FUNGI FROM DEAD ARTHROPODS AND BATS OF GOMANTONG CAVE, NORTHERN BORNEO, SABAH (MALAYSIA)

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Abstract

Borneo is a biodiversity and ecotourism hotspot, yet one of its least-studied ecosystems is their limestone caves. Not many studies have been conducted on the role fungi play in tropical cave ecosystems, and no fungal surveys have been conducted in the caves of Sabah, Malaysia. Here, we assess the mycofloral diversity on bat and arthropod cadavers in one of the most popular ecotourism destinations of northern Borneo, Gomantong caves. Opportunistic sampling of cadavers within the Semud Hitam chamber of Gomantong cave yielded nine dead arthropods and four dead bats. Twenty-four culturable fungi were isolated, of which 14 morphological taxonomic units (MTU) were observed. Twelve of the 14 MTUs underwent molecular characterization of the ITS gene region to confirm identification. All fungi were Ascomycetes except for one Basidiomycete isolate. *Aspergillus* spp. had the highest occurrence (45.8%), followed by *Penicillium* spp. (25.0%), and *Fusarium* sp. (12.5%). *Ceratobasidium* sp., *Diaporthe* sp., *Pestalotiopsis* sp., and *Xylaria feejeensis* were isolated once each. No more than one fungal taxon was isolated from each arthropod cadaver, and not all arthropods yielded culturable fungi. Bat cadavers yielded 14 out of 24 isolates (58.3%), with the highest occurrence of the fungi sampled from their skin. Our results corroborate that bats and arthropods play a role in fungal dispersion and introduction in the cave because their exteriors are likely to harbor fungi they are exposed to in the environment. We also conclude that cadavers are important substrates for fungal growth and proliferation, perpetuating the role of fungi as important decomposers in caves. This study provides a baseline of information of the mycobiome of Bornean caves for future bioprospecting and potential biotechnological applications.

INTRODUCTION

The fungal diversity in caves and their influence on cave ecosystems have yet to be explored in Borneo. Many organisms cannot sustain themselves within the dark, cool, and nutrient-limited cave environment (Gunde-Cimerman et al., 1998). Despite this, fungi are one of the most dominant of all cave organisms with high rates of spore dissemination, colonization capability in various types of substrates, and tolerance to a wide range of pH values (Nováková, 2009; Bastian et al., 2010; Wang et al., 2010; Ogórek et al., 2013; Vanderwolf et al., 2013a). Over 1000 species of fungi stemming from over 500 genera have been found from caves throughout the world (Vanderwolf et al., 2013a). Most cave fungi are Ascomycetes, but Basidiomycetes and Zygomycetes are also found at lesser rates. Many of the fungi within caves are saprophytes that have been isolated from non-cave environments. While many cave-dwelling organisms are true troglobites, very few fungi are specialized in the cave ecosystem. It is the most likely scenario that cave fungi originated from environments external to caves (Zhang et al., 2017).

Fungi are known to interact with a wide array of organisms and play an important role in the greater ecosystems they are a part of, whether as symbionts, parasites, saprophytes, or a food source (Bastian et al., 2010; Arouja and Hughes, 2016). In wild animals, fungi found on ears, lungs, intestines, bladder, kidney, animal dung, brain, and skin may lead to fungal infections (Ainsworth and Austwick, 1955; Seelan et al., 2008, 2009). A few fungal species cause diseases in mammals because their high body temperatures promote fungal growth (Bergman and Casadevall, 2010; Garcia-Solache and Casadevall, 2010). For example, white-nose syndrome (WNS) is a disease that affects hibernating bats. It is caused by a visible white fungus, *Pseudogymnoascus destructans*, that grows on bats' muzzles and wings and has killed millions of bats in North America (Blehert et al., 2009; Lorch et al., 2011; Warnecke et al., 2012). Many non-pathogenic keratinophillic fungi can survive on animal fur, possibly due to less competition from soil fungi with higher saprophytic ability (Rees, 1967). Keratinophillic fungi have been isolated from animals such as cats, dogs, cows, rabbits, horses, rats, and donkeys (Aho, 1983; Bagy, 1986; Ali-Shtayeh et al., 1988). The most common fungi isolated from animals include *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp. and *Mucor* spp. (Aho, 1983; Ali-Shtayeh et al., 1988). Fungal dermatophytes have also been isolated from domestic animals, namely *Trichophyton* spp. and

Microsporum spp., which shows that they are important in the transmission of disease-causing fungi (Ali-Shtayeh et al., 1988). Some keratinophilic fungal species isolated from domestic animals are pathogenic to humans and animals, namely *A. fumigatus*, *Stachybotrys chartarum*, *Scopulariopsis brevicaulis*, and *Cephalosporium acremonium* (Bagy, 1986). Isolation of fungi from animals, especially those from biodiversity-rich ecosystems like the tropics, may lead to the important discovery of novel bioactive compounds (Higginbotham et al., 2014). They showed that fungi isolated from sloth hair have anti-malarial, anti-bacterial, and anti-cancer bioactivity.

Although previous studies on the fungal biomes of bats in Borneo have been conducted, they were not done on bats that were captured in or near a cave environment (Seelan et al., 2008, 2009). Around the world, a plethora of studies on cave fungi from a variety of different substrates have been conducted, including bats, bat guano, invertebrates, soil, rocks, walls, water, and air (Vanderwolf et al., 2013a). In another study, thirty bats from four caves and one mine in the United States yielded 182 fungal isolates (Johnson et al., 2013). These fungi were mainly from the division Ascomycota, while Basidiomycota only made up 14% of the isolates. The most common genera isolated from the bat wings were *Cladosporium*, *Fusarium*, *Mortierella*, and *Penicillium*. A study on cave walls, ceilings, and sediment from six caves led to the discovery of 675 fungal isolates composed mainly of Ascomycota, suggesting that common cosmopolitan asco-mycetes will likely dominate studies that utilize culture-dependent methods (Zhang et al., 2014). While different hosts and substrates in caves result in varying assemblages of fungi, it has been suggested that the specific environmental characteristics of the cave itself plays a significant role on the type of fungi isolated (Johnson et al., 2013; Vanderwolf et al., 2016a).

Arthropod-associated fungi and entomopathogenic fungi have been isolated from caves from many regions of the world (Gunde-Cimerman et al., 1998; Santamaria and Faille, 2007; Jurado et al., 2008; Yoder et al., 2009; Polovinko et al., 2010; Bastian et al., 2010; Porca et al., 2011; Vanderwolf et al., 2016a). Cave invertebrates can thrive in the nutrient-limited cave environment because they are able to utilize a broad range of food sources for sustenance (Smrž et al., 2015). Fungal conidia can act as a food source for cave insects, as demonstrated with *Folsomia candida* (Smrž et al., 2015). Cave isopods were shown to prefer saprophytic fungi growing on bat guano as one of their food sources because it is a source of polyunsaturated fatty acids, an essential nutrient. A world review of cave fungi showed that 201 species of fungi from 89 genera had been isolated from arthropods (Vanderwolf et al., 2013a). Most of these were Ascomycetes and Zygomycetes. Many entomopathogenic fungi in caves are specialized to infect specific hosts. For example, *Rhachomyces* spp. are infectious to carabid beetles that are highly specialized to the cave environment (Santamaria and Faille, 2007). Cave entomopathogenic fungi may also be generalists by nature. For example, the known generalist insect pathogen, *Beauveria bassiana,* was isolated from multiple dead insects in the caves of West Siberia and made up 68% of all isolates (Polovinko et al. 2010). Furthermore, arthropods may carry fungal spores that are pathogenic to other cave fauna (Vanderwolf et al., 2016b). Despite the importance of cave fungal studies that have been conducted on all the major continents of the world, none have been conducted in Malaysian Borneo (Vanderwolf et al., 2013a).

The Gomantong cave system (5°31'30"N 118°04'15"E) is located in the 3,297 hectare Gomantong Forest Reserve, Kinabatangan, Sabah (North Borneo), and it is part of the largest limestone outcrop in the area, Gomantong Hill (Lundberg and McFarlane, 2012). Kinabatangan is known for its rich biodiversity, where at least 51 mammal species, including 10 primate species, have been recorded in the area (Boonratana and Sharma, 1997). The area surrounding the forest reserves are almost exclusively utilized for the monocrop production of palm oil. The cave itself is famous for its swiftlet nest farming and birds nest harvesting (Ismail, 1999; Hobbs, 2004; Lundberg and McFarlane, 2012). Since 2012, Gomantong caves get around 13,000 to 15,000 thousand visitors annually, mostly composed of foreigners, according to the Sabah Wildlife Department. At least 13 species of bats have been recorded from this cave, including some of the common *Hipposideros* spp., *Rhinolopus* spp., and *Myotis gomantongensis* (Abdullah et al., 2007). So far, there has not been any entomological or mycological survey studies conducted in Gomantong cave.

With the development of species barcodes in ecological studies, there are now large amounts of phylogenetic data on species isolated in caves (Woese et al., 1990; Barton et al., 2004; Lahaye et al., 2008). However, no such studies on cave mycota have been published on the caves of Sabah. With the help of DNA barcoding, establishing baseline ecological data for fungal species in areas where these types of studies are scant increases the possibility of discovering novel fungi.

MATERIALS AND METHODS

Site Description

Semud Hitam, Gomantong cave was visited twice, on October 6, 2017 and January 23, 2018 (Fig. 1). The cave is composed of two main sections, Semud Hitam (Black Cave) and Semud Putih (White Cave). Birds nest harvesting apparatus are visible throughout the cave, and the harvesting itself is done by employees of the municipality (Lundberg

Figure 1. A. Cave entrance of Semud Hitam, Gomantong Cave. B. Ceiling opening at the back end of Semud Hitam. C. Foreign tourists on boardwalk passage in the cave. Note that the boardwalk was built for ease of access for people as piles of bat guano and running water streams cover the cave floor.

and McFarlane, 2012). Because Semud Hitam is easily accessible by tourists due to its boardwalk, it is by far the most visited part of the cave (Fig. 2). Some sections of the cave have their walls written on or vandalized by visitors. Hundreds of bats are visible in the ceiling of the cave. A strong pungent smell of ammonia is always present due to the floor of the cave being covered in heaps of bat guano. A diverse array of invertebrates is found throughout the cave on guano piles, walls, ceilings, and even the walkway itself (Lundberg and McFarlane, 2012). At the end of the boardwalk, a large portion of the cave is exposed to the canopy and allows sunlight into the whole back end of the cave. In this back section of the cave, past the boardwalk, there are patches of grassy areas with a plethora of organic litter. Cave temperature and relative humidity were measured during both visits in the cave light zone, twilight zone, and dark zone (Table 1).

Sampling

Opportunistic sampling of bat and arthropod cadavers was undertaken and their positions within the cave were recorded (Fig. 3). All of the insect cadavers were collected aseptically with sterilized tweezers and sealed within sterile centrifuge tubes. The distance from the cave entrance and light zone of each sample were also recorded during sampling (Table 2). The arthropod cadavers were stored in a cooler filled with ice until transportation back to the laboratory where samples were stored at 4 °C. Bat cadavers were in the early stages of decomposition and had no obvious signs of fungal growth. They were identified on site and subsequently swabbed using sterile cotton swabs on five different body parts (i.e. anus, ear, skin, mouth, and hair). The swabs were inoculated into sterile centrifuge tubes containing 900 µL PBS buffer until further processing.

Fungal Isolation

In the laboratory, arthropod cadavers were identified to at least the genus level by using dichotomous keys (Imes, 1992; Chinery, 2005). Any hyphae visibly growing from the cadaver were inoculated onto Potato Dextrose Agar (PDA) incorporated with the antibiotic streptomycin sulfate (40 µg/mL). Isolations were performed in triplicate using the three-point method until pure isolates were produced. Bat cadaver swab samples were serially diluted 10-fold up to 10^{-4}. Of the 10^{-2} to 10^{-4} dilutions, 0.1 mL aliquots were spread on the PDA plates. Dilutions were performed in triplicate using sterile distilled water. All inoculated plates were incubated for 3−7 days at room temperature (25 °C) and in the dark. Isolates were grouped into morphological taxonomic units (MTU) based on the colony morphology and micro-morphological characteristics, i.e. colony color, texture, growth patterns, hyphae, conidia size and shape, and conidiophores.

Figure 2. A. Vandalism on the cave wall by tourists. B. One of the swiftlet nest harvesting apparatus. Care takers of the swiftlet nest farms reside in shacks immediately outside the cave.

Table 1. Temperature and relative humidity in Gomantong cave. The approximate distance from cave entrance and light zone were also recorded.

Date	Light Zone			Twilight Zone			Dark Zone		
	Temp, °C	RH, %	Dist., m	Temp, °C	RH, %	Dist., m	Temp, °C	RH, %	Dist., m
Oct 6, 2017	29	93	5	26	100	35	26	100	70
Jan 23, 2018	30	92	5	29	92	35	27	100	70

Identifications were carried out by comparing the morphological characteristics of the fungi to universal identification keys described by Raper and Fennell (1965), Klich (2002), and Domsch et al. (2007).

PCR Amplification and Sequencing

Molecular identification was performed on all arthropod cadaver fungi isolates and on six bat cadaver fungi isolates. The E.Z.N.A DNA Fungal Kit (Omega Bio-Tek, USA) was used to extract DNA from pure cultures of isolates according to the manufacturer's instruction. The 5.8S sequences were amplified with primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT TGA TAT GC-3') (White et al.; 1990) (Vilgalys Mycology Lab). PCR amplification was carried out in a total volume of 50 µL with the following reagents from Promega (USA): 2 µL of each primer (10 pmol/**µ**L), 0.25 µL *Taq* DNA polymerase (5 units/µL**)**, 10 µL PCR Buffer (5X), 4 µL MgCl$_2$ (25mM), 1 µL dNTPmix (10mM), 2 µL DNA template (~25 ng/mL). PCR cycles were performed in a Bio Rad T100 Thermal Cycler. For amplification, the conditions were 95 °C for 3 min of the initial denaturation, followed by 35 cycles of 94 °C for 30 s of denaturation, 53 °C for 30 s of annealing, and 72 °C for 1 min of extension. A final extension of 72 °C for 10 min was added to complete the process. The PCR products were then electrophoresed in a 1 % agarose gel for 30 mins and subsequently stained with gel red for visualization. Next, the PCR products were purified using Column-Pure PCR Clean-Up Kit (Applied Biological Materials, Inc., Richmond, BC) according to the protocol of the manufacturer. DNA sequencing was performed using the BigDye Terminator v3.1 on a ABI3500 sequencer (Applied Biosystems). Sequencing service was provided by MyTACG (Taiwan). The ITS forward and reverse primers were used in the cycle sequencing. The resulting reads were aligned to obtain the full-length amplicon sequence (BioEdit version 7.0.5.3) and submitted to GenBank. Once the sequencing was completed, the ITS barcode sequences generated from all isolates were queried against NCBI nu-
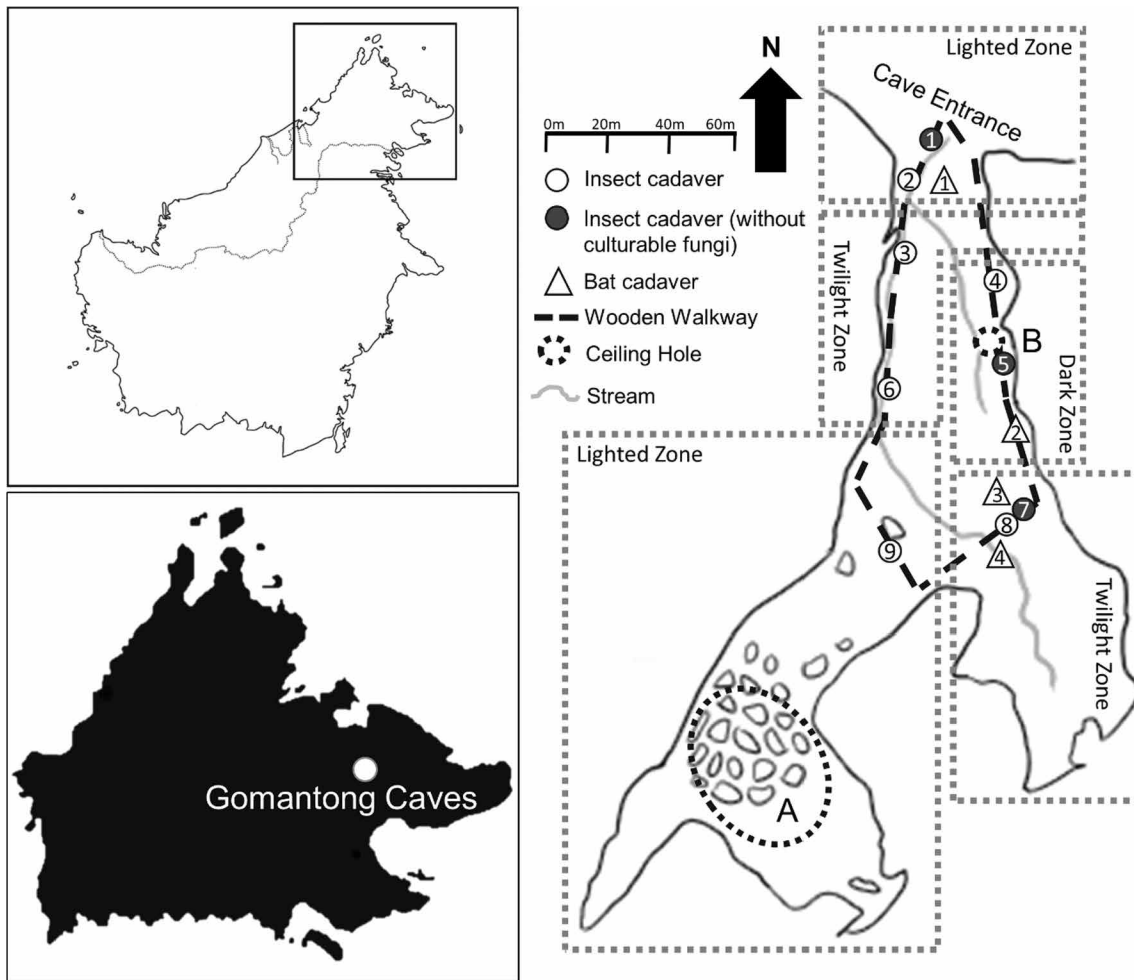
Figure 3. Layout of Semud Hitam, Gomantong Cave, Sukau, Sabah Malaysia. The cave entrance is located in the north end of the cave. The cave is accessible via a wooden walkway that was built for ease of access for tourists and swiftlet nest farmers. The tall mouth of the cave entrance and the presence of two large ceiling apertures (A and B) make the cave relatively well-lit during the afternoon hours. There are areas that do not receive direct sunlight and other areas that are completely dark hidden between walls, nooks, and crevices. All 14 cadavers were collected and their relative distances from the cave entrance measured and labelled by light zone.

cleotide sequence using basic local alignment search tool (BLASTn) to ascertain their closest relationships (Zhang et al., 2000).

RESULTS

Fungi are prevalent in the cave environment, and cave fauna, wind, water, and humans all play important roles in spore dispersion and translocation in and out of the cave. In the current study, a total of 24 axenic fungal isolates were obtained from six out of nine arthropod cadavers and all four bat cadavers sampled (Table 2). Ten pure cultures were isolated from arthropod cadavers and 14 from bat cadavers (Table 3). Isolated fungi were separated

Table 2. Distance of arthropod and bat cadaver samples from the entrance and number of fungal isolates obtained.

Light Zone	Sample ID	Sample species	Distance from Entrance, m	No. of Fungal Isolates
Lighted	A01	*Periplaneta americana*	8	0
	A02	*Thereuopoda* sp.	20	2
	A09	*Periplaneta americana*	108	1
	B01	*Cynopterus brachyotis*	19	8
Twilight	A03	*Periplaneta americana*	28	2
	A06	*Periplaneta americana*	63	1
	A07	*Periplaneta americana*	91	0
	A08	*Trigonilus corallinus*	93	1
	B03	*Balionycteris maculata*	85	2
	B04	*Chaerephon plicatus*	94	1
Dark	A04	*Trigonilus corallinus*	33	3
	A05	*Thereuopoda* sp.	45	0
	B02	*Hipposideros diadema*	76	3

Table 3. Fungi isolated per insect and bat cadaver species. Body parts sources are listed for bat cadaver hosts.

Host Species	Fungal Species	Source	No. of isolates
Arthropod			
Periplaneta americana	*Aspergillus flavus*	Wings	1
	Aspergillus luteovirescens	Abdomen	1
	Ceratobasidium sp.	Thorax	1
	Fusarium solani	Thorax	1
Thereuopoda sp.	*Fusarium solani*	Abdomen	1
	Penicillium sclerotiorum	Abdomen	1
Trigonilus corallinus	*Aspergillus sclerotiorum*	Body	1
	Diaporthe sp.	Body	1
	Fusarium solani	Body	1
	Penicillium shearii	Head	1
Bat			
Balionycteris maculata	*Aspergillus restrictus*	Skin, Hair	2
Cynopterus brachyotis	*Aspergillus flavus*	Skin	1
	Aspergillus ochraceus	Ear	1
	Aspergillus restrictus	Anal, Skin, Ear	3
	Penicillium citrinum	Skin, Oral	2
	Pestalotiopsis sp.	Oral	1
Hipposideros diadema	*Aspergillus restrictus*	Hair	1
	Penicillium paxilli	Skin, Hair	2
Chaerephon plicatus	*Xylaria feejeensis*	Oral	1

Table 4. Molecular characterization based on ITS barcode similarities to the NCBI database.

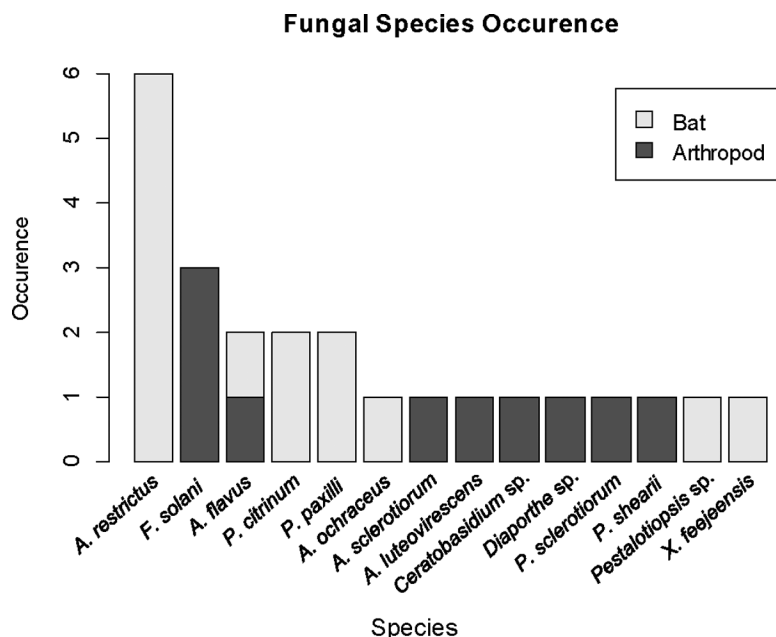| Isolate	NCBI Identification	E value	Identification, %	Host Species
GMT01	*Fusarium solani*	0.0	97.4	*Thereupoda* sp. (A02)
GMT02	*Penicillum sclerotiorum*	0.0	98.2	*Thereupoda* sp. (A02)
GMT03	*Fusarium solani*	0.0	99.8	*Periplaneta americana* (A03)
GMT04	*Ceratobasidium* sp.	0.0	96.2	*Periplaneta americana* (A03)
GMT05	*Diaporthe* sp.	0.0	98.8	*Trigonilus corallinus* (A04)
GMT06	*Aspergillus sclerotiorum*	0.0	100	*Trigonilus corallinus* (A04)
GMT07	*Fusarium solani*	0.0	99.0	*Trigonilus corallinus* (A04)
GMT08	*Aspergillus luteovirescens*	0.0	99.8	*Periplaneta americana* (A06)
GMT09	*Penicillium shearii*	0.0	99.7	*Trigonilus corallinus* (A08)
GMT10	*Aspergillus flavus*	0.0	100	*Periplaneta americana* (A09)
GMC05	*Penicillium paxilli*	0.0	100	*Hipposideros diadema* (B02)
GMC06	*Penicillium paxilli*	0.0	100	*Hipposideros diadema* (B02)
GMC09	*Xylaria feejeensis*	0.0	99.7	*Chaerephon plicatus* (B04)
GMC10	*Penicillium citrinum*	0.0	99.5	*Cynopterus brachyotis* (B01)
GMC14	*Aspergillus flavus*	0.0	100	*Cynopterus brachyotis* (B01)
GMC15	*Penicillium citrinum*	0.0	100	*Cynopterus brachyotis* (B01)

Fungal Species Occurence

Figure 4. Fungal species occurrence from bat and arthropod cadavers in Gomantong Cave. *A. restrictus* was the mode (n=6), followed by *F. solani* (n=3).

into 14 MTUs based on their macro- and micro-morphology. Sixteen out of the 24 pure isolates underwent molecular characterization. Molecular analysis was prioritized for isolates that could not be identified solely on morphology and to corroborate identification of cryptic taxa at the species level. The ITS barcode PCR amplicons were about 600 bp in size for all of the 16 isolates. After DNA sequencing, the BLASTn data from the 16 isolates resulted in 11 operational taxonomic units (OTU) (Table 4). The results indicated that all OTUs belonged to the division Ascomycota except for *Ceratobasidium* sp., from the division Basidiomycota. The majority of the isolated strains belonged to the order Eurotiales and the family Aspergillaceae. For the *Ceratobasidium* sp. and *Diaporthe* sp. isolates reported in this study, ITS gene sequences were not enough to differentiate the isolates up to the species level. The fungal genera isolated were *Aspergillus* (45.8%), *Penicillium* (25.0%), *Fusarium* (12.5%), *Ceratobasidium* (4.2%), *Diaporthe* (4.2%), *Pestalotiopsis* (4.2%), and *Xylaria* (4.2%). *Asper-*

gillus restrictus was isolated a total of six times, accounting for 25% of all isolates (Fig, 4).

We observed that different species of fungi were present on different species of arthropods (Figs. 5 and 6). Four species of fungi were isolated from *Periplaneta americana* (Cockroach) and *Trigonilus corallinus* (Asian/Rusty millipede) cadavers. *Thereuopoda* sp. (Gantcave/Cave centipede) recorded only two species of fungal isolates.

Four different species of bat cadavers were collected during sampling for this study. *Cynopterus brachyotis* (Lesser short-nosed fruit bat) yielded five species of fungi, which was the most for any one sample in this study. Two species of fungi were isolated from *Hipposideros diadema* (Diadem leaf-nosed bat). Only one species of fungi was isolated from each *Balionycteris maculata* (Spotted-winged fruit bat) and *Chaerephon plicatus* (Wrinkle-lipped free-tailed bat) cadaver. The bats' skin had the highest di-

Figure 5. Dead arthropod samples in order of distance from entrance. A. A01, *P. americana*. B. A02, *Thereuopoda* sp.. C. A03, *P. americana*. D. A04, *T. corallinus*. E. A05, *Thereuopoda* sp.. F. A06, *P. americana*. G. A07, *P. americana*. H. A08, *T. corallinus*. I. A09, *P. americana*.
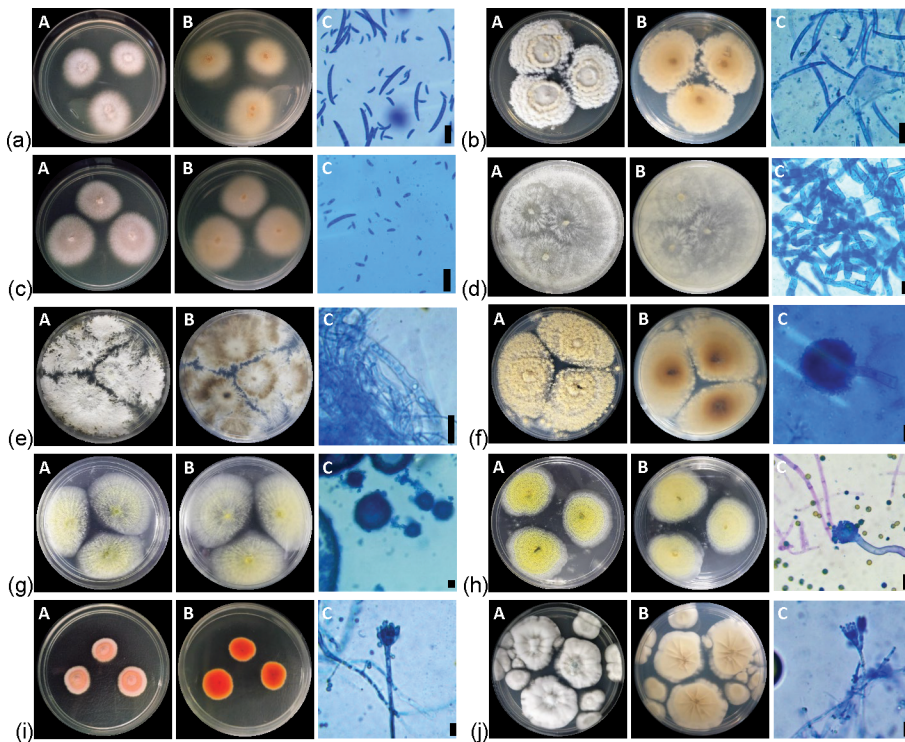
Figure 6. (a) *Fusarium solani* GMT01; (b) GMT03; (c) GMT07; (d) *Ceratobasidium* sp. GMT04; (e) *Diaporthe* sp. GMT05; (f) *Aspergillus sclerotiorum* GMT06; (g) *Aspergillus terricola* GMT08; (h) *Aspergillus flavus* GMT10; (i) *Penicillium sclerotiorum* GMT02; (j) *Penicillium shearii* GMT09. A. Colony surface on PDA. B. Colony reverse on PDA. C. Micromorphological characteristics (*i.e.* conidia, conidiophores, hyphae). Scale = 10 μm.
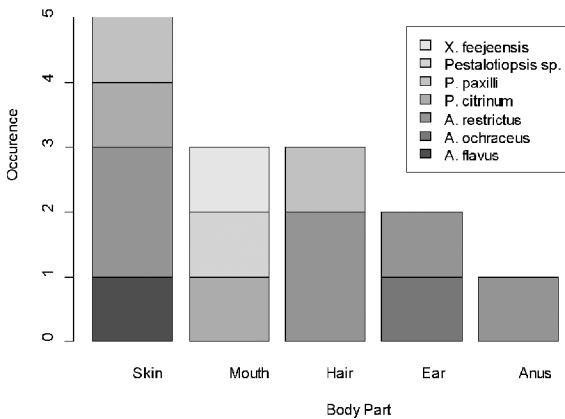

Figure 7. Number of fungal isolates per body part. The skin of bats had the highest fungal isolates (n=5, 4 species), and anus had the lowest (n=1, 1 species).

versity and occurrence for any of the body parts with four species of fungi from five isolates (Fig. 7). The different fungal isolates from all bat cadavers are shown (Fig. 8).

DISCUSSION

Ours is the first study that describes the occurrence of fungi in a cave from Sabah, Malaysia. Fourteen fungal isolates were found on bat cadavers and ten fungal isolates from arthropod cadavers in this study. Both the bat and arthropod cadavers had similar fungal diversity, each yielding eight different species of fungi. Twenty-three of the 24 fungal isolates found in the study were ascomycetes, the remaining one being *Ceratobasidium* sp., a basidiomycete. The most frequently isolated fungal genera in this study were *Aspergillus*, *Penicillium*, and *Fusarium*, which are ubiquitous in non-cave environments (Domsch et al., 2007). Findings from this study are congruent with previous reports where the Ascomycota, especially fungi from genera *Aspergillus*, *Fusarium,* and *Penicillium*, are the dominant division of fungi isolated from cave environment (Nováková, 2009; Voyron et al., 2011, Vanderwolf et al., 2013a). A study conducted previously on cadavers and skeletons of various fauna from cave and mine environments found that 12 out of 39 fungal isolates were identified as Mucoromycota (Nováková et al., 2018). *Mucor* is considered as one of the dominant genera of fungi isolated from cadavers throughout the various stages of decomposition along with *Aspergillus*, *Penicillium*, and *Candida* (Sidrim et al., 2009). The lack of Mucoromycota in this study may be explained by difference in media type used in our study compared to theirs. Using exclusively PDA and MEA during incubation and isolation in this study likely gave preference for rapidly growing ubiquitous fungi, especially *Aspergillus* and *Penicillium*.

Most studies on cave fungi utilize culture-dependent methods of isolation before proceeding to morphological or molecular characterization (Vanderwolf et al., 2013a; Man et al., 2015; Zhang et al., 2017; Nováková et al., 2018; Visagie et al., 2019). Because of limited finances, manpower, and time, this initial study exclusively utilized culture-based methods of isolation. Culture-independent community-based studies on cave fungi are scant, but they have been conducted in recent years (Zhang et al., 2014; Zhang and Cai, 2019). Culture-based methods tend to produce results that over-represent rapidly growing cosmopolitan fungi. Whether fastidious fungi will grow and be observed in culture is heavily influenced by temperature, length of incubation, type of medium used, and aerobic conditions (Bills, 1995; Collado et al., 2007; Unterseher and Schnittler, 2009; Tristan et al., 2012). Culture-dependent methods are known to only reveal around 0.6−8.0% of total the total fungal species in a sample (Hibbett et al., 2009; Hawksworth and Lucking, 2017) so culture-dependent methods are greatly limiting and hinder our understanding of the overall role fungi play in the ecosystems they inhabit. Community based culture-independent methods can generate millions of raw sequences at a time, yielding in the hundreds to thousands of fungal OTUs per sample (Winter et al., 2017; Zhang and Cai, 2019). On the other hand, Zhang and Cai (2019) reported that culture-dependent and culture-independent methods tend to show similar fungal diversity, but culture-independent methods are more likely to find uncommon fungi in their re-
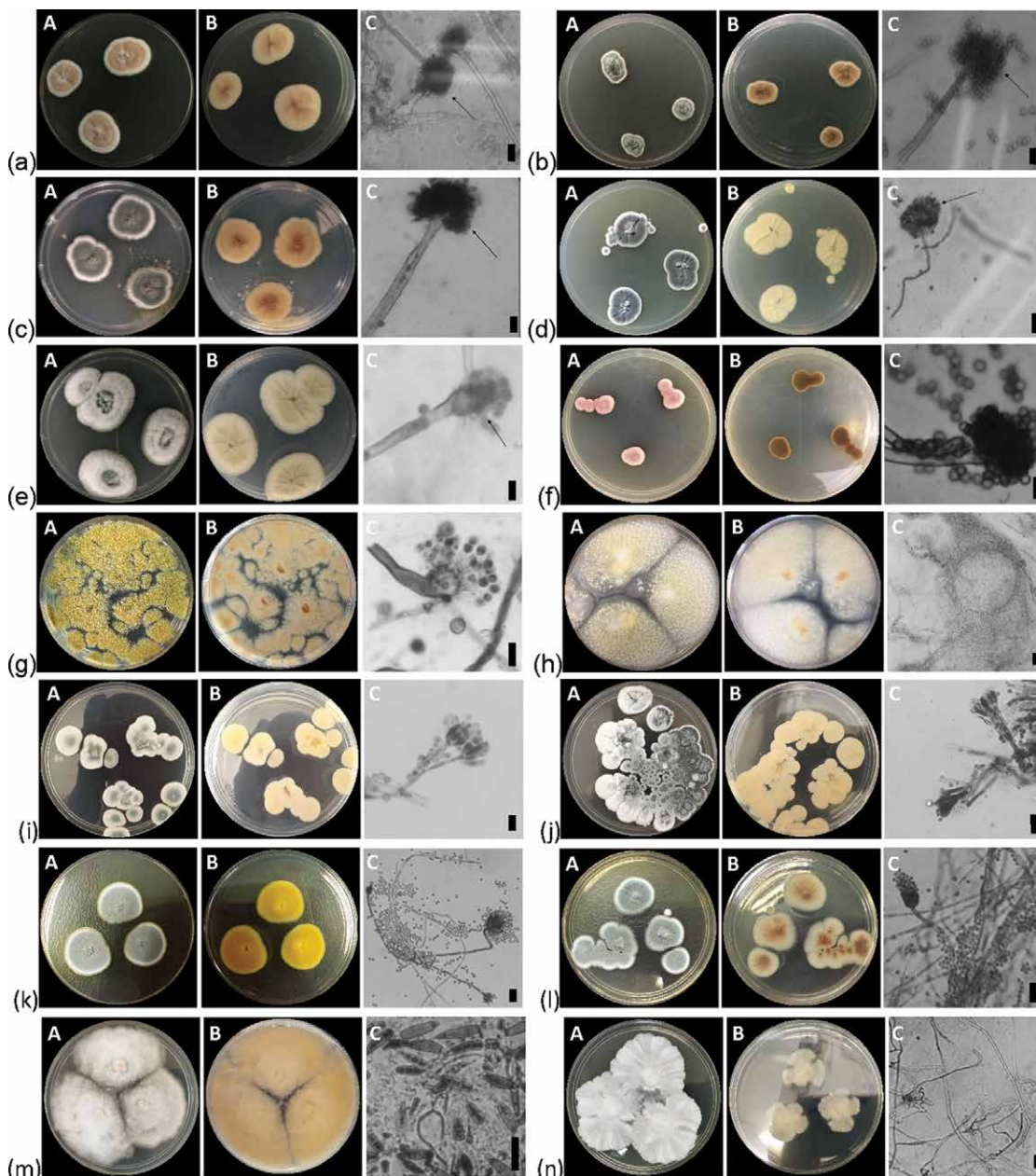
Figure 8. (a) *Aspergillus restrictus* GMC01; (b) GMC02; (c) GMC03; (d) GMC06; (e) GMC07; (f) GMC11; (g) *Aspergillus flavus* GMc13; (h) *Aspergillus ochraceus* GMC12; (i) *Penicillium citrinum* GMC09; (j) GMC14; (k) *Penicillium paxilli* GMC04; (l) GMC05; (m) *Pestalotiopsis* sp. GMC10; (n) *Xylaria feejeensis* GMC08. A. Colony surface on PDA. B. Colony reverse on PDA. C. Micromorphological characteristics (*i.e.* conidia, conidiophores, hyphae). Scale = 10 μm.

spective substrates. They also found that around 3.6−12.0% of the total OTUs in each respective cave were exclusive to that cave only, although most of these OTUs were unidentified to the genus level. Thus, fungal studies utilizing culture-based methods are still primary and will remain necessary as long as they are required to describe new strains and species, in addition to being cost-effective and more readily available.

Bats can travel faster and traverse larger distances than arthropods, which may account for the higher number of fungal isolates as seen in this study. In this study, six out of the nine fungal species isolated from bats (71.4% of isolates) were isolated from the two frugivorous bat hosts compared to the two insectivorous ones. Although our sample size is not large enough to statistically conclude which group of bats are more efficient as fungal carriers and dispersers, our current data shows that frugivorous bats carried a higher fungal load than insectivorous bats. In a previous study in Sarawak-Borneo, 17 *Aspergillus* spp. from six species of bats, namely *Aspergillus restrictus*, *A. sydowii*, *A. niger*, *A. clavatus*, and *A. japonicus* were recorded (Seelan et al., 2008). They reported that the anus and ear yielded the largest number of fungal isolates and showed high fungal diversity. It was noted that various substrate types in wild animal populations resulted in various types of mycoflora, and that the mycoflora found on bats are highly correlated to the food consumed (higher in fruit-eating bats than insect-eating bats) and their roosting site (Seelan et al., 2008). Although fungi and fungal spores exist throughout the cave environment, bats are likely key regulators for the mycoflora of caves, as they are the key transporters in and out of the caves and contribute to guano and carcass deposition (Vanderwolf et al., 2013a, 2013b).

Most studies on bat mycoflora have focused on bat fur (Larcher et al., 2003; Beguin et al., 2005) and *P. destructans* related surveys (Blehert et al., 2008; Gargas et al., 2009; Puechmaille et al., 2011; Johnson et al., 2013; Zukal et al., 2014). Although there have been bat cadavers sampled for fungal isolation in these studies, none have specifically

studied fungal diversity exclusively on cadavers until Nováková et al. (2018). Prior to our study, 67 species of fungi have been reported from bat cadavers found in caves throughout the world, with *Crysosporium merdarium* bring the most frequently isolated (Nováková et al., 2018). Only five other studies had reported fungi from bat cadavers in caves (Zeller, 1996; Wibbelt et al., 2010; Voyron et al., 2011; Vanderwolf et al., 2013b, 2016a, Nováková, 2018). None of the eight fungal species identified from bat cadavers in our study were reported in prior studies on cadavers in caves, and thus are reported for the first time here. None of them were in the genus *Chrysosporium*, although this is not surprising as fungi from this genus were previously isolated from long-dead decomposed bats.

The bat cadavers in our study were in the early stages of decomposition and were still identifiable morphologically *in situ*. Nováková et al. (2018) sampled cadavers from both early and late stages of decomposition, even carcasses of only fur and bone, which may have also contributed to the difference in mycobiome from bat cadavers in both studies. Because fungal growth rates are faster on dead bats than live bats, the increased number of bat cadavers in a cave will likely affect fungal diversity found on live bats in the same cave especially after a mass-mortality event (Vanderwolf et al., 2016a).

Along with bats, studies on the insect diversity of Gomantong cave remain scant, with no published reports on arthropod-associated fungi or entomopathogenic fungi in Gomantong cave. The presence of fungi is expected on cave arthropods because many fungi are entomophilous, entomogenous, or entomopathogenic (Ogórek et al., 2013). The arthropods collected in our study were not commonly sampled in previous cave studies, and all three different species of arthropods yielded culturable fungi. Various cave butterflies, crickets, diplopods, harvestmen, moths, mites, and spiders have been previously cultured for fungi, none of which were sampled in our study (Kubátová and Dvorák, 2005, Vanderwolf et al., 2016b; Nováková et al, 2018). The genera *Aspergillus* and *Fusarium* tied for having the highest number of isolates for arthropod hosts (n = 3). But all three *Fusarium* isolates were identified as *F. solani*, whereas the three *Aspergillus* isolates were identified as the three different species, i.e. *A. flavus*, *A. luteovirescens*, and *A. sclerotiorum*. *Penicillium* was the only other genus isolated multiple times (n = 2). *Aspergillus*, *Fusarium*, and *Penicillium* are known to have entomogenous species recorded from previous surveys, and it is not surprising to find these taxa in our study (Jurado et al., 2008; Bastian et al., 2010; Vanderwolf et al., 2016b). Whenever *Aspergillus*, *Fusarium*, and *Penicillium* fungi are isolated from cave environmental samples, arthropods should always be considered as major vectors, dispersers, and hosts. One of the most frequent entomopathogenic fungi to be identified in prior cave studies, *Isaria furinosa*, was not isolated from any arthropod cadavers in this study (Kubátová and Dvorák, 2005). A reduction in the arthropod population in caves, naturally or artificially, could be a way to reduce fungal abundance in the cave or control fungal contamination to other areas within a cave system.

The different species of fungi documented on different arthropod cadavers may be due to differences in movement patterns, feeding location, diet, aggregation and interaction with other individuals, and other external factors. Insects are known to feed on fungi (Šustr et al., 2005; Jacobs et al., 2017). Guano, which is a known substrate of cave fungi, are also feeding grounds for mites that eat guano inhabiting bacteria and fungi (Smrž et al., 2015). Similar arthropods in Gomantong cave, where most of the cave floor is covered in heaps of guano, could be dispersing fungal spores throughout the cave unintentionally. Cockroaches are well known omnivorous scavengers and are likely feeders on sundry organic matter in guano heaps. These same cockroaches would be unintentionally inoculating fungi on bat guano within the cave, as well as dispersing fungal spores already proliferating on the guano to other areas of the cave that can act as substrates, i.e. dead wood (Marcot, 2017), sediment (Taylor et al., 2014), and cave walls (Bastian et al., 2009).

All 14 taxa of fungi isolated from this study have saprophytic properties. Eight of them have been isolated from cave environments prior to this study (Vanderwolf et al., 2013a). *A. luteovirescens*, *A. ochraceus*, *Diaporthe* sp., *P. sclerotiorum*, *P. shearii*, and *Xylaria feejeensis*, have not been isolated from the cave environment prior to this study. *Ceratobasidium* sp. and *Diaporthe* sp. were not able to be identified at the species level based solely on their ITS barcode. It is unlikely to get a good representation of the overall mycobiome of any cave by evaluating a limited type of substrate or host, i.e. only sampling cadavers. Microbial distribution in caves is heavily determined by the susceptibility of the host, bio-receptivity of the substrate, and environmental conditions (Cuezva et al., 2009; Jurado et al., 2010).

Aspergillus and *Penicillium* accounted for the highest proportion of fungal diversity in this study, constituting five and four different species out of a total of 14, respectively. They also constituted 17 of the 24 total isolates (70.8%). *Aspergillus restrictus* was isolated six times (25%) from three different species of bats. *Aspergillus flavus* was the only species isolated from a bat and an arthropod cadaver, *C. brachyotis* and *P. Americana*, respectively. *Aspergillus* spp. are commonly known saprophytes from soil and plant debris (Domsch et al., 2007). *Aspergillus* and *Penicillium* are the first and second most reported genera in cave mycological studies aside from the genera *Geomyces* and *Histoplasma*, which have a high occurrence due to the many studies focusing specifically on WNS and histoplasmosis, respectively (Vanderwolf et al, 2013a), which is consistent with the finding that *Aspergillus* spp. and *Penicillium* spp. are some of the most frequently-isolated fungi from cave soil, rocks, and bat guano (Lorch et al., 2012; Man et al., 2015). *Aspergillus* spp. and *Penicillium* spp. were some of the most commonly isolated fungi recovered from bat wings, fur, and skin in North American caves (Johnson et al., 2013; Vanderwolf et al., 2013b). In fact, a variety of *Aspergillus* and *Penicillium*

species have been isolated from dead bats and arthropods in caves as well (Voyron et al., 2011; Nováková, 2018). The fungi identified from cadavers in this study are likely to be found growing on other environmental substrates within the cave, making it easy for the fungi to utilize the cadavers as a carbon source as soon as death occurs.

Fusarium solani (12.5%), *Ceratobasidium* sp. (4.2%), and *Diaporthe* sp. (4.2%), were isolated from arthropod cadavers and *Pestalotiopsis* sp. (4.2%) and *Xylaria feejeensis* (4.2%) were isolated from bat cadavers in this study. These taxa of fungi are known plant pathogens and plant endophytes, which suggests a strong influence of the surrounding plant diversity on the fungal communities in Gomantong cave. *Ceratobsidium* sp. is the only basidiomycete of all the isolates in this study. Basidiomycetes are less common in the cave environment, but they are the second most frequent division to be isolated (Vanderwolf et al., 2013a). All of these isolates have been reported in the cave environment prior to this study, except *Diaporthe* sp. and *X. feejeensis*, and thus, they are reported for the first time here (Vanderwolf et al., 2013a). *Xylaria* spp. are fast-growing fungi usually found in healthy plant tissue (Petrini and Petrini, 1985; Davis et al., 2003), and fungi from this genus have been isolated in the cave from guano, soil, and wood prior to this study (Vanderwolf et al., 2013a). *Fusarium solani* was the second most isolated taxa in this study, which is congruent with prior findings because this species has been widely reported in caves and is considered a natural part of the cave ecosystem (Bastian et al., 2009). *Pestalotiopsis* sp. has garnered increased attention in recent years because they produce many important secondary metabolites (Strobel et al., 1996, 2002; Aly et al., 2010; Xu et al., 2010; Maharachchikumbra et al., 2011).

Since these fungi are plant pathogens and endophytes, it may seem unlikely that these fungi would be isolated from bat and arthropods cadavers. However, it is conceivable that the bats and arthropods interacted with these fungi or their spores while traversing the cave or the surrounding forest environment. Bats exit the cave on a regular basis due to their feeding habits and likely interact with *Pestalotiopsis* sp. and *X. feejeensis* while foraging food in the forest. The arthropods could have picked up *Ceratobasidium* sp., *Diaporthe* sp., and *F. solani* mycelia or spores while feeding on decaying organic material, either near the cave entrance or the rear cave floor where plant life is abundant. Cave invertebrates also tend to reside in heaps of guano that are known reservoirs for fungi (Šustr et al., 2005; Nieves-Rivera et al., 2009; Nováková, 2009). Another possibility is that the fungi colonized the cadavers after death, which could happen due to spore dispersal by environmental means, other motile fauna, or human influence.

The geological features of Semud Hitam may have a direct effect on its mycota. The cave has a large cave opening that is about 80 m high and about 30 m wide (Lundberg and McFarlane, 2012). Also, in the back end of Semud Hitam, there is a large opening in the ceiling. The sunlight allows for there to be grassy patches exclusively in this part of the cave area (B, Fig, 1; ceiling hole A, Fig. 3). The presence of autotrophs on the cave floor itself is expected to affect the overall fungal diversity of Gomantong cave, and greater fungal diversity is expected near both the cave entrance and back opening (Shapiro and Pringle, 2010; Kuzmina et al., 2012; Mulec et al., 2012). These openings expose a good portion of the cave to precipitation and sunlight and serves as another point of entry for ambient spores, water, organic content, and airflow. Recent studies have suggested that the fungal communities outside the cave play a major role on the fungal diversity within the cave (Zhang and Cai, 2019). Another source of water, organic matter, and spores stems from rainwater being vertically filtered through the soil and rock above the cave (Ikner et al., 2007). During both visits, temperature and relative humidity data did not vary by much (Table 1). But, there was a discernible decrease in temperature and increase in humidity going from the lighted zones to the dark zones. Air temperature and humidity play a significant role in the microbial diversity in the environment (Ogórek et al., 2013). The dark zone of the cave had the lowest temperatures and relative humidity of 100% during both visits. Gomantong cave showed high relative humidity similar to caves in temperate regions (Nováková et al., 2018).

Gomantong cave, especially Semud Hitam, is a popular ecotourism destination for foreigners and locals. Human visitation may affect cave fungal diversity in a number of ways. There is significant evidence showing that increased human traffic into a cave system will cause contamination of indigenous fungal species by non-indigenous microorganisms (Porca et al., 2011; Griffin et al., 2014). Humans are also responsible for introducing nutrients into a cave (Ikner et al., 2007; Chelius et al., 2009, Shapiro and Pringle, 2010; Pusz et al., 2015). Previous reports have correlated human visitation to lower levels of fungal diversity, but interestingly caves with no human visitations show extremely low fungal abundance (Shapiro and Pringle, 2010). Vandalism in Gomantong cave can be seen on cave walls (Fig. 2A), and previous studies have shown that it affects microbial diversity, although localized to those specific areas (Shapiro and Pringle, 2010). We cannot say how human visitation has changed the mycobiome of Gomantong cave prior to human visitation as the cave has been utilized for swiftlet nest farming for multiple generations. Nonetheless, the increased levels of ecotourism in recent decades have likely affected the microbial diversity in the cave either through spore translocations or nutrient introductions, especially along the boardwalk. All of the samples in this study were collected near or on the boardwalk itself. Comparing the mycobiome of caves with high visitation versus those of low visitation in Sabah has never been done before, and future studies of this nature are highly recommended to better understand tropical cave ecosystems.

Aspergillus flavus and *F. solani* are known opportunistic human pathogens. Although *Diaporthe* sp. was not identified to the species level, it is known that *Diaporthe phoenicicola* causes scleral keratitis in humans (Gajjar et al., 2011). Recent visitors of caves should always be aware of opportunistic fungal pathogens on the rare chance that they present medical symptoms. More surveys need to be conducted to truly evaluate the potential of emerging infectious diseases of fauna residing in the tropical caves of Borneo.

Currently, we are unaware of any deleterious fungal diseases that affect bats and insects in Sabah's limestone caves. No *P. destructans* or *Geomyces* spp. were isolated in this study, likely due to the warmer tropical climate. As the keystone species in many ecosystems, particularly their roles as plant pollinators and insect population control (Mickleburg, et al., 2002; Kunz and Fenton, 2003; Lobova et al., 2009), it is pivotal to monitor the general health of bat populations and to identify any risks that pose a threat to their general wellness. A decline in the population of bats may lead to a cascade of ecological changes that could pose a significant loss to diversity of Bornean bats. Additionally, no obligate entomopathogenic fungi were isolated in this study, but the limited number of sampling days and limited access to all areas of the cave may have played a significant factor. We expect that with more extensive sampling in the future similar entomopathogenic strains that exist in the tropical forests of Borneo would overlap into their caves as well.

Living bats and arthropods are not a food source for most fungi, but their cadavers can be reservoirs for both live fungi and fungal spores. Environmental factors, anthropogenic disturbance, and the natural movement of cave fauna will disperse fungi proliferating on these cadavers into the surrounding environment. Dispersed fungi and their spores will find other suitable substrates to thrive on in the cave, perpetuating further growth. Some saprobic fungi, including ones isolated in this study, can act as opportunistic pathogens to cave fauna and humans (Bastian et al., 2009; Voyron et al., 2011). However, considering their low pathogenic potential to bats and insects, it is likely that the fungi did not play a role in the death of these animals. It is plausible that these animals came in contact with the spores or hyphae from the surface, while traversing the cave environment, or the saprophytic fungi colonized the host cadavers after their deaths. This work corroborates the idea that arthropods and bats contribute to the translocation of fungal spores in and out of the cave and dispersion within the cave itself. There was no evidence of any fungus that required cadavers as their obligatory substrate.

CONCLUSIONS

Our study is the first to report on the mycobiome of a cave in Borneo and serves as a baseline study to propel future interest and develop skills of researchers. Cave fauna, specifically bats and insects, harbor a multitude of fungi on or in their bodies that represent the mycobiome profile of the surrounding environment. The 14 species of fungi reported from 24 isolates in this study very likely account for an extremely small percentage of the total assemblage of fungi residing within Gomantong caves due to the multitude of potential substrates suitable for fungal growth. None of the fungi isolated are obligate cave dwellers since all taxa have been reported from outside the cave environment. Ongoing studies cultivating fungi from various environmental samples from Gomantong cave is currently in progress. We urge that more studies on cave fungi in Borneo be conducted for their enormous biological and industrial potential, and that future studies use both culture-dependent and culture-independent methods.

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