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RESEARCH ARTICLE

Rare occurrence of *Blastocystis* in sea turtles and insects (cockroaches, houseflies, and crickets) from several states in Peninsular Malaysia

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ABSTRACT

Blastocystis a single-celled eukaryotic protist, is known to inhabit the intestines of various hosts, including humans, and has been implicated in a wide spectrum of symptoms, ranging from gastrointestinal issues to skin disorders, thereby establishing its status as an emerging infectious agent. In this study, the prevalence of *Blastocystis* infection was investigated in insects, including cockroaches, houseflies, and crickets, as well as sea turtles. Additionally, the genotypic characteristics of the isolated *Blastocystis* strains were examined, and the evolutionary relationships between *Blastocystis* species found in sea turtles, and animals/humans were determined. Microscopic techniques and molecular methods were utilized in this study. The results showed that four out of 90 insects (4.44%) and one out of 13 sea turtles (7.7%) were infected by *Blastocystis*. Furthermore, detailed observations revealed the presence of characteristic morphological features, such as vacuolar forms in the cockroach, cricket and sea turtle samples and binary fission from cockroach samples, indicative of *Blastocystis*' mode of reproduction. While the ST8 of *Blastocystis* in sea turtles were successfully identified, no subtyping was achieved for the infected insects. This study not only establishes the occurrence of *Blastocystis* infection in sea turtles but also uncovers its ability to infect insects, suggesting a potential reservoir role for these organisms. Overall, this research emphasizes the significance of comprehending the prevalence, genotypic diversity, and evolutionary relationships of *Blastocystis* across various hosts. Such insights are instrumental in developing effective control measures and public health interventions to mitigate the associated symptoms and prevent future outbreaks.

Keywords: *Blastocystis*; insects; Malaysia; protozoan; turtle.

INTRODUCTION

Blastocystis is a unicellular, anaerobic and eukaryotic protist that resides in the intestines of a variety of hosts including humans (Parija & Jeremiah, 2013). The organism is associated with a wide range of signs and symptoms, from nonspecific intestinal symptoms to cutaneous disorders (Tan *et al*., 2010) and it is now known to be an emerging microbial pathogen (Tan, 2008). There are different major morphological forms including granular, vacuolar, cyst and amoeboid (Tan, 2008). The most common mode of transmission is thought to be faecal-oral, but food-borne and waterborne are also considered modes of transmission (Noel, 2005). Noel (2005) also suggested that the mode of transmission can occur via animal-to-human, human-to-animal, animal-to-animal and human-to-human.

This parasite has been extensively studied around the world and in Malaysia. There are various studies of *Blastocystis* in Malaysia such as in human (Anuar *et al*., 2013), animal including goat (Tan *et al*., 2012), cattle (Kamaruddin *et al*., 2020; Razak & Mohammad, 2022), sheep (Razak & Mohammad, 2022), chicken (Farah Haziqah, 2018a), dog, cat (Farah Haziqah, 2018b), and wild rat (Farah Haziqah, 2018c), turkey (Siti Alawiyah *et al*., 2021), primates (Hemalatha *et al*., 2014), quail (Rauff-Adedotun *et al*., 2022), and water (Anuar *et al*., 2013). In Malaysia, the studies of *Blastocystis* in insects (cockroaches, houseflies, and crickets) have not been extensive. There are several studies of this parasite in cockroaches (Suresh *et al*., 1997; Farah Haziqah *et al.,* 2017) but there are no studies on *Blastocystis* in houseflies, crickets and sea turtles.

To date, there are 44 *Blastocystis* subtypes, ranging from ST1 to ST44 (Alfellani *et al*., 2013; Zhao *et al*., 2017; Maloney *et al*., 2019; Maloney *et al*., 2020; Maloney *et al*., 2022). Ten subtypes (STs) are found in humans, ST1-ST9 and ST12 (Ramɒrez *et al*., 2016; Stensvold & Clark, 2016). ST11 was found in elephants, ST12 in western grey kangaroos and giraffes and ST13 was found in quokkas (Parkar *et al*., 2010). ST14 was discovered in cattle in the United States (Fayer *et al*., 2012). ST15 was found in camels from Libya and was also found in sheep from the UK, ST16 was found in the kangaroo and ST17 was found in a gundi from Libya (Alfellani *et al*., 2013). Zhao *et al*. (2017) discovered ST18 from an alpaca, ST19 from a rhesus macaque, ST20 from an ostrich ST21 from a waterbuck and ST22 from a guanaco in China. ST23-ST26 were found in cattle in the US (Maloney *et al*.,

2019). ST27 and ST28 were found in birds (Maloney *et al*., 2020). Additionally, new subtypes have been reported recently. Maloney *et al*. (2021a) identified ST29 in chickens, Maloney *et al*. (2021b) found ST30 and ST31 in white-tailed deer, Higuera *et al*. (2021) discovered ST32 in goats, Baek *et al*. (2022) detected ST33 and ST34 in horses, Maloney *et al*. (2022) identified ST35 in humans, ST36 in bats, and ST37 in rodents. Furthermore, Maloney *et al*. (2022) reported the discovery of ST38 in water voles. In another development, ST39 was reported in wild rhesus macaques in China by Yu *et al.* (2023), ST40 was discovered in muskoxen by Stensvold *et al.* (2023) while ST41 was reported in humans in Colombia by Hernɔndez-Castro *et al.* (2023). *Blastocystis* ST42-ST44 resulted from the division of ST10 into three new STs by Santin *et al.* (2024).

Previous studies have reported two subtypes of *Blastocystis* (ST2 and ST3) in cockroach samples, with the subtypes identified in studies conducted by Ma *et al*. (2020) and Farah Haziqah *et al.* (2017), respectively. These studies likely focused on characterizing the prevalence and diversity of *Blastocystis* subtypes in cockroaches. The studies conducted on houseflies have primarily focused on assessing the prevalence of *Blastocystis* in these insects, rather than identifying specific subtypes (Suresh *et al.,* 1996). Therefore, no subtypes were reported in houseflies in the studies mentioned. As for the cricket, there are no subtypes reported. Similarly for the turtles, most studies have primarily focused on reporting only the prevalence of *Blastocystis* in these reptiles. However, when it comes to subtypes, limited information is available, and the specific subtypes found in turtles remain largely unknown. These studies have not extensively characterized or identified the subtypes associated with *Blastocystis* in turtles. Therefore, the subtypes detected in turtles are mostly unidentified or unclassified at this point.

Hence, this study aims to understand the prevalence of *Blastocystis* sp. found in sea creatures, particularly sea turtles and insects (cockroaches, houseflies, and crickets). Since there is little information about the infection of *Blastocystis* in sea turtles, this study will provide a better understanding and knowledge of the susceptibility of *Blastocystis* infection in sea creatures as well as in insects.

MATERIALS AND METHODS

Ethical approval

The animals used in this study were handled according to the USM Institutional Animal Care and Use Committee (USM IACUC) Health Campus, Universiti Sains Malaysia. Permission to collect turtle faecal samples was obtained from the Fisheries Research Institute (FRI) Rantau Abang, Terengganu and Sea Turtle Research Unit (SEATRU), Institute of Oceanography and Environment (INOS), Universiti Malaysia Terengganu.

Sampling sites

The sampling sites chosen for insects in this study were in Penang Island, specifically Minden (dumpster, sewage site), Sungai Dua (residential area), and Bukit Gambier (food stall, drainage system). The laboratory-bred cockroaches were obtained from the Vector Control Research Unit (VCRU) at USM. The captive crickets were purchased from two different pet stores located in Bayan Lepas, Penang. For the sea turtles, the sampling sites chosen for this study were in Terengganu, specifically at the Chagar Hutang Turtle Sanctuary in Pulau Redang and the Fisheries Research Institute (FRI) in Rantau Abang. These sites were selected for their significance in terms of turtle conservation and fisheries research, respectively.

Study population

A total of 90 insects comprising 30 samples of cockroaches (*Periplaneta americana* Linnaeus, 1758), 30 samples of house flies (*Musca domestica* Linnaeus, 1758), and 30 samples of crickets (*Gryllus* sp.) were examined for *Blastocystis*. Also, a total of 13 turtles including both wild and captive sea turtles were studied. Among the captive turtles, two were Hawksbill turtles (*Eretmochelys imbricata* Linnaeus, 1766), three were green sea turtles (*Chelonia mydas* Linnaeus, 1758), and two were Olive ridley sea turtles (*Lepidochelys olivacea* Eschscholtz, 1829) reared in a pond at the FRI, Rantau Abang, Terengganu. As for the wild turtles, six green sea turtles that landed to lay eggs at the Chagar Hutang beach, Pulau Redang were included in the study.

Sample collection

Dissection was carried out for the insect samples in which the sides of the abdomen were cut on either side of the anus and the complete gut was removed posteriorly. The intestinal content of the insects was immediately soaked in Jones' medium for the cultivation method. However, rectal swabs were conducted for the wild sea turtles right after the egg-laying process whereas the rectal swab was carried out for captive turtles from FRI Rantau Abang during the tank cleaning process.

***In vitro* cultivation**

The intestinal contents and the rectal swab samples were inoculated into the 3 ml of Jones' medium supplemented with 10% heat-activated horse serum in a sterile culture tube, incubated vertically at 25°C for 24 hours before examined using light microscope. The sample was examined at 400x magnification to observe the *Blastocystis* and the isolated parasites were subsequently maintained by sub-culturing once every 3 to 4 days. When there was no growth detected, the sediment was re-suspended in a fresh culture medium for another 48 hours and if the *Blastocystis* forms were absent, the samples were considered negative.

Giemsa staining

Smears were carried out from day-3 positive culture samples. Later, these smears were fixed with methanol, stained with 10% Giemsa and then viewed using a light microscope at 400x and 1000x magnification for the observation of morphological characteristics.

Ultrastructural examination

Further ultrastructural examination of the positive isolates was carried out using scanning electron microscopy (SEM) and transmission electron microscope (TEM) at the Electron Microscopy (EM) Unit, School of Biological Sciences, USM. Thus, the selected day-3 positive culture sample from a turtle was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). However, none of the positive culture samples for insects underwent the electron microscopy examination because the isolates did not grow very well after day 2 of subculturing. The sample was processed following the instructions provided by the Electron Microscopy (EM) Unit, School of Biological Sciences, USM (Siti Alawiyah *et al.,* 2021).

Molecular Analysis

Genomic DNA of *Blastocystis* was extracted by using Nucleospin® DNA stool extraction kit (Macherey-Nagel, German) following the manufacturer's instructions. The *Blastocystis*-specific primer, BhRDr (GAGCTTTTTAACTGCAACAACG; Scicluna *et al*., 2006) was paired with the RD5 (ATCTGGTTGATCCTGCCAGT; Clark, 1997). Both primers were used in a single-step PCR reaction, to amplify a 600 bp region of 18s rRNA. 50 µl reaction containing 25 µl of master mix, 1.0 µl of $MgCl_2$, and 0.5 µl of each primer were used to amplify 2 µl of genomic DNA. The PCR conditions comprised an initial denaturation step at 94°C for 1 minute, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute. The final elongation step was performed at 72°C for 2 minutes. The products of the amplification were then electrophoresed in 1.5% agarose gels with Tris-Acetate-EDTA (TAE) buffer before purification and cycle sequencing by a local commercial company.

Sequence alignment and phylogenetic analyses

The sequences obtained were edited using the BioEdit software. Sequence editing involved trimming, removing ambiguous regions, and ensuring high-quality data. Once the sequences were prepared, a phylogenetic tree was generated using the MEGA 11 software (Tamura *et al*., 2021). The maximum likelihood approach was employed with the Hasegawa-Kishino-Yano model chosen as the substitution model and *Proteromonas lacertae* served as the root for the phylogenetic tree. Subsequently, the reliability of the clades generated by the tree was assessed using bootstrap analysis with 1000 replicates. This analysis provided bootstrap values, indicating the confidence and support for the different clades observed. By following this method, the edited sequences were analysed, and a comprehensive understanding of the phylogenetic relationships among the samples was achieved.

RESULTS

Prevalence of *Blastocystis* infection

Out of 30 samples collected, only two (2/30: 6.67%) samples specifically from the residential area were found to be positive for *Blastocystis* infection in cockroaches. The remaining samples did not show any signs of *Blastocystis* infection. There were no positive samples of *Blastocystis* infection among the houseflies' samples (0/30: 0%). Similarly, for crickets, two out of 30 samples (2/30: 6.67%) examined were positive for *Blastocystis* infection (Table 1).

Out of the 13 study turtles, only one (1/13: 7.7%) was positive for *Blastocystis* infection. The positive sample was found in the wild green sea turtle (*Chelonia mydas*). The remaining turtles were considered negative for *Blastocystis* infection as no signs of the parasite were detected in their samples (Table 2).

Table 1. Prevalence of *Blastocystis* infection in insects

Insects	Sampling location	No. samples	No. insects infected (%)
Periplaneta americana	Lab breed	10	0 (0%)
	Drainage system	10	0 (0%)
	Residential area	10	2 (6.67%)
Musca domestica	Dumpster	10	0 (0%)
	Food stall	10	0 (0%)
	Residential area	10	0 (0%)
Gryllus sp.	Pet store 1	10	0 (0%)
	Pet store 2	10	0 (0%)
	Sewage site	10	2 (6.67%)
Total		**90**	**4 (4.44%)**

Table 2: Prevalence of *Blastocystis* infection in sea turtles

Sea Turtles	No. samples	No. turtles infected (%)
	Captive	
Hawksbill turtle (*Eretmochelys imbricata*)	2	0 (%)
Green sea turtle (*Chelonia mydas*)	3	0 (%)
Olive ridley sea turtle (*Lepidochelys olivacea*)	2	0 (%)
	Wild	
Green sea turtle (*Chelonia mydas*)	6	1 (7.7%)
Total	**13**	**1 (7.7%)**

Morphological characteristics

The positive isolates from cockroaches (Figure 1), crickets (Figure 2) and sea turtle (Figure 3) exhibited the most common form of *Blastocystis* which is the vacuolar form. No other forms of *Blastocystis* were found in these samples.

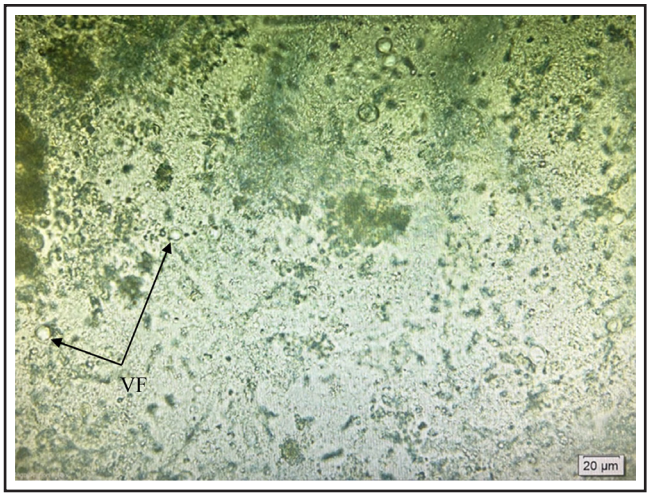

Figure 1. Vacuolar form (VF) of *Blastocystis* in cockroach (arrows) (400x).

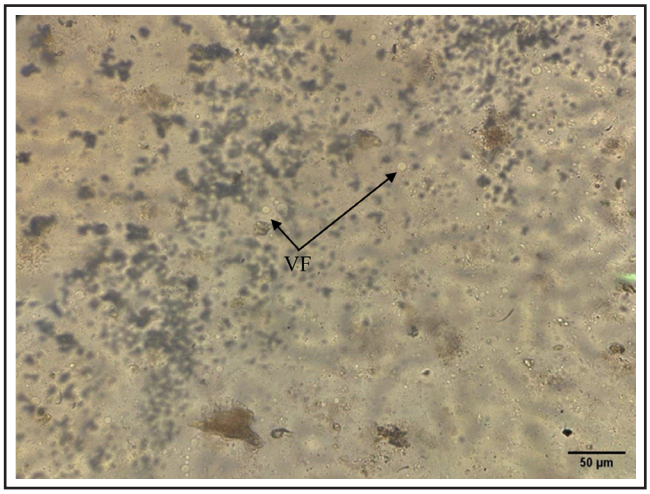

Figure 2. Vacuolar form of *Blastocystis* in cricket (arrow) (200x).

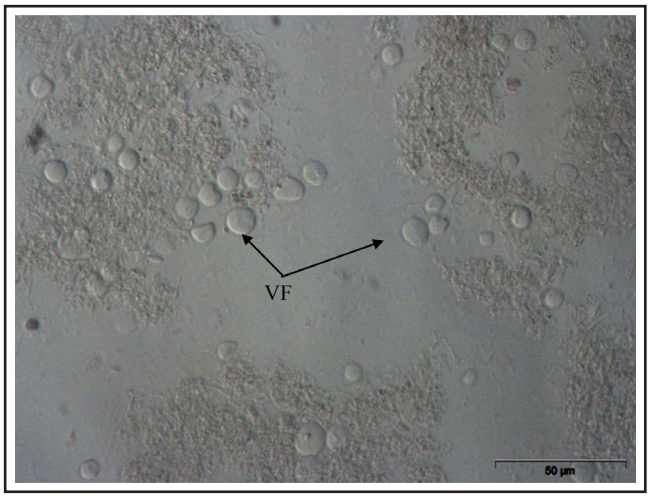

Figure 3. Vacuolar form of *Blastocystis* in sea turtle (arrow) (200x).

Mode of reproduction

It was also observed that the most common mode of reproduction for all the positives isolates was binary fission (Figure 4).

Subtype identification, alignment and phylogenetic analysis

The DNA barcoding method was employed to obtain DNA sequences covering the first 500 base pairs (5'-end) of the *Blastocystis* small subunit (SSU) rRNA genes. Out of the five positive samples detected by *in vitro* cultivation, only one DNA sequence was successfully obtained, indicating the presence of a single *Blastocystis* isolate. Unfortunately, no sequence was obtained for the positive samples from insects (two positive isolates from cockroaches and crickets, respectively).

The obtained DNA sequence was classified as *Blastocystis* ST8 (OR418368) from the wild green sea turtle isolate which reveals its relationship to other *Blastocystis* strains found in various turtle species from different geographical locations and sea snakes. The analysis includes *Blastocystis lapemi* (AY266471) from a sea snake in Singapore, *Blastocystis* from a box turtle in the Philippines (JF750335), *Blastocystis* from a keeled box turtle in Poland (KU146575), and two different individuals of the *Blastocystis* from big-headed turtle species in Japan (KT438714 and KT438713) are closely related to the sample (Figure 5).

DISCUSSION

Insects are widespread vectors of parasitic diseases and they have been proposed as potential reservoirs of the protozoan parasite, *Blastocystis.* This protozoan is a globally prevalent enteric parasite infecting humans and animals since the early 20th century and is associated with health issues that lead to general symptoms (Bogitsh *et al*., 2019) which may include nausea, loss of appetite, abdominal discomfort, bloating, excessive gas, and both acute and chronic diarrhoea (Boorom *et al*., 2008; Coyle *et al*., 2011; Bart *et al*., 2014). However, there are limited studies available on *Blastocystis* in Malaysia especially in insects despite its status as a prevalent gastrointestinal parasite.

The first study on *Blastocystis* in insects conducted in Malaysia was carried out by Suresh *et al*. (1996) on cockroaches and the most recent one, also on cockroaches by Farah Haziqah *et al*. (2017) while a study on cockroaches by Ma *et al*. (2020) was conducted in Northern China. Meanwhile, this study focused on different kinds of insects namely, cockroaches, houseflies, and crickets. Cockroaches can transmit a variety of pathogens which include *Blastocystis*. Cockroaches, well-known pests, frequently consume human faeces, potentially resulting in the spread of enteric protozoan cysts in the surroundings if the faeces are contaminated (El-Sherbini & Gneidy, 2012). Many eggs and cysts of parasites of medical importance have been isolated from cockroaches (Attah *et al.,* 2022).

In this study, positive infection was reported in cockroaches captured from residential areas. During the sampling activity, the residential area was observed to be contaminated with faecal matter and dead rats. It is known that *Blastocystis* infection is primarily transmitted through the oral-faecal route. The presence of faecal matter in the area increases the chances of cockroaches acquiring *Blastocystis* infection. Additionally, the presence of dead mice further contributes to the risk of infection as *Blastocystis* can also be found in the intestines of rats (Defaye *et al*., 2018). The cockroaches could move freely and thus can feed on both contaminated sources which significantly raises their chances of contracting a *Blastocystis* infection.

A previous study by Farah Haziqah *et al*. (2017) reported a high prevalence of *Blastocystis* infection in cockroaches collected from the drainage system site with a prevalence of 40.4% (61/151). Conversely, no cockroaches collected from the water drainage system in this study were infected with *Blastocystis.* This difference can be attributed to the fact that cockroaches captured from this

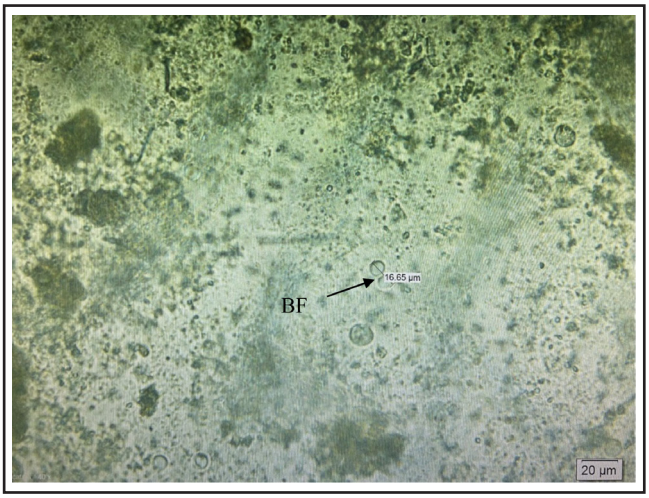

Figure 4. Reproductive form of binary fission (BF) of *Blastocystis* sp. observed from cockroach (arrow) (400x).

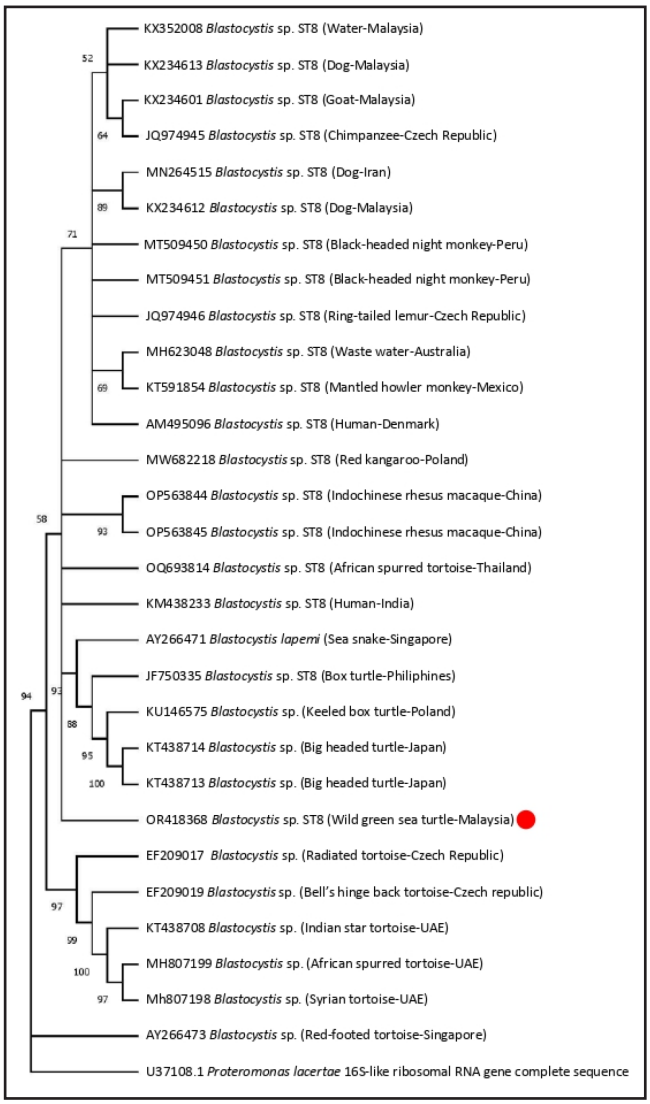

Figure 5. The phylogenetic tree in this study represents the relationship between *Blastocystis* SSU rRNA gene nucleotide sequences isolated from wild green sea turtles. The tree was constructed using the maximum likelihood (ML) method, and the branches are denoted by red-filled circles. The bootstrap values (expressed as percentages of 1000 replicates) as shown at branch points.

study were specifically from a water drainage system, whereas Farah Haziqah *et al*. (2017) focused on cockroaches collected from a drainage system next to a septic tank, which typically harbours a higher level of contamination. Water drainage systems primarily handle the control and removal of excess water such as rainwater and surface runoff. Although there may be some contaminants present, the chances of *Blastocystis* infection are less significant.

Furthermore, all the laboratory-bred cockroaches were free from *Blastocystis* infection as they were not exposed to sources of contamination for this protozoan having been housed in controlled environments that closely replicate their natural habitats, and they were kept in a close box, fed with appropriate food sources, water, and shelter to ensure their survival and reproductive success. Laboratories maintain strict cleanliness and hygiene protocols to minimize the risk of disease transmission. Cockroaches used in research are typically reared in isolated colonies to prevent cross-contamination between different colonies or with the outside environment.

The prevalence of *Blastocystis* infection in crickets can vary depending on their habitat and diet. In the case of wild crickets, their exposure to various contaminated areas and potential sources of infection increases their likelihood of acquiring *Blastocystis*. In this study, low prevalence was reported on *Blastocystis* infection in the wild crickets as they were collected in an open area which was close to the sewage sites. It is projected that the presence of faecal material in the sewage sites further increases the risk of infection for wild crickets, as they may encounter contaminated surfaces or ingest the protozoan cysts through direct exposure to faecal materials. The captive crickets obtained from the pet stores that have a more controlled environment, none of the crickets examined were positive for *Blastocystis*. These crickets were housed in a container and were fed with only vegetables and bread. As they were confined within the container and solely relied on the provided food source, the exposure to potential sources of *Blastocystis* infection was limited making them unlikely to acquire *Blastocystis* infection as compared to the free-roaming, wild crickets. There was a previous study conducted on cricket; however, the results of the study were negative for all the samples (Cian *et al*., 2017). This present study was the first to report an infection of *Blastocystis* in cricket.

Houseflies are recognized as important mechanical vectors that can transport and spread a wide range of pathogens, including bacteria, protozoa, helminth eggs and viruses (Gioia *et al*., 2022). They acquire these pathogens from unsanitary sources such as garbage, sewage, and other unclean environments (Al-Aredhi, 2015), and are responsible for transmitting diseases such as poliomyelitis (Gudnadoƭttir, 1961), cholera (Fotedar, 2001), salmonellosis (Olsen & Hammack, 2000), and various gastrointestinal infections. In this study, a total of 30 samples of houseflies were captured from three distinct areas: the dumpster area, food stall, and residential area. All the houseflies examined in this study tested negative for *Blastocystis* infection. This could be attributed to the fact that the samples were not exposed to cysts of *Blastocystis*, which are typically found in faecal material. Another reason for not finding *Blastocystis* in houseflies in this study may be attributed to the fact that *Blastocystis* primarily inhabits the intestines of humans and animals, while houseflies prefer environments that are rich in decomposing organic matter and waste which may not be suitable for the growth of the parasite as *Blastocystis* requires specific conditions to survive and reproduce. The chosen sampling sites in the studies were likely located far away from faecal materials, which are the primary source of *Blastocystis* infection through the faecal-oral route. Although *Blastocystis* can be transmitted through various modes, including contaminated water and carrion, it is important to note that the absence of *Blastocystis* on houseflies in these studies may simply be due to the selected areas being free from sources of contamination for *Blastocystis.*

To date, *Blastocystis* ST has been identified in insects by two studies, both of which focused predominantly on cockroaches. The ST identified include ST3 (Farah Haziqah *et al*., 2017) and ST2 (Ma *et al*., 2020). ST3 is very common in humans, and it is found in the digestive tracts of people worldwide whether they have symptoms or not (Rojas-Velɀquez *et al*., 2018). Meanwhile, the most recent study was conducted in a zoo located in Northen China by Ma *et al*. (2020). Among the cockroaches examined, 82.8% were infected with *Blastocystis*, and all positive samples belonged to ST2. This high infection rate in cockroaches was the highest reported among similar studies. The study also found that out of the total golden monkey samples, 48.7% tested positive for *Blastocystis*, with three subtypes (ST1, ST2, and ST3) identified. ST2 had the highest prevalence at 44.4%. Genetic analysis of *Blastocystis* subtypes revealed that golden monkeys and cockroaches share the dominant ST2, indicating the potential for mutual transmission between these animals. This phenomenon may be attributed to the extensive mobility of cockroaches, which could enhance the transmission of *Blastocystis* to other animal species. As a zoonotic parasite, *Blastocystis* poses a higher risk of transmission to humans from non-human primates like golden monkeys, particularly in close contact situations such as in zoo settings (Ma *et al*., 2020).

However, the ST could not be determined in the positive samples from insects in this study. This outcome could be attributed to DNA degradation and insufficient sample concentration. Improper storage or handling may have led to DNA degradation, rendering it undetectable during gel electrophoresis. Additionally, the low concentration of DNA in the samples could have contributed to the lack of band formation during the molecular analysis (Dilley *et al.,* 2021).

Sea turtles are currently listed as critically endangered species and are protected by organizations such as the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species on Wild Fauna and Flora (CITES). Consequently, it is crucial to prioritize the conservation of sea turtles to prevent their extinction. However, the sea turtle's health condition also needs to be taken into consideration. This study reported a very low prevalence of *Blastocystis* infection and was the first to report such an infection in sea turtles in Malaysia. Notably, all the screened sea turtles appeared physically healthy and showed no signs of weakness. The infection may have been acquired through the consumption of contaminated water, influenced by human activities. It is important to acknowledge that *Blastocystis* can be present in water sources that have been contaminated and *Blastocystis* ST8 has been detected in water sources (Attah *et al.,* 2023). *Blastocystis* infection has been associated with a range of manifestations, including intestinal symptoms and skin disorders (Tan, 2008). Diarrhoea and abdominal pain are frequently reported as the most common intestinal symptoms in humans linked to *Blastocystis* infection in previous studies (Tan, 2008, 2010). These symptoms can also manifest in animals infected with *Blastocystis*, particularly those with weakened immune systems, posing significant health risks. If a sea turtle has a compromised immune system, *Blastocystis* infection can potentially result in severe health issues. Therefore, it is essential to implement measures aimed at preventing unnecessary problems in the future and safeguarding sea turtles from the threat of extinction.

Blastocystis in turtles is still poorly studied, and the available research reveals a significant gap in our understanding of the subtypes found in these reptiles. Most studies investigating *Blastocystis* in turtles have led to the identification of unknown subtypes. For instance, a study conducted at the GdaƆsk Zoo in Poland detected *Blastocystis* in five turtle samples, but the parasites did not match any of the known mammalian and avian subtypes (RudziƆska *et al*., 2021). Similarly, another study conducted in French zoos also resulted in the detection of an unknown subtype in two

positive samples from turtles, and a study by Cian *et al*. (2017) found six positive samples of *Blastocystis* in turtles, all of which belonged to an unknown subtype (AbuOdeh *et al*., 2019). However, there is one known subtype (ST8), which was identified in a box turtle (*Terrapene carolina*) (Accession no. JF750335).

Based on the phylogenetic analysis, it is suggested that *Blastocystis* ST8 and these related strains share a common ancestor but have diverged, potentially due to factors such as geographical location or host-specific adaptations. It is important to note that *Blastocystis* ST8 is classified as a subtype, and its close relationships with other *Blastocystis* strains in turtles from various regions highlight the potential for shared evolutionary histories within this subtype.

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