due to data dependence (Friedman and Alm, 2012), we constructed co-occurrence networks using combined compositional data on bacterial and fungal species (Fig. S7). However, no direct interaction was found between FSSC and *P. boydii* in nesting beach or hatchery sands.

4. Discussion

Previous reports have shown that fungal pathogens present in nesting beaches can infect sea turtle eggs and cause massive mortalities (Phillott et al., 2004; Sarmiento-Ramírez et al., 2014). Relocated eggs that come into contact with sand can also be contaminated (Phillott and Parmenter, 2001; Patino-Martinez et al., 2012; Sidique et al., 2017). To determine if reusing sand has an effect on disease prevalence, we determined the relative differences in microbial composition and FSSC abundance in sand across seven sea turtle hatcheries and neighbouring nesting beaches in Peninsular Malaysia. Our findings revealed that all hatcheries that practiced sand reusage shared a microbiota that was clearly different from those of neighbouring nesting beaches. By relocating hatchery (i.e. not reusing sand), hatchery P exhibited the lowest relative abundance of FSSC and at the same time, had highest hatching success of the hatchery nests when compared to other hatcheries. We also qualitatively observed fewer eggs that

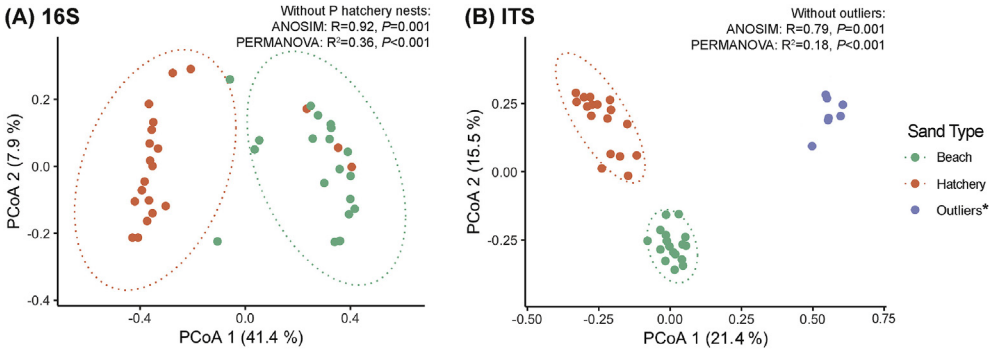


Fig. 4. Principal Coordinate Analysis plots measured by Bray-Curtis distance of (A) bacterial and (B) fungal communities. Each dot represents one site and different colours denote sand type. Dotted lines represent 95% confidence interval within each category (hatchery or nesting beach). Multivariate statistical analyses ANOSIM and PERMANOVA were used to test the community differences between sand types. *Asterisk denotes a subset of samples containing high relative abundance of *Inocybe* sp.

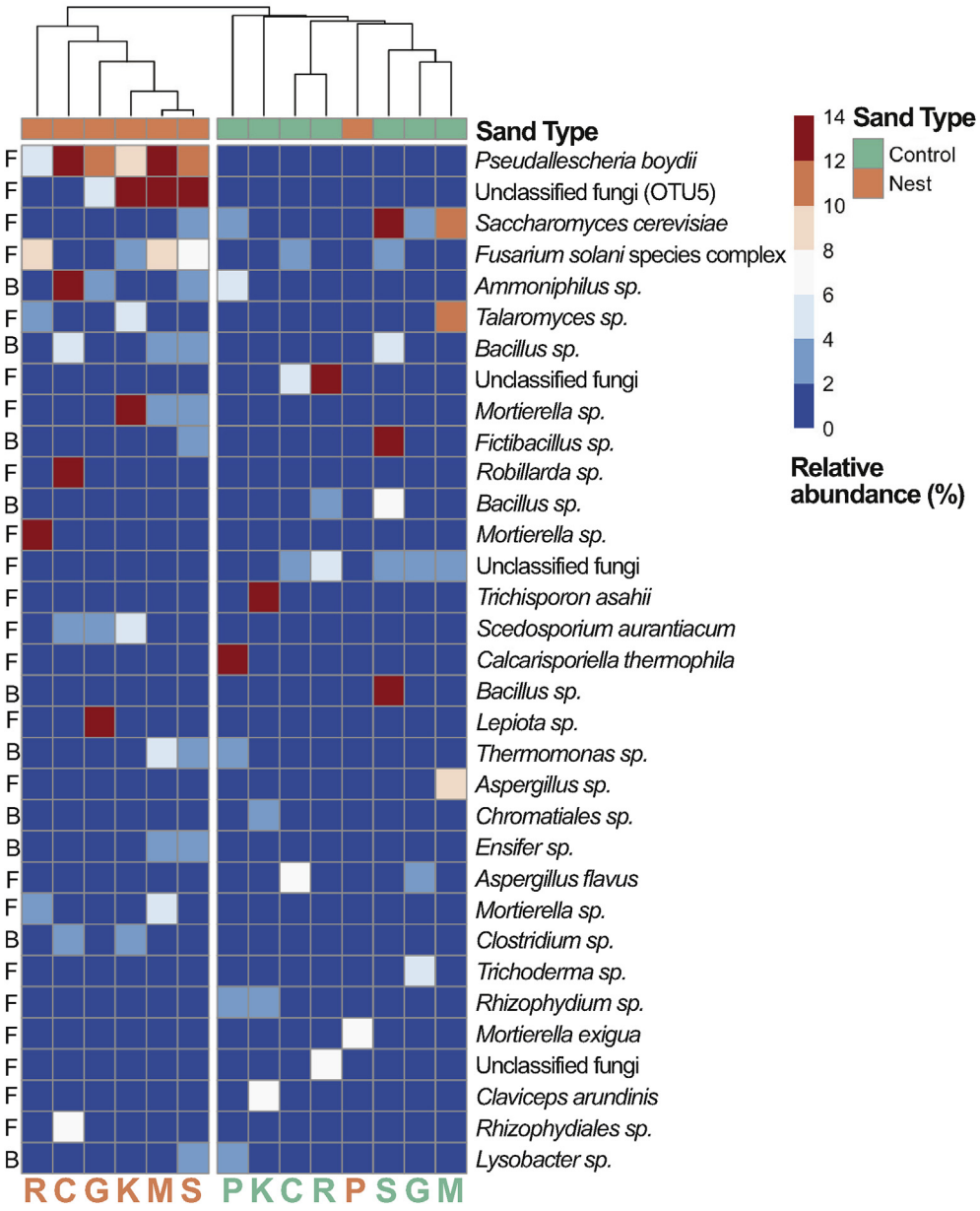


Fig. 5. Heatmap of relative abundance (%) of top ten major bacterial and fungal OTUs from each site totalling 33 OTUs. Colour of each box denotes the mean relative abundance of OTU in corresponding sites. F, fungal; B, bacterial.

exhibited STEF symptoms in hatchery P. Nonetheless, as sample replication cannot be performed at site P, additional surveys should be carried out to strengthen the current results. In addition, higher abundances of several *Bacillus* spp. and FSSC were observed in the nesting beaches of hatchery S, the only *in-situ* hatchery sampled in this study (Fig. 5). One possible explanation is that high nesting turnouts altered the microbiota more drastically in a short nesting beach (approximately 350 m) with high nesting density (mean of 1150 nests per year; Chan, 2010).

The enclosed hatchery area may be a nutrient-rich environment for potential pathogens. Similar scenarios have been observed for other fungal diseases in animals and plants. The fungal pathogen that causes white-nose syndrome in bats, *Pseudogymnoascus destructans*, was detected in the soil of ground environments where the bats hibernate (Lindner et al., 2011). In crop fields, pathogenic *Fusarium* spp. continue their life cycle in crop residues or debris, which then serve as sources of inoculum in the following season (Cobo-Díaz et al., 2019). Reusage of sand will thus become a reservoir for FSSC or other potential pathogens to accumulate and infect eggs in subsequent nesting seasons. Our assessment of microbial abundance was determined post-hatchling emergence and nest excavation to avoid egg disturbance. Hence, the relative abundance of FSSC in hatcheries was a result of infection *a posteriori*. A more detailed assessment of FSSC abundance beginning from oviposition, incubation and up to nest excavation should provide a clearer understanding on STEF establishment. A previous study suggested that fungal hyphae spread from diseased eggs to adjacent viable eggs (Phillott and Parmenter, 2001), signifying that initial establishment is key to successful infection. Initial fungal density could be reduced by applying suitable sand treatments to increase hatching success (Bézy et al., 2015).

The major factor affecting fungal community composition in our study was inter-site environmental differences, which is consistent with a previous report that diversity and dominance in fungi can be

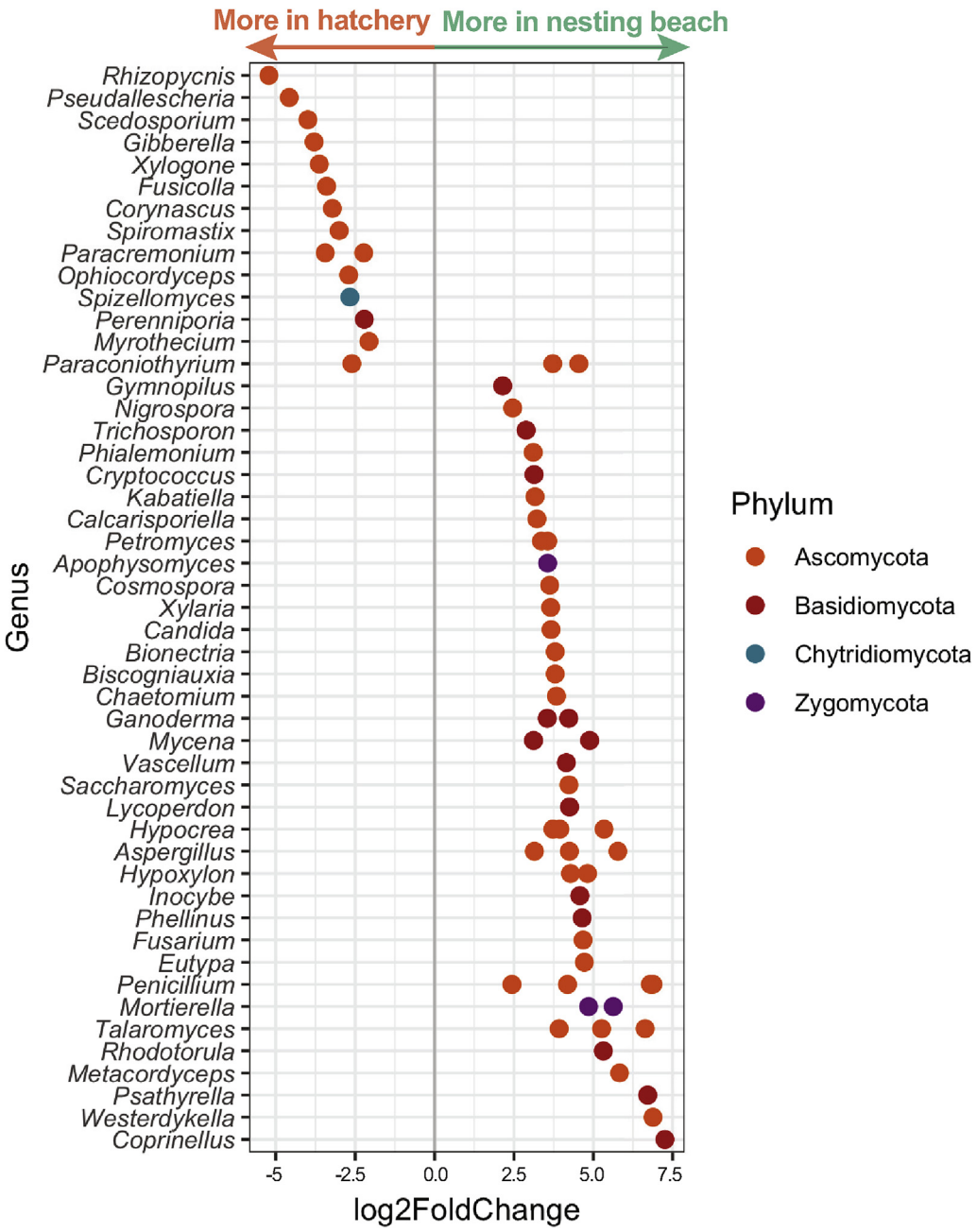


Fig. 6. DESeq2 analysis of differentially abundant fungal OTUs in different sand types. Each dot denotes one OTU. OTUs that were unable to be classified at genus level were excluded.

more heterogenous between closer sites than it was for bacteria (Peay et al., 2016). Nevertheless, we identified distinct differences in sand microbiota between hatcheries and surrounding nesting beaches; FSSC and *P. boydii* were more abundant in the sand of hatcheries than nesting beaches. The outcome of this study may inform hatchery managers in terms of sand usage. In addition to accumulation of pathogens, we speculate that the differences were due to disparate particle size distribution of the substrates which incubated the eggs (Sessitsch et al., 2001). Other possible factors include environmental fluctuations on the nesting beach (Bézy et al., 2015) and the addition of cloacal fluid during oviposition (Phillott et al., 2002). Further experiments are needed to specifically detangle these confounders.

FSSC is commonly found in the environment (Zhang et al., 2006), and has been found along with other *Fusarium* species in natural nesting beaches. We detected a higher abundance of FSSC across hatcheries and in some of the nesting beaches, suggesting that additional factors may contribute to the overall accumulation of this species complex. Differences in the geographical distribution of *F. falciforme* and *F. keratoplasticum* were observed. Previous reports in some of the main sea turtle nesting beaches in the Indian and Atlantic Oceans have shown that *F. keratoplasticum* is predominant (Sarmiento-Ramírez et al., 2014, 2017), but the current study and a recent survey (Sidique et al., 2017) found a higher isolation frequency of *F. falciforme* in Malaysia. More studies on differences in environmental factors are needed to understand the prevalence of pathogenic species across these nesting regions. Our study further revealed different abundances in FSSC species across

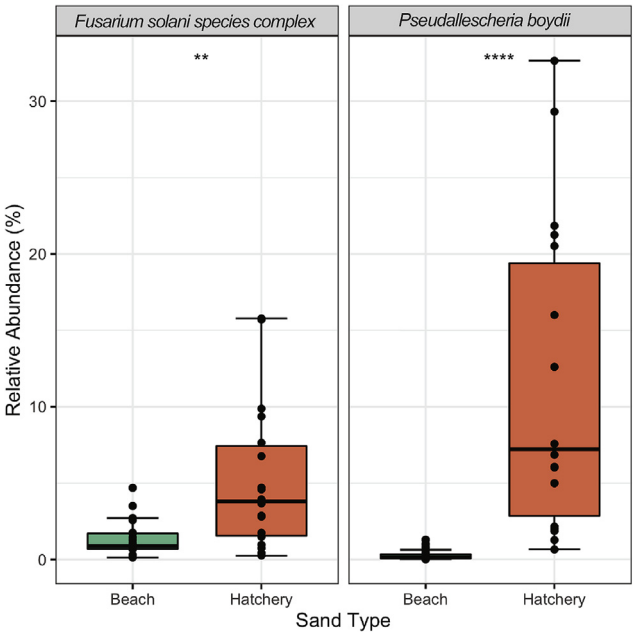


Fig. 7. Boxplots showing relative abundance (%) of *Fusarium solani* species complex and *Pseudallescheria boydii* between two sand types. Statistical significance was calculated using unpaired-Wilcoxon test (**$P \leq 0.01$; ****$P \leq 0.0001$). Samples from hatchery P were removed from analysis due to evidently different community structures with other hatchery sand samples.

Peninsular Malaysia. Of the hatcheries surveyed, only the Melaka (M) hatchery had a higher relative abundance of *F. keratoplasticum* (~5% of sequences; data not shown) and higher isolation frequency (Sidique et al., 2017). The origin *F. keratoplasticum* in these environments has been associated with human activities (Zhang et al., 2006), but whether this was the true source is unclear, as all sampled hatcheries have restricted access.

Various studies have shown that both FSSC and *P. boydii* are frequently found in fungal-infected sea turtle eggs (Phillott et al., 2001; Sarmiento-Ramírez et al., 2017). Co-occurrence of these two fungi and the higher prevalence of the latter suggest that *P. boydii* is a more sensitive biomarker for identifying FSSC-contaminated nests, and possibly a fungal pathogen for sea turtle eggs. To date, it has not been proven that *P. boydii* infects sea turtle eggs, but it has been shown to infect veterinary animals (Elad, 2011). A high relative abundance of *P. boydii* was detected in hatchery nests that reused sand, and this warrants further investigation of the species' role in the infection. Interestingly, our co-occurrence network analyses suggest that FSSC and *P. boydii* did not directly interact. This may also suggest that the high occurrence of *P. boydii* may be a post-effect of FSSC infection. Since *P. boydii* is a common soil microbe (Gilgado et al., 2005) and an opportunist pathogen that causes chronic diseases in humans (Travis et al., 1985; Gilgado et al., 2005), we speculate that FSSC infects turtle eggs and *P. boydii* uses the nutrients from dead eggs to proliferate. In addition, the lack of interaction between these two species also suggests that FSSC alone can cause egg infection, which is consistent with the inoculation experiment in Sarmiento-Ramírez et al. (2010). Moreover, it is worth noting the presence of *Scedosporium aurantiacum*, a member of the *P. boydii* species complex (Gilgado et al., 2005), that was highly abundant in some of the hatchery sands (Figs. 5 and 6). This fungus was also previously isolated from hatchery sands (Rosado-Rodríguez and Maldonado-Ramírez, 2016) and diseased eggs (Sarmiento-Ramirez et al., 2017).

Previous findings demonstrated that higher fungal abundance is associated with lower hatching success in *arribada* nests (Bézy et al., 2015). The low hatching success was a ramification of the high microbial activity that caused high temperatures and oxygen deprivation in nests (Clusella Trullas and Paladino, 2007; Bézy et al., 2015). The hatchery nest environment is analogous to that of *arribada* nests. However, whether similar conditions occur in hatcheries warrants further study. Moreover, we found that pathogenic bacteria such as the *Salmonella* and *Pseudomonas* spp. were significantly more abundant in hatchery than beach sands. These species were previously isolated from sea turtle eggs that failed to hatch (Al-Bahry et al., 2009) and recreational beaches (Esiobu et al., 2004; Yamahara et al., 2012), potentially increasing the infection risk to both hatchery personnel and eggs.

5. Conclusions

This study delineated the microbiota and quantified FSSC abundance in the nests of sea turtle hatcheries. Our key findings are that STEF occurrence most probably increased from the practice of reusing sand for egg incubation. This suggests that both turtle eggs and hatchery staffs suffer an increased risk of the infection by FSSC or other opportunistic microbes. Hence, we recommend that sea turtle hatcheries change sand after every nesting season to reduce potential pathogen inoculation, thereby maximizing hatchling production. This work emphasizes that hatchery management must be stringent to maximize conservation impact. For disease control and prevention, future attention needs to be paid to the causes and consequences of microbe abundances and interactions at different stages of infection, not just in nests but also the nesting beaches, due to rapid changing environments.

Author contributions

IJT conceived the study. DZH, YFL, SNS, and IJT designed the study. DZH and SNS performed the sampling. DZH, YFL, and WAL carried out the experiments. DZH and YFL analysed the data. DZH, YFL, and IJT wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.100964.

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