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RESEARCH

Characterising the mycobiome of healthy and wounded reef sharks in the Seychelles archipelago

Alice B. Iddon[1,2] · Rupert Ormond[3,4] · Christopher R. Clarke[5] · Mauvis Gore[3,4] · Catriona Millar[4] · Alessia Bani[1] · Joe D. Taylor[6] · Mark Bulling[1] · Kara Rising[1] · Michael Sweet[1]

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Abstract Sharks frequently sustain cutaneous injuries throughout their lifetimes, yet subsequent infections are surprisingly rare, and wounds appear to heal rapidly. This has led scientists to explore the potential mechanisms behind shark skin's putative 'superior' capacity for fending off infection. Interestingly, research has turned to the skin-associated microbiome for the answer. Although exploration of the bacterial microbiome has led to its proposal as a potential key factor in wound healing, the role of the fungal microbiome (or mycobiome) remains significantly overlooked. Here, we endeavoured to characterise the fungal communities colonising the skin of 46 blacktip reef sharks (*Carcharhinus melanopterus*) and one sicklefin lemon shark (*Negaprion acutidens*) in order to determine whether the presence of wounds corresponded to shifts within their fungal communities. We found that there were no significant differences between the fungal consortia harboured by insulted and intact skin, mirroring findings related to their bacterial counterparts. Further, we demonstrated fluctuating taxonomic profiles and the lack of a substantial 'core' community of fungal members shared across individuals, highlighting the need to disentangle the role of stochastic versus deterministic processes in shaping fungal communities in the shark skin microbiome. Finally, exploring co-occurrence patterns highlighted 21 positive associations between 13 fungal genera. We encourage future research to uncover the role of rare fungal amplicon sequence variants (ASVs) and investigate the ecological consequences of co-occurrence networks in order to elucidate the role of the skin mycobiome in shark health.

Keywords Shark skin microbiome · Mycobiome · Elasmobranch health · Wound ecology · Microbial co-occurrence networks

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✉ Alice B. Iddon
aiddon@sruc.ac.uk

✉ Rupert Ormond
rupert.ormond.mci@gmail.com

Michael Sweet
m.sweet@derby.ac.uk

[1] Aquatic Research Facility, Nature-Based Solutions Research Centre, University of Derby, Derby, UK

[2] Centre for Epidemiology and Planetary Health, Rural and Veterinary Innovation Centre, Scotland's Rural College, Inverness, Scotland, UK

[3] Marine Conservation International, Aros, Isle of Mull, Argyll, Scotland, UK

[4] Centre for Marine Biodiversity and Biotechnology, Heriot-Watt University, Riccarton, Edinburgh, Scotland, UK

[5] Danah Marine Research, Beach Palace, North Obhur, Jeddah, Saudi Arabia

[6] UK Centre for Ecology & Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, UK

Introduction

The ability to recover from wounds whilst thwarting infection appears a crucial adaptation for elasmobranchs (sharks, skates and rays) (Chin et al. 2015). The behavioural ecology of this group predisposes them to frequent injury throughout their lifetime, with copulation, predation attempts and parturition each often resulting in lesions of varying severity (Womersley et al. 2021). Despite the pathogen-rich nature of the marine realm, shark skin appears unparalleled in its

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innate capacity for rapid wound healing and its ability to resist infection even when skin integrity is severely compromised (Pratte et al. 2022).

The mechanistic defence conferred by the dermal denticles (tooth-like structures embedded in the epidermis) has been proposed as the main factor preventing infection in sharks (Pogoreutz et al. 2019; Perry et al. 2021). Research has shown that this is likely facilitated by the creation of a flow boundary that prohibits opportunistic pathogens from settling on the skin surface (Chien et al. 2020). Further, the associated mucus layer is also known to contribute to immune defence through the provision of host-derived antibacterial and regenerative compounds (Kerr et al. 2023). Shark skin mucus specifically protects against pathogenic infection whilst simultaneously conferring conditions that promote microbial colonisation (Caballero et al. 2020). These endogenous mechanical and biological host properties also facilitate a functionally beneficial microbiome that further promotes host health (Black et al. 2021; Doane et al. 2020; Doane et al. 2017). This cutaneous microbiome has been identified as a crucial component in restoring skin integrity within the wounded tissue of many organisms, including sharks (Canchy et al. 2023). Resident members of the skin microbiome are thought to stimulate the release of host-derived antibacterial compounds upon insult, whilst simultaneously synthesising their own antagonistic molecules and outcompeting any potential pathogens for space and nutrients (Canchy et al. 2023).

Research has already begun to shine a light upon the capacity of key bacterial members of the elasmobranch microbiome to fend off infection. For example, bacterial members of key genera, such as *Rhodococcus* isolated from the skin of sharks and *Pseudoalteromonas* from rays and skates, have been shown to produce molecules exhibiting activity against pathogens of both bacterial and fungal origin (Caballero et al. 2020; Ritchie et al., 2017). Further, secondary metabolites produced by bacterial members, including the genus *Marinobacter*, obtained from thresher sharks (*Alopias vulpinus*), have also been indicated as playing a key role in reducing host inflammatory responses (Doane et al. 2017). Interestingly, when exploring across individuals (and different health states), the bacterial fraction of the shark microbiome appeared to be conserved, suggesting strong selection and retention of key members (Pogoreutz et al. 2019).

Although the prokaryote community of elasmobranch skin health state has been explored in relative depth, the resident fungal community (or mycobiome) has received considerably less attention. Indeed, only a handful of animal microbiome studies have attempted to characterise which fungal species are present, and the majority of those have focused solely on their role as agents of mycotic disease(s) (Santus et al. 2021; Costa et al. 2022). Even fewer have examined the potential benefits of the mycobiome, but there is certainly evidence that many will also exhibit similar levels of bioactive compounds as their bacterial counterparts (Zhang et al. 2016).

Interestingly, what was initially considered to be a synergistic relationship between the fungal species and host can become virulent following exposure to stress (Kelly & Salinas 2017). As in all organisms, wounds can result in an altered microenvironment that provides opportunities for otherwise commensal fungi to become pathogenic, resulting in what are known as mycoses (Iliev and Leondardi 2017; Jacobsen 2023; Nucci and Marr 2005). Indeed, the very mechanisms used by microbes to establish and maintain themselves within a community may conversely serve as mechanisms of virulence (Flowers & Grice 2020). This phenomenon appears to occur as a result of immunocompromised skin triggering previously innocuous microbes to enter a 'survival state'. This can sometimes manifest as single-taxa domination within the microbiome (Flowers & Grice 2020). Within vertebrate systems such as humans and rats, integument trauma has been shown to facilitate the transition of commensal members within the *Candida* genus to become parasitic and highly enriched within the skin mycobiome (Hall and Noverr, 2017; Sanjar et al. 2020). The fungal composition of post-injury skin also exhibited an overall reduction in both richness and diversity compared to healthy individuals (Hall and Noverr, 2017; Sanjar et al. 2020).

However, wounds may not only adversely impact the nature of commensal fungal communities, but also present conditions vulnerable to colonisation by external opportunists (Dowd et al. 2011). Fungal pathogens are ubiquitous in the marine realm and appear to exploit immunocompromised hosts spanning a broad taxonomic range (Pang et al. 2021; Amend et al. 2019). For example, secondary infection within abraded tissue by the fungus *Loboa loboi* has been identified as the probable cause of Lobomycosis in the Atlantic bottlenose dolphin (*Tursiops truncatus)* (Higgins 2000). Similarly, infection of *Ophiocordyceps* spp. within juvenile edible crabs (*Cancer pagurus*) is thought to depend on the presence of cuticle wounds resulting from predation attempts or conspecific conflict (Smith et al. 2013).

Due to the profound influence of the skin microbiome on organism health and susceptibility to disease, uncovering the factors driving microbial dynamics within threatened species presents a research priority (West 2022). Considering the rapid population declines of elasmobranchs worldwide (Hyde et al. 2022), coupled with the increased transmission of disease agents potentiated by expected increases in oceanic temperatures (Costa et al. 2022), studies targeting the role of commensal microbes in disease resistance, and the factors shaping these communities, are needed. This study therefore aimed to determine initially whether the fungal consortia associated with shark skin are conserved or whether it varies in correspondence with skin health

state. Further, as there is preliminary evidence indicating that shark skin-associated fungi may be bioactive (Zhang et al. 2016), investigating fungal composition across healthy and wounded shark skin may further our understanding of the drivers of microbial communities, with potential applications for monitoring shark health in the future. Finally, we aimed to establish whether a shared core fungal community was present across the sharks sampled, and (if found), extrapolate to what extent it might participate in wound healing and antibiotic resistance in sharks.

Methods

Sampling

Skin swab samples were obtained from 46 blacktip reef sharks (*Carcharhinus melanopterus*) and one sicklefin lemon shark (*Negaprion acutidens*). Owing to the opportunistic nature of sampling, skin swabs from a single representative of *N. acutidens* were retained. This species occupies a similar ecological niche to blacktip reef sharks and therefore may contribute additional representation to the dataset. No interspecies comparisons were intended. Sharks were caught with line and pole from five locations around the Amirante Islands, Seychelles (5.4180 °S, 53.2962 °E): one (Dock) on the fringing reef of D'Arros Island and four (Tippers, Fouquet, Benjamin and Ressource) around the rim of the nearby St. Joseph Atoll, the Tippers site being on the outer reef of the atoll, and the others within the central lagoon. The largest distance separating locations was six kilometres (between Dock and Ressource), with the closest sampling points being only a few hundred metres from each other within the St. Joseph's lagoon (Pogoreutz et al. 2019).

Skin swabs were taken from the skin covering the gills and from the left upper body below the first dorsal fin, hereafter referred to as the back of the animal. For the purpose of standardisation, both sample types were obtained from the left side of the body unless otherwise stated. Gill samples were categorised as either 'healthy' ($n=27$) or 'wounded' ($n=20$) depending on whether the skin around the gills was structurally intact or exhibited open wounds, respectively. Wounds were localised to the gill area only, and swabs from injured skin were obtained directly from the compromised site (Fig. 1); skin swabs taken from the back were collected as a control for each shark. Each individual had a back and gill sample with the exception of three sharks: individuals '44', '5' and '30'. Three samples were collected from shark 44 and shark 5, who each had an extra sample from the right side of the body, taken from the gills and back, respectively, whilst a single sample was submitted for shark 30, taken from the gills on the left side. Swabs were preserved in RNAlater solution (Thermo Fisher Scientific, United Kingdom) and frozen immediately at − 20 °C. The sampling methods adhered to ethical guidelines and have been described previously: Pogoreutz et al., (2019). In addition to the health status of the skin, the sampling location, sex, area of the body (back or gills), species and sampling date pertaining to each sample were recorded.

Extracting fungal DNA from skin swabs

Aseptic techniques were implemented to conduct the DNA extractions using the Qiagen DNeasy PowerSoil Pro kit (Qiagen, Germany), following the manufacturer's guidelines. A laboratory blank was prepared following the same procedure minus the application of a skin swab, in order to account for any contamination during the extraction process. Following DNA extraction, PCR with general fungal primers was performed to validate the presence of fungal DNA in the samples, using ITS1F (5'-TCCGTAGGTGAA CCTGCGG-3') and ITS4B (5'-TCCTCCGCTTA TTGATA TGC-3') (Martin and Rygiewicz 2005). For the PCR reactant, 25 µL was prepared for each sample using 12.5 µL Taq DNA Polymerase Master Mix RED, 1 µL ITS1F primer, 1 µL ITS4B primer, 9.5 µL nuclease-free water and 1 µL DNA. The PCR protocol for fungi amplification was as follows: 94 °C for 3 min, 35 cycles of denaturation (94 °C for 60 s), annealing (56 °C for 60 s) and extension (72 °C for 2 min), and an additional final extension step at 72 °C for 7 min. Subsequent gel electrophoresis using 5 µL of each PCR product was performed on 1.5% agarose gel to ensure successful amplification of fungal DNA.

The extracted DNA samples were sent to Barts and The London Genome Centre at Queen Mary University of London for Illumina MiSeq high-throughput sequencing to target the ITS2 region with primers 5.8S-Fun (5'-AACTTT YRRCAAYGGATCWCT-3') and ITS4-Fun (5'-AGCCTC CGCTTATTGATATGCTTAART-3') (Taylor et al. 2016). The PCR protocol consisted of an initial denaturation step at 96 °C for 2 min, followed by 27 cycles of denaturation at 94 °C for 30 s, 58 °C for 40 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. The resultant amplicons were cleaned using a bead-based kit prior to sequencing (AMPure XP, Beckman Coulter, USA).

Bioinformatics and data analysis

Library preparation

The demultiplexed FASTQ files from the Illumina MiSeq sequencing were processed in the package *DADA2* (version 1.8; Callahan et al. 2016). Firstly, the forward and reverse reads were specified, and the primer sequence

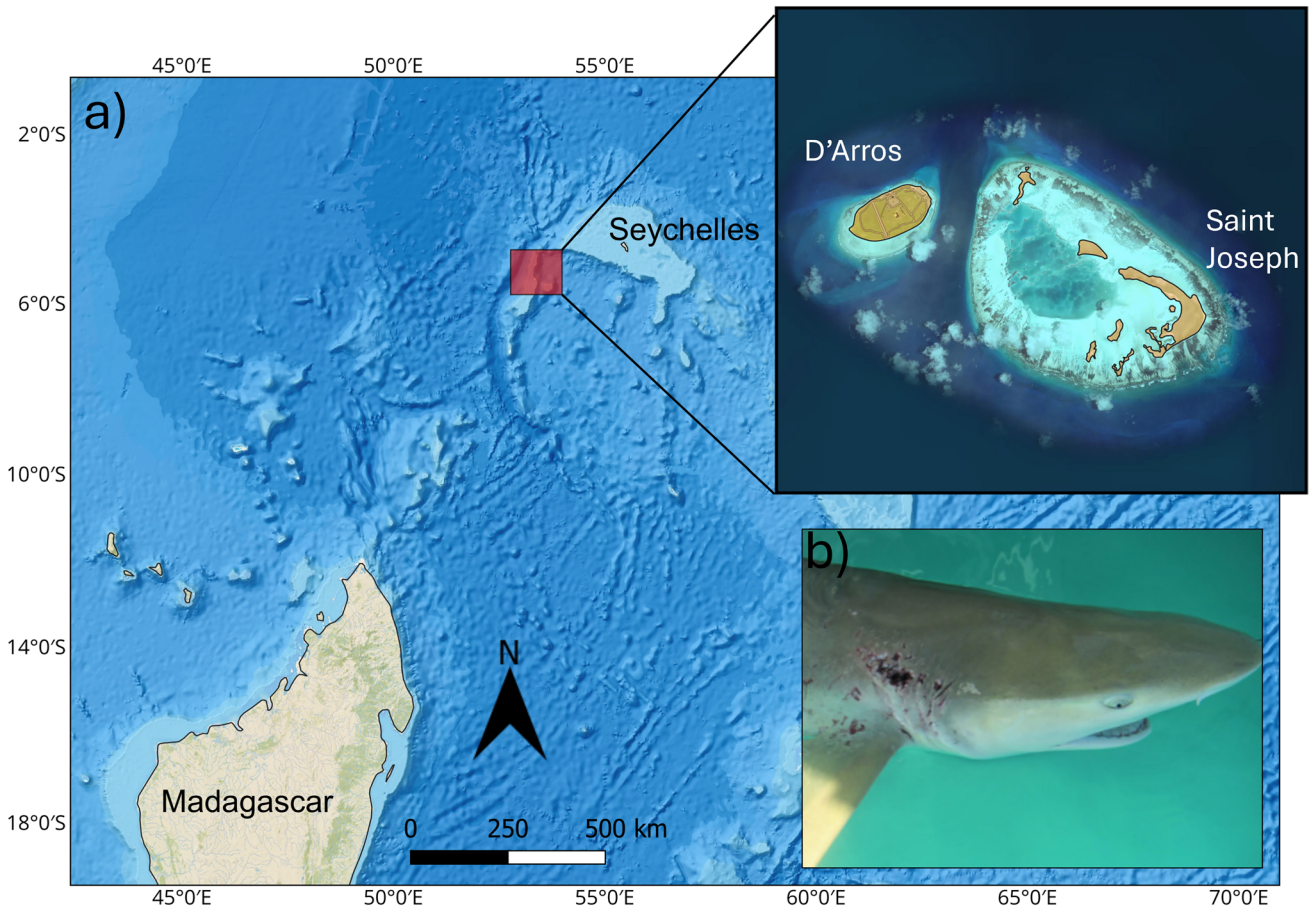

Fig. 1 General sampling area and gill wounds exhibited by blacktip reef sharks (*Carcharhinus melanopterus*) in the Amirante Islands, Seychelles: **a** D'Arros Island and St Joseph Atoll, encompassing the five sampling locations, **b** an example of a wounded blacktip reef shark with lesions localised to the gill area, photographed at the time of sampling (photograph taken from Pogoreutz et al. 2019)

orientations were acquired using the *DNAstring()* function from the *Biostrings* package (version 2.50.2; Pagès et al. 2013). Ambiguously called bases were then removed using the *filterAndTrim()* function. *Cutadapt* (version 4; Martin 2011) was used to trim the primers from the forward and reverse sequences. Reads shorter than 100 nucleotides after trimming were discarded by specifying the parameter '—minimum length 100' to remove non-target reads. The quality scores of the filtered sequences were checked using the *PlotQualityProfile()* function, and poor quality reads were subsequently removed using the *filterAndTrim()* function based on a maximum expected error rate of two.

The error models for the sequence data were generated using the *learnErrors()* function within *DADA2* to identify any errors introduced during the PCR process and sequencing. Denoising was then performed using the *dada* function (version 1.8), which inferred amplicon sequence variants (ASVs) without relying on traditional clustering approaches. Chimeric sequences were identified and removed using the *removeBimeraDenovo()* function within *DADA2*. Taxonomy was assigned to the resulting ASVs using the RDP classifier (Wang et al. 2007) against the UNITE database (Nilsson et al. 2019; version 9 all Eukaryotes).

Data preparation for downstream analysis

Amplification of the ITS2 rRNA region was conducted on 95 skin samples, and one extraction blank, taken from 20 sharks exhibiting gill injuries and 27 visibly healthy sharks ($n = 47$). Data analysis was conducted using the *phyloseq* package (McMurdie and Holmes 2013) in RStudio (version 4.3.0). A total of 1,186 ASVs were returned initially, corresponding to 4,696,105 total reads. Plotting the rarefaction curve using the *rarecurve()* function from the *vegan* package (R package version 2.6–4) (Oksanen et al. 2022) indicated that sufficient sampling depth was achieved and that the true diversity of ASVs within the samples was represented (see Supplementary Fig. 1). Removing reads representing non-target taxa left 1,106 fungal ASVs.

Table 1 Samples available for downstream analysis from 45 blacktip reef sharks (*Carcharhinus melanopterus*) and one sicklefin lemon shark (*Negaprion acutidens*) taken from five locations around the Amirante Islands, Seychelles ($n = 77$)

Sampling location (site name plus whether on St. Joseph's Atoll or D'Arros Island)	No. sharks	Healthy gill samples ($n = 23$)	Wounded gill samples ($n = 20$)	Back samples ($n = 34$)
Fouquet (St Joseph Lagoon)	10	6	4	5
Benjamin (St Joseph Lagoon)	3	0	2	4
Ressource (St Joseph Lagoon)	11	6	3	9
Tippers (St Joseph Outer Reef)	11	3	9	10
Dock (D'Arros Island Reef Flat)	11	8	2	9

Following normalisation using CSS from the *metagenomeSeq* package (Paulson et al. 2013), decontamination using the decon() function within the *microDecon* package (McKnight et al. 2019) and removal of samples containing 0 reads, 77 samples comprising 1,079 ASVs remained. Two samples belonging to a single shark were removed during this process, meaning that this individual was omitted from subsequent analysis. The remaining samples represented 46 sharks (45 blacktip reef sharks and one sicklefin lemon shark) and comprised 43 samples from the gills and 34 from the back (Table 1). These data formed the basis for downstream analysis. Additionally, analyses excluding the samples from the single sicklefin lemon shark were repeated and confirmed that the inclusion of this individual did not confound the statistical inferences in this study.

Statistical analysis

The relative abundance of taxa across all samples was visualised using the *microeco* package (Liu et al., 2021). To discern whether patterns existed in the taxonomy and abundance of microbial members at the sample level, the *estimate_richness()* function from the *phyloseq* package (McMurdie and Holmes 2013) in *RStudio* (version 4.3.0) was used. Tests of Shannon indices were conducted on the raw sequence counts to investigate whether fungal composition varied in the level of richness and/or evenness exhibited between the levels of the categorical variables: wound status, sex, location and swab site.

A Shapiro–Wilk test was employed using the built-in *R* function *Shapiro.test()* to determine whether the data pertaining to each of the categorical variables were normally distributed. All but the sampling location satisfied the assumption of normal distribution ($p > 0.05$); therefore, a Kruskal–Wallis rank-sum test of Shannon indices was used via *R*'s *Kruskal.test()* function to explore the alpha diversity between sampling locations. For swab site, sex and wound status (gill samples only, $n = 43$), a Levene's test was applied to ensure equal variance using *leveneTest()*, before performing t-tests of Shannon indices using the *t.test()* function in *R*.

An additional subset of 58 samples pertaining to 29 sharks was created for the purpose of a pairwise comparison of alpha diversity between gill samples and the corresponding back samples taken from the same shark. Samples were paired by grouping back and gill samples based on the unique ID number assigned to each shark. Only sharks with both a back and gill sample available were considered; 17 sharks had only a single sample remaining in the dataset following quality filtering and were subsequently excluded from this analysis. The paired subset was split into two groups based on the wound status of the gills: 'wounded', comprised of 28 samples from 14 sharks exhibiting gill injury and 30 samples from the 'healthy' group consisting of 15 individuals. Once the conditions for normal distribution using *Shapiro.test()* and equal variance using *LeveneTest()* were satisfied, paired 'two-sided' *t*-tests were conducted using the *t.test()* function on the merged alpha diversity values for the separate groups.

PERMANOVAs were carried out using the *adonis2* function from the *vegan* package (version 2.6–4; Oksanen et al. 2022) to assess whether the fungal composition differed significantly between samples based on the categorical variables. To visualise the community composition patterns, the package *microViz* (Barnett et al. 2021) was used to plot Bray–Curtis distances.

Lastly, associations between fungal members were investigated to infer potential ecologically significant relationships. To investigate whether fungal genera demonstrated co-occurrence patterns across the samples, fungal ASVs were grouped by genus (99 taxa spanning 77 samples), where ASVs without a genus assignment were automatically grouped by the next available taxonomic classification, and a probabilistic co-occurrence analysis was carried out using the *cooccur()* function from the *cooccur* package (Griffith et al. 2016).

Results

Sequence run metrics and taxonomic composition

The total number of reads returned initially was 4,696,105, with a minimum sequencing depth of 1,316. Following normalisation, the dataset comprised 12,060 total counts, which

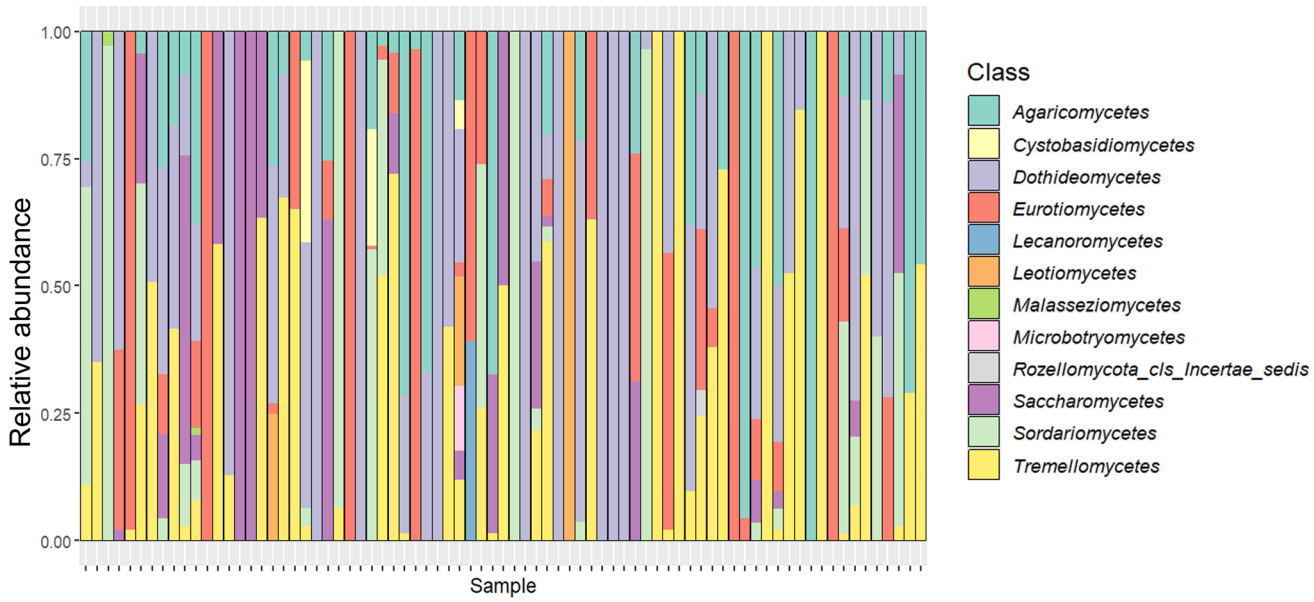

Fig. 2 Class-level relative abundance showing 12 fungal classes spanning 1,079 fungal ASVs across all shark skin samples ($n = 77$)

corresponded to 1,079 unique ASVs used in the downstream analysis. Fungal taxa were assigned to three phyla, the majority of which belonged to *Ascomycota* (63.6%), followed by *Basidiomycota* (36.4%), and the remaining 0.1% were assigned to *Rozellomycota*.

The twelve classes represented within the dataset were *Dothideomycetes* (27.5%), *Tremellomycetes* (22.0%), *Eurotiomycetes* (13.8%), *Agaricomycetes* (13.3%) and *Sordariomycetes* (11.2%), with the remaining lower-abundance classes making up 3.5% of the total sequences collectively. Composition disparity at the class level across samples is shown in Fig. 2. The fungal ASVs were assigned across 34 orders, with the most abundant orders, *Capnodiales* and *Eurotiales*, contributing 12.6% and 12.5% to the total sequences, respectively.

Out of the 73 families exhibited within the dataset, *Trichosporonaceae* had the highest abundance, accounting for 18.3% of the microbial composition, followed by *Eurotiales* (12.5%).

Despite the absence of any dominant taxa consistent across the dataset, there were a number of ASVs that appeared to be more common amongst individual samples. For example, ASV2 (identified as a *Cutaneotrichosporon* sp) contributed 17.2% of the total read counts, ASV36 (*Aspergillus* sp) 9.9%, ASV3 (*Cladosporium* sp.) 7.9%, ASV11 (*Alternaria* sp.) 7.9%, ASV420 (*Sarocladium* sp) 5.0%, ASV50 (*Yarrowia*) 3.3%, ASV12 (*Xylodon*) 3.2%, ASV47 (*Penicillium*) 2.6%, ASV19 (*Hortaea*) 2.5% and ASV72 (*Trichoderma*) 2.5%.

Surprisingly, the majority of ASVs documented displayed low frequency within the data, and many individual ASVs occurred within a single sample only. The maximum frequency of any ASV belonged to ASV2 (*Cutaneotrichosporon* sp), which occurred within 31 separate samples across the dataset. Only two ASVs qualified as possible 'core' members. However, this was established using a liberal 30% occurrence 'standard' (Neu et al. 2021). Regardless of the generous nature of this characterisation, ASV2 and ASV5 did occur within 40.3% of samples ($n = 31$) and 39.0% of samples ($n = 30$), respectively. Both 'core' ASVs corresponded to the genus *Cutaneotrichosporon*.

The dataset demonstrated great disparity within the number of ASVs contained in each sample; the minimum number of ASVs found within a given sample was three, whilst the median number was 16, and the maximum number of ASVs retrieved from a single sample was 63.

Impact of categorical variables on diversity and community composition

The total distribution of fungal ASVs across all 77 samples exhibited relatively high evenness, returning a mean Simpson's evenness value of 0.9115 (±0.0074 SE) (see Supplementary Table 1). This indicates a balanced community structure with similar abundances throughout the dataset.

There were no significant differences detected in Shannon diversity of fungal communities between wounded and healthy gill samples ($t = -0.55572$, df = 41, p-value = 0.5814) (Fig. 3a). There were also no significant differences retrieved in the t-tests of Shannon indices between sampling locations ($t = -0.26878$, df = 75, p-value = 0.7888) (Fig. 3b), or for sex

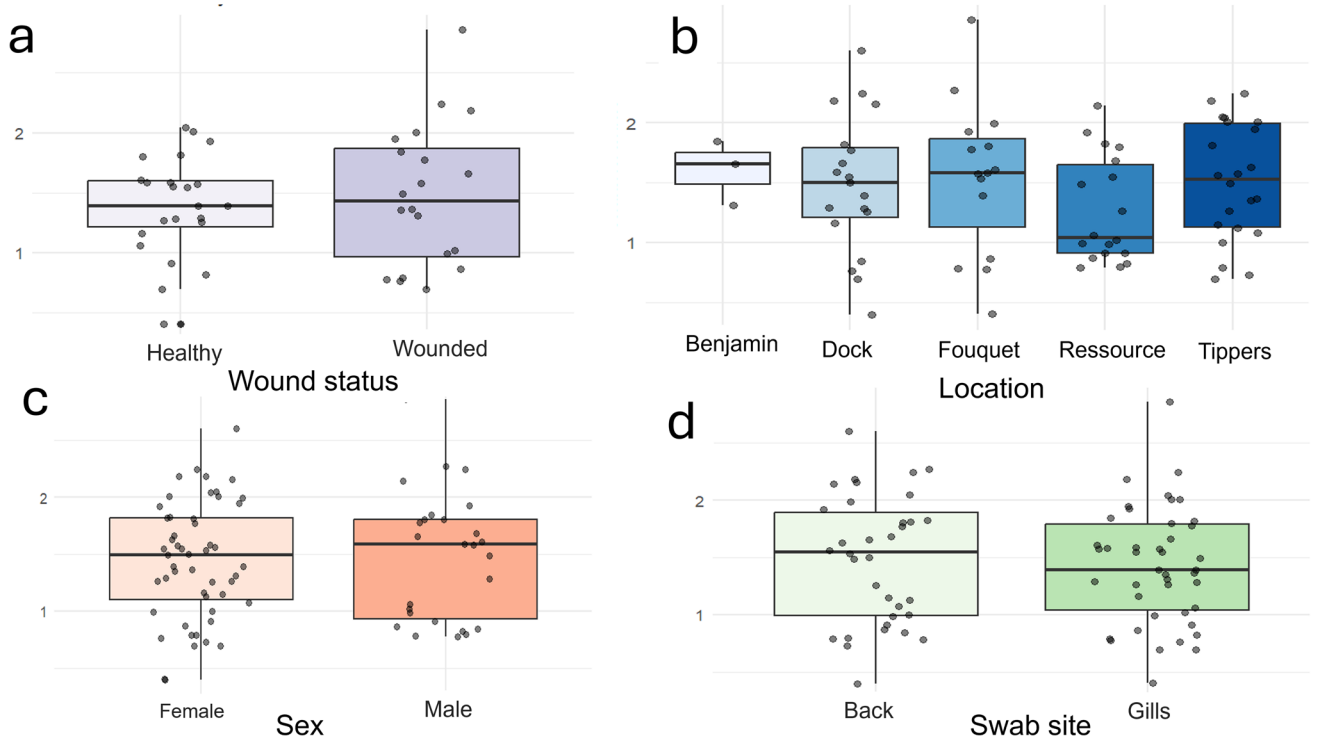

Fig. 3 Box plots of alpha diversity by Shannon Index, **a** wound status: wounded ($n=20$) and Healthy ($n=23$), **b** location: Benjamin ($n=3$), Dock ($n=19$), Fouquet ($n=15$), Ressource ($n=18$) and Tippers ($n=22$), **c** sex: female ($n=51$) and male ($n=26$), **d** swab site: back ($n=34$) and gills ($n=43$)

(male vs female) ($t=-0.26878$, df = 75, p-value = 0.7888) (Fig. 3c), or for swab site (back vs gills) (t = 0.45644, df = 75, p-value = 0.6494) (Fig. 3d).

For the paired data ($n=58$ samples), pairwise t-tests of Shannon indices did not show a significant difference between the microbial composition harboured by wounded gills and the corresponding back sample ($t=0.42851$, df = 11, p-value = 0.6765). Similarly, healthy gill samples and back samples obtained from the same shark also did not exhibit a significant difference in Shannon indices ($t=-0.94046$, df = 15, p-value = 0.3619).

Despite clusters within the Bray–Curtis dissimilarity plots, these did not correspond to any of the categorical variables explored (Fig. 4). There were no statistically significant differences in beta diversity between locations (adonis2 PERMANOVA; pseudo-$F=0.9768$, partial $R^2=0.05147$, p-value = 0.578) (Fig. 4a), across wound status (adonis2 PERMANOVA; pseudo-$F=0.8326$, $R^2=0.0199$, p-value = 0.908) ($n=43$) (Fig. 4b), sex (adonis2 PERMANOVA; pseudo-F = 0.8923, partial $R^2=0.01176$, p-value = 0.722) or between samples taken from the gills and back (adonis2 PERMANOVA; pseudo-$F=1.2166$, partial $R^2=0.01596$, p-value = 0.098).

Fungal co-occurrence

The analysis recovered 4,851 pair combinations, of which 4,777 (98.47%) were removed due to the combinations of these genus pairs being expected to co-occur in less than one sample. Random co-occurrences were those that did not differ significantly from the number of expected associations and deviated by less than 10% of the total number of samples. Instances of negative co-occurrence were not found. Of the remaining 74 pairs, 21 showed positive co-occurrence patterns (28.4% of the analysed pairs), whilst most genus pairs exhibited random associations (71.6% of the analysed pairs, see Supplementary Table 2).

Significant positive associations ($p<0.05$) were exhibited by 13 genera, whereby genus pairings were classed as having a positive co-occurrence when the number of observed co-occurrences exceeded the number expected based on the probability model. Of these genera,

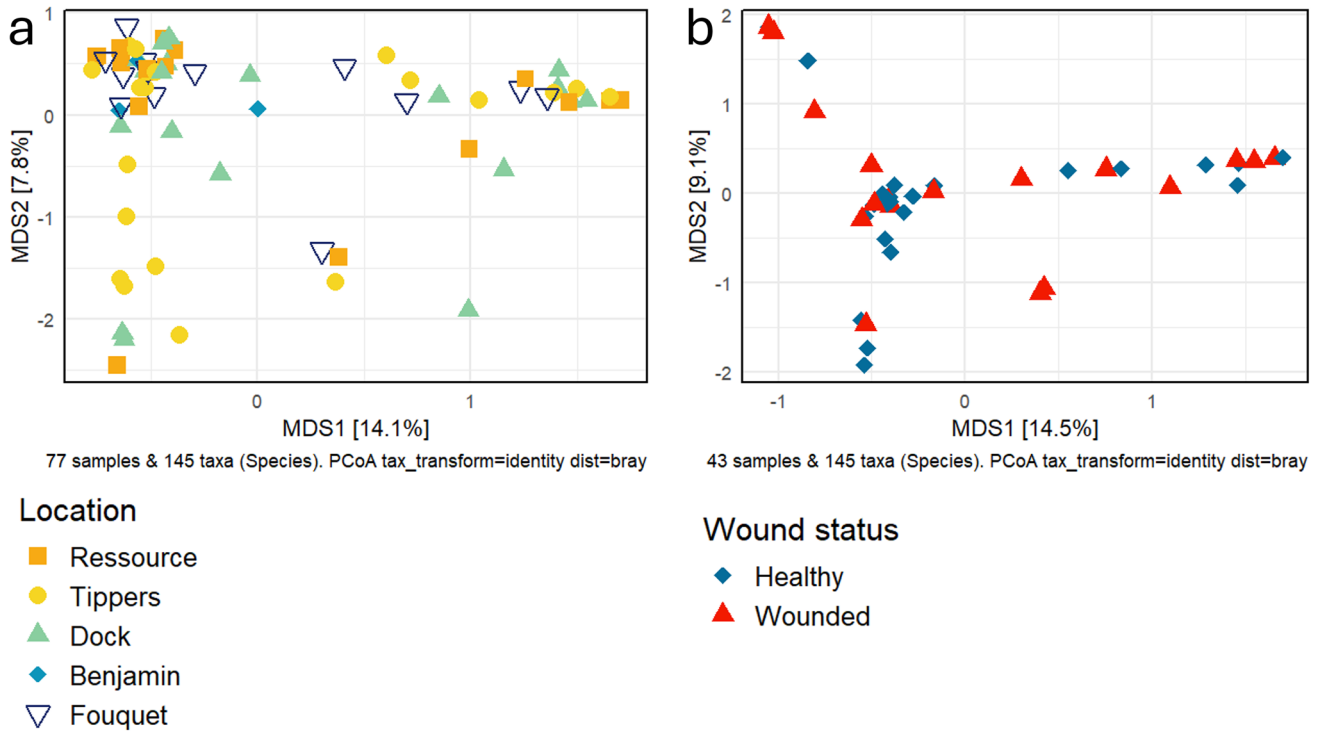

Fig. 4 Bray–Curtis dissimilarity values of species-level communities across **a** location: Benjamin ($n=3$), Dock ($n=19$), Fouquet ($n=15$), Ressource ($n=18$) and Tippers ($n=22$), and **b** wound status: Wounded ($n=20$) and Healthy ($n=23$)

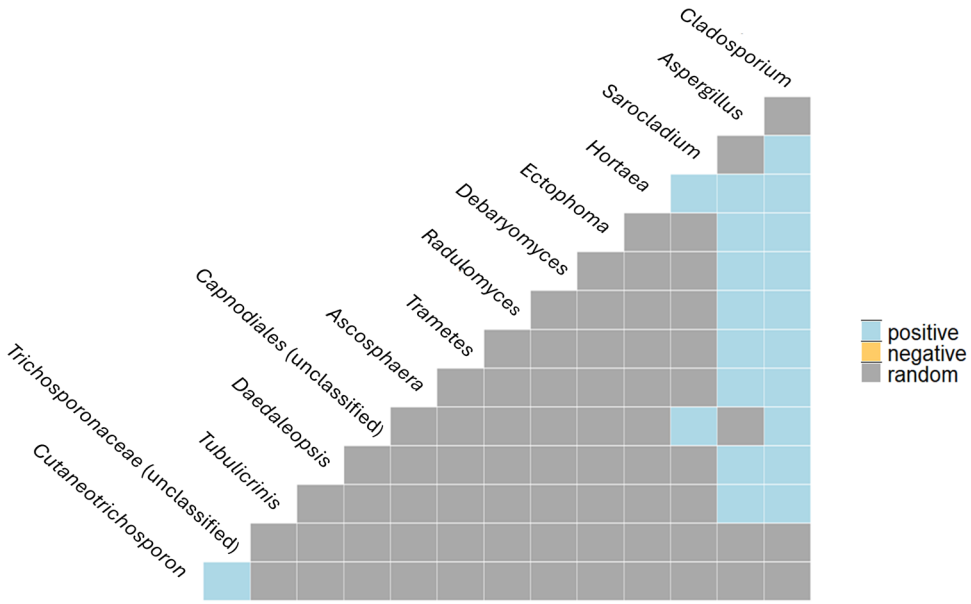

Fig. 5 Mapping of pairwise associations for fungal genera ($n=14$) retrieved from shark skin samples ($n=77$) taken from healthy and wounded reef sharks in the Amirante Islands, Seychelles

Aspergillus and *Cladosporium* had the highest number of positive co-occurrences in the dataset, exhibiting eight and 10 instances of positive associations, respectively (Fig. 5). Both genera co-occurred significantly with *Trametes*, *Radulomyces*, *Debaryomyces* and *Ectophoma*. Despite exhibiting analogous co-occurrence with other genera, they did not co-occur positively with each other.

Discussion

To date, very few host-associated microbiome studies have considered the microeukaryote community and the factors affecting the structure and composition of fungal members (Baker et al. 2017). To our knowledge, this study is the first to highlight the skin mycobiome of reef sharks in the Seychelles. Further, we attempted to contrast apparently healthy mycobiomes with those of sharks with noticeable lesions to determine whether it was possible to identify causal or pathogenic fungi. Surprisingly, there was no significant shift in fungal diversity or community composition with wound status, sex, location or swab site.

This suggests that, despite compromised skin integrity, the presence of wounds across the sharks did not result in or facilitate an altered state vulnerable to the exploitation of commensals or the proliferation of non-indigenous fungi (Canchy et al. 2023; West et al. 2019). This is indeed surprising, as lesions often result from, or potentiate, a shift in the general microbiome towards one of a diseased state, a dysbiosis or pathobiome as it is often referred to (Misic et al. 2014). Further, even in the absence of disease, significant differences in the abundance and identity of specific microbes were still to be expected, as well as reductions in the number of taxa present, as both have been identified as indicators of infection following skin trauma (Johnson et al. 2018). However, the present study mirrors and lends support to a previous investigation which looked at the same set of samples but targeted the prokaryote community. That study found that there was similarly no significant difference in the microbial consortia between healthy and injured sharks (Pogoreutz et al. 2019).

Although the categorical variables considered within this study did not account for significant differences in microbial composition between samples, there did appear to be clustering within the NMDS plots. This suggests that there may be other environmental or host-derived factors outside of those considered that may be shaping differences in the fungal microbiome structure of these sharks. For example, host age has been shown to affect the microbial composition in both humans and wild animals (Lavrinienko et al. 2018). Blacktip reef sharks and sicklefin lemon sharks are sympatric species exhibiting site fidelity to relatively small home ranges within the waters of D'Arros Island and St Joseph Atoll (Lea et al. 2020). Indeed, work using acoustic tags showed that only very rarely did a blacktip reef shark cross the one-kilometre-wide channel between D'Arros Island and St. Joseph's Atoll. This was suspected to be due to the risk of predation by larger sharks, including bull sharks (*Carcharhinus leucas*), that occur there (Lea et al. 2020). Similarly, Papastamatiou et al., (2009) have reported an average home range size of 0.55km^2 for blacktip reef sharks inhabiting a remote tropical atoll in the Pacific. As discrete environmental differences over short geographic scales may influence the available colonising microbes (Ma et al. 2022), the consideration of site-specific effects on microbial structure is warranted. Further, sampling location had been found to be associated with significant differences in the composition of the prokaryote samples obtained from these same individual sharks (Pogoreutz et al. 2019). Therefore, it was unexpected that a similar finding was not replicated for the shark mycobiome. It may be that fungal spores or fragments are able to disperse more readily within the water column than some of the bacterial taxa found on sharks, as it has been noted that planktonic fungi are able to travel considerable distances via oceanic currents (Burgaud et al. 2022). Alternatively, it may be possible that distinct microenvironments, within each location (such as pH, salinity and temperature), may have affected the fungal composition of shark skin, as discussed in Montemagno et al., (2024). Therefore, future investigations exploring drivers of mycobiome community structure should consider a wide range of environmental factors across a higher resolution of spatial scales.

Although the concept of core microbiomes is somewhat contentious in various biological fields (Sweet and Bulling 2017), determining whether patterns or trends exist can allow us to hypothesise about the possible function of certain microbial members or associates. Similar to many studies and across numerous taxa, we were unable to highlight a substantial core fungal community across skin samples. However, ASV2 and ASV5 were identified via the relatively loose assignment of a 30% occurrence standard (Neu et al. 2021). Interestingly, both were assigned to the yeast genus *Cutaneotrichosporon* of which representative species have been identified as commensals of the bottlenose dolphin (*T. truncatus*), highlighting a potential role in the skin microbiome of large marine predators (Buck et al. 2006; Garcia-Bustos et al. 2024). Certain terrestrial commensals of the genus have notable roles in disease resistance and have been shown to inhibit the activity of pathogens responsible for white-nose syndrome in bats, for example (Nguyen and Kalan 2022). Conversely, *Cutaneotrichosporon* sp. have also been identified as potential opportunistic pathogens of humans and animals (de Almeida et al. 2017). Although this is certainly an interesting finding, inferring true functionality of these ASVs within the shark skin mycobiome is not currently possible. Specifically, strain-level resolution of these taxa (and therefore obtaining cultural isolates) is vital to explore ecological functionality in more depth (Liao et al. 2023).

It is also worth noting that other fungi, such as those from the genera *Aspergillus* and *Penicillium*, were also relatively common and amongst the taxa contributing to the top 10 most abundant ASVs, based on the relative abundance of read counts. These are often the most commonly described genera in the marine realm (Gladfelter et al 2019), and

members from both have previously been isolated from the gills of other shark species, such as white sharks (*Carcharodon carcharias*) (Zhang et al. 2016). Further, such fungi have been shown to have bioactive properties against the likes of Methicillin-resistant *Staphylococcus aureus* (*MRSA)*, *Escherichia coli* and brine shrimp larvae (Zhang et al. 2016). We could therefore hypothesise that such fungi may confer beneficial functions through antimicrobial and antibiofouling properties that have led to their incorporation within the shark skin microbiome. Additionally, *Cladosporium*, *Alternaria* and *Sarocladium* (which were also identified as some of the more abundant genera in our samples) have been previously shown to harbour bioactive compounds with antimicrobial properties (Mohamed and Ibrahim 2021; Shaaban et al. 2012; Eskander et al. 2020). Interestingly, and likely to be of increasing importance in the future, members of the genus *Yarrowia* have been found to possess genes encoding for heavy metal tolerance (Bankar et al. 2018). Bioaccumulation of heavy metals, which enter marine environments through coastal industrial activity, is suggested to have sublethal effects for sharks when they are acquired in high concentrations (Doane et al. 2023a; Shipley et al. 2021). Thus, heavy metal tolerance may be an important service provided by commensal microbes for sharks in the Anthropocene era. Many other ASVs identified and described (such as *Cutaneotrichosporon*, *Xylodon* or *Trichoderma*) are much less understood. However, what little information we have on these genera hints at further antimicrobial activity. The genera identified here may therefore form the basis for future bioactivity assays to explicitly assign function to individual taxa.

Despite some dominance, the apparent lack of a true core or stable community suggests a more even distribution of taxa and the predominance of rare ASVs. Although abundant taxa are commonly identified as being important influences regarding functionality (Neu et al. 2021), rare taxa may still provide ecologically important functions (Tong et al. 2019). Indeed, these rare taxa have been shown to mediate microbial dynamics through the production of antagonistic metabolites, in addition to contributing valuable functions to the metagenomic repertoire (García-Sánchez et al. 2023). In contrast to abundant fungal communities, rare communities (those composed of low-frequency ASVs) do not exhibit strong compositional shifts in response to environmental perturbation and readily replace abundant taxa during times of stress (Xiong et al. 2021). Further, communities harbouring dominant taxa in some systems are said to be shaped predominantly by stochastic factors, such as random dispersal, whereas communities composed of rare members are driven by biotic and abiotic factors and represent host-selected taxa (Xiong et al. 2021). Therefore, the role of rare ASVs in shaping the shark skin mycobiome and the subsequent influence of these communities on the wound healing capacity within sharks present an area requiring further exploration.

As it stands, we cannot discount the possibility that the high variability and lack of structuring within this dataset were influenced by the stochastic nature of fungal microorganisms present within the water column, rather than exclusively representative of a host-determined community (Sen et al. 2022). Mazzella et al. (2025) reported a strikingly similar pattern from an investigation into the fungal composition of four species of marine sponge (phylum Porifera) across the Mediterranean, where the observed fungal consortia were also characterised by a lack of a stable 'core' community and fluctuations in taxonomic profiles. The authors suggested that host-associated fungal communities were likely transient in nature, mirroring the ambient marine consortia, rather than signifying a beneficial host-selected mycobiome (Mazzella et al. 2025). Furthermore, *Aspergillus, Cladosporium, Alternaria* and *Penicillium*, which were amongst the notable taxa returned in the present study, also comprised some of the most relatively abundant genera exhibited by marine sponges. The concordance of patterns across phyla, given that sessile invertebrates occupy disparate niches to those of vagile predatory sharks, is surprising. These findings together hint at the possibility of a more generalised role of microeukaryotes occupying living surfaces in the marine environment (James et al. 2024). This begs the question as to whether the shark skin mycobiome is composed of a random assortment of ubiquitous marine fungi, or whether it is ecologically shaped by discrete patterns, not yet able to be resolved with metabarcoding alone (Mazzella et al. 2025).

An important caveat to note is that it was not possible to compare the fungal communities obtained from shark skin versus those from the surrounding seawater in the present study. Control seawater samples were taken and filtered, and filter papers were preserved at the time of sampling, but these were used exclusively for the sister microbiome study (Pogoreutz et al. 2019). It is therefore recommended that in further work, sufficient seawater samples be taken to explicitly distinguish between skin-associated microbes and those from the surrounding water column, in order to leverage ecological inferences.

The examination of fungal co-occurrence, though the network appears relatively sparse, indicated genus-level patterns within the shark skin mycobiome. Recent studies have underscored the importance of microbial co-occurrence over taxonomic identity with regard to microbiome functionality (Barnes et al. 2020; Doane et al. 2023b). A metagenomic analysis of the whale shark (*Rhincodon typus*) microbiome by Doane et al (2023b) uncovered two potential ecological niches fulfilled by co-occurring family networks that may perform functions pertaining to energy provision for other keystone microbial members.

The identity and abundance of microbial members were found to shift in correspondence with whale shark aggregation sites, but functionality was maintained via microbiome architecture (Doane et al. 2023b). Additionally, the findings from the current investigation mirror those found in a study conducted on the amphibian skin microbiome, whereby fungal taxa exhibited only positive or random interactions and did not show any negative associations with other genera (Rebollar et al. 2020). Therefore, we may glean some inference into the commensal fungal dynamics on the skin of sharks and highlight potential genera that may be of interest when investigating network-mediated functionality. With this in mind, it may be that despite the lack of a substantial shared 'core' microbial community in our current study, the ecological function of the microbiome may still be conserved through bioactivity resulting from the co-occurrence of interacting microbes (Tian et al. 2020), thus alluding to the possibility of functional redundancy within the microbial community, as has been suggested for bacterial communities from the skin of leopard sharks (*Triakis semifasciata*) which demonstrated consistency in gene function regardless of variation in the abundance of bacterial genera (Doane et al. 2023a).

Metagenomic assessments should be undertaken to uncover the genetic contributions and potential functionality of the shark mycobiome, particularly in reference to maintaining skin health and elevating wound healing. Further, microbiome functionality is context-specific and driven by dynamics spanning microbial kingdoms (Chen et al. 2018). Strong co-occurrence patterns have been established between fungi and bacteria (Harrison et al. 2021), and such cross-kingdom interactions are believed to result in the provision of secondary metabolites that aid host functions (Harrison et al. 2021). Elucidating the nature of multi-kingdom interactions within shark skin may, therefore, also aid us in uncovering the mystery behind their elevated capacity for wound healing.

Here, we present the first comprehensive overview of the variability in the skin mycobiome of blacktip reef sharks (and one sicklefin lemon shark) and, through comparisons of assemblages found on healthy and injured skin, have broadened our understanding of the underexplored fungal communities associated with shark skin. Contrary to what has been observed across other vertebrate systems, here we have demonstrated that the fungal consortia of shark skin mirror that of the prokaryotic community, and are conserved across individuals regardless of the presence of mechanical insult. However, further environmental and host-related factors need to be considered to elucidate the drivers shaping the fungal microbiome in sharks. At present, it is unclear whether, and to what extent, fungal communities are stochastic in nature or host-mediated, and inferences into the functional consequences of the patterns highlighted remain speculative. We signpost avenues for future investigations to explore the functional potential of shark skin-associated microbes, by homing in on the roles of rare ASVs and uncovering the ecological implications of co-occurrence, both between fungal members and across taxonomic kingdoms. Such insights may further our understanding of the role that the shark skin microbiome plays in wound healing and infection resistance within this vulnerable group.

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Author contributions CC, MAG and RO conceived the study and provided funding. MAG, CM and RO conducted fieldwork. ABI and KR performed DNA extractions. ABI performed PCRs, statistical analysis and data visualisation, and wrote the original draft of the manuscript. JDT processed raw sequence data. JDT, AB and MB provided guidance on data analysis. MS supervised the project. All authors edited the manuscript.

Data availability The sequencing data generated in this study have been deposited to the NCBI Sequence Read Archive (SRA) and are publicly available under the accession number: PRJNA1155150.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Samples were obtained within the scope of a letter of approval from the Seychelles Ministry of the Environment. All sharks sampled were released unharmed.

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